SOME PROPERTIES OF GABA - ACTIVATED CHLORIDE CHANNELS IN MAMMALIAN NEURONES

by

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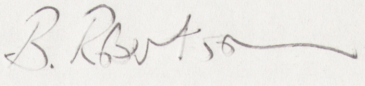
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The early experiments on the study of inhibitory currents in the hippocampus were carried out in collaboration with Dr. Graham Collingridge, and I am grateful to him for helping me begin that project.

The remainder of this thesis is my own original work, and I certify that none of the work presented in this thesis has previously been submitted for a degree or other examination.



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ABSTRACT

The properties of GABA-activated chloride channels in mammalian neurones were investigated with voltage clamp techniques, in an effort to describe some of the biophysical and pharmacological characteristics of these channels. Spontaneous inhibitory postsynaptic currents in pyramidal neurones were used to characterize GABA channels in central neurones. The basic properties of these inhibitory events, and the actions of some clinical anaesthetics and other drugs on their conductance changes were investigated. The effects of anions other than chloride on the time course and voltage sensitivity of these currents were explored.

The characteristics of GABA-activated chloride channels in mammalian dorsal root ganglion neurones were examined using voltage jump relaxation techniques. The actions of analogues structurally similar to GABA were investigated. Some clinical anaesthetics were also effective in directly activating GABA channels in dorsal root ganglion neurones. The anaesthetics also were capable of 'blocking' these channels. GABA channels in drg neurones are more permeable to iodide and bromide ions than chloride, but fluoride and propionate are considerably less permeable. Iodide and bromide exerted anomolous effects on chloride efflux at negative potentials. Foreign anions also altered channel kinetics.

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SECTION A:

GENERAL INTRODUCTION

SECTION A: GENERAL INTRODUCTION

It is the aim of this work to describe some of the basic characteristics of chloride channels activated by the neurotransmitter γ-aminobutyric acid (GABA), in freshly isolated neurones from the mammalian central and peripheral nervous systems. Activation of these channels causes inhibition in the nervous system, and it is becoming apparent that these channels may be involved in the aetiology of important clinical disorders such as epilepsy. Additionally, the kinetics of these channels is markedly affected by clinical anaesthetics. In spite of the importance of GABA channels, we still know very little about the fundamental biophysical characteristics of these complexes in mammalian neurones.

In this section, some of the background to the scientific study of inhibition, the mechanism of GABA receptor/channel activation and current theories of the basis of action of anaesthetic compounds will be presented.

The earliest experimental basis for our present concepts of neural inhibition were carried out in the first half of the 19th century. In 1838, Volkmann noted that stimulation of the vagus nerve had an inhibitory action on the heart - unfortunately however he rejected this phenomenon as an experimental artefact (Brazier 1959). A few years later, in 1845, Eduard and Ernst

Weber published similar results obtained after stimulation of vagi with a voltaic pile, in a manuscript entitled "Experiments which prove that the vagus nerves, stimulated with a rotary electromagnetic apparatus, slow and interrupt the heart beat" (see Fulton 1930). Despite these pioneering studies, the nature of central inhibition was still mysterious. In the 18th century Robert Whytt had made special study of reflex activity in decapitated animals, and had also commented on the phenomenon of conscious inhibition of reflex activity. However, though this work may have led to speculations about the involvement of central inhibition in reflex action, many still believed that inhibition was a peripheral phenomenon, and there were several theories hypothesizing the existence of inhibitory nerves to muscles (see Brazier 1959).

The firm establishment of a central inhibitory mechanism originates from the work of Ivan Sechenov. By using Turcks reflex (i.e. the withdrawal of the frogs foot from a noxious stimulus) as a measure of reflex time, Sechenov observed the effects of placing salt crystals on the exposed frog brain. He found that crystals placed in certain regions of the brainstem blocked the reflex action, and later extended and confirmed these studies using electrical stimulation and sectioning of the brain. Sechenov concluded that there were inhibitory centres in the central nervous system, which could be activated by incoming sensory fibres, and these centres could subsequently influence reflex excitation (Sechenov 1863).

The consolidation of what has become our present ideas on central inhibition was principally due to the researches of Charles Scott Sherrington. Sherrington devised a unifying hypothesis of 'central inhibition' and 'central excitation' to explain his many observations on reflex activity in mammals (see Sherrington 1906). Sherrington also incorporated two other important theories in his scheme of nervous system function (see Sherrington 1925). The first was the concept of the 'synapse' which he held to be a special functional connection from one nerve cell to another: Sherrington's support for the neurone theory significantly advanced acceptance of this hypothesis over the alternative reticular theory, which was popular at that time. (The reticular theory proposed that nerve cells were anatomically united with one another rather than individual entities connected physiologically - see Granit 1966). Sherrington also included the concept of chemical transmission between neurones into his theories of reflex activity (Sherrington 1925), although it was to be some time before the notion of chemical neurotransmission was universally accepted.

The next important phase in the understanding of inhibition in the central nervous system came with the development of more refined electrophysiogical techniques, enabling experimenters to record from single neurones, while directly and preferentially stimulating the excitatory and inhibitory connections associated with them. These techniques were used from the 1950's onward with considerable success by Eccles' and his

collaborators. There was at this time a growing body of evidence from Dale and his colleagues to suggest that transmission across synapses was chemical in nature (Dale 1937). In this hypothesis, the action potential in the presynaptic terminal causes the release of a tiny quantity of the chemical transmitter, which then diffuses across the gap between neurones, and then causes an ionic current to be generated across the postsynaptic membrane. Eccles believed at that time that synaptic transmission was in fact electrical, and that 'action currents' of the nerve impulse directly influenced the polarization of the postsynaptic cell membrane. Eccles was particularly impressed by the rapid nature of synaptic transmission (often less than 1ms) and believed that release of chemical transmitters was too slow to account for the rapid communication between neurones (Eccles 1946).

It was not until the techniques of intracellular recording were sufficiently well developed that it was possible to test directly the validity of electrical or chemical theories of synaptic transmission. Eccles siezed the oppurtunity afforded by the developments of glass microelectrodes by Ling & Gerard (1949) which had been successfully applied to the study of neuromuscular transmission by Fatt & Katz (1950), and reported the first intracellular recordings from nerve cells in the central nervous system (Brock, Coombs & Eccles 1951). This short paper demonstated the existence of excitatory postsynaptic potentials in motoneurones and the results were consistent with Eccles' electrical hypothesis. The electrical

hypothesis was to finally meet its end as a viable alternative to chemical transmission when Eccles and his colleagues investigated inhibitory synaptic transmission. Previously Brooks & Eccles (1947) had proposed on the basis of the electrical theory that synaptic inhibition would generate a postsynaptic depolarization, but the chemical theory predicted a hyperpolarization of the neurone. The intracellular recordings revealed that synaptic inhibition was clearly due to a hyperpolarization of the neurone (Brock, Coombs & Eccles 1952). Eccles had discovered the inhibitory postsynaptic potential, and instantly became converted to the chemical theory of synaptic transmission (see Eccles 1970, 1982). (Shortly after Dale (1954) reviewed the chemical theory and was particularly pleased at Eccles' conversion). Subsequently Eccles and his co-workers went on to study the ionic basis of IPSPs (e.g. Coombs, Eccles & Fatt 1953, 1955), their generation by interneurones in the CNS (e.g. Andersen, Eccles & Loyning 1964a,b) and their pharmacology, in an attempt to find the neurotransmitter responsible for inhibitory synaptic action (e.g. Curtis 1959). It is to this work that we now turn.

The neutral amino acid γ-aminobutyric acid (GABA) was not discovered to be an important constituent of mammalian brain until 1950, and was reported as such in two papers from separate laboratories (Awapara, Landua, Fuerst & Seale 1950, Roberts & Frankel 1950). Since GABA is found in few other tissues, its high concentration in the brain suggested that it may have had some special function

there. To be considered as a synaptic transmitter, GABA had to meet several criteria (see e.g. Phillis 1966):

(1). The chemical must be present in the neurones proposed to release it.

(2). The neurones must have the necessary enzymic machinary to synthesize and release the substance, and the precursors and intermediates involved in its production should be demonstrable.

(3). The compound should be detected in the extracellular solution around the neurone after stimulation.

(4). When the substance is applied artificially to the postsynaptic cell, it should mimic the action of the synaptically released transmitter.

(5). There should be mechanisms to terminate and/or take up transmitter after its action is completed.

(6). Pharmacological agents which interact with the endogenous transmitter should have identical effects on the artificially applied suspected transmitter.

Some of the most powerful evidence suggesting that GABA is an important inhibitory transmitter came from microiontophoretic application experiments where the amino acid and its antagonists were assayed by using the firing pattern of single neurones. GABA depresses the firing of almost all cortical neurones, and causes a membrane hyperpolarization associated with an increase in membrane chloride conductance (see Curtis & Johnston 1974 and Krnjevic 1974 for literature survey). (However in more recent experiments on central neurones, it has been found that GABA has other actions see e.g, Newberry & Nicoll

1985 and Section E in the present work). The depressant effects of GABA can be decreased by parallel application of the convulsant antagonists bicuculline and picrotoxin (e.g. Curtis, Felix & McLennan 1970, Hill, Simmonds & Straughan 1972). As a result of intensive research over the last twenty years it is now believed that GABA is the transmitter mediating most inhibitory synaptic potentials in the mammalian CNS.

There is a great deal of clinical interest in the examination of GABA-mediated neurotransmission, especially since disorders of this system have been implicated in various neurological disturbances. There is evidence that inhibitory transmission is disturbed in epilepsy (e.g. Meldrum 1975), with possibly a preferential decrease in the number of inhibitory synaptic terminals (e.g. Ribak, Hunt, Bakay & Oertel 1986). It has also been suggested that the GABAergic system is disturbed in Huntingtons chorea and Reyes Syndrome (Barbeau 1973, Lloyd & Dreskler 1979), schizophrenia and Parkinsons disease (Lloyd, Sheman & Hornykiewicz 1979). GABA receptors are also likely to be intimately involved in the actions of benzodiazepines (see e.g. Bowery 1984), which are amongst the most widely prescribed drugs in the world, and barbiturates, which are extremely important clinically as anaesthetics and anticonvulsants (see e.g. Johnston 1983). All these reasons provide a very strong impetus for characterizing the synapses and receptors that use GABA as a transmitter. Indeed there is an enormous literature on

this neurotransmitter, especially on the actions of benzodiazepines at this site. Despite this, there have been very few studies of the basic properties of the GABA receptor/channel complex in mammalian preparations. Electrophysiological studies at the channel level can provide information about the permeability properties of the channel (and hence give an indication of pore "size"), and analysis of drug effects on channel kinetics can provide clues to the mechanism and possibly the site of action of the compound. Such results could provide useful clues for the design of more effective drugs for the control of neurological disorders or anaesthesia, and also to increase our understanding of inhibitory mechanisms in the nervous system.

Chemical neurotransmitters bind to specific receptors on the postsynaptic membrane, and these receptors are part of, or are closely coupled to, ion channels. The gating or opening of these ion channels induced by the binding of the transmitter allows ions to flow down their electrochemical gradients leading to a current flow across the cell membrane. This conductance change is finite however, and the channels usually remain open only for a few milliseconds before closing again. Present theories of the sequence of events leading to channel activation are based on the hypothesis of del Castillo & Katz (1957) who suggested by analogy with enzyme kinetics, that the binding of an agonist leads to a conversion of the receptor/channel to an inactive form,

which then changes to an active, open channel:

$$T + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} TR^*$$

(The rate constants are labelled according to more recent convention).

The ionic current (I) generated by the agonist is given by:

$$I = [TR^*].\gamma.(V - Vnull)$$

where $[TR^*]$ is the number of open channels, γ is the elementary channel conductance, and (V - Vnull) is the difference between the cell membrane potential (V) and the equilibrium potential (Vnull) for the permeant ion(s). At equilibrium the number of open channels is given by:

$$[TR^*] = N.\ \beta/(\alpha + \beta)$$

where N is the total number of receptor/channels.
At low agonist concentrations β is generally assumed to be small compared with α. The closing rate α is considered to be the rate limiting step in channel closure (e.g. Magleby & Stevens 1972b, Neher & Sakmann 1975).

The decay of small spontaneous conductance changes at synapses is exponential, and is characterised by the time constant of decay τ, which is equivalent to $1/\alpha$ if transmitter concentration falls rapidly. Study of the time course of inhibitory synaptic currents can therefore give an indication of basic channel kinetics. If the activated channel complex exists in equilibrium with the inactive intermediate state, then a sudden perturbation of the system will result in a 'relaxation' to the new equilibrium. This relaxation will have time constant τ, equal to $1/(\alpha+\beta)$. Results presented here show that τ for GABA is sensitive to membrane potential, becoming shorter with hyperpolarization - this means that it is possible to use voltage jump methods to obtain a measure of α and β (Adams 1975, Neher & Sakmann 1975, Sheridan & Lester 1975). In the second section of the experimental work reported here, this technique was applied to GABA receptor channels.

There are more direct methods available to examine single channel behaviour. The first of these is called noise or fluctuation analysis, which deconvolves the current signal generated by the statistical superposition of the opening and closing of many channels. When all of the important criteria for valid deconvolution are met, this analysis yields a measure of the single channel conductance γ, and channel open time. The second technique, known as patch clamp, allows the most direct measure of channel kinetics and conductance presently possible. Patch clamp allows the experimenter to resolve

the opening and closing of single channels which appear as discrete on-off steps of current. This technique was also employed in an attempt to measure the properties of GABA-activated channels in drg neurones.

In the present work some emphasis was placed on the effects of some anaesthetics on GABA channels, and consequently it is appropriate that some historical introduction be made to this area of study.

There have been many theories advanced to explain the fundamental basis of anaesthetic action. For instance Claude Bernard proposed that anaesthetics reversibly coagulated the protoplasm of neurones thereby disrupting nervous function (see Henderson 1930). Other early membrane theories suggested 'hardening' or 'blistering' of cell membranes (see Halsey, Green & Wardley-Smith 1980). In 1893 Richet proposed the empirical rule that the lower the solubilty of an anaesthetic in water, the greater its potency (Verworn 1911). This hypothesis was later extended by the (independent) work of Hans Horst Meyer and Charles Ernest Overton, who found a strong correlation between the potency of an anaesthetic and its lipid solubility. Meyer proposed that the state of anaesthesia commenced when the anaesthetic sustance reached a certain molar concentration in the membrane lipids (see Meyer 1899, 1937). These studies were the first of the 'unitary' hypotheses of anaesthetic action, and because of the strong correlation between lipid solubility and potency many subsequent studies focussed on various kinds

of disruptions of cell membrane lipids as the means of anaesthetic action (see e.g. Mullins 1954, Lee 1976, Trudell 1977). Since the function of membrane excitability lies mainly in protein complexes in the lipid membrane, disruptions of lipids are thought to perturb the functions of the proteins. Despite the attractiveness and tremendous popularity of the 'lipid theories' of anaesthetic action, there is presently a growing body of evidence to suggest that anaesthetics may affect membrane proteins directly (see e.g. Richards et al. 1978, Franks & Lieb 1982, Miller, 1985). This has arisen partly because lipid theories place certain thermodynamic restrictions on the effects of temperature or high pressure on the potency of anaesthetics, and these are not met experimentally (e.g. Richards, Keightly, Hesketh, & Metcalfe 1980). Additionally, specific sites for certain anaesthetic substances have been found in proteins and membrane channels (see Section E), and more selective receptor sites for the anaesthetics are necessary to explain the marked differences in potency of isomers of anaesthetic molecules (see e.g. Butler 1950, Richards & Hesketh 1975).

It may be more satisfactory theoretically to think of complex anaesthetic molecules binding to specific regions of membrane proteins, and consequently disturbing the normal transitions or functions of the protein by an allosteric mechanism, but clear hard evidence for this is still scarce. We also know very little about the nature of these sites or precisely how the anaesthetic molecule

'allosterically' disturbs protein function.

Our ideas on how anaesthetics affect the workings of the nervous system have also become more refined, and we now believe in selective actions of anaesthetics in the nervous functions. Sherrington proposed that synaptic transmission was much more sensitive to anaesthetic action than axonal conduction (Sherrington 1906), and Winterstein (1919) reached a similar conclusion. That this was the case was clearly demonstrated by Larabee & Posternak(1952), who compared the effects of some clinical anaesthetics on both axonal and synaptic transmission through the stellate ganglion of the cat. Inhibitory synaptic transmission appears to be profoundly affected by anaesthetics. In one of the earliest studies, Eccles & Malcolm (1946) showed that pentobarbitone greatly prolongs the dorsal root potential in the frog spinal cord - which is considered to be mediated by GABA. My own interest in the effects of anaesthetics on GABA channels is directed two ways. Firstly in an attempt to understand more about the mechanisms of anaesthesia on the nervous system, and secondly to use these substances as probes of the GABA channel at the biophysical level: that is to try and understand how the channel may be activated and modulated by these extremely potent molecules.

The first part of this thesis presents the results of experiments directed towards characterising postsynaptic GABA channels in central neurones. I have used spontaneous inhibitory postsynaptic currents (IPSCs) as a measure of GABA channel properties and examined the effects of foreign anions and some clinical anaesthetics and other drugs on GABA channel function. The second section deals with results of experiments aimed at examining GABA channel properties on a peripheral neurone. Further studies were made on the effects of foreign anions on the permeability and kinetic properties of these channels. Additionally experiments were carried out on the properties of these channels when agonists other than GABA (including anaesthetics) open the channel.

SECTION B :

GABA CHANNELS IN THE HIPPOCAMPUS

SECTION B: INTRODUCTION.

There have been many electrophysiological studies of GABA activated channels in a tremendously wide variety of vertebrate and invertebrate preparations, ranging from the helminth Ascaris (e.g. Martin, 1985) to the amphibian spinal cord (e.g. Curtis, Phillis & Watkins, 1961), including locust muscle (e.g. Cull-Candy 1984), lobster stretch receptor neurones (Adams, Constanti & Banks 1981) and mammalian ganglionic (Adams & Brown 1975) neurones. These studies have provided a wealth of information on the general properties of GABA-activated chloride channels. The work on peripheral and invertebrate nervous systems was carried out partly to obtain some clues as to the nature of GABA-ergic inhibitory neurotransmission in the mammalian CNS; particularly as disorders of this system are clinically relevant (see Section A). Until the last few years, the difficulties in obtaining an isolated preparation of mammalian CNS prevented a rigorous electrophysiological analysis of GABA-activated channels, and we had to extrapolate from results obtained in simpler, more accessible preparations. However, in vitro techniques of cell culture and tissue slicing became sufficiently sophisticated to allow experimenters to record from isolated CNS neurones and to use voltage clamp techniques and many of the other methods that had been used so successfully on e.g. invertebrate neurones and

squid giant axons to determine basic channel properties.

The ability to culture central neurones permitted visual control over electrode placement and direct, accurate application of agonists and drugs (even in some cases to particular nerve cell regions (e.g. Barker & Ransom 1978). This has been very successful in providing insights into the pharmacology and biophysics of transmitter and voltage activated channels. Barker's group has used this approach over a number of years in the study of GABA-activated channels (see e.g. MacDonald & Barker 1981 for review). One of the major criticisms that might be levelled at this work however is that neurones de-differentiate during the culture process, and the expression of channels in these cultured cells may be quite different from that normally obtained in vitro. Further discussion of this problem will be made later.

Since much of the biophysical data on GABA-activated channels had been obtained on cultured neurones, it was thought it would be worthwhile to examine some of the characteristics of these channels in freshly isolated central neurones. To this end, brain slice techniques were used to obtain a preparation of CNS neurones. This would allow study of a (hopefully) more appropriate physiological model of the intact, living CNS and the results could be compared with those obtained from neurones in tissue culture.

Biochemists have been using tissue slice techniques since Warburg's studies in the 1920's. Warburg developed tissue slices for the measurement of respiration of cancer cells (see Krebs 1981) and detailed examination of aerobic and anaerobic metabolism. McIlwains group was one of the poineers in the preparation of brain slices for biochemical and electrophysiological studies in the 1960's, and it was not long before electrophysiologists realised that the brain slice preparation had tremendous potential for studying the electrical workings of the nervous system. Early studies by Li & McIlwain (1957) and Yamamoto & McIlwain (1966) on isolated cortex showed that neurones could be successfully isolated and maintained in vitro, and that the electrical properties of these neurones were very similar to those previously observed in vivo. These early experiments led to a rapid expansion of brain slice techniques for electrophysiology, and a wide variety of different preparations have appearred, including olfactory cortex, cerebellum, visual cortex (see texts edited by Kerkut & Wheal 1981 and Dingledine 1984).

The brain slice technique offers several advantages over in vivo methods for the student of nervous system physiology. These are:

(1). Direct control of the environment of the neurone. This gives enormous advantages for the application of known quantities of drugs, and alteration of the concentration of permeant ions.

(2). The need for anaesthesia, which is a vital requirement for many *in vivo* studies, is avoided. This advantage is particularly important since many anaesthetics alter channel properties and hence the normal characteristics of the neurone. Many of the experiments reported here involved the application of anaesthetics to neurones, hence the need for 'non - anaesthetised' controls.

(3). Chemicals and drugs which would not penetrate the blood-brain barrier *in vivo* can be applied to neurones.

(4). The slice preparation allows a much greater degree of visual control in the placement of microelectrodes. For instance, in the transverse hippocampal slice, it is possible to place electrodes into dendritic or somatic regions of cells quite easily. In contrast, *in vivo* work requires precise stereotaxic measurements for accurate impalement.

(5). The *in vitro* slice is mechanically very stable. There are none of the problems of movement due to heart beat or respiration which can make whole animal experiments difficult.

(6). Slices offer simplified neuronal organisation for stimulation of particular pathways. It becomes possible to localize stimulation, and avoids *en masse* discharge of large parts of the nervous system. (This of course can be a major disadvantage for particlar experiments, since the diverse interconnections between various parts of the nervous system is lost, and interpretation of results in terms of the workings of the whole nervous system could be

very misleading).

(7). The isolated slice preparation makes electrophysiogical recording easier than in the intact nervous system. For instance, microelectrodes do not have to penetrate deep into tissue, this avoids excessive electrode tip capacitance and gives a more favourable situation for voltage clamp with a single microelectrode. In vivo reduction of tip capacitance has to be achieved by carefully shielding microelectrodes, which is very time consuming and less adequate than being able to eliminate the source of the unwanted tip capacitance directly.

The transverse hippocampal slice was the preparation chosen for the experiments on GABA-activated channels reported here. The hippocampus (Greek for sea horse) is phylogenetically one of the oldest parts of the nervous system, and is part of the limbic system. It is believed to be involved in memory consolidation (in particular the tranformation of short term into long term memory), error evaluation, smell, emotion, and cognitive and spatial mapping (see e.g Green 1964, Sahgal 1980). The anatomy of the hippocampus was beautifully described by Ramon y Cajal (1911) and Lorente de No (1934). These studies revealed that the hippocampus had a relatively simple structure, consisting of a chain of three neuronal regions: the CA1, CA3 and dentate gyrus, folded round in a sort of 'swiss roll' structure. (CA is the abbreviation for Cornu Ammonis, or Ammons horn). The cell bodies of the pyramidal cells in CA1 and CA3 lie in the same plane,

in a single compact layer (the stratum pyramidale), with their dendrites placed on either side of this region to receive afferent traffic from other regions. However it was not until comparatively recently that it was shown that the hippocampus also had a well organized lamellar structure. Lomo (1971) and Andersen, Bliss & Skrede (1971) showed that stimulation of the fibres in a particular part part of the hippocampus resulted in excitation of cells in a narrow strip of tissue in the same plane. This antomical advantage meant that the hippocampus was ideally suited to slicing techniques, since a transverse slice perpendicular to the long axis of the hippocampus will preserve the integrity of the chain of neuronal regions. Skrede & Westgaard (1971), working in Andersen's laboratory, took advantage of this organisation and isolated transverse slices of the hippocampus and reported that all three neuronal groups were connected and could be electrically activated in the transverse slice.

The simple anatomy of the hippocampus meant that it was the subject of some important neurophysiological studies in vivo. Kandel, Spencer & Brinley (1961) were the first to observe large and prolonged IPSPs in pyramidal cells induced by stimulation of the fimbria, and because this response was present even after deafferentation of the fimbria, Spencer & Kandel (1961) concluded that there was a recurrent inhibitory pathway

onto the hippocampal pyramidal neurones. In two classic papers, Andersen, Eccles & Loyning (1964a,b) suggested that the IPSP was generated on, or close to the soma of the pyramidal cell and that this IPSP was generated by interneurones (most probably basket cells). These papers were the first to identify inhibitory cells in the CNS, and laid the foundations for studies of GABA-mediated IPSPs and the present results on hippocampal IPSCs.

On the basis of the silver stain anatomical methods of Cajal and Lorente de No, Andersen et al.(1964b) suggested that the basket interneurone, with its somatic terminals, was the most likely mediator of the recurrent IPSP. More recent anatomical studies, employing stains for glutamic acid decarboxylase (GAD) (Storm-Mathisen 1977, Ribak, Vaughn & Saito 1978), GAD antibodies (Storm-Mathisen, Leknes, Bore, Vaaland, Edminson, Huang & Ottersen 1983) and GABA transaminase (Nagai, McGeer & McGeer (1983) have also shown that interneurone/GAD positive terminals lie both on pyramidal somata and axon initial segments (as well as some dendritic regions).

Pharmacological studies (Biscoe & Straughan 1966, Curtis, Felix & McLennan 1970, Segal, Sims & Smissman 1975) suggested that GABA was the neurotransmitter causing the recurrent IPSP in hippocampal pyramidal neurones. The evoked IPSP is reduced by the specific GABA antagonists bicuculline and picrotoxinin. Iontophoretic application of GABA to the cell soma results in a hyperpolarization (Andersen, Dingledine, Gjerstad, Langmoen & Laursen 1980) and conductance increase (e.g.

Dingledine & Langmoen 1980, Ben-Ari et al. 1981). The IPSP reverses at the chloride equilibrium potential (Alger & Nicoll 1980, Ben-Ari et al. 1981). By taking advantage of the somatic location of inhibitory terminals, one is presented with an almost ideal opportunity to study GABA-mediated neurotransmission in the CNS with voltage clamp techniques. However, since it would be extremely difficult, if not quite impossible, to impale a CA1 neurone with two separate microelectrodes (one for recording voltage, the other for passing current), it is necessary to employ a single microelectrode or 'switch' clamp. In this system, originally devised by Wilson & Goldner (1975), the microelectrode is switched at a high frequency between sensing membrane voltage and passing current. This means that it is only necessary to place one microelectrode into a neurone to voltage clamp ionic conductances - providing the conductance change is not too large or too fast. This technique has been used to voltage clamp hippocampal cells by e.g. Johnston, Hablitz & Wilson 1980. In the hippocampal slice, it is relatively easy to impale the ~ 40μm soma of a CA1 pyramidal cell by moving the electrode through the stratum pyramidale region. Since the inhibitory conductance changes associated with the recurrent IPSP are probably somatic in origin, the single electrode clamp is able to record GABA-mediated conductances relatively faithfully.

Alger & Nicoll (1980) had reported the existence of spontaneous depolarizations in chloride-loaded CA1 neurones, and these were similar to IPSPs observed in cat

hippocampus in vivo (Nicoll, Eccles, Oshima & Rubia 1975). These authors presented strong evidence that these IPSPs were due to spontaneous firing of interneurones and release of quanta of GABA onto pyramidal cells. Since the interneurones fired spontaneously, this meant that it was not necessary to activate the recurrent inhibitory pathway by stimulating the hippocampus artificially. This therefore avoided contamination by extracellular field potentials and activation of excitatory pathways while recording IPSCs. Spontaneous release of transmitter also meant that the IPSCs would probably mimic a 'delta' function of transmitter release. Packets of GABA would be released onto the postsynaptic sites resulting in a fairly synchronous opening of channels, and the decay of the current would then be analagous to the exponential distribution of channel closing times. In this fortunate situation it is then possible to interprate results in terms of GABA channel kinetics in the freshly isolated CNS, and the hope is that these phenomena are similar to those occuring in the living mammalian nervous system.

In this section the results of experiments directed at characterising the properties of IPSCs, and the effects of some anaesthetics and drugs on these inhibitory conductance changes will be given. Additionally the actions of foreign anions on channel properties are presented.

SECTION B: METHODS.

For the experiments reported in this section, adult Wistar rats of both sexes and approximately 150g in weight were used. Rats were chosen as the experimental animal because of the convenient size of their hippocampus and their availability. Animals of about 150g were used due to the relative ease of opening up the skull - the skull becomes much harder in older rats and the dissection is consequently slower. An illustration of the hippocampus in the rat brain is shown in Fig. 1. Animals were stunned by a sudden blow to the head or upper body, and their necks were dislocated. The head was removed high up at the neck, and the skin covering the top of the skull cut and peeled away. The skull was then cut along the line of the sagittal suture, being careful to keep the scissors and the head parallel. Following this the parietal bones and dura were peeled away using large tweesers. The cerebellum was removed and the brain sectioned coronally just behind the coronal suture. After cutting the cranial nerves, the brain was transferred onto filter paper in a petri dish filled with cold Krebs solution (the filter paper helped to keep the brain in position during further dissection). Cold, oxygenated Krebs (~ 10^0C) was used throughout the dissection in order to: a) to make the brain 'firmer' and therefore easier to handle and b) to reduce neurone activity during the hypoxic stress of dissection. The

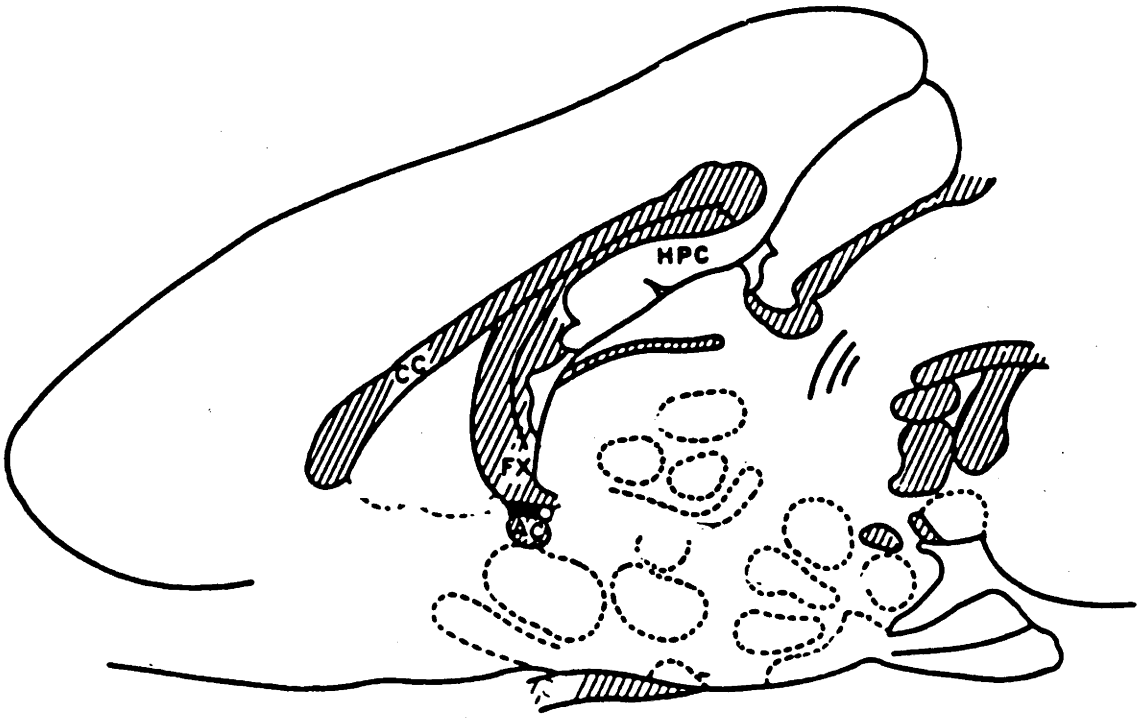


FIGURE 1: A section through the rat brain showing the position of the hippocampus (HPC) in the rat brain. The hippocampus lies roughly between the corpus collosum and the cerebellum, and lies at a slight tangent to this sagittal section.

midbrain was removed and the brain was positioned vertically in the rostro-caudal axis. The brain was then cut along the midline and the blunt end of a wooden toothpick placed under the fimbria. The hippocampus was first loosened by carefully moving the toothpick from side to side, and the hippocampus was gently raised out and completely separated from the rest of the brain. Any extra unwanted tissue adhering to the hippocampus was removed with the toothpick. One of the two hippocampi was transferred to a piece of moistened filter paper on the stage of a hand-operated tissue chopper. Slices perpendicular to the long axis of the hippocampus were taken from the middle of the hippocampus, advancing 400µm after each slice was made. After making 6-12 slices, the hippocampus was transferred to a clean petri dish and the slices were carefully separated by pouring cold Krebs over them and by teasing them apart with a fine paintbrush. Individual slices could then be transferred to the recording chamber using the paintbrush, or preferably a Pasteur pipette which had been broken back and heat polished. The use of the pipette avoided the problem of touching the cells at the surface of the slice, especially since these cells often remained viable and could be used experimentally.

Hippocampal slices were left in the recording chamber for at least an hour before recording was attempted. The cells appeared to be 'dead' if microelectrode recordings were made before the hour elapsed. A similar phenomenon has been noted by several other authors and has been

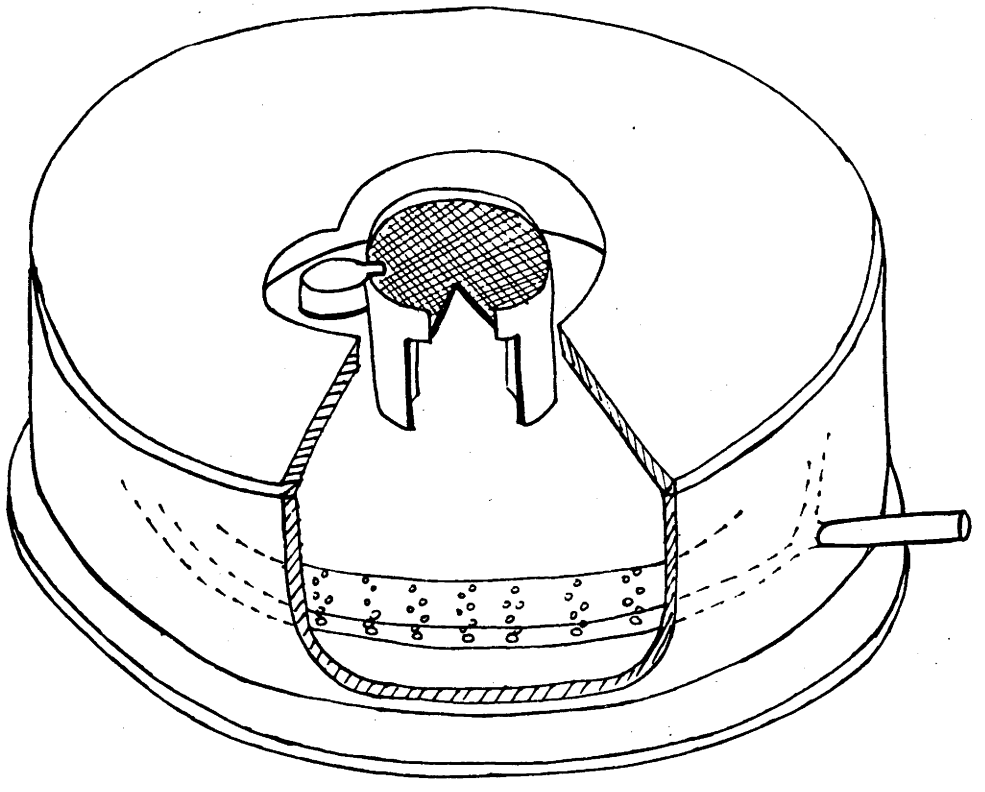
variously ascribed to a form of 'spreading depression' or profound morphological changes in the tissue (e.g. Teyler 1980).

Slices were illuminated from above using a fibre optic light source. Under these condition the cell body layers (dentate gyrus, CA3 and CA1 regions) appear as dark bands, contrasting well with the lighter regions of dendrites and myelinated fibres. A Wild M4 dissecting microscope was used to visualize the preparation and under the 25x power identification of the stratum pyramidale in the CA1 region was very easy in healthy slices. Slices were supported in the recording chamber on ordinary ladies nylon stocking which had been stretched and glued onto a 2cm plastic 'O'-ring. Dark, preferably black, stocking was used since this provided the best contrast against the pale hippocampal slices.

RECORDING CHAMBER.

The chamber for intracellular recording and maintenance of slices was similar to that designed by Spencer, Gribkoff, Cotman & Lynch (1976) and was manufactured by Josef Biela Engineering (Anaheim, California). The chamber used is illustrated in Fig. 2. Slices were supported in the central chamber, with a side chamber attached for the removal of bathing solution during continous perfusion. Water in the outer chamber was heated to 25^{0}C and bubbled with 95% O_2 /5% CO_2 to

FIGURE 2: Schematic diagram of the 'interface' recording chamber used for the hippocampal experiments. Several slices lay on a nylon net in a central chamber, and perfusate was aspirated from this recording chamber from a second smaller chamber attached to the side. A carbon dioxide/oxygen mixture was bubbled through perforated tubing in the outer chamber, which contained water. This provided an oxygen rich, humidified atmosphere in the larger outer chamber. Gaseous anaesthetics were applied in the same inlet as the carbon dioxide/oxygen supply. All of this bath was constructed from clear perspex, which was thoroughly washed and dried between experiments.



provide a humidified, oxygenated atmosphere for the interface slices.

A note on 'interface' versus 'submerged' slices.

There are currently two different ways of maintaining brain slices *in vitro* for electrophysiological recording. The first, often called the 'interface' system, is that employed in the experiments reported here and simply means that the slice rests upon a perfused surface (usually a net) and solution creeps up through the slice to the top surface which is exposed to the atmosphere. The slice then effectively lies at an air-water interface.

The other commonly used system involves submerging the slices in the recording bath, completely surrounding them with the perfusate.

Both systems have advantages and disadvantages, and the experimenter has to weigh each before choosing the system best suited to their particular requirements. The principal advantage of the interface system is that it reduces to a minimum the solution level above the slice without allowing the slice to dry out. This means that the microelectrode transmural capacitance is considerably reduced, which is a prerequisite for achieving high switching rates with the single microelectrode voltage clamp. The biggest drawback is that the slice is perfused adequately only on its underside - this greatly reduces the speed and efficiency of solution changeovers, since a long delay is introduced during which a new solution, e.g.

containing a drug, has to diffuse up to the slice surface (see e.g. Nicholson & Hounsgaard 1983 for discussion). This problem of diffusion/equilibration time was the major obstacle during the anaesthetic and drug studies, and was particularly acute during the snake toxin experiments.

The submerged slice has the advantage that solution changeover times are much shorter, because the slice is perfused on two sides and diffusion of substances into the tissue is enhanced considerably. However, microelectrode capacitance is greatly increased (~1pF for each 1mm immersion). The system chosen was the interface bath, since we were principally interested in obtaining a fast voltage clamp with low noise, to optimize the recordings of inhibitory postsynaptic currents.

RECORDING TECHNIQUES.

The standard bathing medium contained (in mM); NaCl 124; KCl 5; $NaHCO_3$ 26; Na_2HPO_4 1.25; $MgSO_4$ 2; $CaCl_2$ 2; d-glucose 10 and was maintained at pH 7.4 by bubbling with 95% O_2/5% CO_2 (obtained from C.I.G., Sydney as Carbogen). Fresh solutions were made up from solid chemicals directly before the experiment. The slices were perfused from an oxygenated reservoir by a peristaltic pump (Gilson, France) via a gravity-feed reservoir at a rate of approximately 1 ml per minute. This flow rate permitted stable electrophysiological recordings whilst slowly changing the cell environment.

Thirty minutes were allowed between each solution changeover and the first recording of any effects of the change. Conventional intracellular recording techniques were employed. Microelectrodes typically had resistances of 17-50 MΩ when filled with 3M KCl or 3M CsCl. The microelectrodes were manufactured usind a Brown-Flaming puller (P77, Sutter Instrument Co. Ltd.), using 1mm (o.d.) 'Omegadot' glass tubing (Clark Electromedical Ltd.). Microelectrodes were held in a W.P.I. electrode holder which in turn was mounted directly into the headstage of the voltage clamp amplifier. Under microscopic control, the electrode was positioned in the stratum pyramidale region of the CA1 area using a Narishige micromanipulator which supported the whole microelectrode/headstage assembly. Very occasionally, cells could be impaled by hand using this manipulator alone, but generally an hydraulic stepping microdrive (Narishige) was employed. The electrode was advanced in 3μm steps, and cell penetration was aided by brief 'zapping' with the Axoclamp 'buzz' facility. The buzz switch is a device which rapidly overcompensates the microelectrode capacity neutralization, probably resulting in an extremely rapid oscillation of the electrode tip, helping the electrode to pierce the cell membrane.

Cells could be recorded from for up to 5 hours, but usually it was extremely difficult to impale and hold cells with the low resistance microelectrodes favoured here. Many hours could be spent trying to impale and successfully hold cells with 20 MΩ elecrodes. However the advantages

of rapid chloride loading of cells and fast clamp switching rates when a cell was impaled with such an electrode usually outweighed the frustration experienced.

The voltage clamp system used was the Axoclamp 1 (Axon Instruments Inc., Burlingame California). This system allows voltage clamping with a single microelectrode by electronically switching at a high frequency between current passing and voltage recording modes. Particular attention was paid to a high gain monitor of the headstage voltage to ensure that the voltage across the electrode tip had completely settled after a current passing episode. Clamp switching rates of between 10 and 17 kHz were used throughout the hippocampal studies (see Fig. 3). The clamp feedback gain was set to a maximum consistent with stability and good voltage control. No phase lag or lead was used (see Finkel & Redman 1983). The 10x membrane current output of the voltage clamp was led to a 100x gain stage where a 1 sec A.C. coupling was also introduced, and then to the analogue to digital converter of a 6809 (Motorola) based microprocessor (Biosystems Ltd. N.S.W.). Sampling of current traces was usually at 6.25kHz, and filtered at 1-3kHz. Membrane voltage was recorded on another channel of the A-D converter, providing synchronous records of membrane current and voltage.

With electrodes of lower resistance, spontaneous depolarising potentials would appear within a very few seconds. These would often reach 10 to 20 mV in amplitude and be large enough to trigger action potentials (see Fig. 4). In stable cells these spontaneous postsynaptic

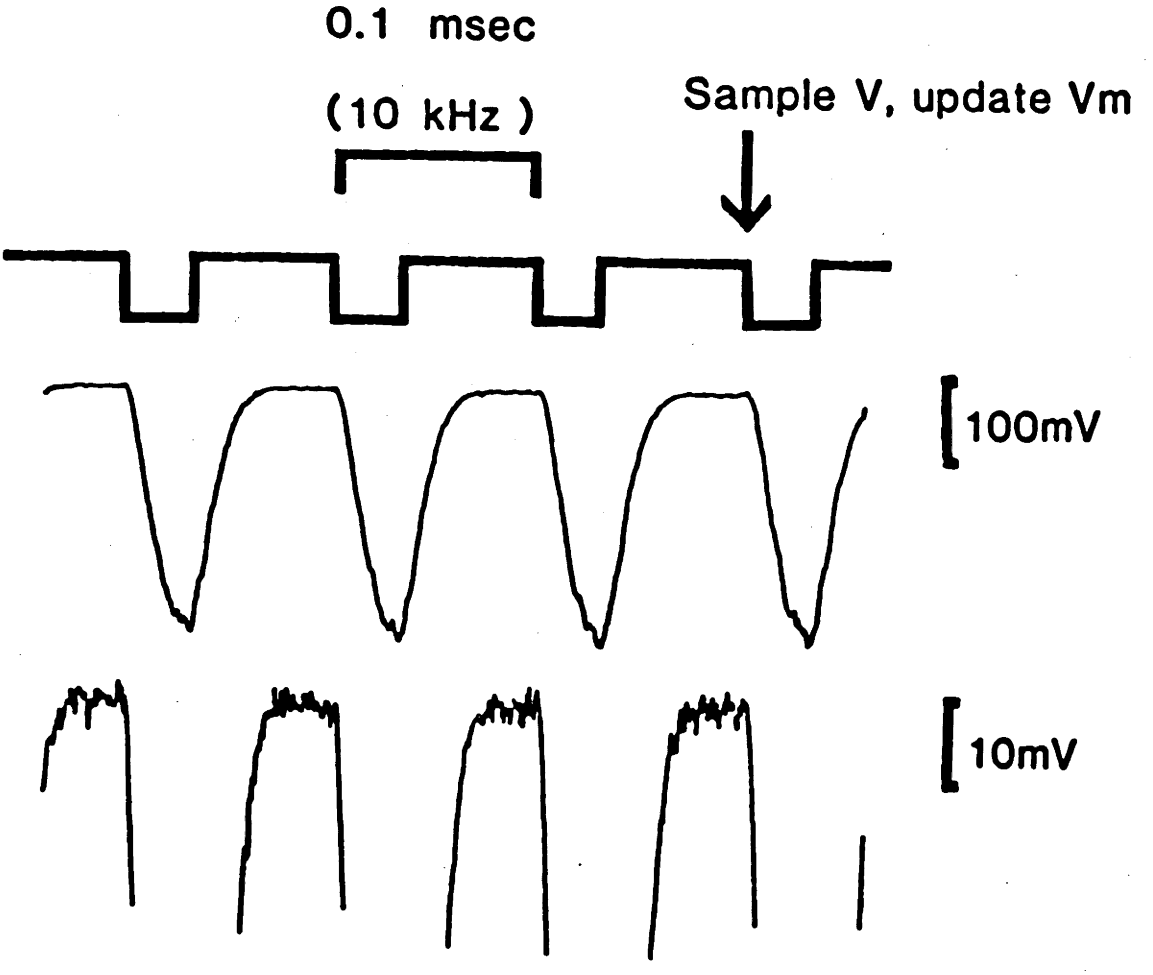


FIGURE 3: A high and low gain recording of the voltage clamp headstage voltage. The clamp was switching at 10kHz, and passing inward current. Note that the voltage settles completely between current passing episodes.

potentials could be voltage clamped, giving rise now to spontaneous postsynaptic currents. A current threshold trigger level could be set with the 6809 data acquisition routine and currents captured and displayed on a Tektronix 505 oscilloscope screen for more detailed visual inspection. If spontaneous currents were judged to be suitable they were then written onto magnetic disc. The whole process was then repeated with the next current. Usually 20-50 spontaneous currents were stored for each part of the experiment. For instance in control solution at different voltages, or prior to drug application. The amplitude, rise time and decay time constant of the currents were then measured off-line after the experiment.

SECTION B: RESULTS.

Inhibitory postsynaptic potentials.

After penetration of a CA1 neurone in the cell body region with an electrode containing a chloride salt, spontaneous depolarizing potentials could be observed. If the electrode resisitance was less than ~50MΩ, these potentials appeared very rapidly and grew in size until their amplitude stabilized a few seconds or minutes later. In general, the lower the resistance of the microelectrode, the larger and more rapidly the potentials would appear. With higher resistance electrodes (~ 60MΩ) potentials would appear very slowly (after 20 minutes) and remain very small - if they were visible at all. The frequency of these potentials varied greatly between neurones. Frequency could be as low as 0.1Hz or in excess of 50Hz - in the latter case it became extremely difficult to capture clearly resolvable individual events (see Fig. 4).

Spontaneous postsynaptic potentials were observed only when microelectrodes contained the electrolytes potassium chloride, caesium chloride or caesium sulphate (the latter at positive membrane potentials). No similar depolarizations were observed in CA1 neurones when electrodes contained potassium methylsulphate, potasssium sulphate, potassium citrate or potassium acetate. Very, very occasionally a small (<0.5mV) hyperpolarizing 'bump'

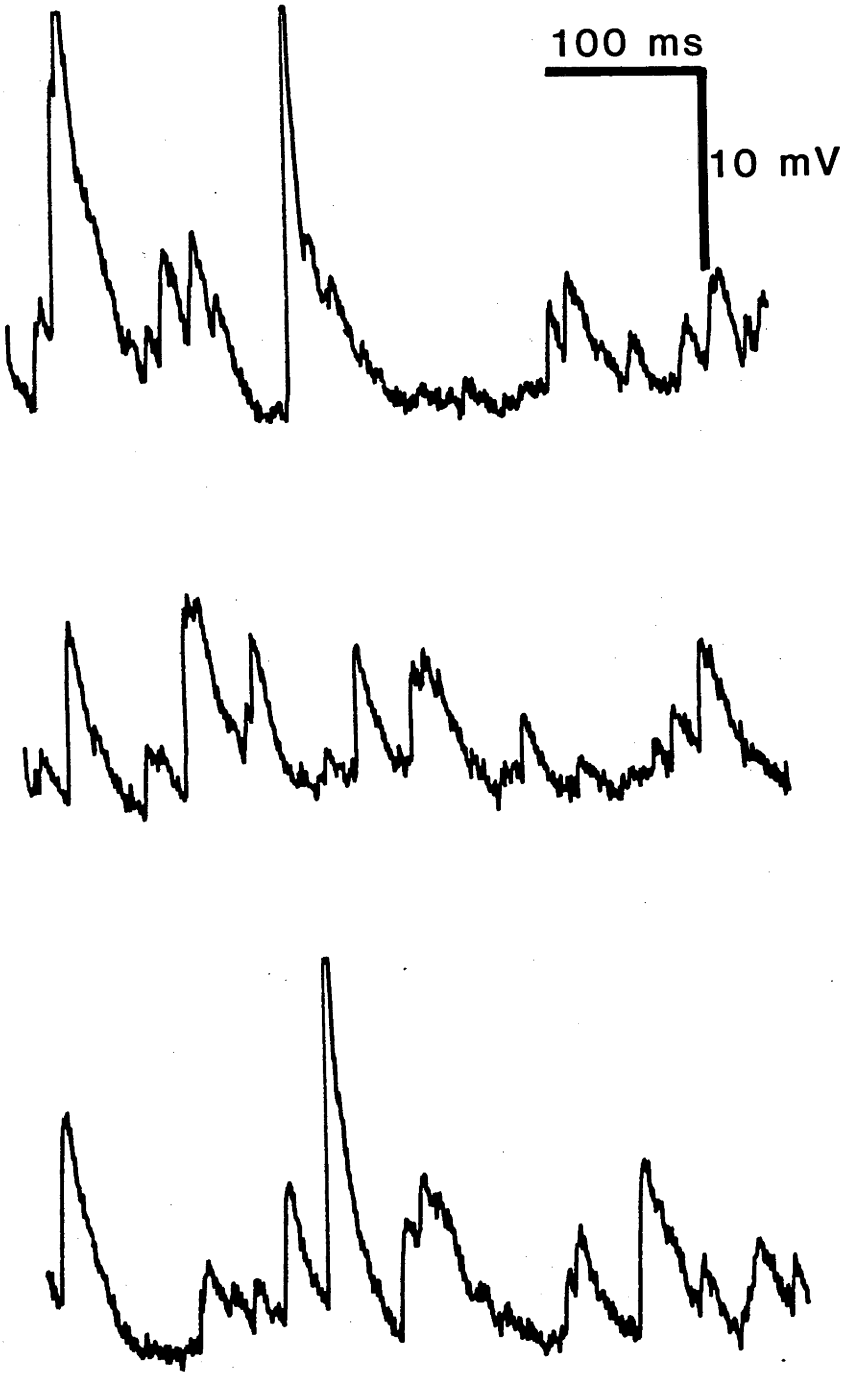


FIGURE 4: Spontaneous inhibitory postsynaptic potentials. Note the high frequency and wide range of IPSP amplitudes in this cell.

was observed with these large anion containing electrodes, but these were too small and infrequent to be usefully analysed. Since the spontaneous postsynaptic potentials were only observed at hyperpolarized potentials when chloride containing electrodes were used, it was concluded that they were generated by leakage of chloride from the microelectrode. The diffusional leakage is a consequence of the low electrode resistance: the hydrostatic pressure of the electrolyte in the electrode/electrode holder assembly causes efflux from the electrode tip (Fromm & Schultz 1981). The fibre incorporated in the electrode glass prevents the formation of a meniscus at the tip which would tend to reduce the leakage of electrolyte. Efflux of chloride ions from the electrode tip could be aided by passing negative current through the electrode. This had the additional advantage of stabalising the cell after penetration, and was carried out routinely.

The spontaneous postsynaptic potentials were much slower than the so-called 'd'-spikes often observed in hippocampal neurones (see e.g. Kandel & Spencer 1961), and d-spikes were not affected by the cells chloride gradient. The spontaneous potentials were abolished by perfusion of the specific GABA antagonist bicuculline (1-10μM), and became larger and longer when cells were exposed to solutions containing pentobarbitone (100μM). Perfusion of the sodium channel blocker tetrodotoxin (0.5-1.0μM) produced a reduction in frequency and amplitude of the potentials, with only the smallest amplitude group of potentials remaining.

These results, (apart from the persistence of smaller potentials in solutions containing TTX) are very similar to those obtained by Alger & Nicoll(1980), which suggested that these spontaneous depolarizations are reversed 'inhibitory' postsynaptic potentials (IPSPs) generated by GABA secreted from the terminals of inhibitory interneurones in response to presynaptic action potentials. Nicoll, Eccles, Oshima & Rubia (1975) also observed spontaneous depolarizing potentials in the cat hippocampus in vivo - these potentials depended on an outwardly directed chloride gradient and were markedly prolonged by pentobarbitone.

INHIBITORY POSTSYNAPTIC CURRENTS.

When cells displaying spontaneous depolarizing IPSPs were voltage clamped using the switched single electrode voltage clamp, transient inward currents were seen. These currents corresponded to the IPSPs in frequency, dependence on chloride loading and sensitivity to bicuculline, and may therefore be called inhibitory postsynaptic currents (IPSCs). The IPSCs were characterised by a rapid (<1ms) rising phase, followed by an exponential decay with a time constant of several milliseconds. With voltage clamp switching rates of 10 to 17kHz, most of the IPSCs were well clamped, with only the largest currents producing a significant voltage deflection in the rising phase of the current. This

voltage deflection was over before the current decayed to 80% of its peak value. Sample IPSCs and corresponding voltage traces are shown in Fig. 5. Occasionally, smaller, rounded (rising phase >2ms) IPSCs were observed, and some cells showed only this kind of current. These IPSCs were discarded, since it was likely that they were generated on unclamped regions of the pyramidal cell, e.g. from dendritic regions in a somatic recording. Detailed study was made only on the rapid type IPSCs, which were observed in 90% of the cells penetrated.

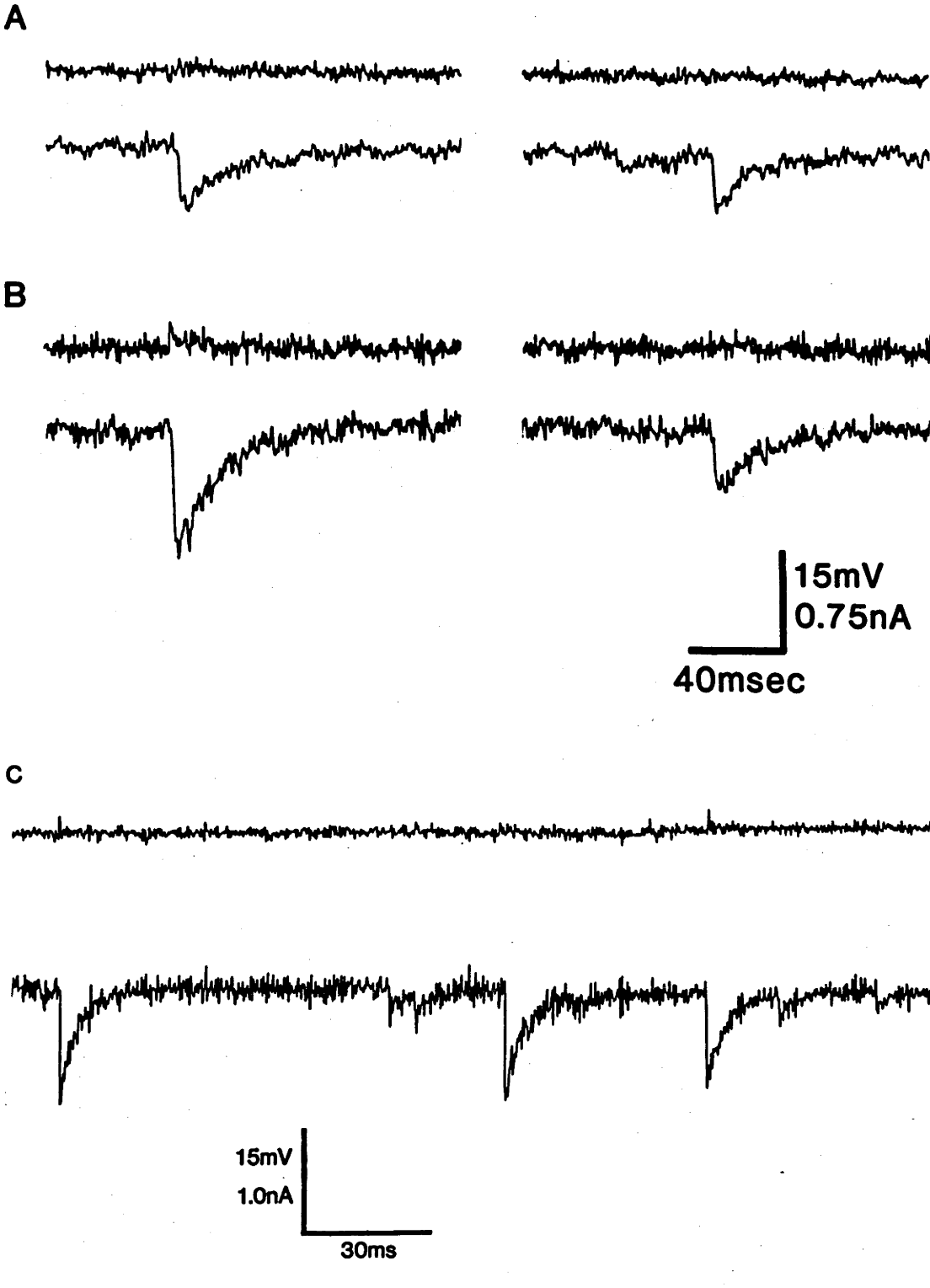
IPSCs were often observed to occur in 'bursts', with several currents summating together to produce an action potential (see e.g. Fig. 6). These bursts may have been due to repetitive firing of inhibitory interneurones.

IPSCs varied considerably in amplitude, even in a single cell, with some appearing just out of the baseline noise (~ 0.1nA) while others could be as large as 3nA. Typical amplitude histograms are shown in Fig. 6. The very large IPSCs were impossible to clamp completely, and their decay phase often deviated from a single exponential It is possible that these were generated by multiple quanta released asynchronously.

IPSC amplitude became stable within a very short time after electrode penetration, the delay presumably being due to the establishment of a new chloride equilibrium potential. Amplitudes of IPSCs during a 15min recording period are shown in Fig. 7.

It was a matter of some concern that IPSCs were observed only when caesium chloride or potassium

FIGURE 5: Spontaneous IPSCs and corresponding voltage traces from three cells. Cell A: Currents recorded at -90mV with a potassium chloride electrode, Cell B: IPSCs at -67mV with caesium chloride electrode, Cell C: IPSCs in a longer sweep, recorded at -56mV. The voltage trace for all three cells shows good clamp control, except occasionally where small voltage transients occur during the rising phase of the IPSCs. The current and voltage traces were obtained at clamp switching rates between 10 and 13.5kHz.


A
B
15mV
0.75nA
40msec
C
15mV
1.0nA
30ms

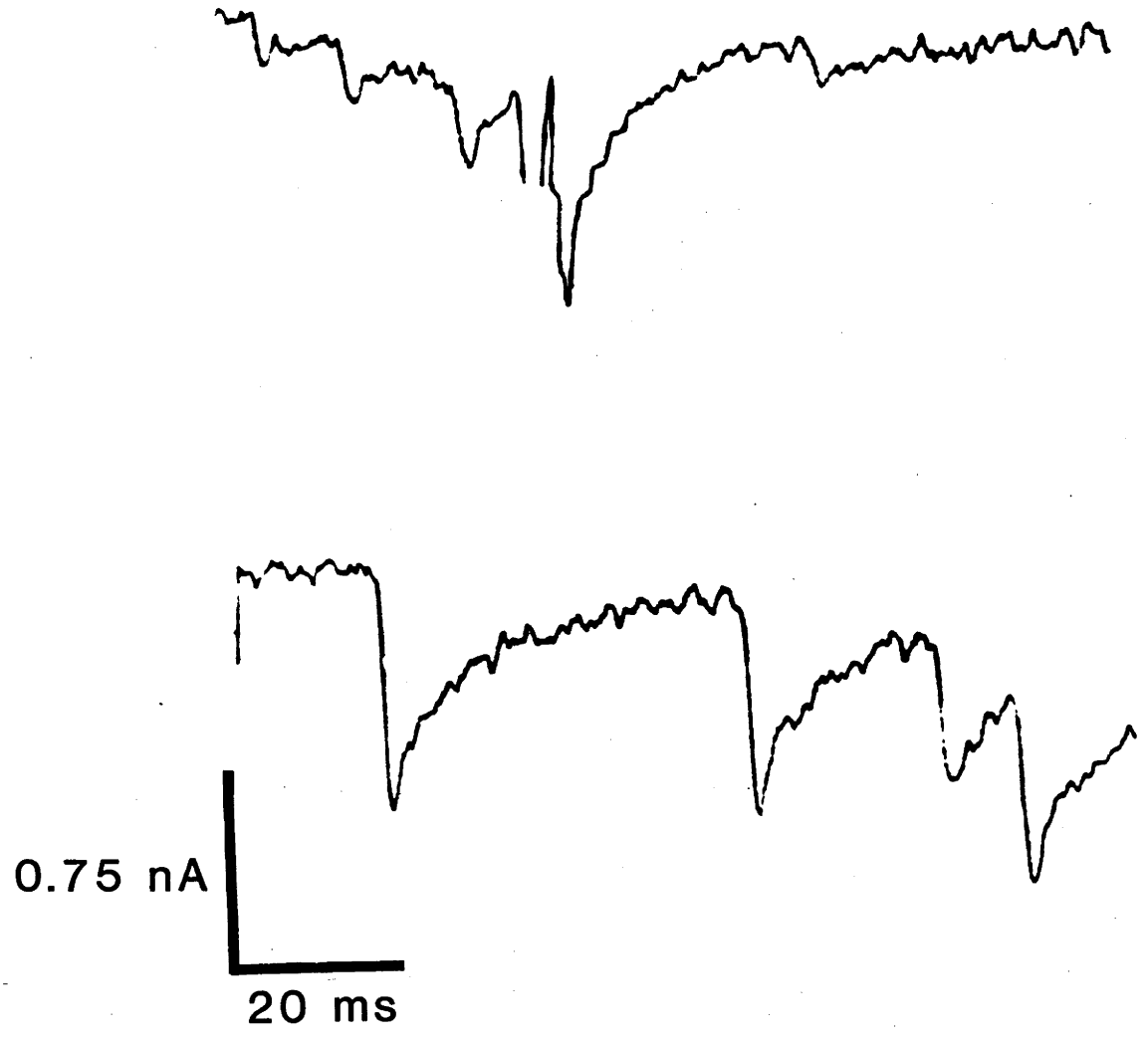
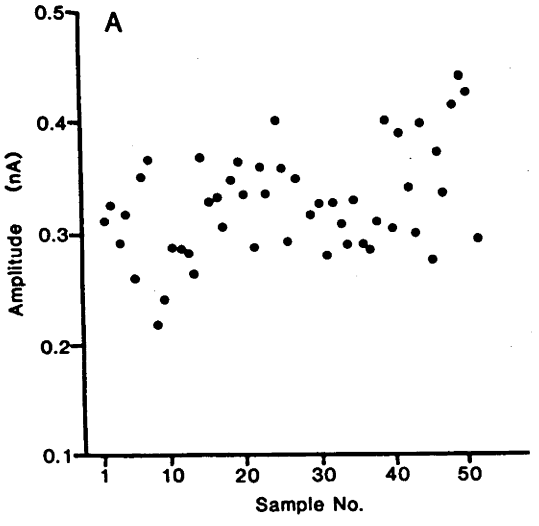
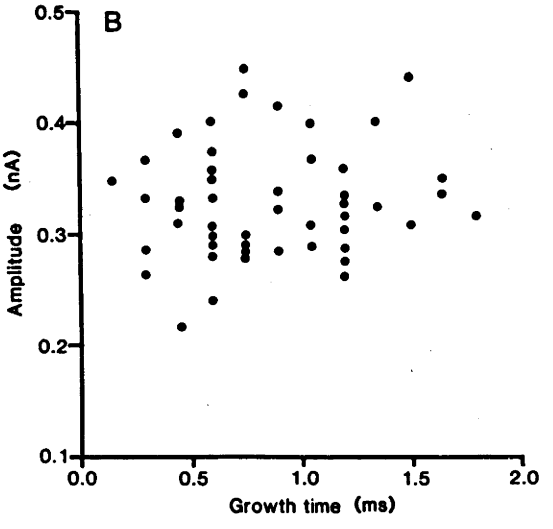
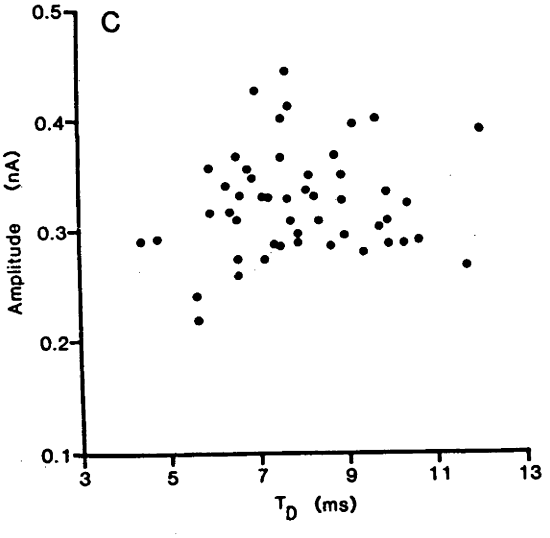


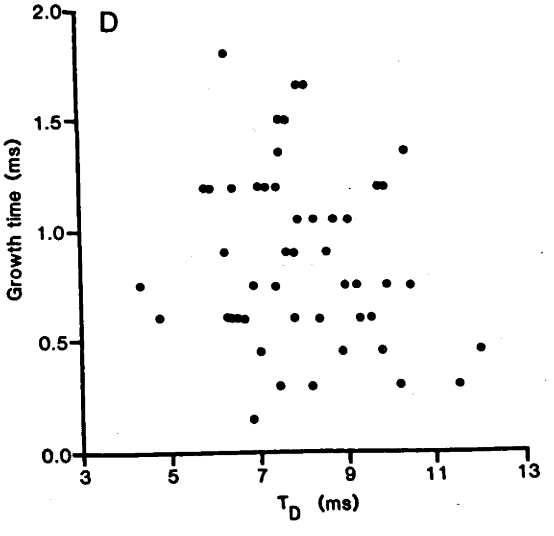
FIGURE 6: Two traces of membrane current, showing the high frequency of IPSCs. In the top trace, summation of IPSCs produced an action potential, which could not be resolved well by the single electrode voltage clamp. The lower trace again shows summation of IPSCs in another cell.

FIGURE 7: Scatter plots of the correlation between IPSC amplitude and recording time and other IPSC parameters. A) Amplitude of IPSCs from the time of penetration to the final IPSC collected 15 minutes later. B) There is no significant correlation between IPSC amplitude and growth time. C) The time constant of decay (τ_D) was not related to the amplitude of the current. D) Growth time and decay time were not related. All of these graphs were obtained from a single cell, recorded at a potential of -97mV.


A
Amplitude (nA)
0.5
0.4
0.3
0.2
0.1
1
10
20
30
40
50
Sample No.


B
Amplitude (nA)
0.5
0.4
0.3
0.2
0.1
0.0
0.5
1.0
1.5
2.0
Growth time (ms)


C
Amplitude (nA)
0.5
0.4
0.3
0.2
0.1
3
5
7
9
11
13
T_D (ms)


D
Growth time (ms)
2.0
1.5
1.0
0.5
0.0
3
5
7
9
11
13
T_D (ms)

chloride electrodes were used. They were not seen when sulphate, methylsulphate, citrate or acetate were used as the anion. With caesium chloride electrodes, it was possible to polarize the membrane to positive potentials, this resulted in reversed, i.e. outward IPSCs due to inward movement of chloride. With caesium sulphate electrodes, IPSCs could be seen only at positive potentials: however the time course of these currents were similar to those recorded with CsCl electrodes at the same potentials. This suggests that chloride loading does not alter the kinetic properties of the GABA-activated channels, at least at positive membrane potentials. Further aspects of the results obtained with foreign anions (i.e. anions other than chloride) will be discussed below.

Most of the IPSCs had growth times (20% to 80% of peak height) below 2msec. The distribution of growth times for the same cell as in Fig. 7 is shown in Fig. 8. One of the standard tests for space and temporal clamp is to examine events for correlations between the amplitude, growth time and rate of decay of postsynaptic potentials/currents (see Rall 1977). A strong correlation would be expected if the conductance change was 'filtered' by the parallel combination of membrane resistance and capacitance in neuronal regions between the site of the conductance change and the (somatic) recording site. In Fig. 7 scatter plots of IPSC growth phase, amplitude and time constant of decay (τ_D) are illustrated. These figures suggest that IPSCs were

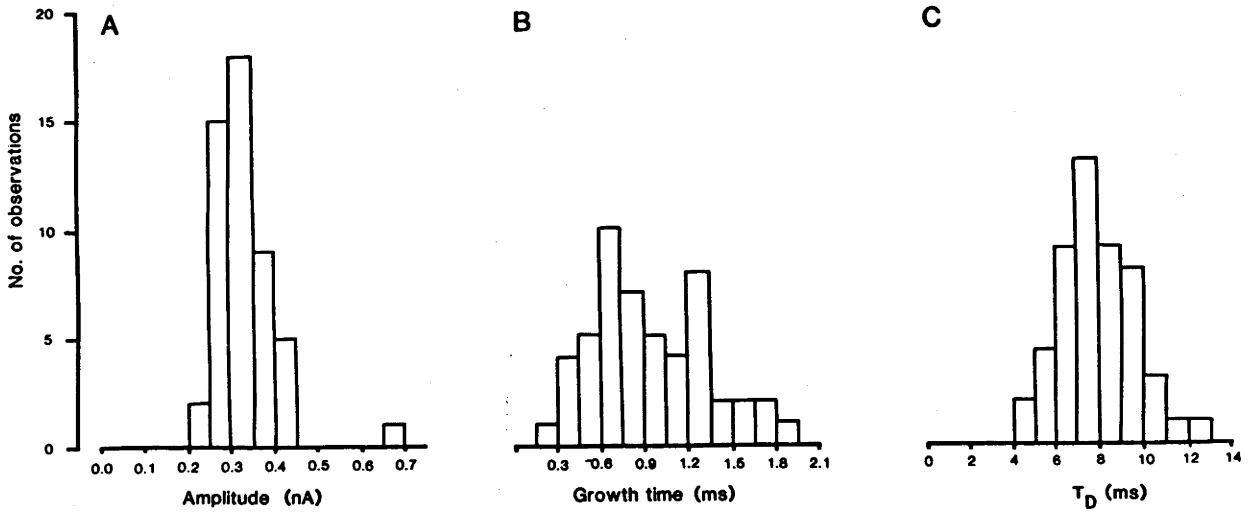
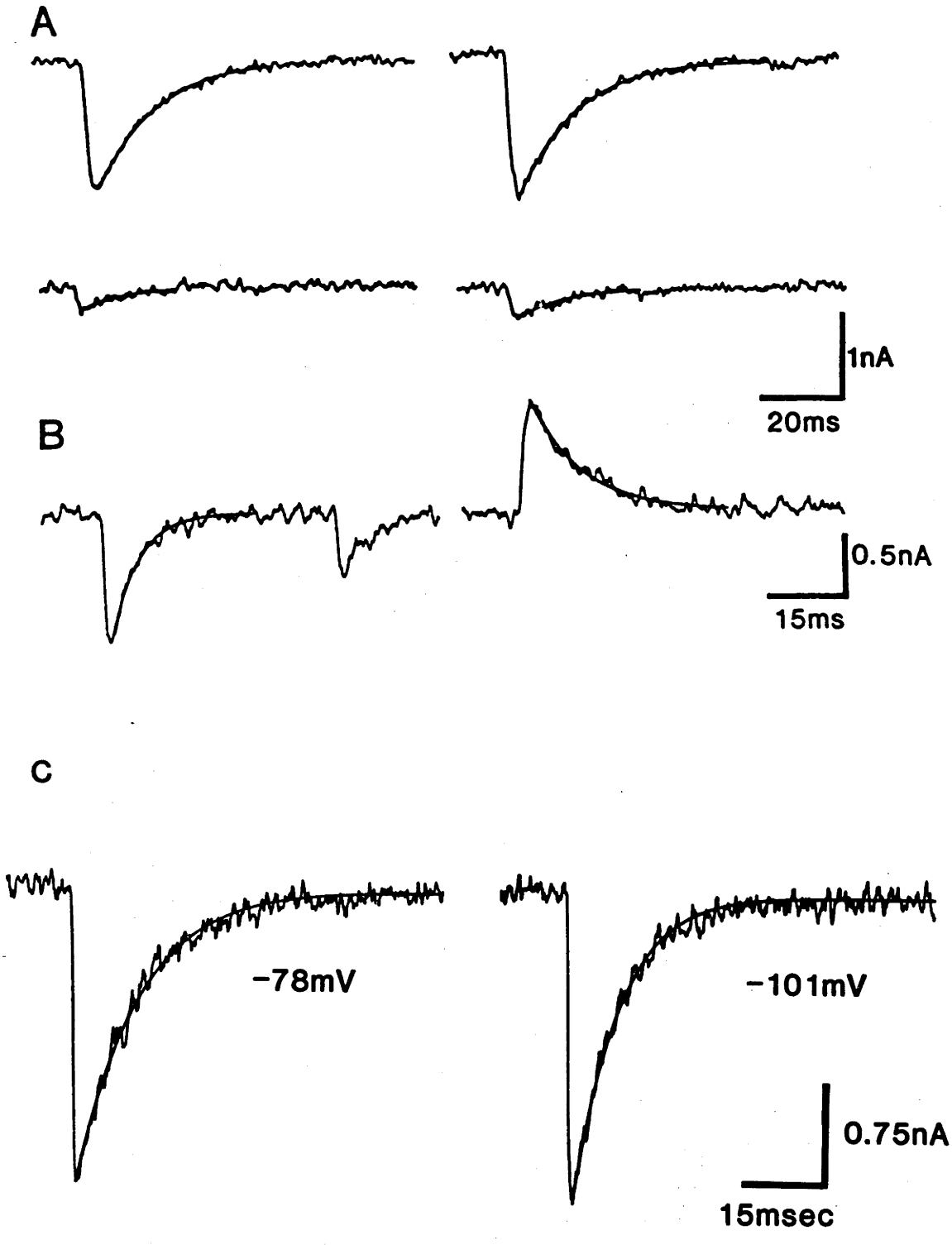


FIGURE 8: Frequency histograms for the amplitude (A), growth time (B) and decay time constant (τ_D) of IPSCs recorded at -97mV. The temperature was 25^0C.

FIGURE 9: Records of IPSCs at different potentials and temperatures, with computer-generated exponential fits to their decay phases. A) Large and small IPSCs at -99mV, recorded at 25^0C. B) IPSCs in another cell at 32^0C. Note the faster decay of these currents. The time constant of decay of the inward current is 5ms at -58mV, while the outward current has a τ_D of 9.8mV at -12mV. C) IPSCs at -78 and -101mV in another cell - hyperpolarizing the membrane accelerates the decay phase.


A
1nA
20ms
B
0.5nA
15ms
C
-78mV
-101mV
0.75nA
15msec

generated in regions of the cell which were adequately voltage clamped.

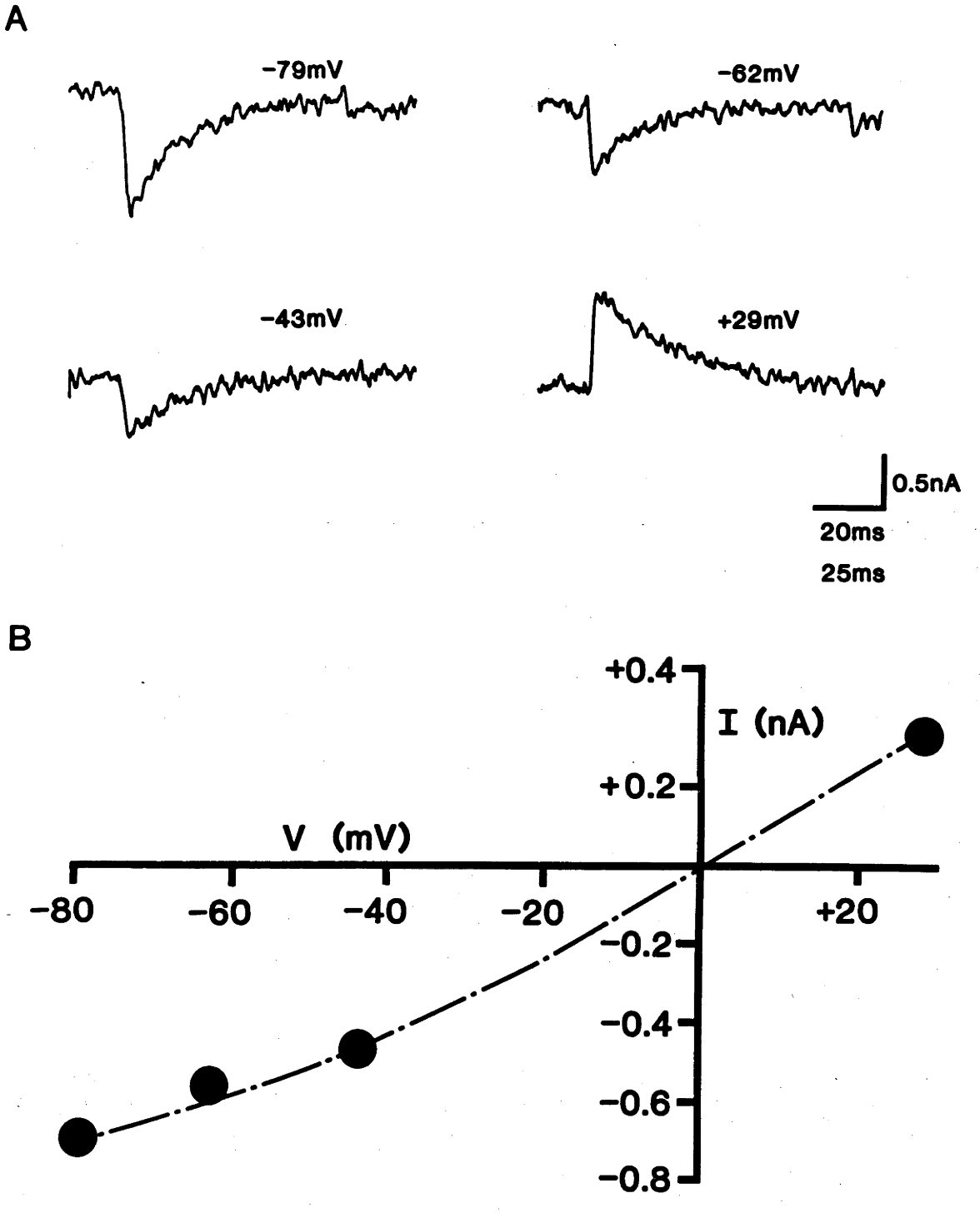
The decay phase of the IPSC could be well fitted by a single exponential, with time constant τ_D. Examples of computer-generated exponential fits are shown in Fig. 9. Note that the smaller currents could be fitted as well as the larger currents. The decay of both inward and outward currents could be described by a single exponential. At a given membrane potential and temperature, the range of IPSCs τ_ds in any cell was comparatively small, as can be seen in Fig. 8. Cell to cell variation in τ_D was also fairly small, this is illustrated in Fig. 12. τ_D in 9 cells voltage clamped at -80mV (25^{0}C) was 11.7±1.4ms (x ± 1 x S.D.).

Some of the very large (>2.5nA) IPSCs and those with slow rising phases had decays that were not adequately described by a single exponential. These currents are not included in the present analysis.

Effects of Membrane Potential.

Changes in membrane potential caused alterations in both the amplitude and time course of the IPSCs. Sample IPSCs which were recorded (with a caesium chloride electrode) at different potentials are shown in Fig. 10. It can be seen that the amplitude of the current decreases as the membrane potential approaches zero due to the reduced driving force for chloride and the decay phase is

FIGURE 10: The dependence of IPSC amplitude and time course on membrane potential. Currents were recorded with a CsCl containing electrode, enabling the membrane to be depolarized to values to record outward currents. A) The time constant of decay of IPSCs is prolonged as the membrane is depolarized. Membrane potential is given alongside sample IPSCs. The horizontal calibration bar is 20ms for currents at negative potentials, and 25ms for the current at +29mV. Note that IPSC amplitude is progressively reduced as the membrane is depolarized, until the current finally reverses. B) The current-voltage relation for the same cell as in A) The symbols are the average value of 8 to 24 individual currents recorded at each potential, and the standard error bars are smaller than the symbols.


A
-79mV
-62mV
-43mV
+29mV
0.5nA
20ms
25ms
B
+0.4
I (nA)
+0.2
V (mV)
-80
-60
-40
-20
+20
-0.2
-0.4
-0.6
-0.8

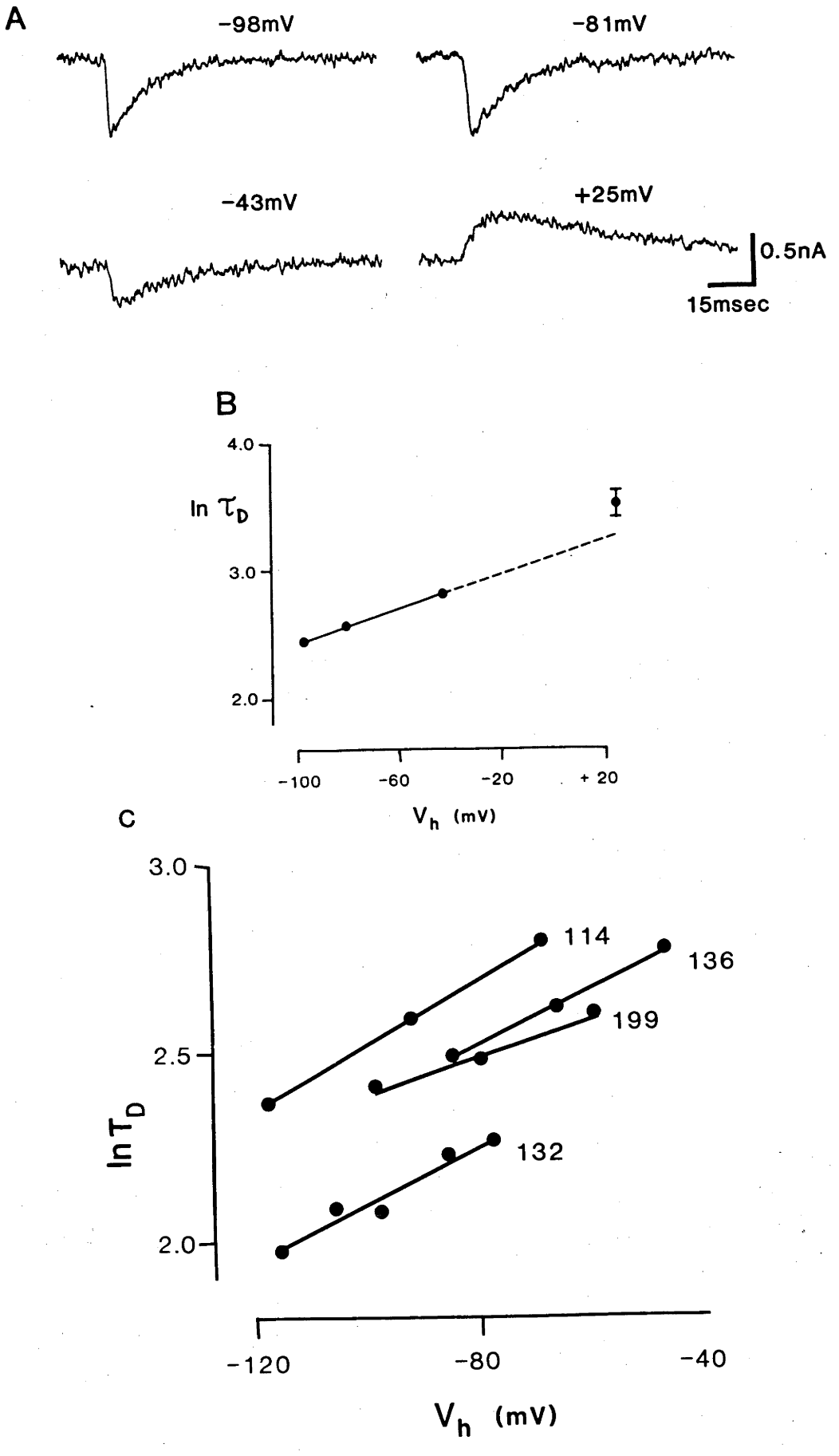
slower at more positive potentials. For example, in Fig. 9B, the IPSC recorded at -58mV has a decay time constant of 5.0ms, while the sample IPSC at -12mV has a time constant of 9.8ms. Similar results are seen in Figs. 9A,C, 10 and 11. The acceleration of IPSC decay phases at increasingly negative potentials was a general feature of the hippocampal studies. Only very occasionally was a cell encountered which had minimal dependence of τ_D on membrane potential. Fig. 11 shows the change in time course of IPSCs with voltage for four different cells. In each cell the decay became slower as the membrane was depolarized. Pooled results of 17 different cells with IPSCs recorded at a variety of different potentials are plotted semi-logarithmically in Fig. 12. All records were obtained at 25^{0}C ($\pm$1^{0}C). It can be seen that the relationship is well described by a single straight line i.e. there is a logarithmic relationship between τ_D and membrane potential. The straight line through the data points is the linear regression line:

$$\ln\tau_D = \ln(\tau_D)_0 + V/H$$

where V is membrane potential, $(\tau_D)_0$ is 20.5ms and H, the 'volt constant' is 128mV. An exponential relationship between τ_D and V was also seen in each of these 17 cells examined individually, and the average H value in this case was 146 $\pm$ 9.6mV ($\bar{x} \pm$ SEM).

In a few cells (e.g. Fig. 11) there appeared to be a significant deviation of τ_D from a simple logarithmic

FIGURE 11: Effect of membrane potential on the decay time of IPSCs. In the cell in parts A and B, depolarization slows the decay and prolongs rise time. This is particularly clear for the IPSC at +25mV. B shows the logarithmic relationship between τ_D and membrane potential over a 120mV range. The point at +20mV is slightly longer than would be expected from a simple logarithmic relationship. C) Time constant of decay against membrane potential in four separate cells. The 'H' value for the cell is given alongside each regression line. This illustrates the range of τ_D values between cells and the different degrees of voltage sensitivity expressed by various cells.


A
-98mV
-81mV
-43mV
+25mV
0.5nA
15msec
B
4.0
ln τD
3.0
2.0
-100
-60
-20
+ 20
Vh (mV)
C
3.0
2.5
2.0
ln TD
114
136
199
132
-120
-80
-40
Vh (mV)

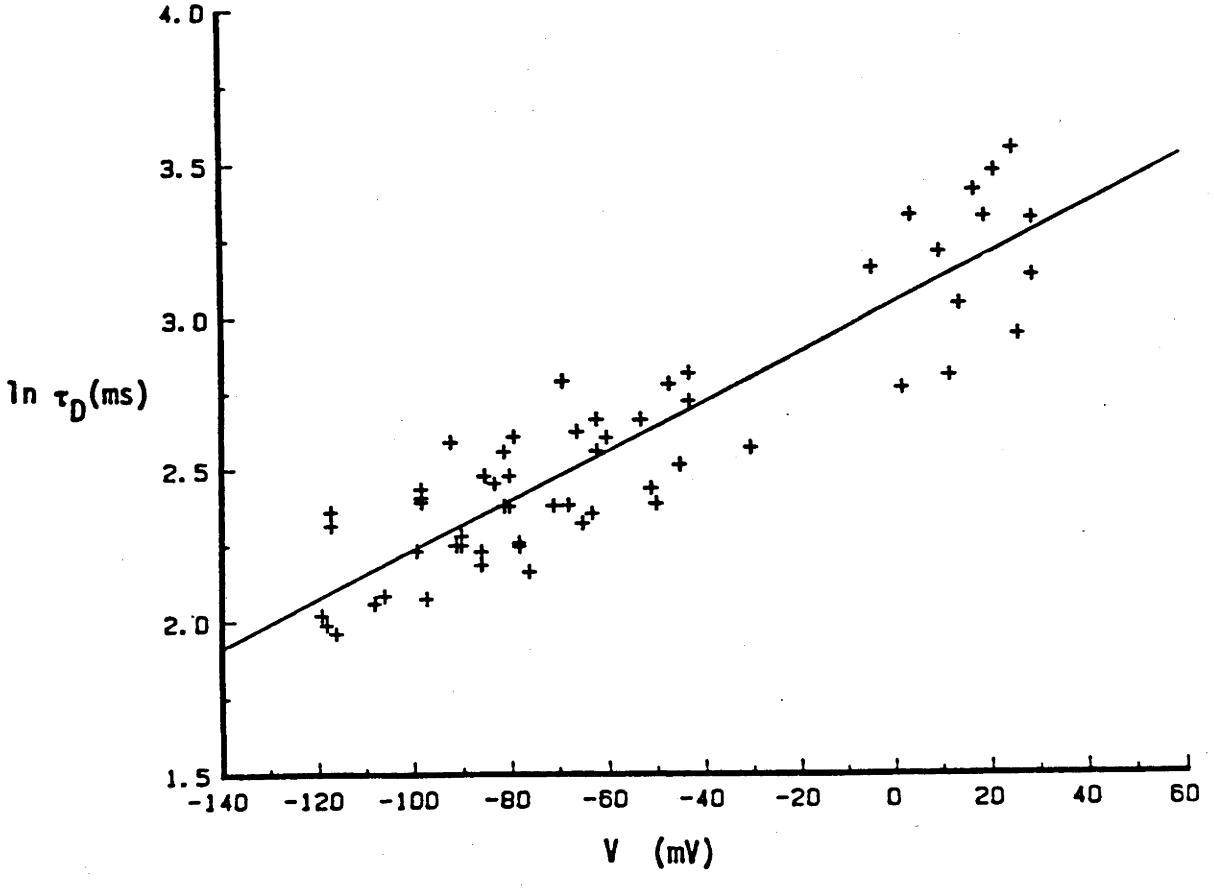


FIGURE 12: The dependence of the time constant of decay of IPSCs on membrane potential for seventeen cells. The τ_D depended logarithmically on membrane potential, and the regression line is $\ln\tau_D = \ln\tau_D(0) + V/H$. H is 128mV and τ_D at 0mV is 20.5ms.

relationship at positive membrane potentials. However, this would have to be examined much more rigorously over a larger potential range to be completely convinced of the validity of this result.

In some cells, the growth phase of the IPSCs was also affected by changes in membrane potential, with longer growth phases appearing at more positive potentials (Fig. 11A and 13). In four of seven cells in which this relationship was studied in detail, the growth time became greater at more depolarized values.

Effect of temperature on IPSCs.

The vast majority of the experiments reported in this section were performed at room temperature (21-25^{0}C). This 'unphysiological' temperature had the advantage that the currents were slower, allowing better voltage control in clamp mode. However, it was of interest to examine the temperature dependence of the time course of the IPSCs, and the voltage dependence of τ_D as characterised by 'H'. Therefore several experiments were carried out at 30-35^{0}C. Unfortunately, the experimental apparatus did not allow temperature scans to be performed quickly, so it was not possible to obtain control data at a variety of temperatures. Fig. 9B shows IPSCs recorded at 32^{0}C. The decay phases were much faster at the higher temperatures, but remained a single exponential. From five cells at 32^{0}C and a range of membrane potentials an H

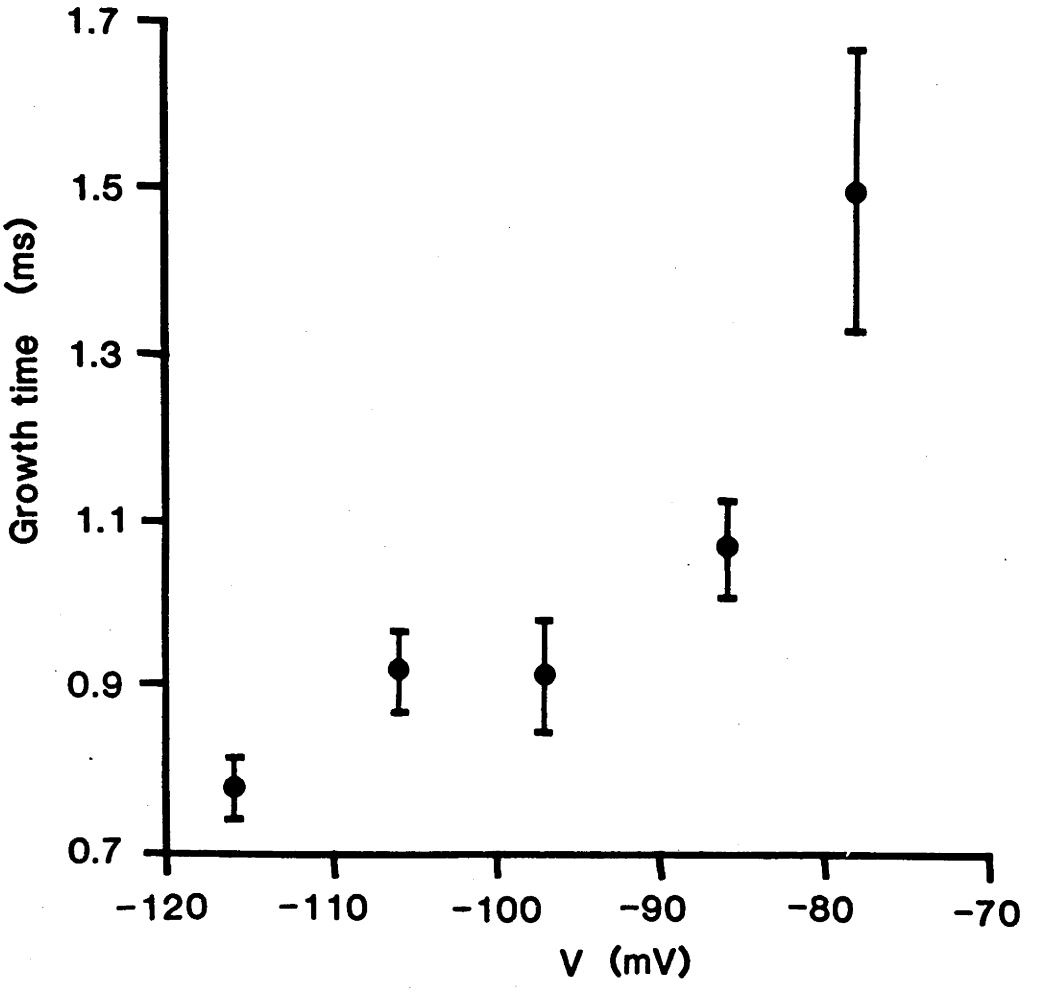


FIGURE 13: The dependence of growth time of IPSCs on membrane potential in one cell. In this particular cell, the growth time increased as the membrane was depolarized.

value of 208 ± 103mV was obtained, and a $\tau_D(0)$ of 8.3 ± 0.8ms. A comparison of $\tau_D(0)$ values at 25°C (19.0ms) and 32°C gives a Q_{10} value of 3.3 for τ_D. It would obviously have been much more satisfactory to examine the temperature dependence of τ_D and H in more detail, and it would also be useful to examine the Q_{10} of the IPSC growth phase.

Tetrodotoxin and Cadmium.

Alger & Nicoll (1980) have reported that spontaneous IPSPs were abolished by TTX, suggesting that these potentials were generated by action potentials in presynaptic neurones. We were interested in examining this further. Preparations were exposed to TTX solutions (0.5 - 1.0 μM) to examine wether quantal currents were discernable. TTX reduced IPSC frequency and modal amplitude, but did not result in a complete abolition of currents. IPSPs and IPSCs were observed in 12 cells in TTX solutions. Figs. 14 and 15 show some IPSCs recorded in a solution containng 1μM TTX. Although there was a decrease in average IPSC amplitude (to ≈ 1/3 of control values, n = 3 experiments), there was no change in the time course. The decay phase remained exponential, with time constants and growth phases similar to values before TTX. In spite of the presence of TTX, there was still a large spread in IPSC amplitude (see Fig. 14). The dependence of τ_D on membrane potential was similar in

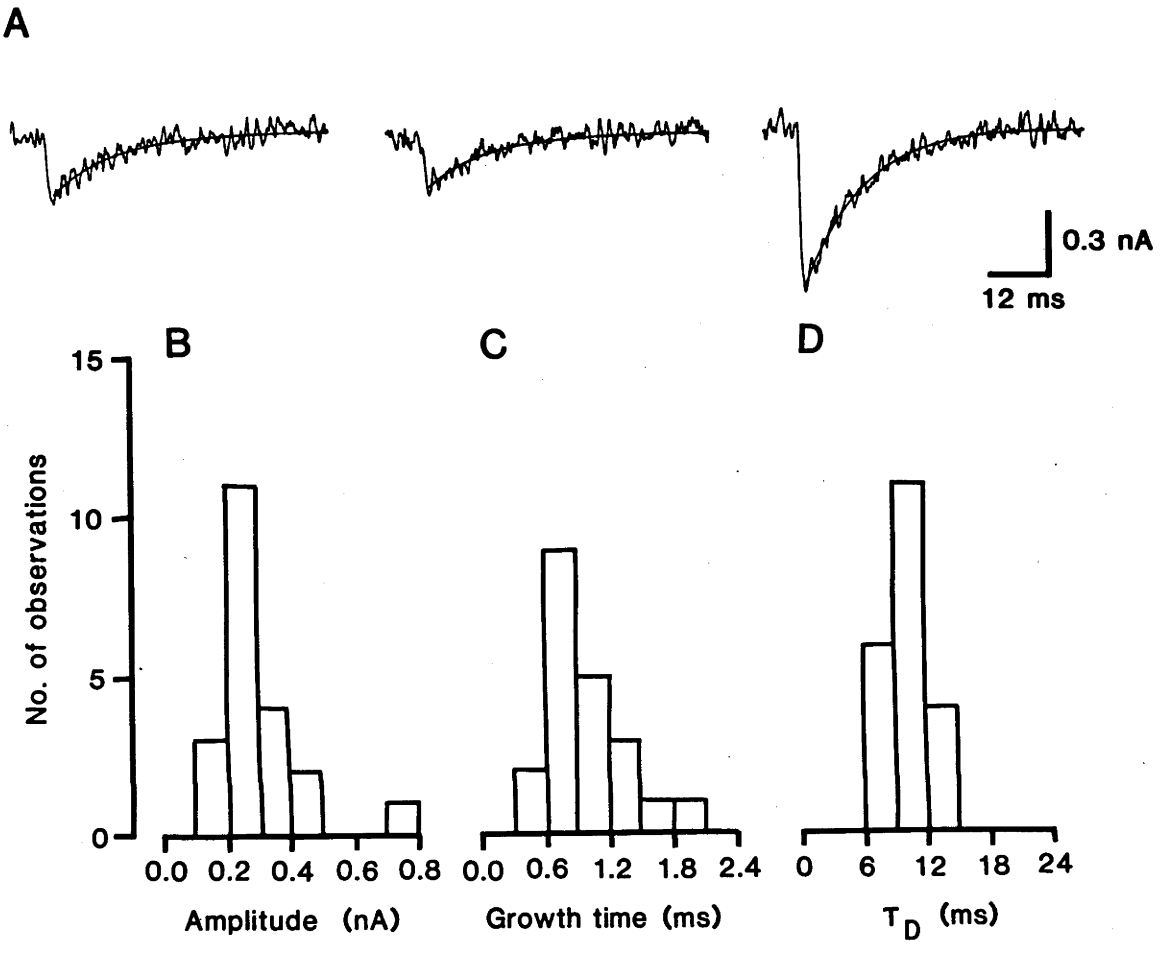


FIGURE 14: IPSCs recorded from one cell in a solution containing 1μM tetrodotoxin. Clamp potential -87mV. A) shows the range of amplitudes of IPSCs, with exponential fits to their decay phase. Frequency histograms of IPSC amplitude (B), growth time (C) and decay time constant (D).

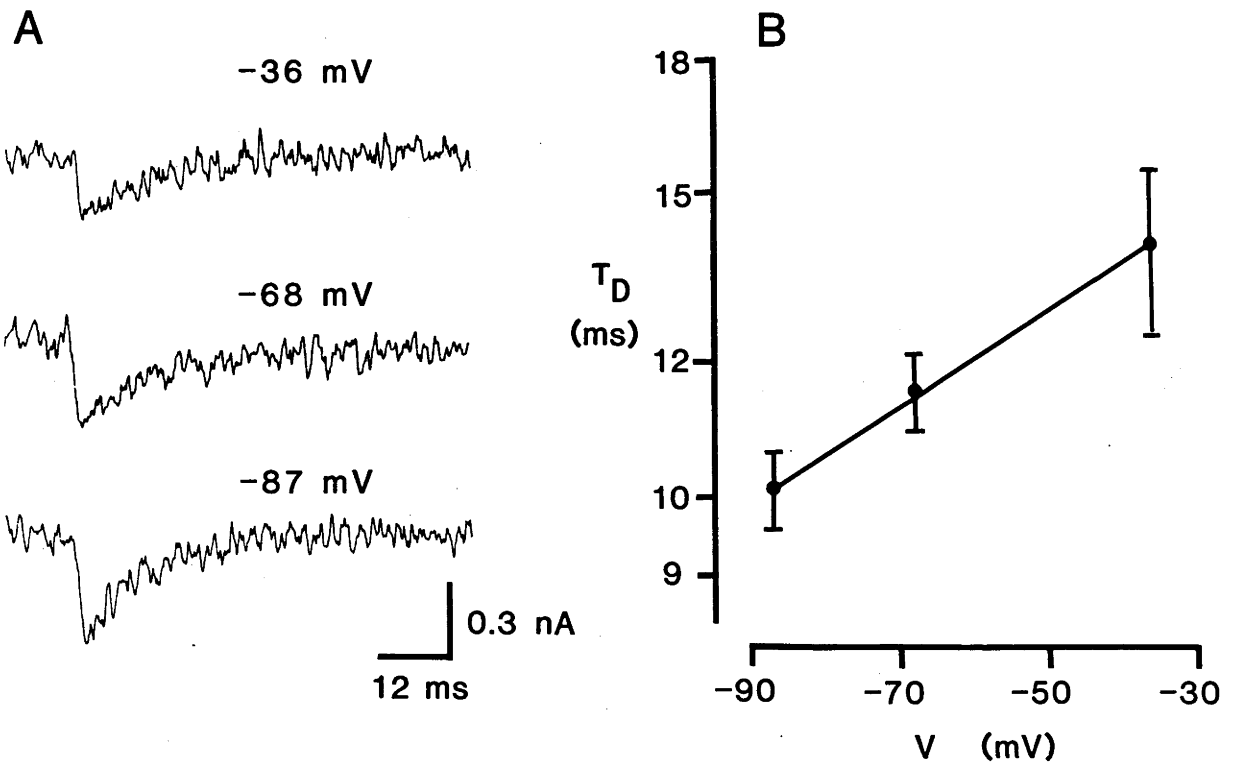


FIGURE 15: Quantal IPSCs have voltage sensitive decay phases. Individual currents recorded at three potentials in a 1μM TTX solution are shown in A. Note the relatively small amplitude of these currents. B) The voltage sensitivity (H) of this cell is 160mV, which is within the range of values obtained for IPSCs in normal solution. (Same cell as in Fig. 14).

TTX solution (Fig. 15), and the H value was 160mV in this particular cell. Effectiveness of TTX was confirmed by trying to stimulate the pyramidal neurone to fire a spike - no spikes were observed in the presence of TTX.

In some experiments 0.2mM cadmium chloride was added to the standard or TTX-containing solution in order to block calcium currents in presynaptic terminals and hence suppress transmitter release. Small IPSCs were still observed in Cd solutions however, supporting the argument that the smallest group of IPSCs are due to spontaneous secretion of 'quanta' of GABA.

THE EFFECTS OF SOME ANAESTHETICS ON IPSCs.

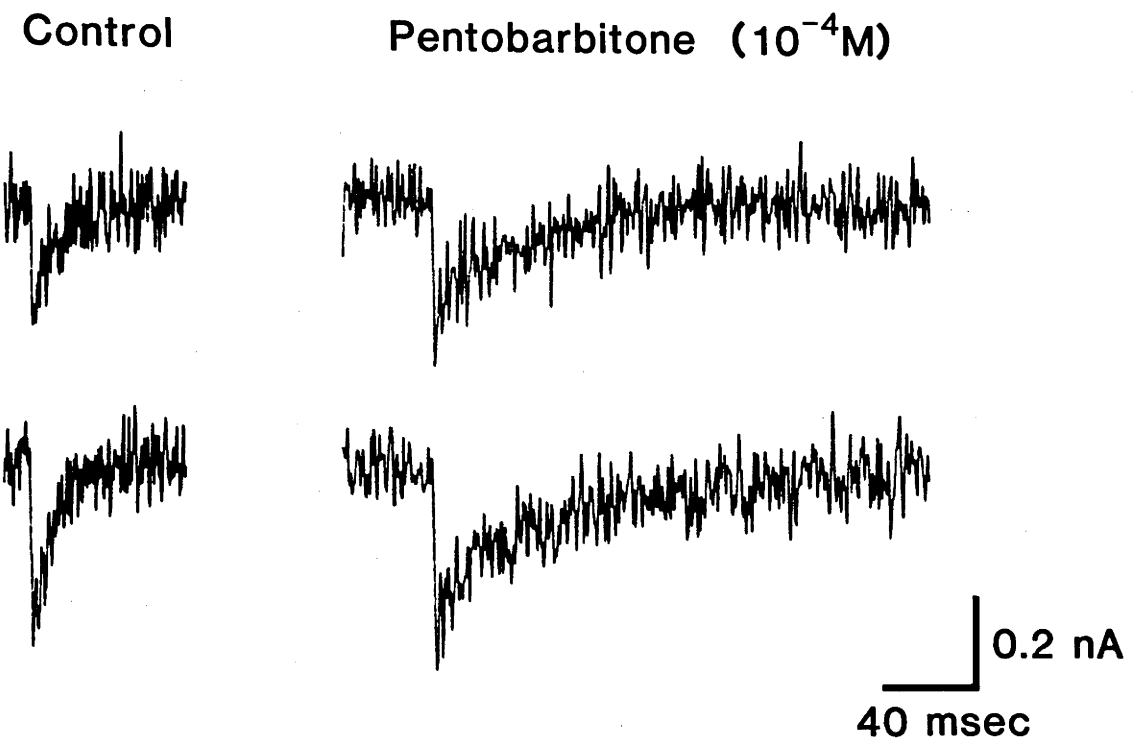
Pentobarbitone.

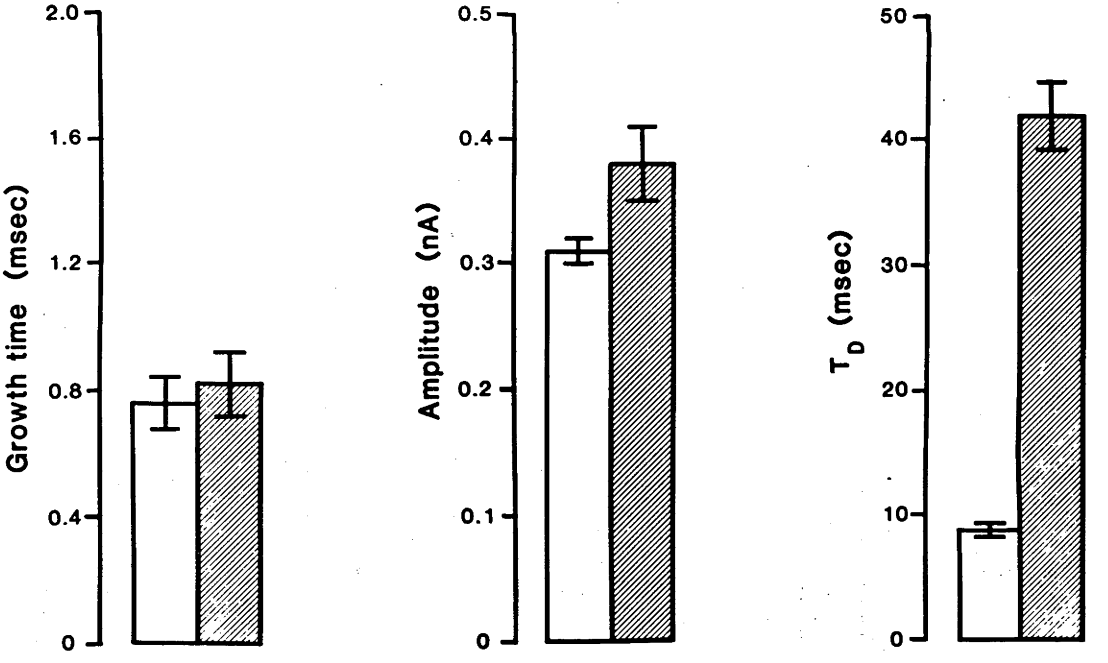
Nicoll et al. (1975) found that both evoked and spontaneous IPSPs were increased in amplitude and greatly prolonged by systemic administration of pentobarbitone. This suggested that the underlying conductance change may have been prolonged by the drug - this turned out to be the case. Bath application of pentobarbitone (50 - 200 μM) resulted in a marked prolongation of the decay phase of IPSCs, which remained a single exponential except at the very highest concentration of the drug. Prolongation of the decay phase was clearly seen during

the first 10 minutes after solution changeover. The effect was stabilized by 20 minutes and data was collected for analysis after 30 minutes. The prolongation remained as long as the drug was present and could be fully reversed after washing out for thirty minutes. Fig. 16 shows two different cells exposed to 100μM pentobarbitone and Fig. 17A shows sample traces from one cell with two concentrations of the drug. It was extremely difficult to hold a cell long enough to expose it to the three concentrations of the drug, since 30 minutes were required between each application at a particular concentration.

Pentobarbitone prolonged IPSC decay in all 15 cells studied. Fig. 17B shows the relative lengthening effect of 50, 100 and 200μM pentobarbitone. 50μM increased τ_D more than three fold, 100μM almost 7 fold and 200μM approximately 8 fold. The ampitude or rise time was hardly affected at 50 or 100μM, e.g. amplitude before 100μM pentobarbitone 0.31nA, 0.38nA after; rise time 0.76ms before and 0.83ms after pentobarbitone (see also Fig. 16B, which compares growth times, amplitudes and τ_D in control solutions and 100μM pentobarbitone). However, with 200μM pentobarbitone the IPSC amplitude was usually decreased. Although no detailed study was made, IPSC frequency appeared to be reduced on 2 out of 4 occasions. Cell holding current was decreased in three experiments but was not appreciably changed in 9 other experiments. Further experiments would be desirable to ascertain the magnitude and significance of pentobarbitones effect on resting cell properties.

FIGURE 16: Prolongation of IPSCs by pentobarbitone. The currents presented in the top trace are sample currents recorded in control solution and after at least 30 minutes in 100μM pentobarbitone. The histograms in the lower part of the figure are the results from another cell, showing the effect of 100μM pentobarbitone on the average growth time, the the amplitude and the time constant of decay (τ_D). Control values are open bars, pentobarbitone values are hatched bars. The vertical bars are ± one standard error of the mean.


Control
Pentobarbitone (10^{-4}M)
0.2 nA
40 msec


Growth time (msec)
2.0
1.6
1.2
0.8
0.4
0
Amplitude (nA)
0.5
0.4
0.3
0.2
0.1
0
T_D (msec)
50
40
30
20
10
0

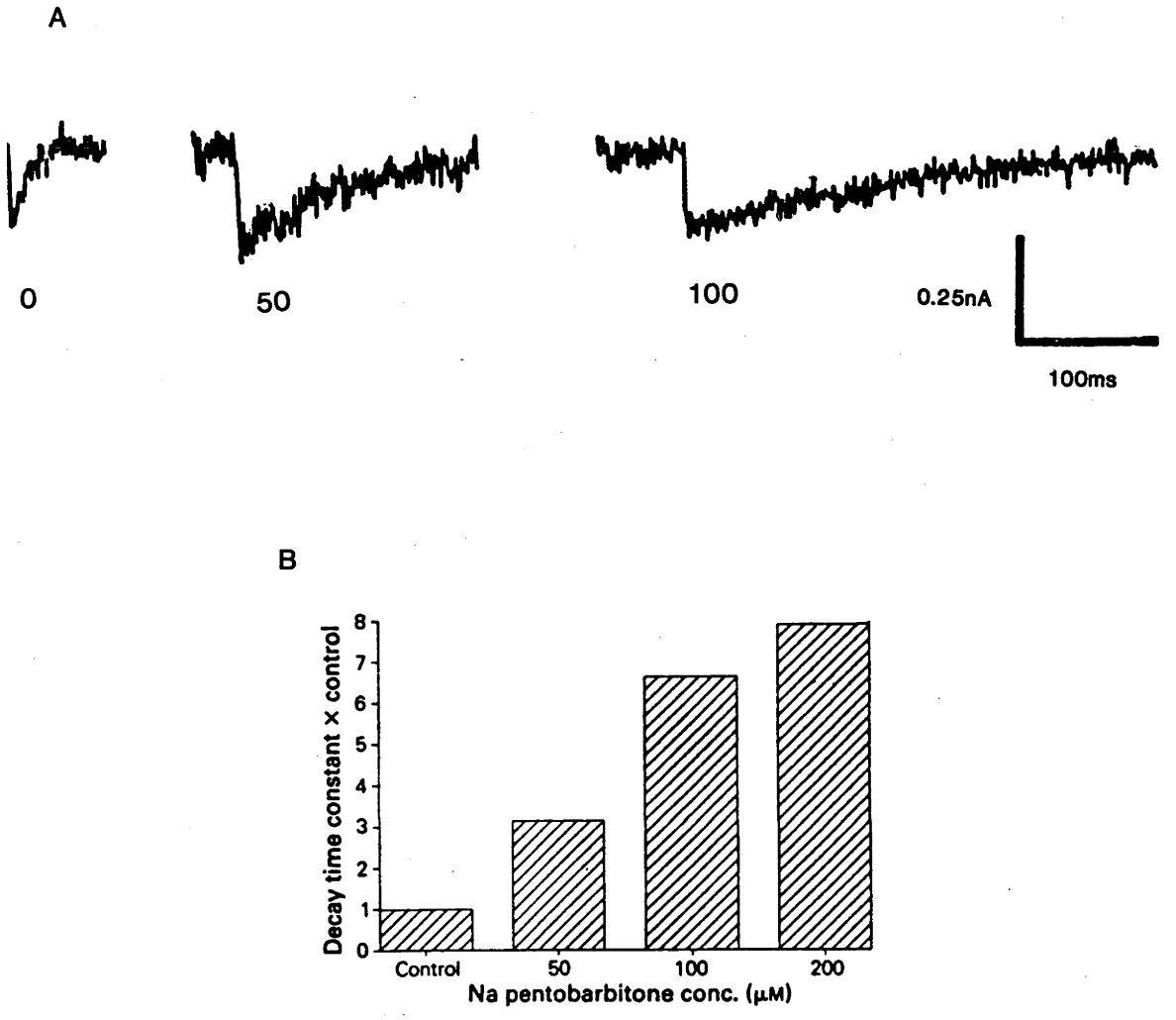


FIGURE 17: A) The action of pentobarbitone on IPSCs. Currents were recorded in control solution and 50 and 100 μM pentobarbitone, and this anaesthetic markedly prolongs the decay phase, with little effect on the amplitude of the currents. Membrane potential -41mV. Clamp switching rate 11.5kHz. B) Results from 5 cells exposed to two or three concentrations of pentobarbitone.

Halothane.

Halothane is an extremely volatile liquid, and early experiments on the actions of this anaesthetic were unsuccessful. This was because halothane was added in solution to the reservoir of perfusate. After 30 minutes in this solution IPSCs were unaffected. It was suspected after a few such experiments that halothane was simply evaporating away through the perfusion tubing before reaching the recording chamber and even sealing the system and bath as much as possible was of little use. Two pilot experiments were then carried out in which approximately 5mls of liquid halothane were added to the outer bath (see Fig. 2), so that the halothane as gas would quickly reach the slices in the central recording chamber. The effect was quite dramatic. No sooner had the injection of the liquid into the outer bath begun than the IPSCs became markedly prolonged. This experiment was repeated again two hours later (after the previous application of halothane had completely washed out/blown off) and a similar result was obtained. In this experiment, halothane also caused a marked increase in frequency of IPSCs. For further experiments, halothane was applied in gaseous form in the O_2/CO_2 supply: this allowed the anaesthetic to be applied very rapidly to the atmosphere around the slices, and was also bubbled into the perfusion solution in the reservoir. Halothane was administered using a Fluotec 3 (Cyprane, England)

vaporizer which allowed a precise percentage of gas to be applied. With this system the lengthening effect of halothane was manifest within a very few seconds, but currents were collected for analysis after a full 5 minutes to allow for full equilibration and stabilisation.

Halothane was applied to a total of 15 cells, and always prolonged IPSC decay. Fig. 18A shows sample IPSCs in a cell voltage clamped at -54mV before and after 3% halothane. The average decay time constant was 9.9ms before halothane and 21.5ms in halothane - a greater than two fold prolongation. A dose-response relation is presented in Fig. 18B. The rapid and accurate method of halothane application meant that a range of concentrations of the anaesthetic could often be examined in a single neurone. Results from two such experiments are shown in Fig. 19. These graphs show that the effects of halothane increased with concentration and were fully reversible. It is interesting to note that there may be some 'desensitization' or tachyphylaxis to the anaesthetic upon a second application (see Fig. 19). Halothane does not greatly affect the amplitude of rise time of the IPSCs, at least in the concentration range studied here, e.g.:

	Amplitude	Rise Time
Control:	0.45nA;	0.68ms.
3% halothane:	0.51nA;	0.91ms.
Control:	0.42nA;	0.87ms.
2% halothane:	0.45nA;	0.92ms.

FIGURE 18: Halothane prolongs IPSC decay phase. A) Control IPSCs and sample currents in 3% halothane are illustrated. Note the different time scales in control and halothane. B) Pooled dose response data for 11 applications of 5 different concentrations of halothane on the IPSC decay phase.

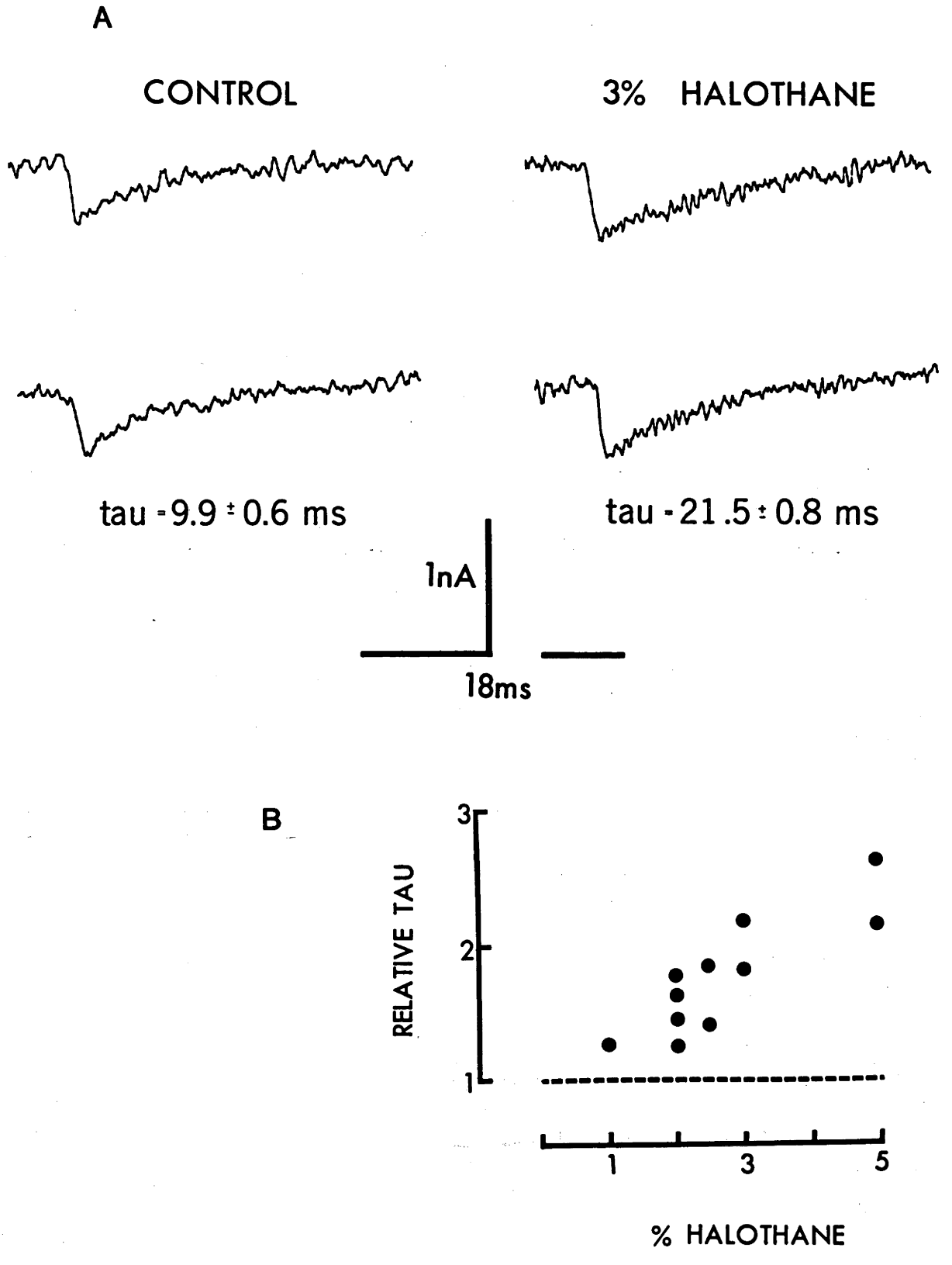
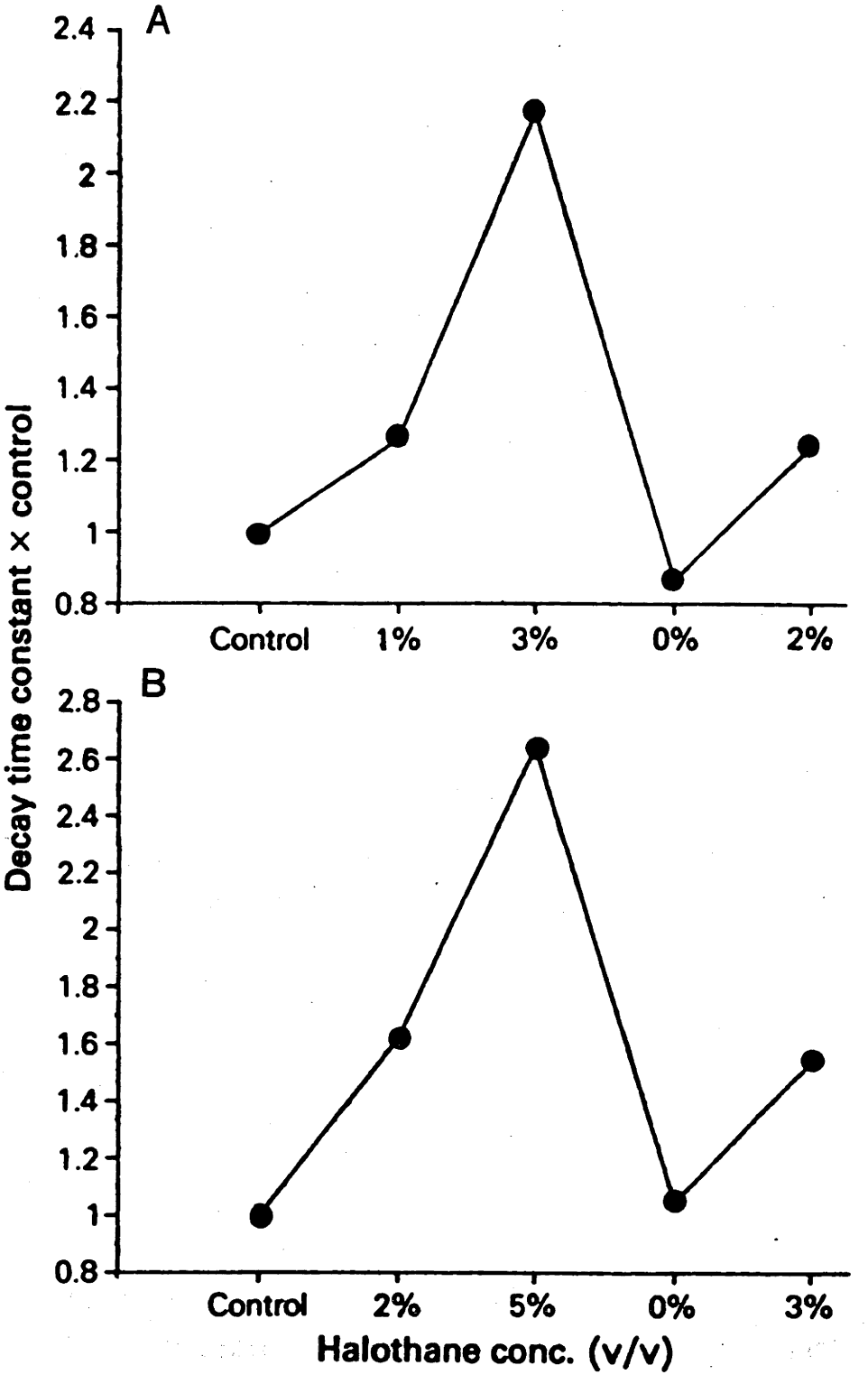

A
CONTROL
3% HALOTHANE
tau = 9.9 ± 0.6 ms
tau = 21.5 ± 0.8 ms
1nA
18ms
B
RELATIVE TAU
3
2
1
1
3
5
% HALOTHANE

FIGURE 19: The action of a range of halothane concentrations on the time constant of decay of IPSCs. Each point is the average decay time of 9 to 35 individual IPSCs. The membrane potential was set at -54mV in A, and -72mV in cell B. The control time constant was 9.9ms in A and 11.5ms in B. Note that halothane became less effective in prolonging decay time after returning to control values in both cells. The reason for this is not yet clear.


A
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
Control
1%
3%
0%
2%
B
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
Control
2%
5%
0%
3%
Decay time constant x control
Halothane conc. (v/v)

KETAMINE.

Ketamine (see structure in Appendix A) is a water soluble dissociative anaesthetic, which accelerates the decay of end plate currents generated by acetylcholine at the motor end plate (Torda & Gage 1977, Maleque, Warwick & Albuquerque 1981), inhibits responses to excitatory amino acids in mammalian central neurones (e.g. Thomson, West & Lodge 1985). However, Scholfield (1980) has reported that ketamine potentiates IPSPs in the olfactory cortex, although other authors find inhibition is unaffected (e.g. Anis, Berry, Burton & Lodge 1983). It was therefore of interest to examine the effects of ketamine on spontaneous IPSCs in the hippocampus.

Ketamine was applied at a concentration of 0.5mM, and prolonged IPSCs, but to a lesser degree than either pentobarbitone or halothane. The effect was examined successfully in three different cells. Sample control and anaesthetic-treated IPSCs are shown in Fig. 20. In the three experiments, 0.5mM ketamine increased the average decay time constant of IPSCs by 57%, 74% and 81%. Amplitude and rise time were not significantly altered (rise time may have been slightly, but not significantly reduced). The increase in decay time constant was completely reversible after 30 minutes perfusion with control solution. No change in holding current was observed during exposure to ketamine.

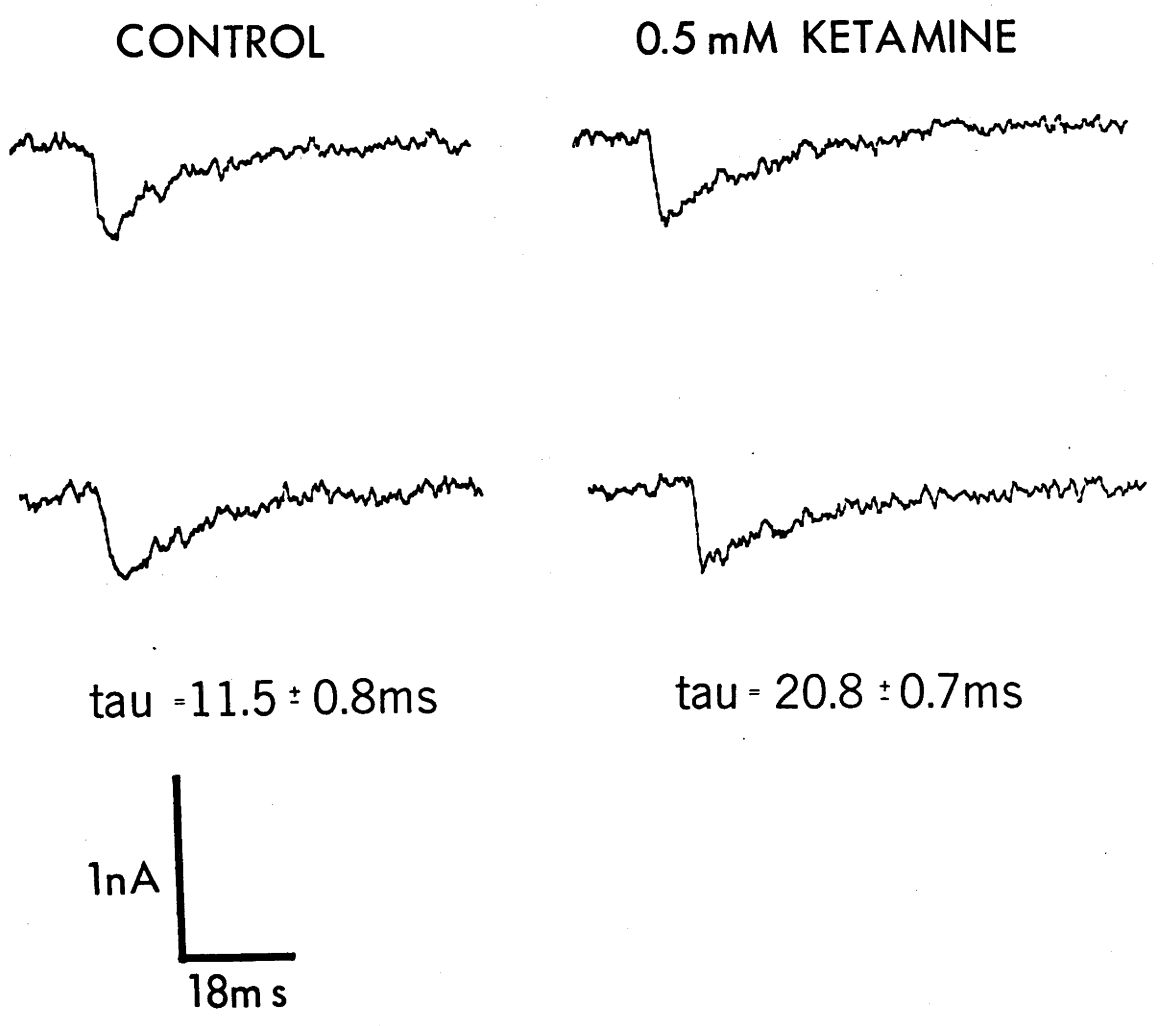


FIGURE 20: Ketamine (0.5mM) prolongs IPSC decay time. Clamp potential -53mV. Values for tau are the average time constant of decay for individual IPSCs in each solution.

ETHANOL.

Ethanol has a depressant action on the central nervous system but the method of action is at present unknown. Since previous results on the effects of ethanol on inhibitory neurotransmission are conflicting, it was thought worthwhile to test the effects of the alcohol on IPSCs. Also, since τ_D of IPSCs is affected by membrane potential and a detailed model has been proposed for alcohol action on channel dipole movement, it was worth testing the applicability of this theory to a GABA-ergic synapse.

Low concentrations of ethanol (10mM) similar to those found in intoxicated subjects (corresponding to a blood alcohol level of 0.05, the legal limit for road users in New South Wales) were tested first. This concentration had no effect on IPSC paramters in two cells so the concentration was increased to 100mM - again no effect was seen. Finally four cells were exposed to 200mM ethanol - which is a concentration sufficient to double end-plate current decay at the neuromuscular junction. The following results were obtained:

	MEAN TAU.		MEAN TAU.
Cell (1). control	13.3 ± 0.6ms;	0.2M ethanol	15.2 ± 0.4ms.
Cell (2). "	10.8 ± 0.4ms;	"	11.2 ± 0.6ms.
Cell (3). "	14.4 ± 0.2ms;	"	14.3 ± 0.3ms.
Cell (4). "	7.1 ± 0.3ms;	"	7.0 ± 0.3ms.

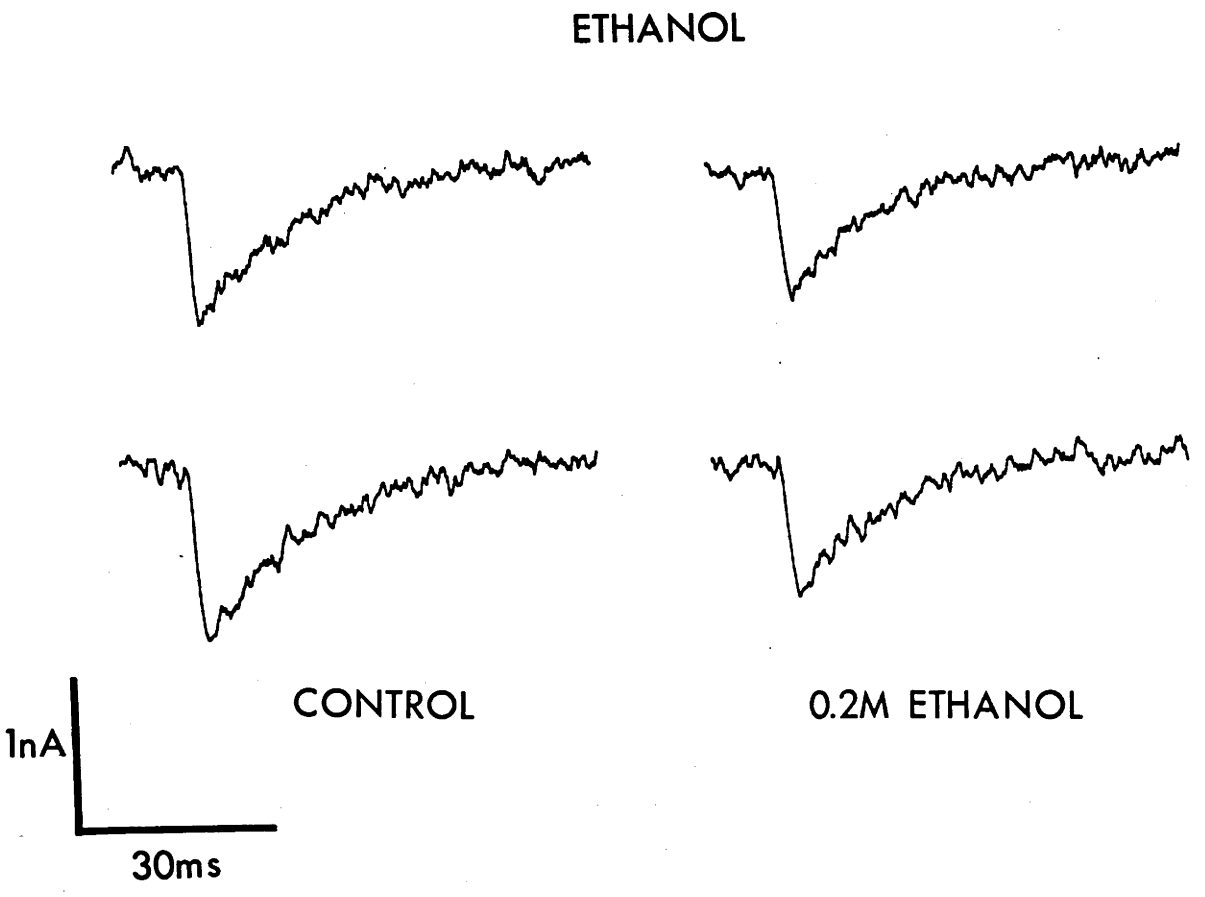


FIGURE 21: 0.2M ethanol was ineffective in altering IPSC amplitude, rise or decay time.

Some of the example IPSCs from these experiments are shown in Fig. 21. There was no consistent, significant effect on IPSC rise time, amplitude or on the cell holding current.

NITROUS OXIDE.

Nitrous oxide was added at 75 to 80% in the oxygen/carbon dioxide supply line, in a manner similar to halothane. IPSC decay was not significantly altered - any slight decrease could have been due to secondary changes in O_2 or CO_2 tension. For instance measurement of the pH of the bathing solution showed a +0.2 to +0.4 pH unit shift when 75 - 80% N_2O was included. The effects of pH or reduced oxygen tension on IPSC properties was not investigated.

The results were:

		MEAN TAU		MEAN TAU.
Cell (1).	control	7.2 ± 0.4ms;	N_2O	6.8 ± 0.4ms.
Cell (2).	"	8.8 ± 0.7ms;	"	8.1 ± 0.8ms.
Cell (3).	"	7.7 ± 0.4ms;	"	7.1 ± 0.3ms.
Cell (4).	"	6.3 ± 0.4ms;	"	5.3 ± 0.2ms.

There were no detectable changes in either resting cell properties or IPSC frequency, rise time or amplitude. Some sample IPSCs from one of these experiment are shown in Fig. 22.

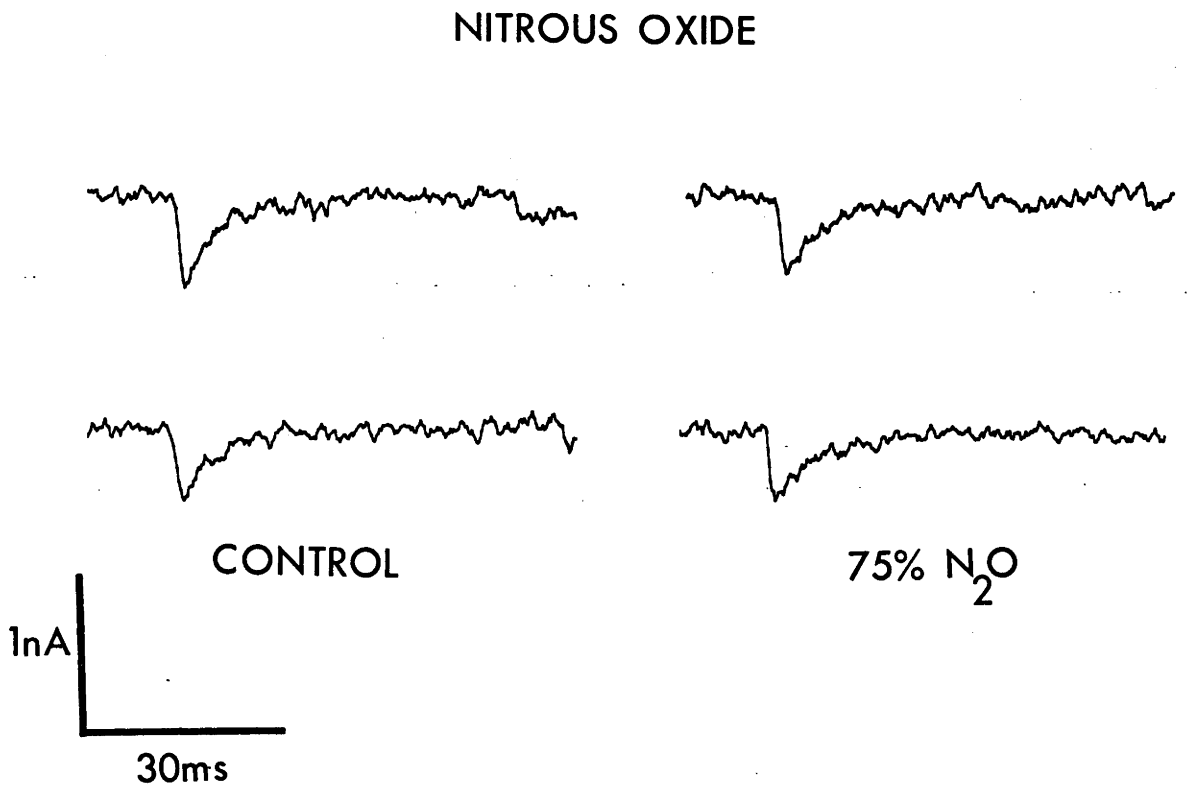


FIGURE 22: 75% N_2O does not alter IPSC decay phase.

CAFFEINE.

Interest in caffeine (see structure in Appendix A) was prompted by the report of Nistri & Berti (1983), who found that this substance was able to selectively enhance or inhibit GABA responses in the frog spinal cord, depending on the concentration applied. A GABA blocking action could have explained the central stimulatory action of this compoud. Caffeine was applied at a 'clinically' relevant concentration of 10μM (10 cells) with no effect on IPSC amplitude or time course. Further exposures to 50μM (2 cells) and 500μM (1 cell) again had no effect. Additionally, normal IPSCs were observed in 7 cells exposed to 5mM caffeine. The amplitude and time course of these IPSCs were normal even after 90 minutes in 5mM caffeine.

THE EFFECTS OF FOREIGN ANIONS ON IPSCs

It was appropriate to examine the effects of foreign anions on hippocampal IPSCs for two reasons: (1). There has been very little work concerning the anionic permeability of synaptic channels in central neurones. Kelly, Krnjevic, Morris & Yim (1969) reported that large anions e.g. citrate, are permeable through GABA channels

in cortical neurones, while Eccles, Nicoll, Oshima & Rubia (1977) suggested that the permeability of GABA channels in hippocampal neurones was similar to those chloride channels activated by glycine in motoneurones, where citrate and other large anions were excluded. (2). Channel kinetics have been shown to be affected by permeant ions in a variety of preparations (see e.g Van Helden, Hamill & Gage 1977) this had never been examined in GABA channels from the CNS.

Foreign anions were injected into neurones by filling microelectrodes with as concentrated solution as possible of the anion under examination, and after a cell had been impaled, diffusion and current injection served to load the cell with the anion (see e.g. Coombs, Eccles & Fatt 1955). This method was used for all of the results reported here. Junction potential errors were calculated and in some cases, also measured experimentally. If corrections for junction potentials were less than ±5mV (which was usually the case), then the measured membrane potential was not corrected.

Impermeant anions

Electrodes were filled with 2M caesium sulphate, 0.6M potassium sulphate, 1M potassium citrate, 3M potassium acetate or 3M potassium methylsulphate, with pH corrected to pH 7.4 as necessary. The electrode holder was filled with normal standard solution to prevent junction

potentials developing at the Ag/AgCl pellet. When electrodes containing any of these electrolytes were used, there was no convincing evidence of spontaneous activity in either membrane potential (during current clamp) or current traces (during voltage clamp). No spontaneous activity was observed even if the cells were polarized to very negative potentials, suggesting that these large anions were impermeable through the synaptic GABA channel in CA1 neurones.

With caesium sulphate electrodes, outward currents only could be observed at positive potentials - no inward IPSCs were ever recorded, even when the membrane potential was made very negative.

Permeant anions

Iodide

When electrodes containing 3M potassium iodide were used, extremely large IPSPs were observed. When these cells were voltage clamped prolonged IPSCs were seen (n = 7) with a time constant of decay approximately three fold longer than IPSCs in cells impaled by chloride electrodes at -80mV. Results of time constant of decay versus membrane potential in two cells clamped at several potentials are shown in Fig. 23. It can be seen that the decay phase of these IPSCs was not markedly affected by membrane potential. τ_D values at ~-40mV are similar to those of IPSCs recorded at -90mV. In one other cell successfully clamped at -135mV, the mean τ_D of 8 IPSCs

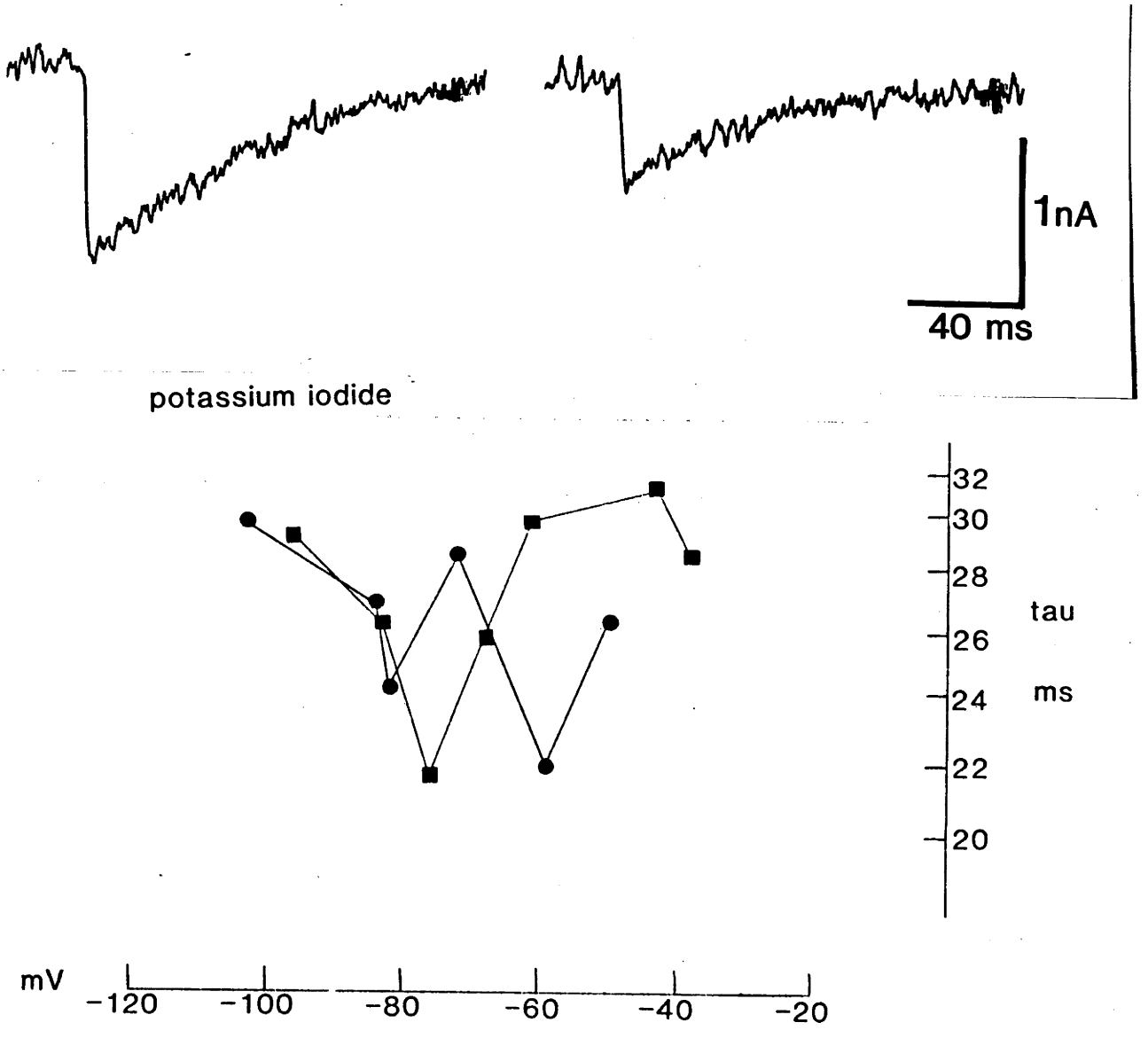


FIGURE 23: The effects of membrane potential on IPSC decay time constant recorded with 3M potassium iodide electrodes in two different cells. Inset shows sample currents carried by iodide at a potential of -26mV from another cell. Mean τ_D was 42 ± 1ms.

was 28 ± 1.6ms. These results suggest that the normal voltage dependence of IPSC decay may be lost when iodide ions pass through the channel.

Bromide

Three cells were successfully voltage clamped with electrodes containing 3M potassium bromide. The duration of these IPSCs did not appear to be significantly longer than currents recorded with a chloride-filled electrode in the same slice, but as Fig. 24 shows, in two of these cells in which currents were collected at four or five potentials, the decay phase was unaffected by changes in membrane potential. In one other cell, mean τ_D was 15.7ms at -70mV and 16.9ms at -53mV.

Thiocyanate

A total of six cells were voltage clamped with electrodes containing 3M potassium thiocyanate. Only two of these cells could be clamped at three or more potentials (see Fig. 25). This anion produced longer duration IPSCs relative to chloride - even at -123mV, IPSC time constant of decay still reached 20.2 ± 0.7ms. Fig. 25 shows that τ_D became slightly longer as membrane potentials were made more positive, the individual data points from other cells show that there is some degree of scatter in τ_D values in thiocyanate.

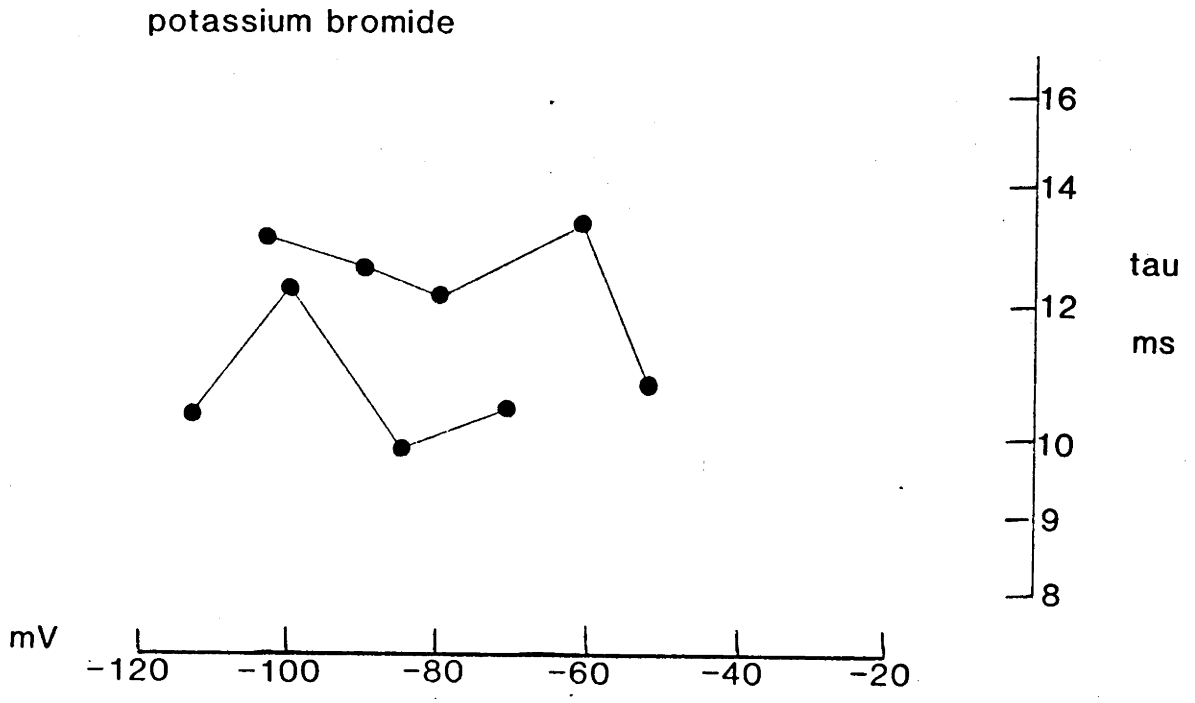
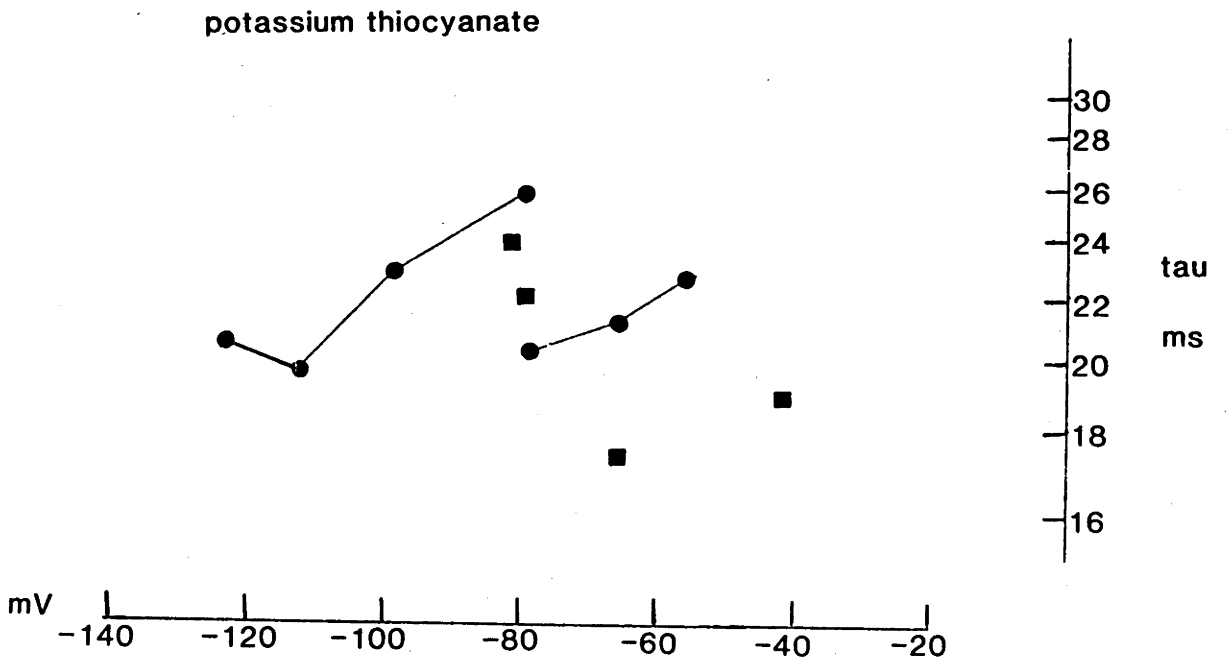
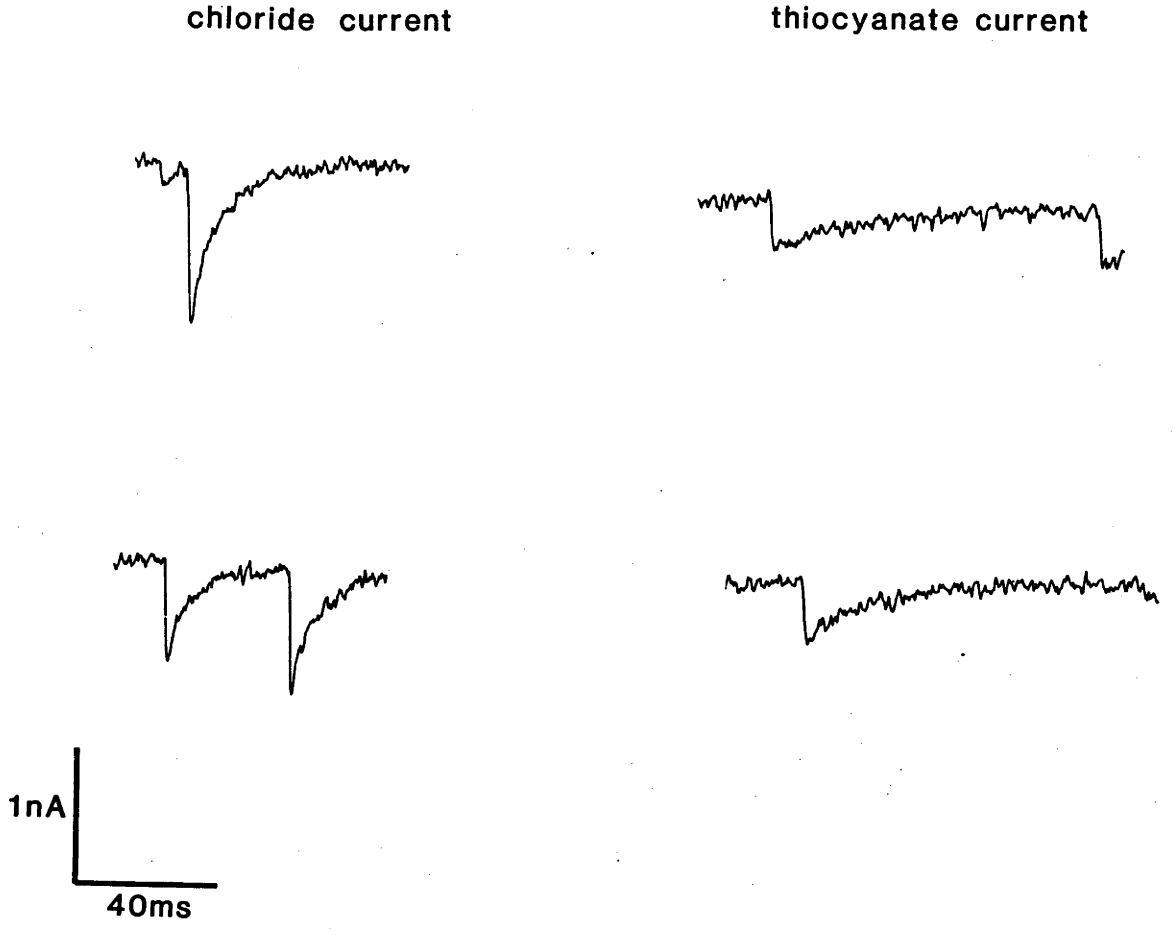


FIGURE 24: The normal voltage sensitivity of IPSCs is abolished in these two separate cells impaled with potassium bromide electrodes.

FIGURE 25: Voltage sensitivity of IPSCs carried by thiocyanate ions. The results from 6 separate cells are shown in the top half of the figure, while the lower half compares the decay phases of currents recorded at -80mV with chloride and thiocyanate containing electrodes.


potassium thiocyanate
30
28
26
24
22
20
18
16
tau
ms
mV
-140
-120
-100
-80
-60
-40
-20


chloride current
thiocyanate current
1nA
40ms

Nitrate

Five cells had spontaneous IPSCs when penetrated with 1.5M potassium nitrate electrodes. Results from two cells recorded at two or more potentials are shown in Fig. 26, and in two other cells τ_D was between 16 and 18ms at membrane potentials greater than -100mV. These results, although few in number, again suggest that τ_D is not as dependent on membrane potential as observed when chloride ions are the charge carrier.

Cyanate

Spontaneous synaptic activity was observed in all 8 cells successfully impaled with electrodes containing 2M potassium cyanate. IPSPs were observed in 4 cells, but these neurones deteriorated irrecoverably before they could be voltage clamped. IPSCs were recorded in the other four cells, but could only be captured at a variety of potentials in one of these cells. The results from this cell are shown in Fig. 27. In this cell the time constant of decay was prolonged as the membrane was depolarized, and τ_D's were within the normal range for IPSCs carried by Cl^- ions, but were slightly shorter in three other cells.

Azide

With electrodes filled with 5M sodium azide (NaN_3) as the electrolyte, spontaneous potentials were observed in two cells and IPSCs were recorded from three other cells. All recordings with azide were very short lived,

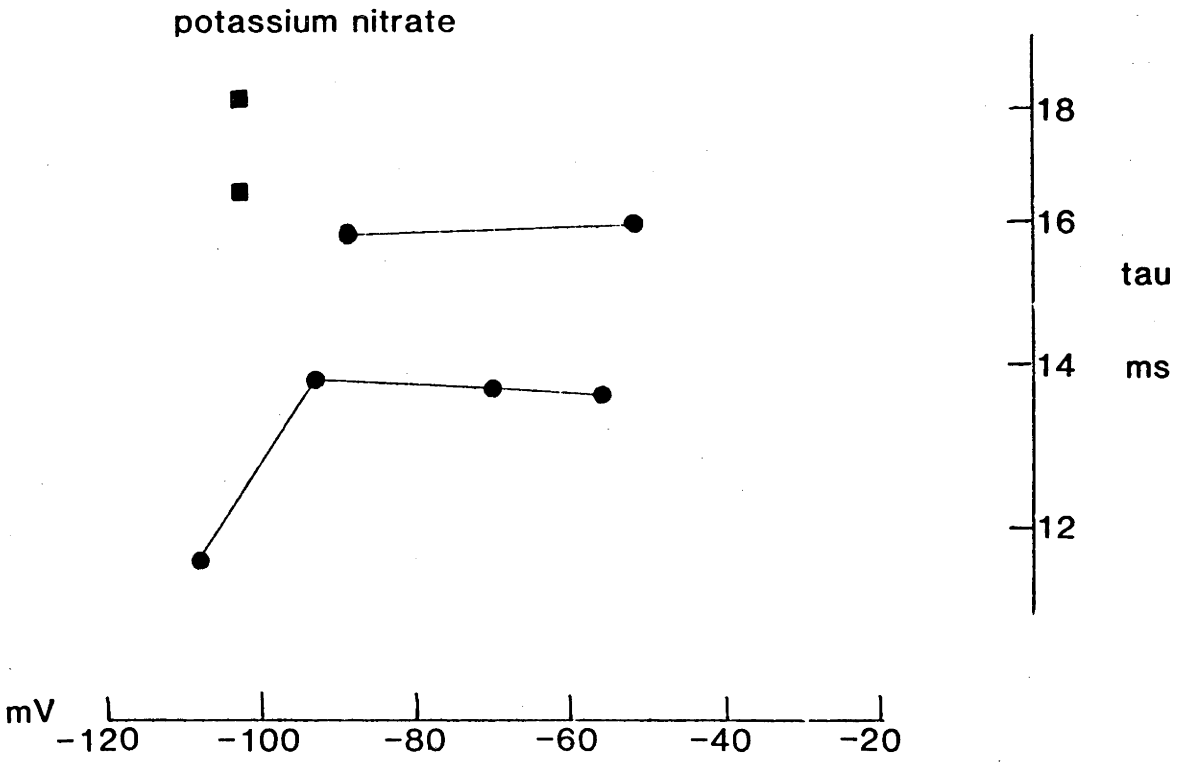


FIGURE 26: IPSCs recorded with a 1.5M potassium nitrate electrode did not appear to be significantly voltage sensitive. Results from 4 separate cells are shown in this figure.

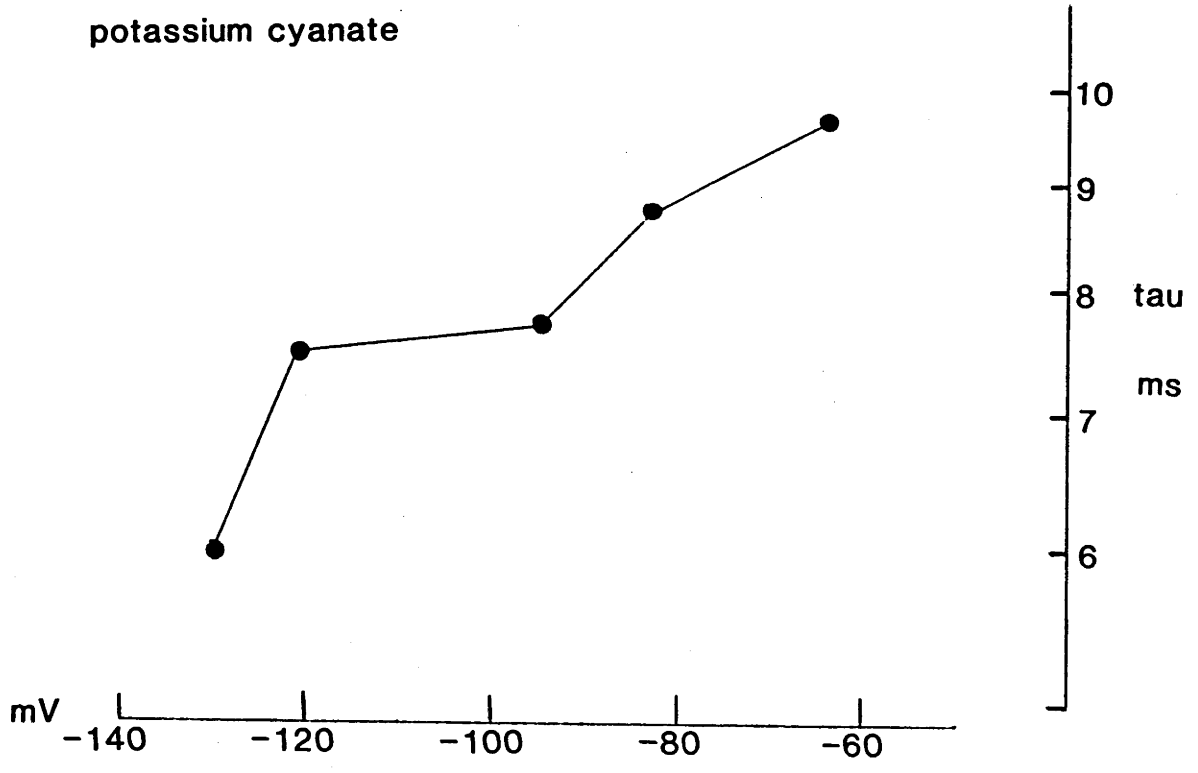


FIGURE 27: IPSCs carried by cyanate ions appeared to have normal voltage sensitivity in one cell.

probably due to the extreme toxicity of this anion (see Discussion in this Section). Due to the shortness of the impalements, it was not possible to examine τ_D over several potentials, but in two cells τ_D was 8.1 ± 0.9ms at -83mV, and 7.9 ± 0.5ms at -80mV.

Chlorate

Spontaneous activity was observed in both cells successfully impaled with 0.5M potassium chlorate ($KClO_3$) electrodes. Only one of these cells could be voltage clamped, but most of the IPSCs were too small to be usefully analysed. However, τ_D was approximately 20ms at -94mV, suggesting that τ_D was longer with chlorate than chloride, at least at this potential.

SECTION B: DISCUSSION.

These experiments were designed to explore some of the properties of GABA receptor/channels in the mammalian central nervous system. This was achieved by combining the techniques of brain slices and single electrode voltage clamping to study spontaneous inhibitory postsynaptic currents in hippocampal CA1 neurones.

Spontaneous inhibitory events in CA1 pyramidal neurones are presumably generated by GABA-ergic interneurones, which project to the cell soma and axon initial segment (e.g. Somogyi et al. 1983). [Inhibitory neurones also project to the dendrites of these cells (see references in Alger & Nicoll 1982), and these may also generate spontaneous IPSCs, but these would appear as small slow events in somatic recordings]. The inhibitory projection to a single pyramidal cell soma is probably very great. Andersen (1966) has calculated that at least 40% of the somatic membrane is covered with basket cell terminals. Miles & Wong (1984) suggested that in the transverse slice preparation, up to fifteen inhibitory interneurones form synapses on a CA3 pyramidal cell. The axo-somatic location of inhibitory terminals is therefore ideally located to block impulse generation. The somatic location of such a strong inhibitory input provided an ideal opportunity for examining the conductance change generated by GABA using voltage clamp techniques, since the frequency resolution of conductance changes falls off

very rapidly with increasing electrotonic distance from the soma (see e.g. Brown & Johnston 1983). Effectiveness of the clamp to resolve IPSCs was further aided by a) using low resistance electrodes, b) experimenting at room temperature, and c) the fortuitous slow time course of the IPSCs themselves. Spontaneous IPSCs are much slower than unitary EPSCs in central neurones, even after correcting for temperature differences (see Finkel & Redman 1983 and Nelson, Pun & Westbrook 1986). Segal & Barker (1984b) have reported that GABA-mediated IPSCs in cultured rat hippocampus have durations similar to, but slightly longer than, those reported here. These authors also performed noise analysis on current responses to exogenously applied GABA, and found good agreement between the measure of channel lifetime from IPSCs and from noise, suggesting that the decay of spontaneous IPSCs does reflect channel lifetime. There is also relatively close agreement between IPSC time constant of decay and the average open time of channels determined by noise analysis in other central neurones (e.g. cultured spinal cord neurones [GABA]: Barker & McBurney 1979, goldfish Mauthner cells [glycine]: Faber & Korn 1980, lamprey Muller cells [glycine]: Gold & Martin 1983a,b) and in invertebrate preparations (lobster muscle [GABA]: Dudel, Finger & Stettmeier 1977, locust extensor tibae [GABA]: Cull-Candy 1986). It appears then that the decay of spontaneous inhibitory currents in the CNS may reflect to a first approximation, channel lifetime, as at the neuromuscular junction (Anderson & Stevens 1973).

It was surprising that spontaneous IPSPs and IPSCs could be observed only at negative membrane potentials when chloride containing electrodes were used. Other authors have observed this phenomenon in CA1 neurones (Alger & Nicoll 1980, Biscoe & Duchen 1985), and neocortical neurones (Galvan, Franz & Constanti 1985). However in CA3 neurones there have been reports of hyperpolarizing inhibitory synaptic potentials with acetate (Miles & Wong 1984) and sulphate (Brown & Johnston 1980,1984) electrodes. These results would suggest that there may be some fundamental difference between the GABA channels in CA3 and CA1 neurones. It is possible that the channels in CA1 neurones show more marked inward rectification than CA3 neurones. There is some evidence that the conductance of single GABA-activated channels in the hippocampus shows marked rectification in symmetrical Cl^- solutions (Gray & Johnston 1985) . This problem would certainly seem to warrant further investigation. It is interesting to note that Gold & Martin (1982) observed that increasing intracellular chloride concentration decreased the single channel conductance of glycine activated channels in the Lamprey brainstem, this is clearly not the case for hippocampal neurones activated by GABA.

Repetitive firing of interneurones.

Frequently a cluster of IPSPs and IPSCs appearred together (see Fig. 4 & 6), and the closeness of these events resulted in significant summation of individual

responses. This summation freqently led to the generation of an action potential. The summation of these spontaneous events may have been due to repetitive firing of a single interneurone, which is believed to cause at least a significant portion of the recurrent IPSP (see e.g. Fig. 6 of Miles & Wong 1984 and Fig. 8 of Andersen et al. 1964b). Since the width of the action potential in interneurones is very short (see e.g. Schwartzkroin & Mathers 1978, Knowles & Schwartzkroin 1981), the repetitive firing of a single interneurone could release a large amount of inhibitory transmitter in a short period, and cause a fairly large inhibitory potential on its own.

Use of IPSCs as an assay of presynaptic function.

It is possible to use the frequency, and also the amplitude of multiquantal IPSPs and IPSCs as an assay of presynaptic function, i.e. as a measure of the firing rate and the transmitter release probablity of interneurones. This has already been done by Nicoll, Alger & Jahr (1980) who used the frequency of spontaneous IPSPs in the hippocampus to measure the effect of a stable enkephalin analogue on transmitter release from interneurones. We have also used IPSCs as an assay of the effectiveness of transmitter release. In a study of the actions of prejunctionally active snake toxins in the hippocampus (Harvey & Robertson, see Appendix D), we found that the action of the toxin β-bungarotoxin was consistent with a

presy naptic mechanism of action in the hippocampus, as previosly suggested by Halliwell & Dolly (1982). It is likely then that recordings of postsynaptic inhibitory events in pyramidal neurones will prove fruitful in the study of the physiology and pharmacology of interneurones.

Quantal IPSCs.

As recently as 1980, Krnjevic concluded that there was little convincing evidence to suggest that transmitter was released in a quantal manner in the CNS. Alger & Nicoll (1980) reported that tetrodotoxin and high concentrations of magnesium entirely abolished spontaneous IPSPs, and Galvan, Franz & Constanti (1985) also found that TTX eliminated IPSPs in olfactory and neocortex. These authors suggested that all spontaneous IPSPs were due to action potentials in interneurones, and there was no evidence for spontaneous quantal secretion, which would be resistant to sodium and calcium channel blockers (as occurs at the neuromuscular junction). The results presented here demonstrate that spontaneous potentials and currents can still be detected in solutions containing TTX and/or Cd^{2+}, which block evoked transmitter release in both central (Kuan & Scholfield 1986) and peripheral (Katz & Miledi 1967) synapses. The IPSCs in TTX were generally smaller than those in control solutions, but the time course, voltage sensitivity and pharmacology were in no respect different from evoked

IPSCs. Johnston & Brown (1980,1984) have also reported TTX resistant IPSPs in CA3 neurones in the hippocampus, and Takahashi(1984) and Kojima & Takahashi(1985) have examined 'miniature' IPSPs in isolated rat spinal cord treated with TTX, and have confirmed that release of transmitter from central terminals is similar to that at peripheral synapses.

The IPSCs recorded in TTX and Cd^{2+} here were usually smaller (but see Fig. 14) than those in control solution, and it is possible that they may have been obscured by the baseline 'noise' in others' experiments. Alternatively, it may have been that the concentrations of TTX and Cd^{2+} used in the present experiments (0.5-1.0μM and 0.2mM respectively) may have been too low to fully block action potentials or calcium dependent release in presynaptic interneurones. This can be tested directly by recording action potentials or voltage activated conductances from interneurones during application of these blockers. No spikes were observed in pyramidal cells in the present experiments with these blockers.

However, taking a mean value of 0.3nA for a 'quantal' IPSC at -100mV would give a peak conductance of 3nS (assuming a null potential of 0mV). This allows upper and lower limits to be placed on how many channels are opened by a single packet of transmitter, by using other published values for the single channel conductance of GABA channels. Values range between 16 & 45pS (see Section E) which would suggest that there are between 66

and 200 channels open at the peak of a 'miniature' IPSC. Miles & Wong(1984) have calculated a value of approximately 300 channels opened following an action potential in a CA3 neurone following a presynaptic action potential. Segal & Barker (1984) have calculated that about 1700 channels are opened at the peak of an IPSC generated by an action potential in a co-culture of pyramidal neurones and interneurones (but this may represent summation of responses from multiple release sites). If we assume that a quantum of GABA opens even say 200 channels, this value is considerably less than the 1000-2000 channels activated by a quantum of acetylcholine at the mammalian motor end-plate (e.g. Colquhoun, Large & Rang 1977), but is similar to the number of channels opened by a single quantum of excitatory transmitter on the motoneurone soma (Finkel & Redman 1983, Nelson, Pun & Westbrook 1986) and by Ach in submandibular ganglion cells (Rang 1981). Studies of inhibitory (but glycine-mediated) conductance changes in other vertebrate nervous systems suggest values of 1000-2000 channels per quantum (goldfish Mauthner cells, Faber & Korn 1982, lamprey Muller cells, Gold & Martin 1983a,c). These results suggest that mammalian central neurones use fewer channels per quantum of transmitter compared to the myoneural junction: this is reasonable from a functional point of view since central neurones have higher input impedance than skeletal muscle fibres, so a smaller number of channels need be opened to give a particular voltage increment. The underlying reason for the smaller number

of channels opened by each quantum is not known, but may be:

(a). The 'vesicle' in central neurones may be smaller and consequently contain fewer transmitter molecules, or there may be a lower concentration of transmitter substance inside the vesicle.

(b). The density of channels on the postsynaptic membrane may be lower.

(c). Central receptors may have a lower affinity for their transmitter, or efficacy of transmitter to gate the channel could be lower.

Or perhaps a combination of any or all of the above reasons is responsible for a smaller quantal conductance change (see also Rang 1981). There is presently little data to allow us to distinguish clearly between these possibilties. However, electron microscopy suggests that vesicles in the synaptic terminals of central neurones are roughly similar in size to peripheral junctions (see Uchizono 1975, and Ceccarelli & Hurlblut 1980), with 'inhibitory' quanta being roughly oblong in shape and with dimensions of ~50 x 38nm, and 'excitatory' vesicles ~50 x 50nm. Since it is likely that both central and peripheral vesicles are in osmotic equilibrium with the extracellular environment there may be similar numbers of molecules of transmitter in both kinds of vesicle. (If GABA were present at even 80mM in a 50nm vesicle, there would be approximately 3,000 molecules of transmitter in the vesicle). The patch clamp studies of Sakmann, Bormann & Hamill (1983) of single channel burst length and

number of gaps per burst has led to the suggestion that the affinity of the GABA receptor (K_D) is actually higher than the nicotinc Ach receptor (K_D for GABA 0.5μM, Ach 100μM), suggesting that there may be no real difference in the binding of the agonists to the receptors. Unfortunately, there is no data yet on the density of receptor/channels in the postsynaptic membrane of central neurones. It will be important to extend these studies, and to determine precisely how and why less channels are opened by central 'quanta'.

The rate of decay of IPSCs was observed to be voltage sensitive, τ_D becoming shorter with membrane hyperpolarization with a volt constant (H) of ~140mV. Membrane potential has similar effects on GABA-activated channels in the muscle (Onodera & Takeuchi 1979, Dudel 1977) and stretch receptor neurones (Adams, Constanti & Banks 1981) of crayfish, and in locust extensor tibae muscle (Cull-Candy 1983, 1986). IPSCs in cultured hippocampal neurones are also shortened by membrane hyperpolarization (Segal & Barker 1984b). However, τ_D from noise analysis of GABA-induced current in cultured spinal cord neurones is apparently unaffected by changes in potential (Barker, McBurney & MacDonald 1981). In the present work there was also some indication that the rising phase of hippocampal IPSCs was voltage dependent, with longer rising phases at more positive potentials. Although this result must be treated with caution, similar behaviour has been observed for GABA-ergic transmission in

invertebrates (Dudel 1977, Onodera & Takeuchi 1979, Cull-Candy 1983,1984). This result would imply that the forward rates leading to channel activation were voltage sensitive.

The direction of the voltage sensitivity of GABA channel lifetime in the hippocampus is opposite to that seen for Ach receptors in the periphery (amphibian neuromuscular junction: Takeuchi & Takeuchi 1959, Magleby & Stevens 1972a, mammalian neuromuscular junction: Colquhoun et al. 1977, parasympathetic ganglion: Rang 1981). There are to date only a few results on the voltage sensitivity of channel lifetime in central excitatory synapses, and contradictory results have been reported. Finkel & Redman (1983) found that τ_D of EPSCs from Ia fibres in the cat spinal cord was shortened by hyperpolarization, but recently Nelson et al. (1986) have found that EPSCs in cultured rat spinal neurones have voltage insensitive decays. Brown & Johnston (1983) found that EPSCs in CA3 pyramidal cells evoked by mossy fibre stimulation were lengthened by hyperpolarization. In these central excitatory synapses the transmitter is probably glutamate (or a similar amino acid), and excitatory currents at glutaminergic synapses in invertebrate preparations are usually shortened by membrane hyperpolarization (e.g. Onodera & Takeuchi 1978, Wachtel 1984, Cull-Candy & Miledi 1982, - but see Anwyl & Usherwood 1975 and Magazanik & Vysocil 1979). It would be of interest to examine the possible voltage sensitivity of excitatory synapses in the CNS in more detail.

It is unlikely that the voltage sensitivity of GABA-mediated IPSCs has any functional significance (e.g. enhancing inhibition at more positive potentials by extending channel open-time), since the temperature dependence of the voltage sensitivity is such that the H value becomes greater as temperature is increased i.e. the channels become less sensitive to membrane potential. So at a body temperature of 37^0C, it is unlikely that potentials in the physiological range would affect τ_D to alter the efficiency of inhibition (see also discussion in Section E). The temperature dependence of the voltage sensitivity has been examined in more detail at the amphibian neuromuscular junction, where increases in temperature reduce the degree of voltage dependence (Anderson & Stevens 1973). This leaves the physiological significance (if any) of the voltage sensitivity of GABA channel lifetime unclear. Lester, Koblin & Sheridan (1978) have speculated that the voltage sensitivity of Ach receptors in Electrophorus electroplaques and neuromuscular junctions serves to strip Ach from receptors to prevent a conductance shunt during the generation of the action potential. There would seem to be no corollary for inhibitory potentials which are usually hyperpolarizing in the CNS.

A note on development of inhibition in the hippocampus.

There have been a few studies of the development of synaptic inhibition in various regions of the nervous system, including the hippocampus, and there appear to be marked species differences as to when inhibition becomes functional in the hippocampus. Purpura, Prelevic & Santini (1968) suggested from electrophysiological and anatomical observations that IPSPs were not fully developed in neonatal kittens, but more recently Schwartzkroin & Altschuler (1977) reported very effective IPSPs in immature kitten hippocampal slices. Additionally a fairly large somatic inhibitory input was identified by these authors electron microscopic studies. Schwartzkroin (1982) has found that inhibition develops fairly late in the rabbit hippocampus, and has suggested that this is due to immaturity of the GABA receptor channels in the postsynaptic membrane. In neonatal animals, GABA produces a depolarization at the pyramidal somata, but after 2-3 weeks produces a hyperpolarization (Mueller, Taube & Schwartzkroin 1984). These authors have proposed that the pyramidal cell changes its GABA receptor type with development, but the significance of this change is not yet known.

It has been suggested that inhibition appears late in the development of the hippocampus of the rat (after postnatal day 6: Harris & Teyler 1983, postnatal days 14-17: Dunwiddie 1981, >8 days: Coyle & Enna 1976). Similarly Barnard et al.(1983) found that mRNA coding for GABA receptors could only be obtained in sufficient

quantities from rat brains 14 days after birth. In contrast, we (B. Robertson & Ian Spence, unpublished observations) have found sponaneous IPSCs in rat CA1 pyramidal neurones in animals as young as 4 days old. These IPSCs were simliar in all respects to those observed in mature rats, except that the time constant of decay was longer than adult values, and decreased to adult values after about three weeks of birth. A similar shortening of synaptic current decay occurs at the neuromuscular junction of developing rats (Brenner & Sakmann 1978, Fischbach & Scheutze 1980), and it has been suggested that the transition from 'slow' currents to 'fast' type kinetics is associated with some change in the geometry of the postsynaptic membrane (Brenner & Sakmann 1983)*. It is possible that the increased open times of GABA channels in developing pyramidal neurones somehow increases the effectiveness of inhibitory neurones, perhaps compensating for a lower number of interneurones or their ability to fire repetitively in young animals.

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* It has very recently been shown that the change from 'slow' to 'fast' channel kinetics at the motor end-plate is due to an alteration in the composition of one of the subunits of the ACh receptor channel complex (Mishina et al. 1986). It would be interesting to determine if a similar substitution occured at GABA receptor/channels, and how this is achieved in the neurone.

Effects of anaesthetics and drugs on IPSCs.

Pentobarbitone

The barbiturate anaesthetic pentobarbitone produced a marked prolongation of IPSC decay at low 'clinical' concentrations. Richards (1972) has calculated that 100-200 μM pentobarbitone is sufficient to produce clinical anaesthesia. The results presented here show that even 50 μM pentobarbitone was sufficient to lengthen IPSCs more than three fold. The powerful actions of pentobarbitone and other barbiturates in enhancing synaptic inhibition have been noted many times before. Nicoll(1972) demonstrated that barbiturates could prolong inhibition at concentrations that did not affect the EPSP in the olfactory bulb, and Nicoll, Eccles, Oshima & Rubia (1975) showed that pentobarbital lengthened the evoked and spontaneous IPSPs in CA3 neurones in the cat hippocampus. Scholfield (1978,1980) has also observed prolongation of IPSPs and inhibitory conductance changes in the guinea pig olfactory bulb.

These results suggest that the open time of GABA channels was being increased by barbiturates. Barker & McBurney (1979) found that GABA-mediated currents in cultured spinal neurones were lengthened by phenobarbitone, and their noise analysis measurements showed that this was due to an increase in channel lifetime. Similar observations have been made in

hippocampal neurones with pentobarbitone by Segal & Barker (1984b). These authors found that pentobarbitone increased channel lifetime with no effect on conductance.

It could be argued that the prolongation of the decay of IPSCs is due to inhibition of the GABA-uptake mechanism: this effect of pentobarbitone has been demonstrated at the GABAergic synapse on the crayfish stretch receptor neurone (Aickin & Deisz 1981). Such an action is considered unlikely to be significant here for the following reasons: Jessel & Richards (1977) found no evidence that pentobarbitone reduces uptake at the concentrations employed here. In our own experiments, application of the uptake blocker nipecotic acid (Krogsgaard-Larsen & Johnston 1975) at concentrations of 10 μM and 1mM caused no significant prolongation of IPSCs (B. Robertson & I. Spence, unpublished observations). Brown & Scholfield (1984) could detect no clear change in the duration of recurrent inhibitory conductance with 0.5mM nipecotic acid. Hablitz & Lebeda (1985) found that nipecotic acid produced only a ~20% prolongation of the fast phase of orthodromically evoked IPSCs in guinea-pig CA1 neurones. Dingledine & Korn (1985) reported that uptake inhibitors only slightly prolonged evoked IPSPs.

Finally, noise analysis studies (Study & Barker 1981, Segal & Barker 1984b) have shown that the increase in decay time constant of IPSCs can be adequately explained by an increase in channel open time alone. However, Mathers 1985 has suggested that pentobarbitone increases macroscopic currents by increasing the opening frequency

of GABA channels, but the concentrations of pentobarbitone used could have led to the contamination of single GABA channel records with channels directly activated by the anaesthetic itself (see Section C).

Unfortunately it was not possible to study presynaptic effects of pentobarbitone in the present study. This was a pity, since barbiturates have been reported to decrease the quantal content of monosynaptic EPSPs in the cat spinal cord (Weakly 1969). Since this may be brought about by a reduction in calcium current in the presynaptic terminals, as has been observed in snail neurones (Nishi & Oyama 1983) and cultured spinal neurones (Heyer & MacDonald 1982a), it would have been interesting to determine if a similar phenomenon occured at inhibitory interneurones. From a teleological point of view, one might expect that an anaesthetic which enhances inhibition postsynaptically would not undermine this effect by reducing the release of the inhibitory transmitter presynaptically. However, the effect of barbiturates on the release of inhibitory transmitters still remains to be determined.

Further discussion of the effects of pentobarbitone on GABA-activated channels will be made later, when other effects of this anaesthetic will be examined.

Halothane

Halothane is a volatile anaesthetic widely used in clinical practice, and has effects on both inhibitory and

excitatory transmission. Halothane prolonged IPSC decay in a dose dependent fashion, and had no significant effect on IPSC amplitude or rise time. Nicoll (1972) found that halothane increased the duration of granule cell inhibition of mitral cells in the olfactory bulb in vivo, and Galindo (1969) had previously reported that 2% halothane potentiated the actions of iontophoretically applied glycine and GABA in the cuneate nucleus. Scholfield (1980) reported that low concentrations of halothane resulted in a 1.5-3 fold prolongation of IPSP duration, and that high concentrations depressed IPSPs, EPSPs and action potentials. In contrast, Yoshimara, Higashi, Fujita & Shimazi (1985) have reported that in their experiments, halothane depressed IPSPs at low doses, with no effect on EPSPs. Low concentrations of halothane have been reported to decrease the effectiveness of excitatory transmission in both the periphery (Gage & Hamill 1975, Elliott, Haydon & Hendry 1984) and in the central nervous system (Galindo 1969, Nicoll 1972, Richards 1973) where the synaptic transmitters are acetylcholine and (probably) glutamate respectively. Conventional 'unitary' hypotheses of anaesthetic action suggests that the anaesthetic substance causes an indiscriminate perturbation of the membrane lipid, e.g. by increasing membrane fluidity (Rosenberg 1977), which may reduce τ_d by accelerating the rate of channel closure, as suggested by Gage & Hamill (1975). This hypothesis in its simple form predicts that all channels would be affected in the same way, which is clearly not the case

for the GABA-activated channel since halothane lengthens rather than shortens the channel lifetime.

Ketamine

Ketamine is a dissociative anaesthetic substance, which has quite a different structure from the barbiturates, and is frequently used for general anaesthesia of children and research animals. Ketamine has been found to potentiate GABA-mediated transmission in the olfactory cortex (Scholfield 1980) and superior cervical ganglion *in vitro* (Little 1982). In the present study, ketamine slightly prolonged IPSC decay, suggesting an enhancement of inhibitory conductance by prolonging channel open time. However, the concentrations of ketamine used in the present study (500μM) is higher than the concentrations measured in the brains of rats after i.v. administration of this substance (360μM, Cohen, Chan, Way & Trevor 1973) and it is possible that during ketamine anaesthesia, actions on GABAergic transmission are of minor importance in comparison to effects on other systems.

Lodge & Anis (1982) and Anis et al. (1983) report that ketamine does not potentiate GABA mediated inhibition, and these authors have shown that ketamine selectively acts on excitatory responses in the cortex mediated by N-methyl-d-aspartate receptors. Since this action is observed *in vivo* at doses lower than those required to produce psychotomimetic effects in man, it is

possible that excitatory synapses are the prime site of action for ketamine (Thomson, West & Lodge 1985). Additionally, excitatory cholinergic transmission at the motor end-plate is markedly depressed by ketamine at concentrations much lower than those employed here (e.g. Torda & Gage 1977, Maleque, Warnick & Albuquerque 1981).

Nitrous oxide

Nicoll et al. (1975) used a combination of 75% N_2O and 3% halothane as a baseline to measure the effects of subsequent applications of pentobarbitone on spontaneous and evoked IPSPs in the hippocampus. I was interested in examining if N_2O had any effect on GABA-mediated transmission on its own, particularly since N_2O is one of the simplest molecules that induces anaesthesia/ analgesia. The results obtained showed that N_2O did not prolong IPSC decay, unlike pentobarbitone, halothane or ketamine, and these results would suggest that N_2O is a good choice as a 'control' anaesthetic when investigating the postsynaptic effects of drugs on inhibitory transmission in vivo.

N_2O antagonizes the 'high pressure neurological syndrome', which is manifested as a series of convulsions as animals are exposed to depth or high pressure (e.g. Brauer 1974). It has been suggested that N_2O potentiates GABA responses under pressure, and alleviates the HPNS. However, Little & Thomas (1986) have reported recently that N_2O can transiently potentiate responses

to GABA in the rat superior cervical ganglion at normal pressures, but depressed GABA responses at 1.5 and 3 atmospheres.

It is possible that N_2O depresses excitatory transmission rather than potentiating GABA responses to produce analgesia. In this connection it is of interest to note that N_2O depresses Ach induced responses in cat pericruciate neurones (Catchlove, Krnjevic & Maretic 1972).

Ethanol

Ethanol was applied to hippocampal slices at concentrations between 10 and 200μM to examine possible actions of this drug on $GABA_A$ mediated transmission. Previous results on the effects of ethanol on inhibitory events are contradictory. Nesteros (1980) has suggested that ethanol enhances GABA-mediated transmission, and Newlin, Mancillas-Trevino, Jorge & Bloom (1981) reported that ethanol increased inhibition in the CA3 region. In contrast, Nicoll(1972) reported that ethanol did not enhance synaptic inhibition in the olfactory bulb, but actually antagonized inhibition. Carlen, Gurevich & Durand(1982) and Gruol(1982) also found that ethanol had no effect on GABA-mediated potentials. In a more recent investigation from Bloom's laboratory, (Mancillas, Siggins & Bloom 1986), systemic administration of ethanol had no consistent, significant effect on GABA responses, and any apparent enhancement was artefactual due to repetitive

iontophoretic application.

The negative results obtained here with ethanol also have implications for the lipid dielectric model proposed by Gage, McBurney & Schneider (1975) to explain alcohol action on motor end-plates. Ethanol has been found to increase channel open time at nicotinic neuromuscular junctions, (Gage et al 1975, Quastel & Lindler 1975) and decrease channel open time at crustacean glutaminergic end junctions (Adams, Gage & Hamill 1979, Wachtel 1984). At the nicotinic end plate, membrane hyperpolarization prolongs channel open time, while hyperpolarization at glutaminergic junctions shortens channel open time. Gage et al suggested that ethanol and other short chain alcohols dissolved in the membrane lipid and increased its dielectric constant. The channel closing mechanism is believed to involve movement of a dipole in the membrane field (Magleby & Stevens 1971b) and rate theory models suggest that movement of a dipole is hindered by an increase in the dielectric of the environment (Glasstone, Laidler & Eyring 1941). Therefore channels that would be stabalised in an open conformation by hyperpolarization (i.e. Ach channels) would remain open for longer in ethanol solution, while channels with the opposite voltage sensitivity (i.e. glutamate synapses in crustacea) would have shorter durations in ethanol. (But see the opposite results of Magazanik & Vysocil 1979). This model predicted that ethanol would shorten GABA-mediated IPSCs in the hippocampus, since channel lifetime here is shortened by membrane hyperpolarization. However, no

effect on τ_d was observed. Similar results to these have been obtained at the neuromuscular junction of the crayfish. Finger & Stettmeier (1984) also found that ethanol has no effect on GABA-mediated IPSCs, and the voltage sensitivity of these channels is similar in magnitude and direction to those in hippocampal neurones. The lack of any effect of ethanol on the voltage sensitive IPSC decay weakens the general applicability of the dielectric theory.

Caffeine

Caffeine is a methylxanthine with well known central stimulatory activity, but the basis for this central action is still obscure. Relatively high concentrations with respect to 'physiological' levels are required to block phosphodiesterase activity and release intracellular calcium (see Snyder 1981). There are other, possibly more realistic theories which suggest that caffeine produces its central stimulatory effects by antagonizing adenosine actions on purine receptors (Snyder 1981), or altering benzodiazepine receptor function (Polc et al. 1981). Nistri & Berti (1983) suggested that these two actions might be linked and proposed that caffeine could be acting at the GABA receptor complex. These authors reported that low concentrations of caffeine enhanced GABA responses in the frog spinal cord, while higher concentrations (up to 1mM) antagonized GABA responses. It was of particular interest to examine any possible

blocking action of caffeine, since there are very few effective 'blockers' of GABA channels, in contrast to the vast number of 'blockers' of the Ach channel for instance. Any blocking action could be investigated quantitatively using the time course of decay of IPSCs as a measure of blocking kinetics. However, caffeine was found to be ineffective on hippocampal IPSCs at concentrations between 10 μM and 5mM. It is possible that this result may reflect a difference between the GABA receptor/channels of central mammalian neurones and those present on amphibian dorsal and ventral roots (see also the general discussion in Section E). Alternatively, it is possible that caffeine was having another action on other channels in the amphibian nerve trunks to apparently influence GABA responses.

THE EFFECTS OF FOREIGN ANIONS ON IPSCs.

The results presented here suggest that the anions sulphate, methylsulphate, acetate and citrate are fairly impermeable through central GABA receptor/channels, while chloride, bromide, iodide, nitrate, azide, cyanate, thiocynate and chlorate ions are significantly permeable. In this regard, these results are similar to those

obtained by Eccles, Nicoll, Oshima and Rubia (1977) for CA1 neurones in the cat hippocampus *in vivo* and more recently by Biscoe & Duchen (1985) recording from CA1 neurones in mouse hippocampal slices. In contrast, Kelly, Krnjevic, Morris & Yim (1969) examined the selectivity of evoked (presumably GABA-mediated) IPSPs in cat cortical neurones, and suggested that these channels were permeable to glutamate, acetate, and other large anions. However, the study of Kelly et al. suffered from artefacts associated with instability of recording conditions and contamination of IPSPs by excitatory potentials. The measurement of spontaneously occuring IPSCs avoids problems associated with field potentials and EPSP contamination, or other components of inhibitory potentials.

The results obtained from hippocampal neurones would indicate that the Cl^- channel activated by the inhibitory channel here has similar permeability properties to glycine-activated Cl^- channels in the mammalian spinal cord, which have been the subject of intensive study *in vivo* (Coombs, Eccles & Fatt 1955, Araki, Ito & Oscarsson 1961, Ito, Kostyuk & Oshima 1962). Ito et al. (1962) found that anions with a hydrated diameter less than chlorate were permeant (neglecting the anomalous behaviour exhibited by bicarbonate and hydrogen sulphide), and excluded large anions such as citrate and acetate.

Unfortunately, it was not possible in the present study to examine the absolute permeabilities of these

anions relative to Cl^- ions, since it would be impossible to determine the concentration of the anions inside the neurone, and the reversal potential would vary according to the different holding potentials. The present study was therefore limited to assessing wether certain anions were permeable or not, and examining the effects of different anions on IPSC decay time course, and where possible, voltage sensitivity.

TIME COURSE AND VOLTAGE SENSITIVITY.

The decay of IPSCs was longer with iodide and thiocyanate (and possibly with nitrate and chlorate ions also) compared to IPSCs generated by chloride, while currents generated by movement of cyanate, azide and bromide ions were all of roughly similar duration or slightly shorter than those in chloride. It is difficult to compare τ_D's for these different anions very accurately, because of the normal scatter in τ_D values with chloride-mediated IPSCs, but the following rough sequence may be derived from the results:

$$I > SCN > ClO_3 > NO_3 > Br \approx Cl > OCN \approx N_3$$

This sequence was constructed by using the average value for τ_D at -80mV for each anion. Since the decay phase is voltage insensitive for some of the anions

tested, this sequence will also be voltage sensitive i.e. the relative positions of the anions will change, depending on the membrane potential chosen. This sequence given above is similar to sequence 1 of the Eisenman series (see Wright & Diamond 1977).

The most reliable results are those obtained with thiocyanate and iodide. Iodide has been observed to produce prolonged IPSCs in other chemically-activated chloride channels, in the buccal ganglion of Aplysia (where Ach is the transmitter, Adams et al. 1982) and at the neuromuscular junction of the crayfish (Onodera & Takeuchi 1979). There have been no other studies of SCN on channel kinetics apart from the present report, but this anion is one of the most potent in the lyotropic series (e.g. Dani, Sanchez & Hille 1983).

Variations in the duration of agonist-induced channel lifetime with different anions have been observed previously in a variety of preparations and further discussion of this effect and possible causes of these actions will be made later (see discussion in Sections C and E).

The voltage dependence of τ_D also depended on the nature of the permeant ion, as has been noted before (Van Helden, Hamill & Gage 1977, Marchais & Marty 1979, Gage & Van Helden 1979, Adams, Gage & Hamill 1982). In the present study, voltage sensitivity was reduced in iodide, bromide and nitrate. This is most clearly illustrated with iodide in fig. 23. Adams et al. (1982) also observed abolition of voltage sensitivity in iodide

solutions, but Onodera & Takeuchi (1979) found that the voltage sensitivity of IPSC decay was similar in chloride and iodide solutions. Because of these unusual effects, further experiments with anion substitution were carried out in dorsal root ganglion neurones, where it is possible to control both the ionic composition of the intra and extracellular environments more accurately and easily. The results and interpretation of these experiments will be made in later chapters.

It proved extremely difficult to record IPSCs mediated by azide ions, since cells died very quickly when this anion was injected. A similar phenomenon has been noted previously (Araki, Ito & Oscarsson 1961) and may be due to the deleterious effects of this ion on cell metabolism (Ling & Gerard 1949). It was also difficult to record from cells for long periods of time with electrodes containing thiocyanate and cyanate, which could also have been caused by an inhibition of cell respiration.

In summary, the study of spontaneous postsynaptic currents activated by GABA in hippocampal neurones has revealed some of the basic properties and characteristics of GABA receptor/channels in mammalian central neurones. The decay of IPSCs is affected by membrane potential, some clinical anaesthetics, and the nature of the permeant anion. How each of these actions are exerted are not yet clear.

SECTION C :

GABA CHANNELS IN DORSAL ROOT GANGLION NEURONES

SECTION C: INTRODUCTION.

The study of GABA-activated channels in pyramidal neurones provided some useful information regarding the voltage sensitivity of the channel open time, its temperature dependence, and data concerning the permeability of foreign anions and the effects of these ions on the kinetic properties of the chloride channel. Also some data on the effects of a few anaesthetics and drugs was obtained. However, I became unhappy with the limitations that the hippocampal slice preparation imposed on the study of the biophysics and pharmacology of GABA channels. For example, it was extremely difficult to change solutions around cells quickly, and to apply precisely known concentrations of drugs to cells because of the considerable diffusion problems in the interface brain slice. Additionally, there are major problems in effectively space clamping the membranes of central neurones due to their extensive dendritic trees (see e.g. Rall 1977, Brown & Johnston 1983, Rall & Segev 1985). Since these dendritic processes have both synaptic GABA receptors and voltage activated conductances (which may also be modulated by GABA - see e.g. Alger & Nicoll 1982 and Gahwiler & Brown 1985), this would lead to incompletely clamped conductance changes during voltage jump or noise analysis experiments which would hinder interpretation of somatic GABA channel kinetics (see e.g. Finkel 1983). Another major problem encountered in

voltage clamping was that relatively high resistance electrodes had to be used to record from CA1 neurones to avoid excessive damage, and this curtailed single electrode clamp switching rates to a maximum of about 17kHz, and allowed only a few nanoamps of current to be passed. Additionally, it was extremely difficult to control the concentrations of internal ions -this meant that it was not possible to measure the relative permeabilities of anions through synaptic chloride channel. Internal anion concentrations depended on microelectrode resistance, and how much current was being passed through the electrode to set the membrane potential at the desired value. A further, perhaps more trivial reason for discontinuing the hippocampal experiments was the frustration experienced in searching for neurones and trying to hold them through various solution changes and balance the advantages of low resistance electrodes against the difficulties in holding cells. For all of these reasons, as well as the desire to examine and compare GABA channel properties in other mammalian neurones, the experimental preparation was changed to neurones from the dorsal root ganglion.

Dorsal root ganglion neurones and presynaptic inhibition.

Sato & Austin (1961) were the first to report electrophysiological recording experiments in mammalian dorsal root ganglion neurones. Subsequently there has

been some interest in this preparation, especially since dorsal root ganglion neurones may be readily impaled, and one can characterise the type of afferent fibre it supports. This is especially useful since it is as yet impossible to record from the central terminations of these neurones in the spinal cord.

Primary afferent neurones are subject to a form of control known as presynaptic inhibition, which causes a reduction in the release of excitatory transmitter from neurone terminals. Frank & Fuortes (1957) were the first to describe reductions in the amplitude of Ia EPSPs without any detectable change in postsynaptic (motoneurone) conductance. Eccles and his collaborators (Eccles, Eccles & Magni 1961, Eccles, Schmidt & Willis 1963a,b) extended this work and suggested that presynaptic inhibition was produced by GABA acting to depolarise primary afferent terminals. In vitro studies showed that GABA application caused depolarization of primary afferent fibres in the amphibian spinal cord (e.g. Curtis, Phillis & Watkins 1961, Schmidt 1963, Davidoff 1972, Barker & Nicoll 1973), and the GABA-mediated depolarization model of presynaptic inhibition has received widespread support (see e.g. Schmidt 1971, Curtis 1978). The cell bodies of primary afferent fibres in the spinal ganglia are also depolarized by GABA, in both amphibia (Hattori, Akaike, Oomura & Kuraoka 1984) and mammals (DeGroat 1972, DeGroat et al. 1972, Feltz & Rasmisky 1974, Gallagher, Higashi & Nishi 1978), and it is believed that studies of GABA receptor channel complexes in these neurones may provide

fundamental information about the mechanisms of presynaptic inhibition (e.g. Feltz & Rasmisky 1974).

Dorsal root ganglion neurones as an electrophysiological preparation.

Dorsal root ganglion neurones, both in freshly isolated and cultured form, have been used by many groups for electrophysiological studies. For reasons discussed above, there have been several studies of GABA-mediated chloride responses in this preparation, and because GABA causes a depolarizing response in these cells then the intracellular chloride concentration has to be maintained by an active chloride pump and electrophysiological investigations into the nature of this pump have begun (Gallagher, Nakamura & Shinnick-Gallagher 1983a, Alvarez-Leefmans, Gamino & Giraldez 1986).

There have been many studies of the voltage-activated conductances present in these neurones (e.g. Kostyuk, Veselovsky & Tsyndrenko 1981, Dunlap & Fischbach 1981, Carbone & Lux 1984). Additionally, there has been a good deal of interest in these neurones as a model for a novel kind of GABA-mediated effect which may be involved in presynaptic inhibition. It has been shown that drg neurones possess $GABA_B$ receptors which are activated by baclofen (β-p-chlorophenyl GABA) and are resistant to the classical GABA antagonists bicuculline and picrotoxin (see Bowery 1983 for a review of the properties of this receptor). Activation of these receptors results in a

change in the voltage activated Ca current (Dunlap 1981, Deisz & Lux 1985) or cause an increase in potassium conductance, as suggested by Desarmenien, Feltz, Occhipinti & Santangelo 1984. A change in either of these currents in primary afferent terminals could greatly alter transmitter release, and $GABA_B$ receptors have been suggested to mediate in another form of presynaptic inhibition.

Many of the studies of channels in drg neurones have been carried out on neurones maintained in cell culture. These cells were amongst the first neurones to be successfully cultured (Crain 1956) and the stability in culture coupled with their relatively large size has made these cells very amenable to study with two microelectrode voltage clamp (e.g. Gallagher et al 1983b). However in cell culture drg neurones begin to develop processes (Ransom, Neale, Henkart, Bullock & Nelson 1978) and since there is the strong possibility that changes in receptor/channel profile occur with time in culture (see e.g. Dunlap 1981, who found alterations in the numbers of $GABA_A$ receptors with time in culture) freshly isolated DRG neurones were used for the present experiments.

The suction electrode technique.

The ability to change the intracellular solution of a cell allows the control of the concentration of permeant ions, and the application of 'blockers' of unwanted ionic conductances to the cytoplasmic surface. The dialysis

technique was originally devised by Baker, Hodgkin & Shaw (1961) and Oikawa, Spyropoulous, Tasaki & Teorell (1961) for squid giant axons, but methods for the perfusion of nerve cell bodies were not developed until much later (Kostyuk, Krishtal & Pidoplichko 1975). The original technique of Kostyuk et al. involved placing a single neurone into a small hole between two chambers - one half of the cell was destroyed, effectively leaving only one layer of cell membrane between the two chambers. Each compartment formed part of the voltage clamp circuit, and the cell could be perfused on either the intracellular or extra-cellular sides. The experiments reported in this section used the alternative cell dialysis technique developed by Lee, Akaike & Brown (1977, 1978, 1980). In this system, a large heat polished micropipette is used to aspirate the neurone, and after rupturing the membrane under the electrode lumen the cell could be perfused and voltage clamped. Adult drg neurones must be enzyme treated to allow high resistance seals to be formed between the electrode and the cell membrane, and this treatment also facilitated the separation of the cell soma from its axon. By using the suction electrode technique in conjunction with the single electrode clamp, it becomes possible to isolate and faithfully measure the chloride conductance change induced by GABA.

The voltage jump relaxation technique.

Results shown in Section B showed that the open time of GABA activated channels in the hippocampus is voltage sensitive. It was considered that it might be possible that GABA channels in neurones in the drg would also have voltage sensitive kinetics, and therefore be amenable to the voltage jump relaxation technique. This method was first employed by Adams (1975), Neher & Sakmann (1975) and Sheridan & Lester (1975) and was derived from perturbation methods originally used in organic chemistry.

In the presence of agonist, channels will exist in an equilibrium between open and closed states. If, during this current, the membrane potential is suddenly stepped to a new value, there will be a 'relaxation' (or re-equilibration) of the channels to a new equilibrium dictated by the new potential. The characteristics of the amplitude and the time course of the relaxation reflect the kinetic processes underlying channel activation.

In this section results will be presented on the basic properties of GABA channels in drg neurones, and how channel kinetics are altered by different agonists (including clinical anaesthetics) gating the conductance. The effects of anions other than chloride on the time course and permeability properties of GABA channels is further investigated, and the basic properties of GABA channels is also discussed.

SECTION C: METHODS.

PREPARATION.

Dorsal root ganglia obtained from adult rats (Wistar strain) and cats (JCSMR animal house) were used for the majority of the results reported in this section. Preliminary experiments on the ganglion preparation were carried out on ganglia obtained from neonatal rats, 3-25 days old. The rationale for using young animals was that there is less connective tissue around the neurones in neonates, facilitating high resistance seals with the suction electrode technique. Another advantage of younger animals is the comparative ease with which dorsal laminectomy could be performed because the vertebrae are much softer than in adult animals. Neurones from younger rats had the normal complement of voltage activated conductances and proved to be very robust, but neonatal drg neurones proved unsuitable for the GABA experiments, since these neurones seldom responded to GABA. However, neurones obtained from adult (~150g) rats responded well to GABA and other GABA-mimetic agonists, and the switch to older rats was made to supplement the experiments with cat ganglia.

Dorsal root ganglia were removed fom rats in the

following manner. Animals were stunned and had their necks dislocated using a long metal rod or scissors. The skin covering the backbone was cut and peeled away, and the muscles attached to the vertebrae were removed. Once the vertebrae were exposed, a dorsal laminectomy was performed using Skidmore bone rongeurs, being careful not to cause damage to the spinal cord or dorsal and ventral roots in the process. The laminectomy extended from about the mid thoracic region to low lumbar segments. The spinal cord was then gently lifted out of the split backbone while cutting dorsal and ventral roots as close to and as high up the cord as possible (thus leaving roots to ease further dissection and handling). Ganglia were dissected out fom between the vertebrae using fine forceps and scissors under an Olympus dissecting microscope at 40x magnification. Chilled standard solution was used throughout the dissection.

Cat dorsal root ganglia were kindly supplied by Dr. Robert Fyffe of the Experimental Neurology Unit at JCSMR. Laminectomies were often performed on pentobarbitone - anaesthetised cats in the course of studies on the anatomy and neurophysiology of the spinal cord. This allowed me to obtain ganglia from these animals up to four times a day. Cat dorsal root ganglia were removed under microscopic control and immediately placed into chilled standard solution. The tough connective tissue sheath surrounding each ganglia was removed under the dissecting microscope with microscissors and fine forceps. Cat

dorsal root ganglion neurones responded well to GABA, and electrophysiological procedures were made easier because of the large diameter of cat neurones.

In the course of this study, dorsal root ganglia from three adult macaque monkeys (Macaca nemestrina and Macaca fascicularis) also became available. The ganglia from monkeys were approximately the same size as cat ganglia, though the individual neurones were generally smaller. It was thought worthwhile to study GABA responses in primate neurones in order to get a physiological preparation as close to man as possible. Unfortunately however, only 4 of over 20 monkey neurones responded to GABA, and even then only very weakly (< 1.6). GABA experiments on this preparation were subsequently discontinued, although other experiments were undertaken on the voltage activated conductances in these primate neurones.

A similar situation was found when ganglia were obtained from rabbits and neonatal and adult guinea pigs; very few of the neurones from these species responded well enough to GABA to be of much use experimentally.

In summmary therefore, there seems to be both developmental and species differences in the GABA sensitivity of dorsal root ganglion neurones. Rat neurones acquire GABA receptor/channels at some point during their development, after 25 days and before 100 days. GABA receptors exist with a fairly high density on

cat and (adult) rat neurones, but have a considerably lower density on rabbit, guinea pig and monkey dorsal root ganglion neurones.

Subsequent to removing most of the thicker connective tissue, ganglia were pinned out by their nerve trunks onto the Sylgard (Dow Corning) base of the recording bath (see Fig. 28) with small insect pins. Under the 80x power of the Olympus microscope, the rough outline of neurones inside the ganglionic capsule could be seen. The preparation was then exposed to enzymes for 1 hour at 30^0C, in order to remove most of the connective tissue around the neurones and thereby cleaning the neuronal membrane for the suction electrode technique. In the initial stages of this work a suitable protocol for enzyme treatment had to be devised - various combinations and concentrations of enzymes and incubation temperatures were tried before the 'optimum' treatment was found: this was a 50:50 mixture of 0.5% trypsin (Type IX Sigma) and 0.5% collagenase (Type 1A Sigma) at 30^0C. Electron micrographs of enzyme treated rat drg neurones are shown in Fig. 30. Enzyme treatment cleaned the membranes of connective tissue, and removed the neighbouring glial (satellite) cells.

After the ganglia had been exposed to the enzyme in standard solution for 1 hour, the enzymes were completely washed out of the bath with 20ml of standard solution

before recording was started. It was then possible to see individual cells protruding from the ganglia quite clearly with 80x magnification. Enzyme treatment was sufficient to permit seals between the cell membrane and suction electrode tips of >20MΩ.

A special recording bath was designed for these experiments and was constructed by the Physiology Mechanical Workshop (see Fig. 28). The preparation was illuminated from below, and the whole bath was mounted on a peltier device with temperature controller. Attempts were made to reduce bath volume and dead space wherever possible in order to obtain rapid solution changes. The bath volume was approximately 0.4ml (most of which was taken up by the cat ganglia) with a maximum of 0.5ml dead space between a solution multiplexer switch and the recording chamber. Quite rapid perfusion rates were achieved - the rise time of a large GABA-induced current response was between 1 and 2 seconds. This fast perfusion had the vital advantage of revealing channel 'unblocking' by some of the anaesthetic agonists. This phenomenon would have been so small and slow as to be invisible if perfusion rates had been slower.

Experiments were performed at 21^0C unless otherwise noted.

SOLUTIONS AND DRUGS.

A standard solution containing (in mM) was

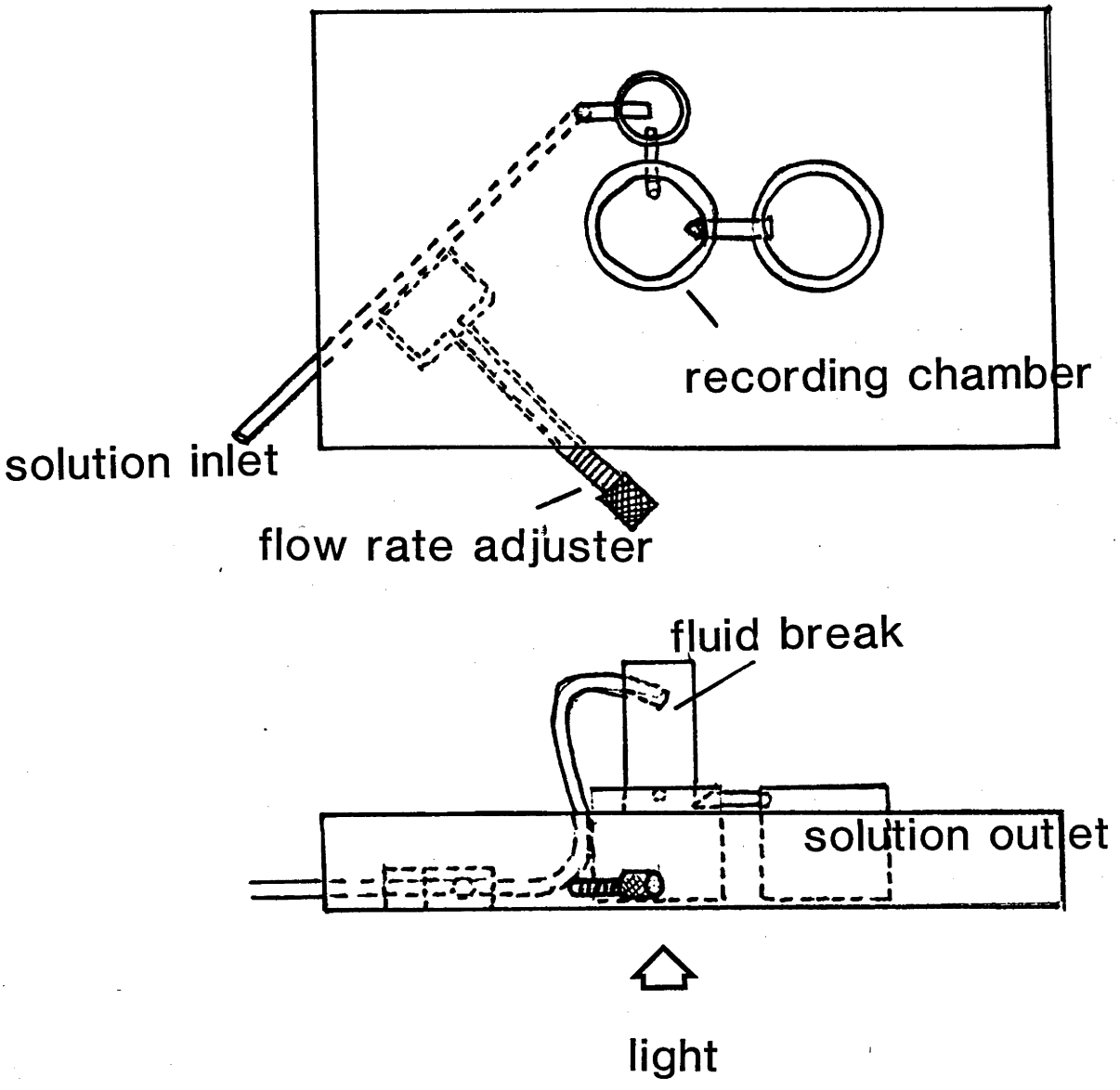


FIGURE 28: Top and side views of the recording chamber used for the dorsal root ganglion experiments. The distance between the solution multiplexer tap (not shown) and the recording bath was deliberately kept short. The main recording chamber (centre) was 2mm deep, and individual drg neurones were moved as close to the solution inlet as possible, to maximize solution change rates. Flow rate was adjusted by means of a screw and block to increase the resistance of the inlet tubing. The whole bath assembly was mounted on a peltier temperature control apparatus.

developed:

NaCl 140; $CaCl_2$ 5; KCl 2; N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 5; glucose 10; pH 7.4.

The most commonly used solution for studying GABA-induced chloride responses was: choline chloride 135; CsCl 5, $MgSO_4$ 10; HEPES 5; glucose 10; pH 7.4.

For the foreign anion experiments, choline chloride was replaced with the appropriate amount of choline bromide or iodide.

External solutions were made up before each experiment.

The routine suction electrode (internal) solution contained (in mM):

CsCl 140; HEPES 5; ethyleneglycol-bis-(β-amino ethytether)-N,N-tetra-acetic acid (EGTA) 2; CsOH 5; pH 7.4. The internal solution was stored at 4^0C.

GABA (γ-aminobutyric acid). (See structure in Appendix A). This was obtained from Sigma and B.D.H., and was made up before each experiment as a 2mM stock before being diluted into the experimental solution.

Muscimol (5-aminomethyl-3-hydroxylisoxazole). (See Structure in Appendix A). This was obtained from Sigma and was made up in a 2mM stock and stored at 4^0C.

Picrotoxinin and Bicuculline methiodide (Structures in Appendix A) were obtained from Sigma and were made up freshly for each experiment at 10 or 50μM.

Sodium pentobabitone (structure in Appendix A) was supplied by Prosana or Abbott (courtesy of Professor D. Curtis).

α-Chloralose (Structure in Appendix A) was manufactured by Fluka and was a gift from Professor W. Levick. Chloralose could be made up to the appropriate concentration after vigorous stirring for thirty minutes.

Piperidine-4 sulphonic acid (P4S) (structure in Appendix A) was a gift from Professor Curtis and was dissolved after prolonged shaking.

Ethylenediamine monocarbamate (EDAMC) (structure in Appendix A) was made up following the procedures of Curtis & Malik (1984): further details are given in the Results.

Taurine and Glycine were purchased from Sigma.

Baclofen (β-p-chlorophenyl GABA) hydrochloride was a gift of CIBA-GEIGY Switzerland. This compound was stored at $4\,^{0}C$ as a 2mM stock.

Etomidate (Janssen Pharmaceuticals) was kindly gifted by Professor D. Curtis, and could be dissolved at low concentrations in the experimental solution after vigorous shaking. The structure of this anaesthetic is shown in Appendix A.

Avermectin B1a was a gift fom Merck Sharp and Dohme Laboratories. (Structure in Appendix A). This substance is insoluble in water, and was made up in 2% dimethyl sulphoxide to give a final concentration of 10μM and 0.1% DMSO for experiments.

Alphaxalone was a gift from Glaxo Group Research, Greenford, Middlesex. The structure is shown in Appendix A. This steriod anaesthetic was dissolved in 0.2mM ethanol and diluted for the experiment to give a final concentration of 0.01mM ethanol.

ELECTRODES AND VOLTAGE CLAMP.

Suction electrodes were fabricated from 1.5mm (O.D.) microhaematocrit borosilicate glass (Vitrex, Modulohm I/S Denmark) in the following manner. Electrodes were pulled on a David Kopf (model 700C) puller (without solenoid) to give an initial tip diameter of about 3μm.
Microelectodes were scored against a glass cover slip under the 20x power of a dissecting microscope. The electrodes were broken back along the score to give an outside diameter of about 80μm. The broken tip was then heat polished on a microforge (Narishige) to give a final electrode diameter of approximately 10μm. Electrode resistance was usually 0.3MΩ when filled with the intracellular solution given above. The suction electrodes were mounted in commercial suction holder (EH-

2S, Clark Electromedical Instruments) machined out to 1.5mm inside diameter. This in turn was mounted directly into the headstage of the ASF-2 current and voltage clamp system (Axon Instruments Inc.). No electrode shielding was employed. Suction was applied from a 50ml glass syringe and portex tubing connected via a three way tap. Usually, suction electrodes could be used to record from several cells before blocking with debris or building up a tissue residue on the tip which prevented proper electrode sealing. A photograph of the suction electrode sealed onto a drg neurone is shown in Fig. 29.

Bath electrodes were made from Clark Elecromedical 1mm glass (fibre filled), bent at $90^0$and filled with a 3M KCl / 3% agar solution by boiling the tubing in the electrolyte solution for at least 10 minutes. Bath electrodes were stored in 3M KCl between experiments, and were mounted in a 1mm (inside diameter) perspex holder with a silver/silver chloride pellet.

Single electrode voltage clamp switching rates of between 60 and 65 kHz were achieved because of the low electrode resistance and through by reducing electrode tip capacitance by drawing the cell up to the solution surface. Clamp feedback gain was set at 100nA/mV and phase lag was introduced to achieve maximal clamp speed while preventing 'ringing'. The clamp was capable of passing ± 240nA, with the voltage traces settling completely in less than 250μs in response to a 100mV hyperpolarizing pulse.

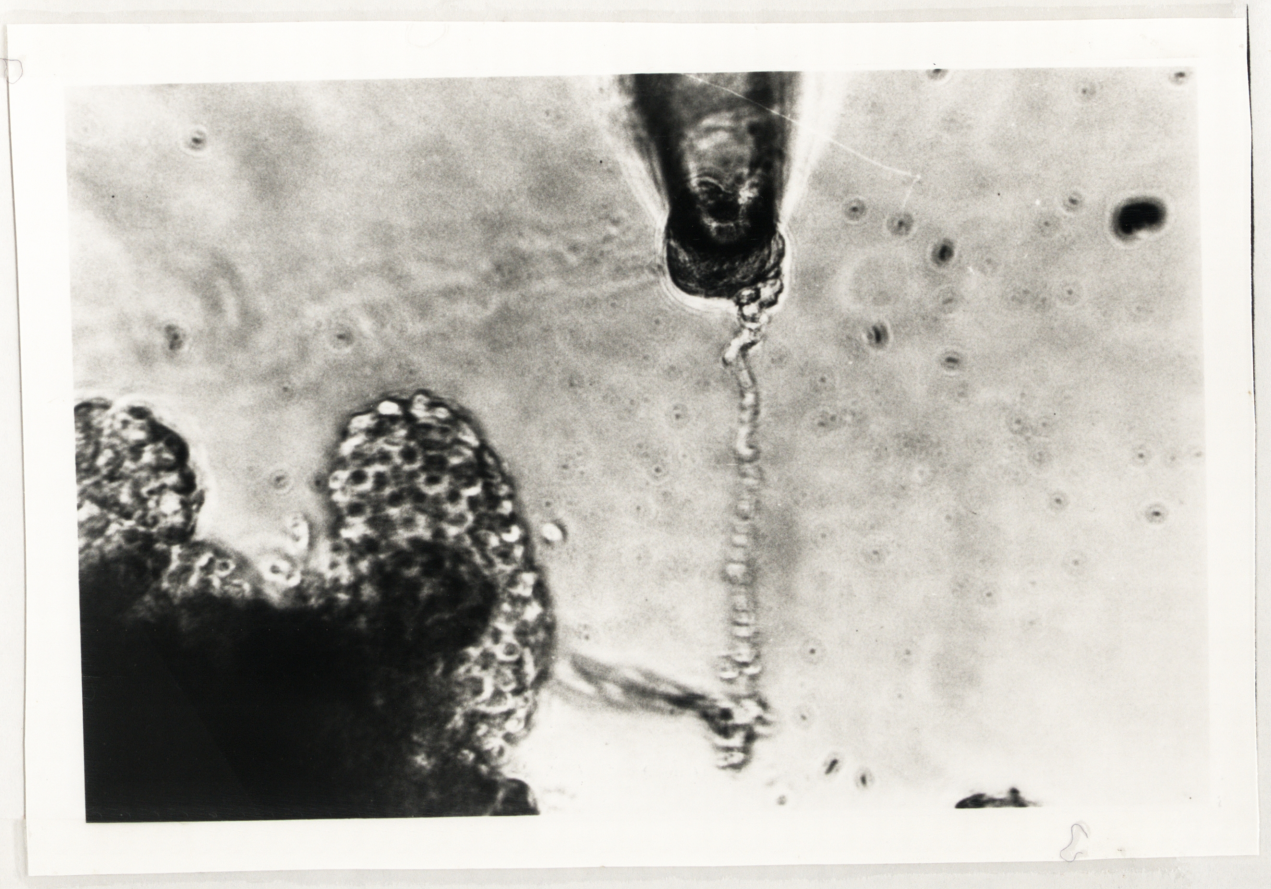
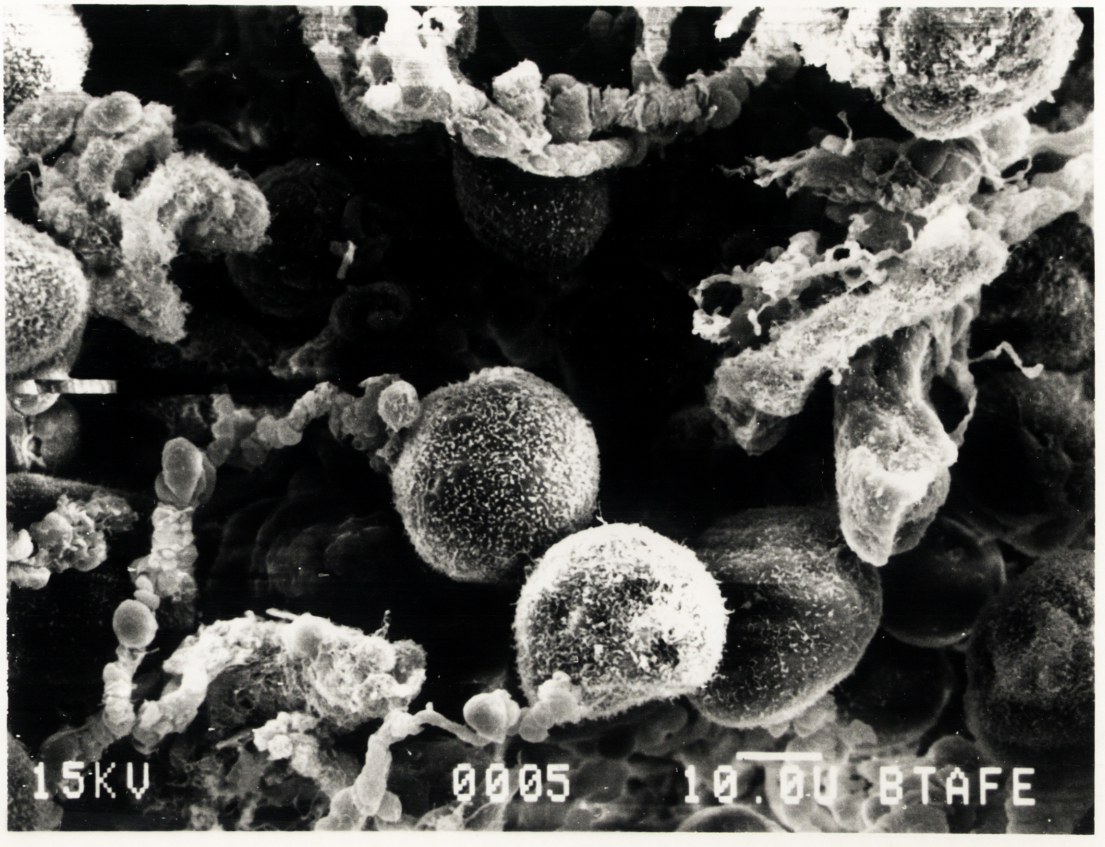


FIGURE 29: A suction electrode (top) sealed onto a cat drg neurone. There is a length of axon attached to the cell body. Observe the size of the electrode lumen in relation to the cell diameter. (~30 μm).

FIGURE 30: Electron micrographs of enzyme treated rat dorsal root ganglion neurones. The somatic membrane is clear of connective tissue and satellite cells. The calibration bar in each picture is 10μm. (These photomicrographs were kindly produced by Katherine Newell).


15KV
0005
10.0U BTAFE


15KV
0004

When the suction electrode tip was pressed against the cell membrane, application of steady suction would generally result in an electrode/membrane seal of 20 to 60MΩ. Often the cell membrane in the electrode lumen would break down spontaneously after the seal formed, providing immediate access to the cell interior, but usually it was necessary to apply a large, brief depolarizing current pulse (100nA) to rupture the cell membrane. The cell soma was then pulled away from the ganglia to the solution surface - this usually resulted in the axon breaking off. Occasionally a length of axon remained attached to the soma, and these cells were rejected if unwanted ionic conductances were present. Cell resting membrane potentials of between -40 and -60mV were obtained in standard solution, and cell input resistances were usually greater than 20MΩ in the standard solution.

Voltage traces were amplified 10x at the voltage clamp amplifier and fed into the analogue to digital converter in the microcomputer. Current traces were amplified 10x at the voltage clamp, and a further 5-10x on a Bioamp system, which also introduced a 1-sec A.C. coupling and a Bessel 4-pole filter set at 2kHz. Current and voltage signals were digitized on line (usually at 400μs per point) and stored on magnetic disc.

Early experiments were done using a 6809 (Motorola) based microprocessor to apply voltage pulses and capture data, but the vast majority of the results reported here

were obtained using a PDP 11/23 microcomputer. An automatic current subtraction of control (leak) traces from agonist induced current traces was performed during the experiment - this enabled immediate assessment of the condition and suitability of a cell for experimentation. Data was subsequently analysed and plotted on a PDP 11/44 computer. The amplitudes of the instantaneous and steady state currents and fitting of exponentials were performed the 11/44 computer. The decay of the current relaxations used a Levenberg-Marquardt algorithm, which minimizes the sum of the squared deviations in generating the best exponential fit to the data points.

SECTION C: RESULTS.

GABA.

Voltage jump experiments were carried out in symmetrical chloride solutions. CsCl was used for internal perfusion - caesium ions blocked all of the potassium conductance in the soma, but could not block all of the potassium channels if there was a significant length of axon attached to the cell. Addition of 10mM TEA chloride to the intracellular solution was still insufficient to block axonal potassium conductance. For this reason, cells were rejected if they had a significant amount of axon attached, or where internal caesium was not sufficient to block all of the outward potassium current remaining in the cell. Outward potassium current resulted in spurious relaxations at positive potentials. This outward current would often be labile and consequently not subtract out properly. The contaminating current also frequently had an unusual and irregular time course, presumably due to voltage and time dependent block of potassium channels.

Preliminary experiments on GABA with voltage jump relaxations were carried out in a 'symmetrical' CsCl solution so that the chloride equilibrium potential was theoretically 0mV. However voltage jumps in the absence of agonist in CsCl solutions showed unusual relaxations.

One likely explanation is that Cs^+ externally is permeable through either sodium, calcium or even potassium channels (or some non-selective channel). This behaviour was seen in 8 cells examined in symmetrical CsCl solutions. Additionally high concentrations of caesium ions were found to directly activate a voltage sensitive chloride conductance, which would contaminate GABA induced responses (see Section D). It was not possible to use the normal NaCl solution externally because of the existence of two classes of inward sodium currents in these neurones (see Robertson & Taylor, Appendix D). TRIS chloride was also used initially, and gave 'leak' currents only, without unwanted voltage activated conductances. The main problem with TRIS ions externally however was due to the powerful H^+ buffering action of TRIS. To set the pH of the external solution correctly several mls of 10M HCl were required. This meant that it was harder to achieve a precisely known concentration for Cl^- externally and it was desirable to have a uniform equilibrium potential for Cl^- when comparing different cells. Finally, and for all of the experiments reported here, choline was used as the main external cation. Choline is a large fairly impermeable molecule and there is no problem adjusting the pH of the external solution since choline is a very weak buffer. Choline also has the advantage that it is fairly easy to obtain the bromide and iodide salts commercially. This latter property is particularly useful for anion substitution experiments. Choline is a weak agonist for muscarinic and nicotinic

receptors but no Ach-activated conductances have yet been observed in drg neurones and no unusual current relaxations were observed in choline solutions. The principal disadvantage of having different cations in the internal and external solutions is that the chloride activity coefficient can be dissimilar in each solution. This means that although the chloride concentration in each solution was 140mM, the presence of different principal cations (as well as the unequal distributions of EGTA, HEPES etc.) would result in quite different activities for chloride, and a non-zero equilibrium potential. In the experiments here, a slightly positive null potential was obtained (see e.g. Figs. 34A & 35) implying that the Cl activity in the internal (predominantly Cs) solution was higher than that in the external, predominantly choline, solution. Measurement of Cl activities with a chloride sensitive electrode (ION83 Ion Analyzer, Radiometer, Copenhagen) gave activity coefficients of 0.71-0.75 for the external solution and 0.86-1.0 for the internal solution, giving a chloride equilibrium potential +5 to +14mV. This compares favourably to the measured equilibrium potential for GABA-activated Cl channels obtained in the present experiments.

GABA Application and Voltage Jumps.

The ionic currents in response to depolarizing and hyperpolarizing voltage jumps in the solution used to

record GABA activated currents (hereafter called the control solution) in the absence of agonist were consistent with a leak conductance only, and showed no sign of voltage activated conductances. Often cells showed 'ragged' currents in response to extreme hyperpolarizing steps, suggesting that the cell became leaky during the voltage pulse. These cells were rejected. A holding potential of -40mV was used for most of the experiments reported here. This potential was chosen because: a) it was sufficiently far from the chloride equilibrium potential to give a good indication of the effectiveness of GABA on a particular cell and b) it was approximately half-way between the maximal hyperpolarizing and depolarizing voltage steps, so that excessively large pulses were not required to step to the positive and negative potential limits.

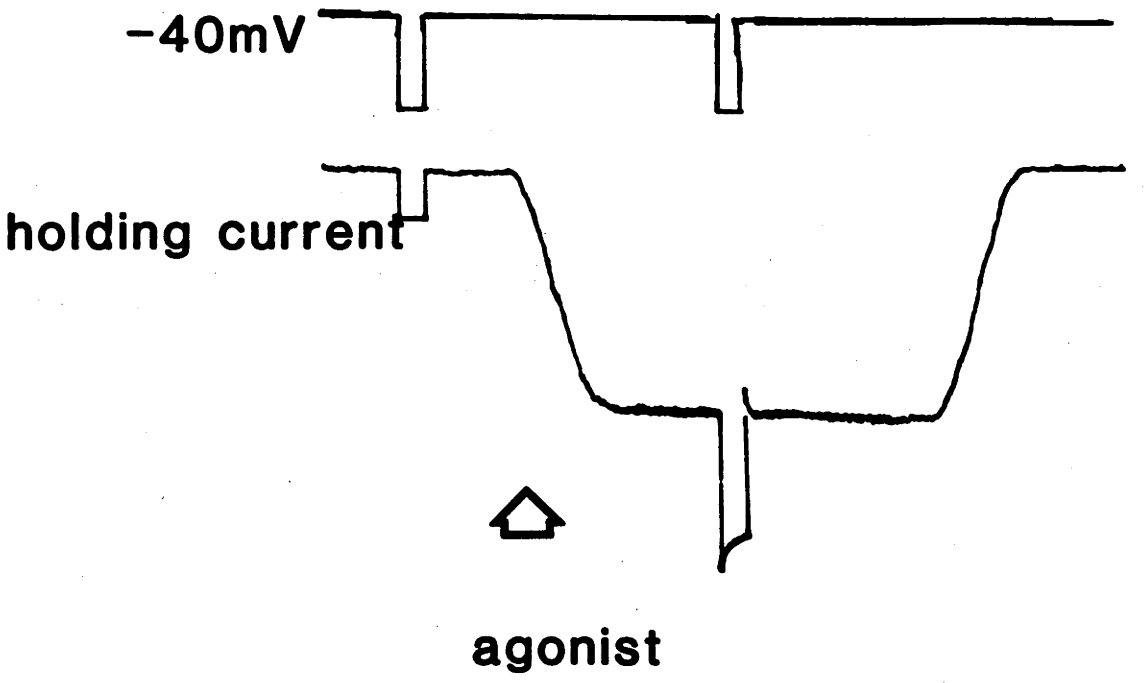
The most frequently used concentration of GABA was 60 µM, which gave ~3nA (-3.25 ± 1.9, x ± S.D., n = 36) of inward current at -40mV in cat neurones, and -1.64 ± 0.8nA (x ± S.D., n = 45) in rat drg neurones. Desensitization was not significant at this concentration, but in rare cases cells were found that responded with several nanoamps, (up to 11nA) to with 60µM GABA and these responses showed marked desensitization. Presumably these cells had a greater density of GABA channels or a longer channel open time than normally observed. Taking 50 µm as an average diameter for the cat neurones used and 35 µm for rat cells (and assuming equal single channel conductances of 25pS, and that the average current was due

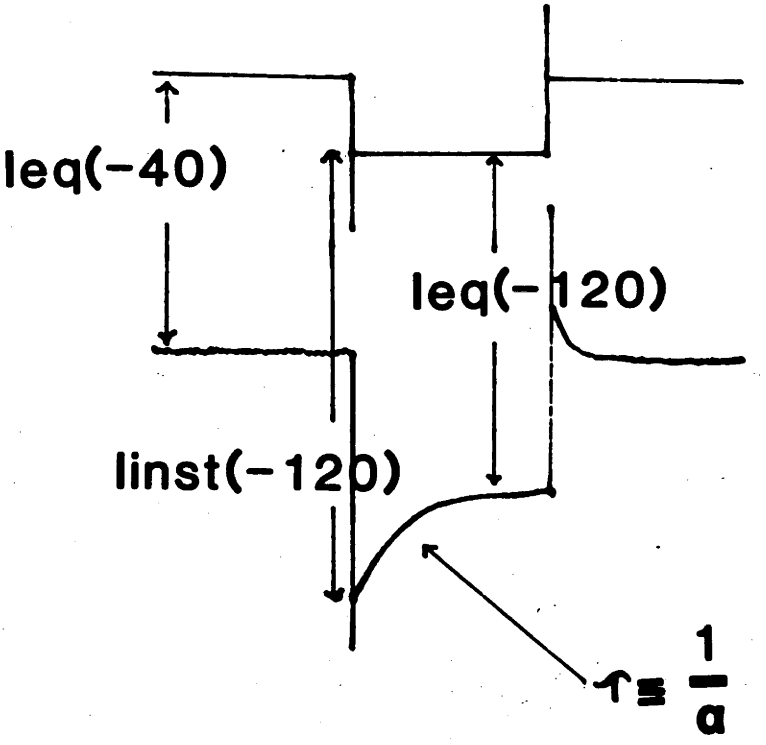
to activation of 25% of the total number of channels), a density of 0.35 GABA channels / μm^2 for cat cells and 0.38 GABA channels / μm^2 for rat is obtained. Not every cell responded to GABA however, yet the proportion of unresponsive cells was only about 10%, in contrast to some of the drg cells obtained from other animals (see above). Results for cat and rat neurones are pooled here, since the time constant of relaxations and voltage sensitivities were identical, as was the pharmacological profile of these cells.

Cells responding with less than 1nA to 60μM GABA were used only in exceptional circumstances, since voltage jump relaxations were correspondingly small and it became difficult to measure and fit small amplitude relaxations accurately.

After suitable voltage jumps were obtained in control solution, and application of the agonist resulted in ~1nA or more inward current at -40mV, an identical sequence of voltage jumps was carried out in the agonist solution (see Fig. 31). The control currents were automatically subtracted away, and typical GABA induced current relaxations were observed. These consisted of an instantaneous current changing to a steady state current with an exponential time course (see Fig. 32). No relaxation was visible with voltage steps very close to the Cl null potential. A series of typical relaxations are shown in Fig. 33. These are the form of relaxation expected for a channel with a voltage dependent rate

FIGURE 31: This figure schematically illustrates the protocol used to obtain voltage jump relaxations with agonists. Depolarizing and hyperpolarizing voltage jumps were made from -40mV in control solution and stored on magnetic disc. Agonist was then applied, and when the agonist-induced current stabilised, the voltage jumps were repeated. The difference between the voltage jumps in control and agonist solutions gave the equilibrium or steady state current at -40mV, and the instantaneous current and the steady state current at other potentials. The current relaxation was analysed to obtain a measure of the time constant of decay.


-40mV
holding current
agonist


Ieq(-40)
Ieq(-120)
Iinst(-120)
τ ≈ 1/α

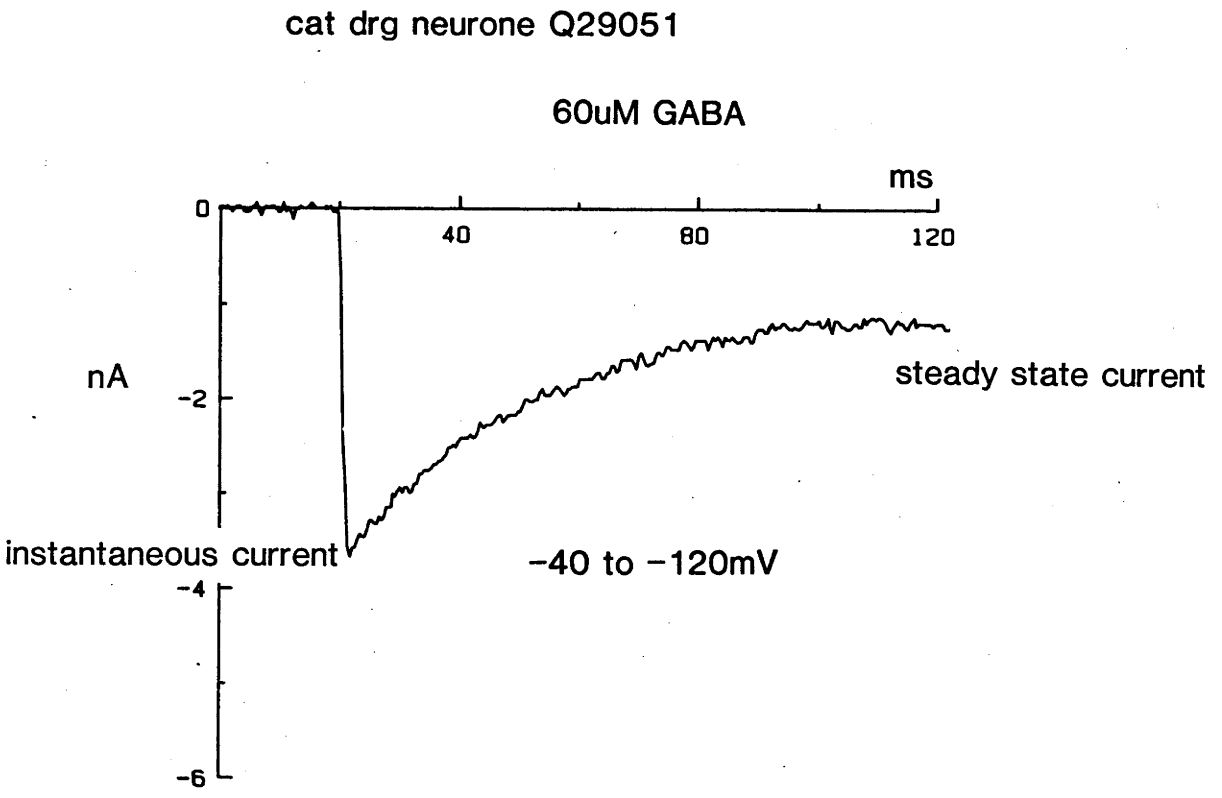
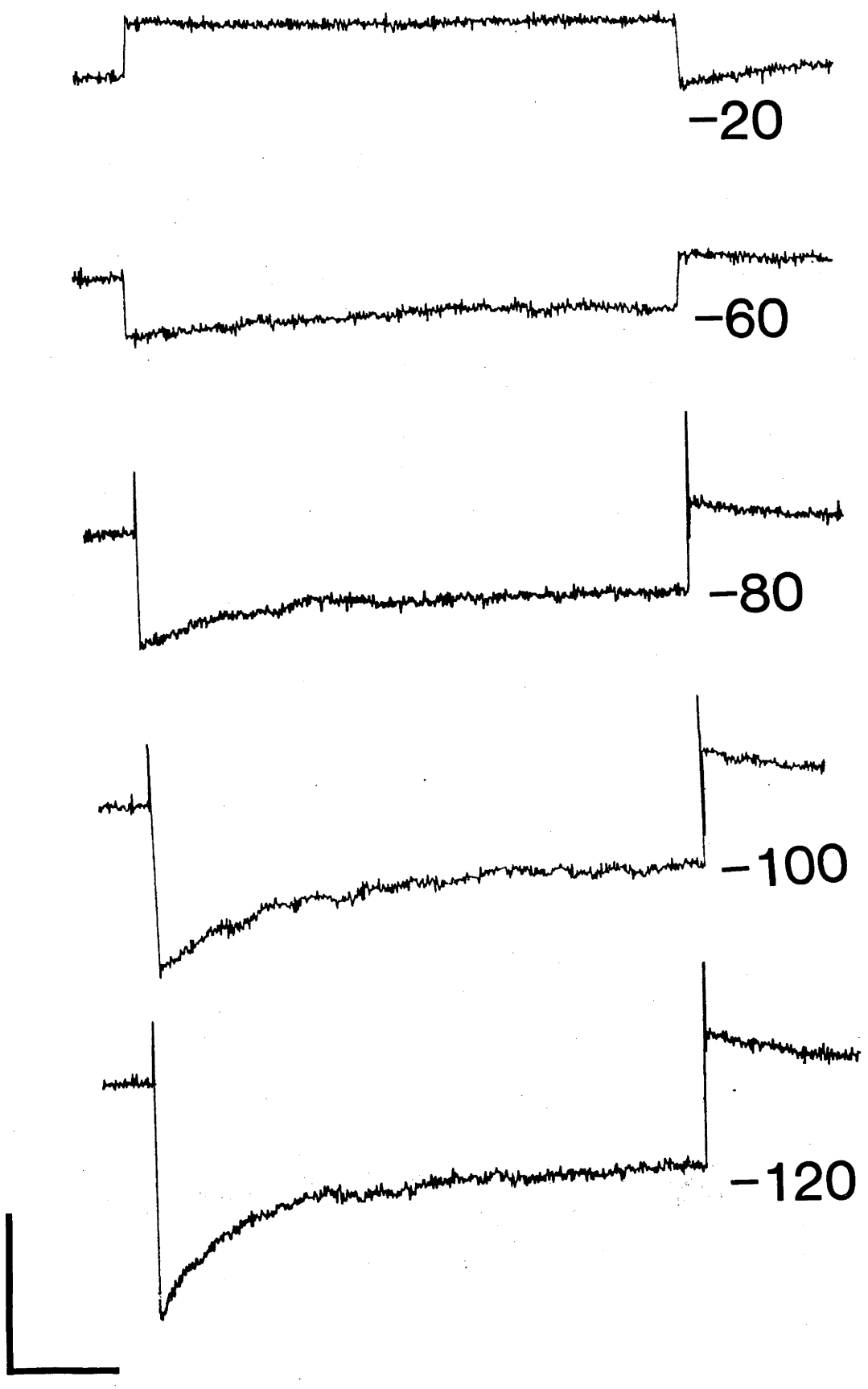
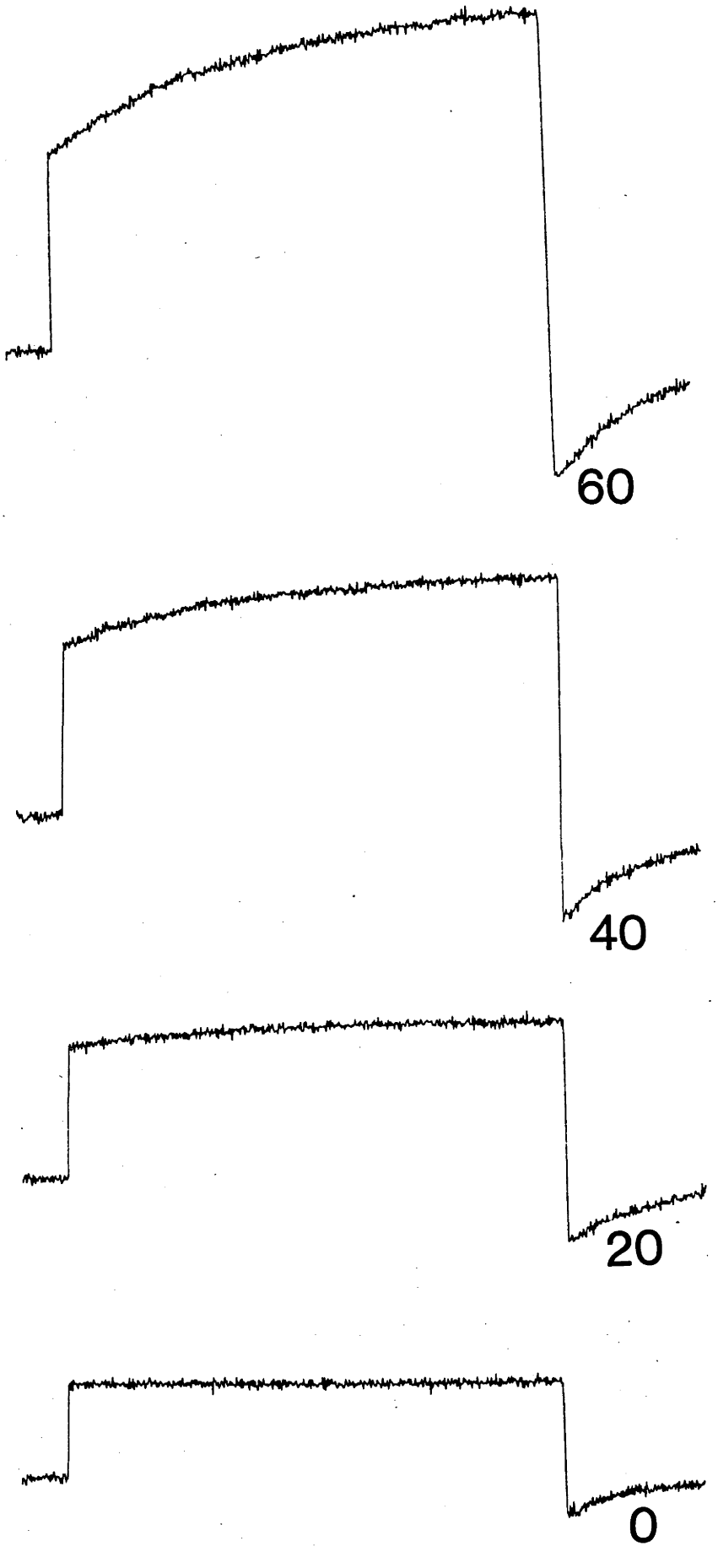


FIGURE 32: A sample current relaxation in response to a voltage step from -40 to -120mV with 60μM GABA. This figure shows the instantaneous current followed by the exponential decay to a new steady state current. The current levels and the decay time course, were analysed on computer.

FIGURE 33: A family of voltage jump relaxations in 60μM GABA, obtained by stepping from -40mV to the potential indicated on the right hand side of the current traces. Steps to negative potentials are shown opposite, positive potential steps are shown on the next page. The vertical calibration bar denotes 1.5nA, the horizontal calibration is 40ms.


−20
−60
−80
−100
−120


60
40
20
0

constant between closed and open states.

Analysis of these relaxations provides three separate measurements. These are:

(a). The instantaneous current - which is the level of current obtained immediately the potential reaches the clamp command value, before the current 'relaxes' to its new equilibrium value. This current provides a measure of the ionic conductance through GABA channels that are already open at the time of the voltage jump.

(b). The steady - state current - the current after the relaxation. This is the equilibrium current for the particular potential, and reflects the new number of open channels.

(c). The time constant of relaxation (τ_D), which is equivalent to (see General Introduction) $1/(\alpha + \beta)$, or $\approx 1/\alpha$ for low concentrations of agonist.

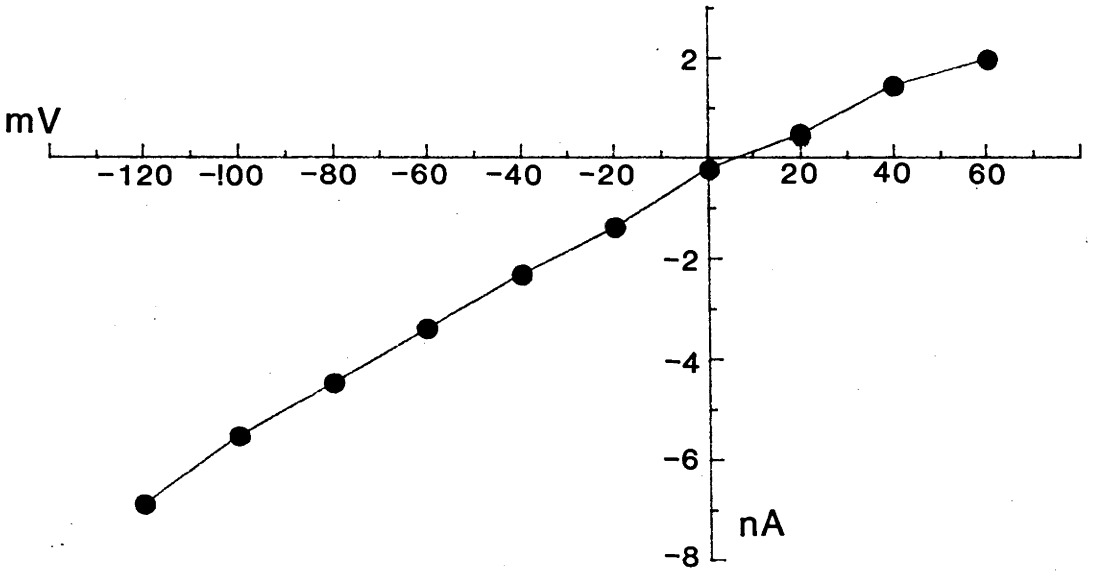
From each relaxation we can therefore obtain macroscopic rates for channel closing and a measure of the permeability/conductance properties of the channel.

Instantaneous Current-Voltage Relation in GABA.

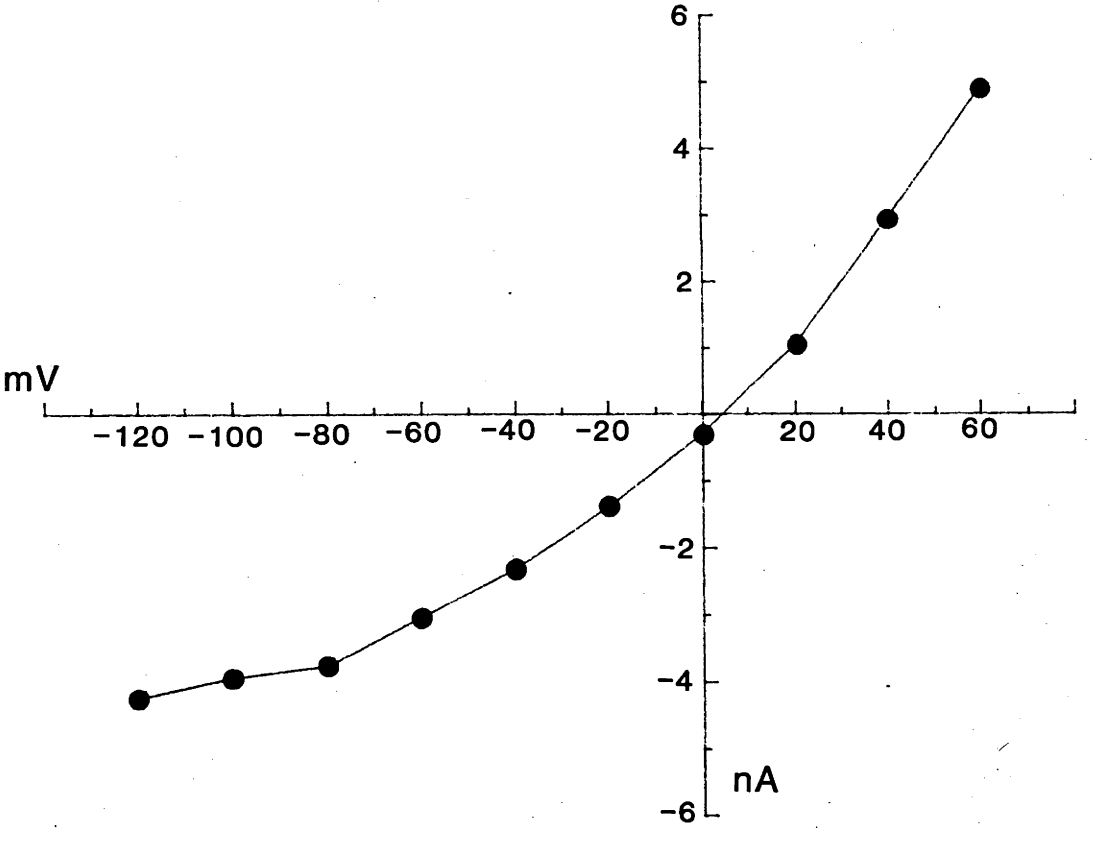
Figs. 34A & 35 show pooled and normalized instantaneous I-V's for individual drg neurones. The I-V is well fitted by a straight line over the whole voltage range examined, and gives a null potential for the GABA-activated Cl channel of ~ +10mV. Goldman-Hodgkin-Katz models predict that channels should have linear

FIGURE 34: A) The instantaneous current voltage relation for a single neurone in 60μM GABA. B) is the steady state current voltage relation for the same cell. The instantaneous I-V is almost linear in symmetrical solutions, while the steady state current is reduced at negative potentials due to the voltage sensitivity of GABA channel lifetime.

A


mV
2
-120
-100
-80
-60
-40
-20
20
40
60
-2
-4
-6
-8
nA

B


6
4
2
mV
-120
-100
-80
-60
-40
-20
20
40
60
-2
-4
-6
nA

instantaneous I-V's in symmetrical solutions, and similarly rate theory predictions suggest that channels with symmetrical energy barriers will also have linear instantaneous I-V's.

The non-zero null potential is explained by the different activity coefficients in the external and internal solutions. (see above).

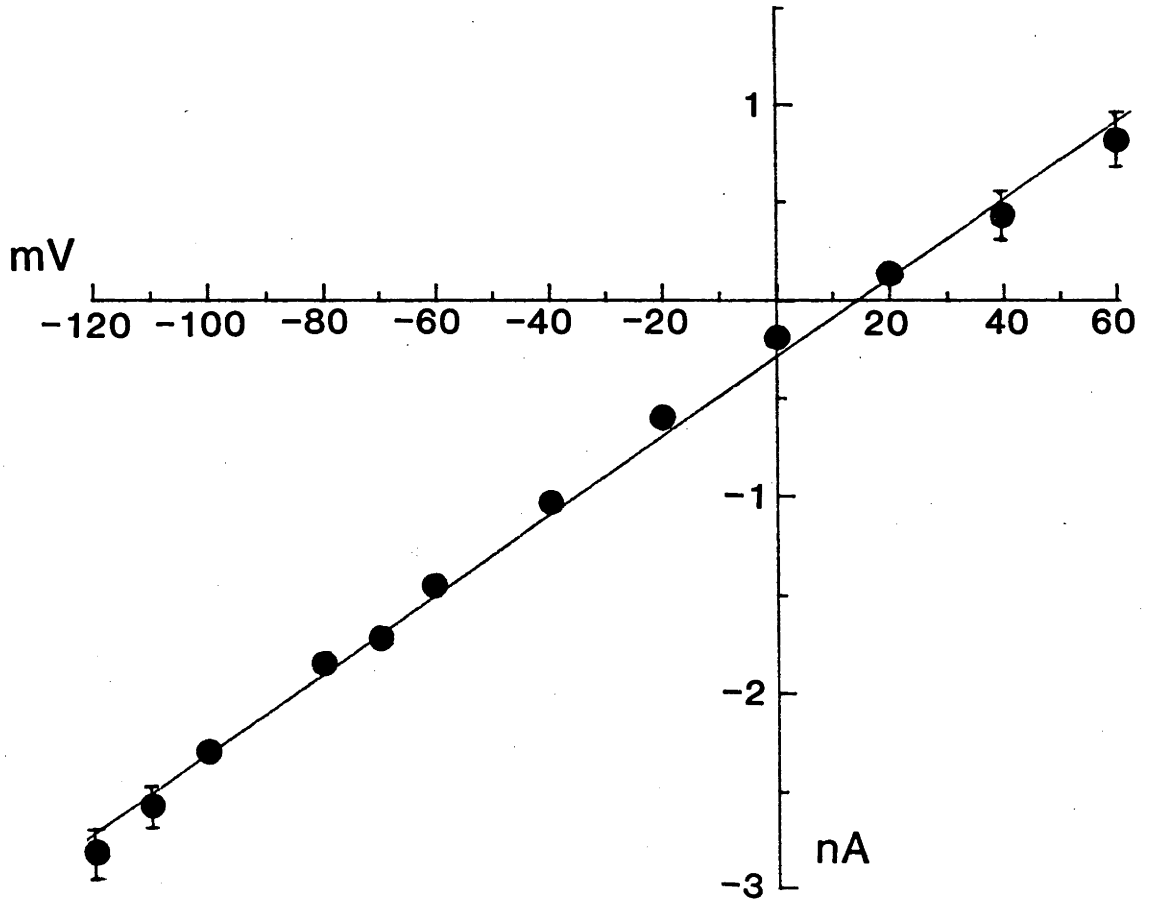
In a few instances the instantaneous I-V relationship curved downwards slightly at positive potentials (see e.g Fig. 47 & 49). The explanation for this is that in these particular experiments the steady state current at -40mV decreased during the course of these depolarizing jumps. This is probably due to desensitization which would tend to reduce the number of conducting channels. Deviation from linearity in the instantaneous I-V was most marked when channel open time/desensitization was significantly increased. (Alternatively it is possible that the individual channel conductance showed rectification in these cells, but this is considered unlikely since in experiments where voltage steps to positive potentials were performed first, no rectification was observed).

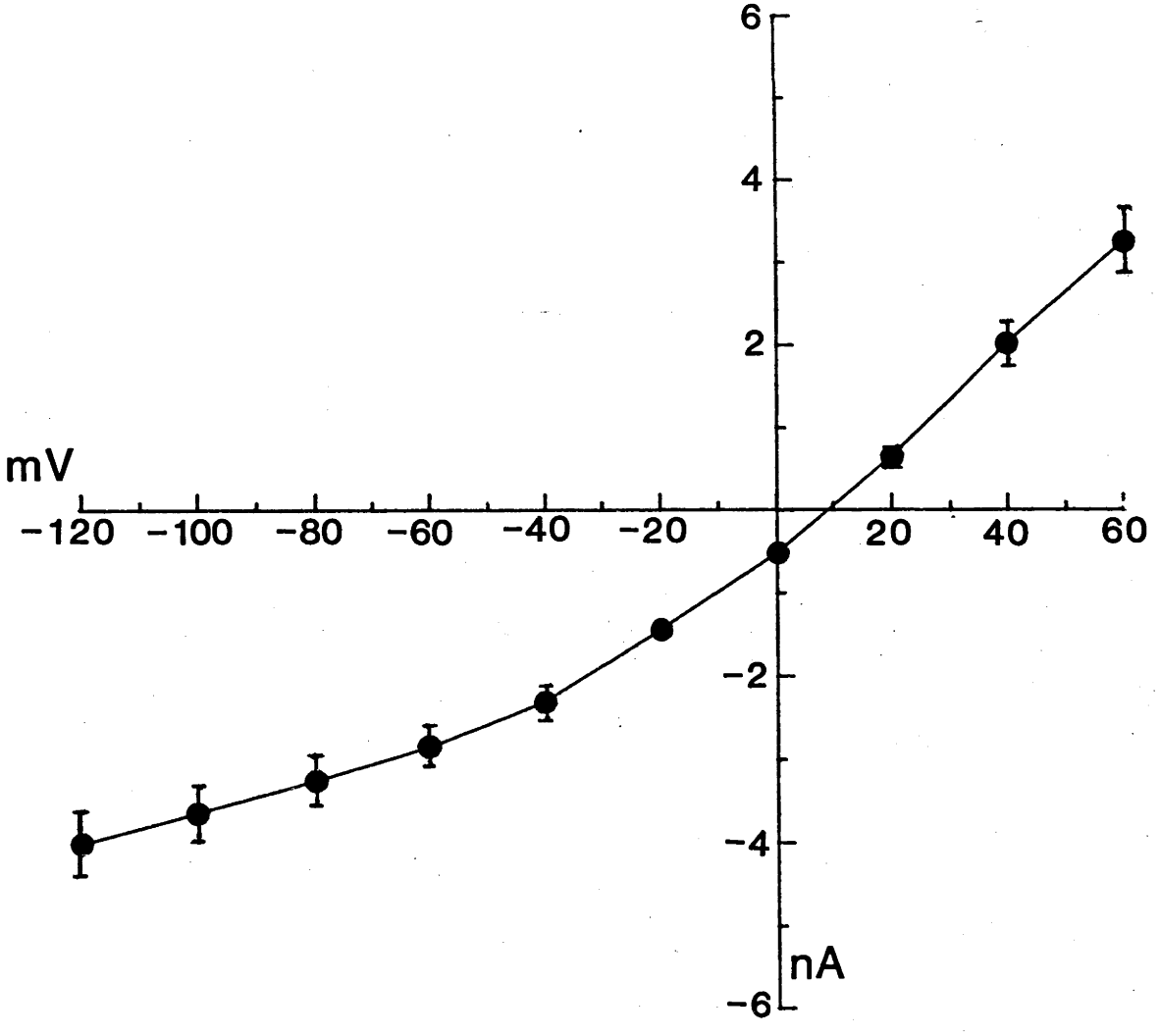
Steady-State Current-Voltage Relation in GABA.

Figs. 34B & 35 show steady state I-V's between -120 and +60mV. As may be expected from previous studies of GABA channels with a voltage-dependent lifetime, the relationship is curvilinear, with less current produced at hyperpolarized than depolarized potentials. The shape of

FIGURE 35: Instantaneous current voltage relation with 140mM chloride on both sides of the cell membrane. Data points are mean ± one standard error of the mean for 8 cells, normalized to -1nA at -40mV.

Steady state current voltage relation for 60μM GABA. Mean of 8 cells, error bars are ± one standard error of the mean.


mV
-120 -100 -80 -60 -40 -20 20 40 60
1
-1
-2
-3
nA


mV
-120 -100 -80 -60 -40 -20 20 40 60
6
4
2
-2
-4
-6
nA

the curves in Figs. 34B & 35 is consistent with the result that the lifetime of GABA activated chloride channels is shortened by membrane hyperpolarization. The curve can be fitted by an expontial function and correlates with the voltage sensitivity of the time constant of decay

Time Constant of Relaxation.

The relaxations of the agonist-induced current to the new steady state value were well fitted by a single exponential, and were characterised by the time constant of decay τ_D. The most reliable measures of time constants were obtained at the extreme positive or negative potential steps, since these relaxations were the largest. Relaxations at potentials nearer -40mV were often more difficult to fit, because of their smaller amplitude and poorer signal to noise ratio. Plots of τ_D versus V_m are shown in Figs. 36 & 37 for 60μM GABA. These figures show that τ_D is exponentially related to voltage and can be fit by an equation of the form:

$$\tau_D = (\tau_D)_0 \exp V/H$$

If τ_D reflects $1/\alpha$ and is equal to the channel lifetime (or burst duration) then we can say that the open time of the channel changes e fold for an ~ 190mV shift in potential, this is defined as the value of 'H', the constant of voltage sensitivity.

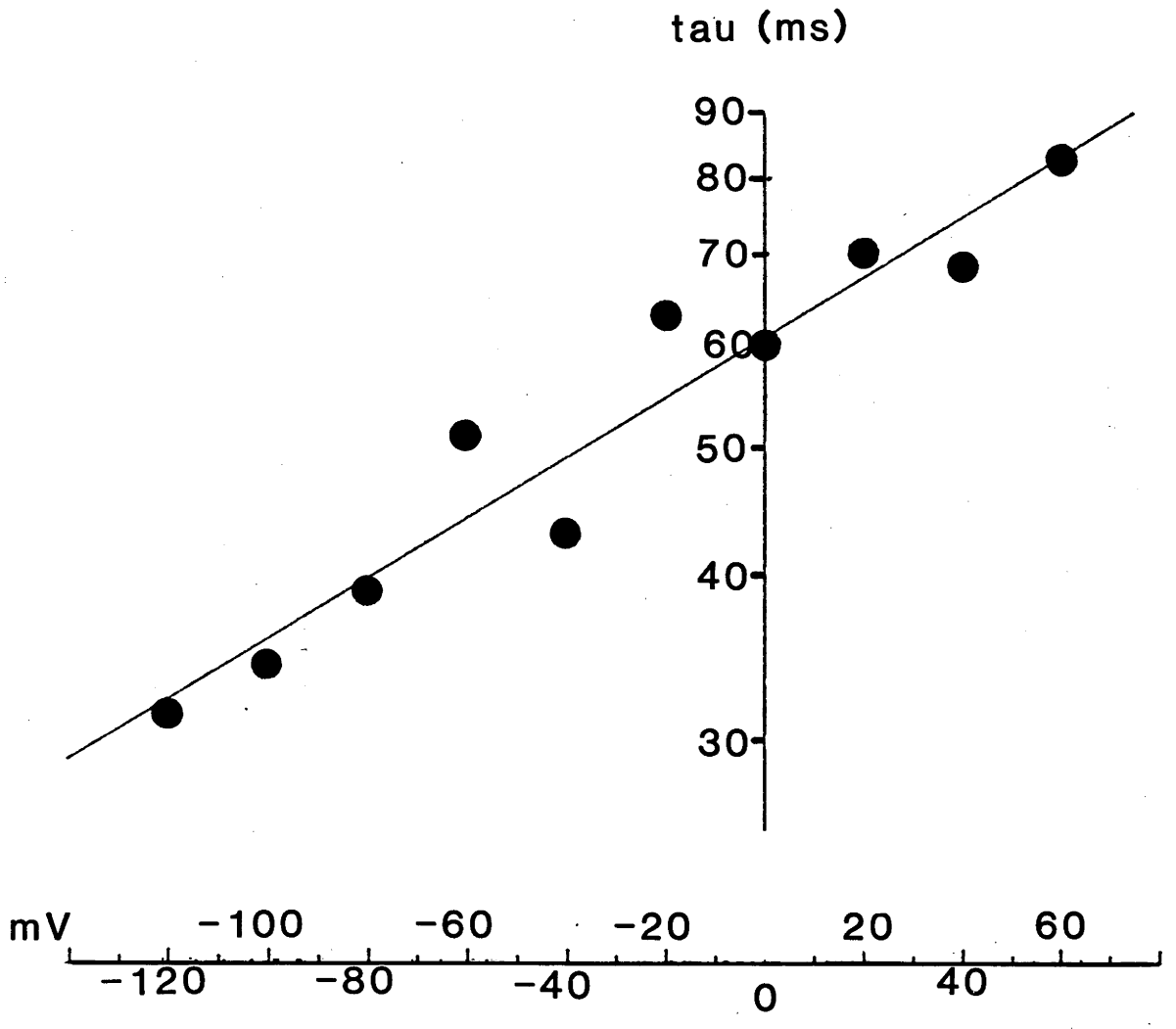


FIGURE 36: The voltage sensitivity of the time constant of decay (τ_D) of current relaxations in 60μM GABA. Points are the mean of 8 measurements. The scatter at potentials near -40mV is due to the poorer signal to noise ratio of these smaller relaxations. The regression line has a correlation coefficient of 0.964, and gives a $\tau_D(0)$ of 60ms. The H value is 188mV.

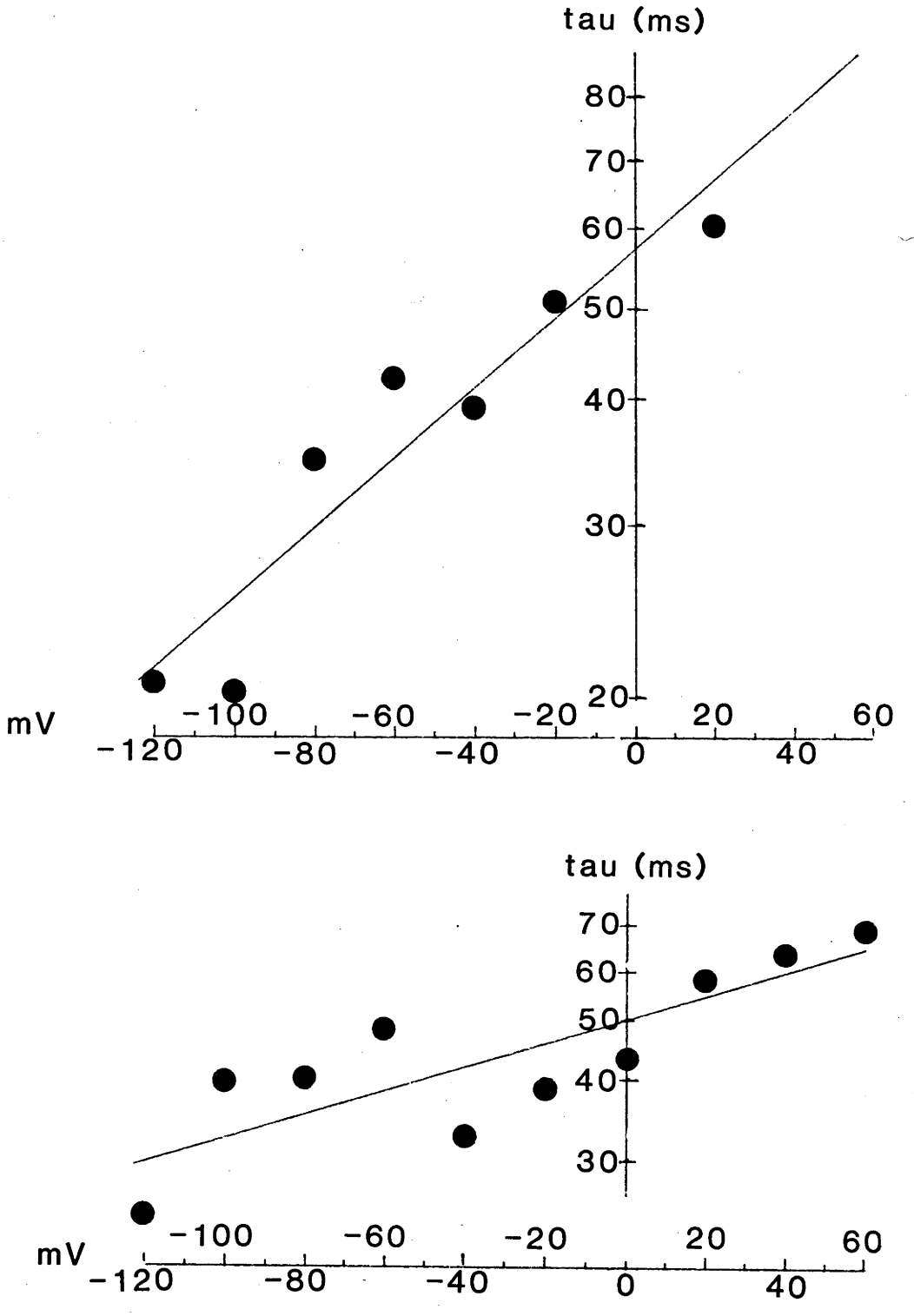


FIGURE 37: The voltage dependence of the time constant of decay in 60μM GABA for two individual cells are shown. The voltage sensitivities (H values) are 123mV for the top figure and 228mV for the lower figure.

The steady state current at -120mV was ~ 60% of the instantaneous current after a step from -40mV, and taking a mean τ_D value of 50ms at -40mV and 33ms at -120mV, we can determine the voltage sensitivity of β from the ratio of the time constants at -120mV and -40mV and the steady state current to the instantaneous current at -120mV (see Neher & Sakmann 1975). The value of $\beta(-120)/\beta(-40)$ from this is 1.1, suggesting that β has little voltage sensitivity.

Measurement of τ_D from analysis of voltage jumps to values of τ_D obtained from preliminary noise analysis experiments revealed a close similarity at -40mV, since in two experiments τ_D from noise was ~ 40ms at -40mV, but conductance varied between 10 and 20pS.

Effects of varying agonist concentration and the actions of GABA-like agonists.

(1) Blockers.

GABA induced currents could be completely blocked by 10-50μM bicuculline methiodide or 10-50μM picrotoxinin. This behaviour was seen in 10 cells with bicuculline and 6 cells with picrotoxinin. Use of these two specific antagonists strongly suggests that only $GABA_A$ type chloride channels are being activated. Blocking by these antagonists was completely and rapidly reversible.

(2) Effects of varying GABA concentration.

In 12 cells it was possible to examine agonist induced relaxations over a limited range of GABA concentrations. Data from these experiments is shown in Figs. 38 and 39. With increasing concentrations of GABA, desensitization became a greater and greater problem, presumably because more channels were being opened and more were available to be absorbed into the desensitized state. Hyperpolarizing steps were performed as close to the peak of the response as possible.

In Fig. 38, τ_D at -120mV is plotted against GABA concentration, revealing that τ_D is dependent on the concentration of GABA, which is expected since:

$$\tau_D \propto 1/(\alpha + \beta)$$

β is dependent on the agonist concentration.

Points at lower concentrations were particularly hard to obtain, since 10 and 30μM GABA produced only a very small conductance change, and relaxations were small, slow and consequently difficult to measure. Extrapolation of the line in Fig. 38 to 'zero' concentration gives a τ_D value of ~50ms. No accurate figures for τ_D(-120mV) could be obtained at concentrations much higher than 1000μM since desensitization at this concentration was so rapid as to be within the time course of the GABA-induced relaxation itself. Note that concentration is plotted logarithmically. It is clear that there is evidence for a 'foot' and a plateau in the graph. A

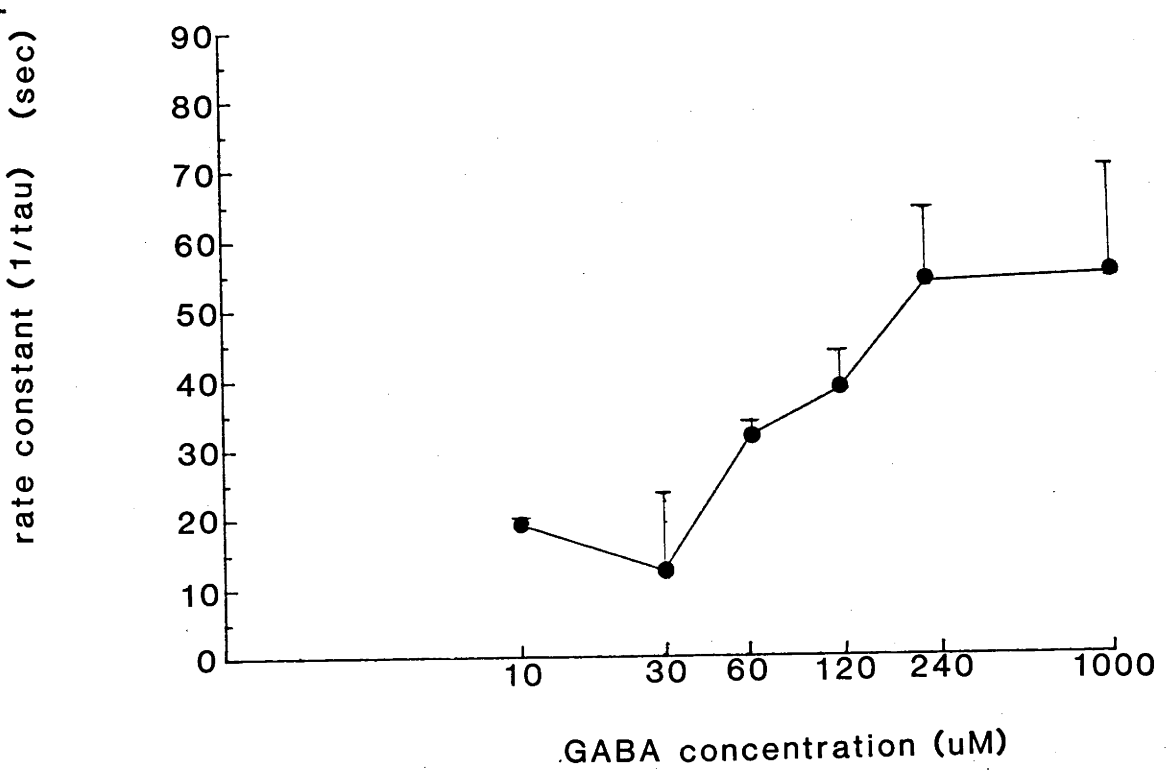


FIGURE 38: The relationship between the time constant of decay of the relaxation (plotted as rate/second) and GABA concentration. (Note that the abcissa is plotted logarithmically for convenience only). Each point is the mean of 3 - 12 determinations from 12 cells.

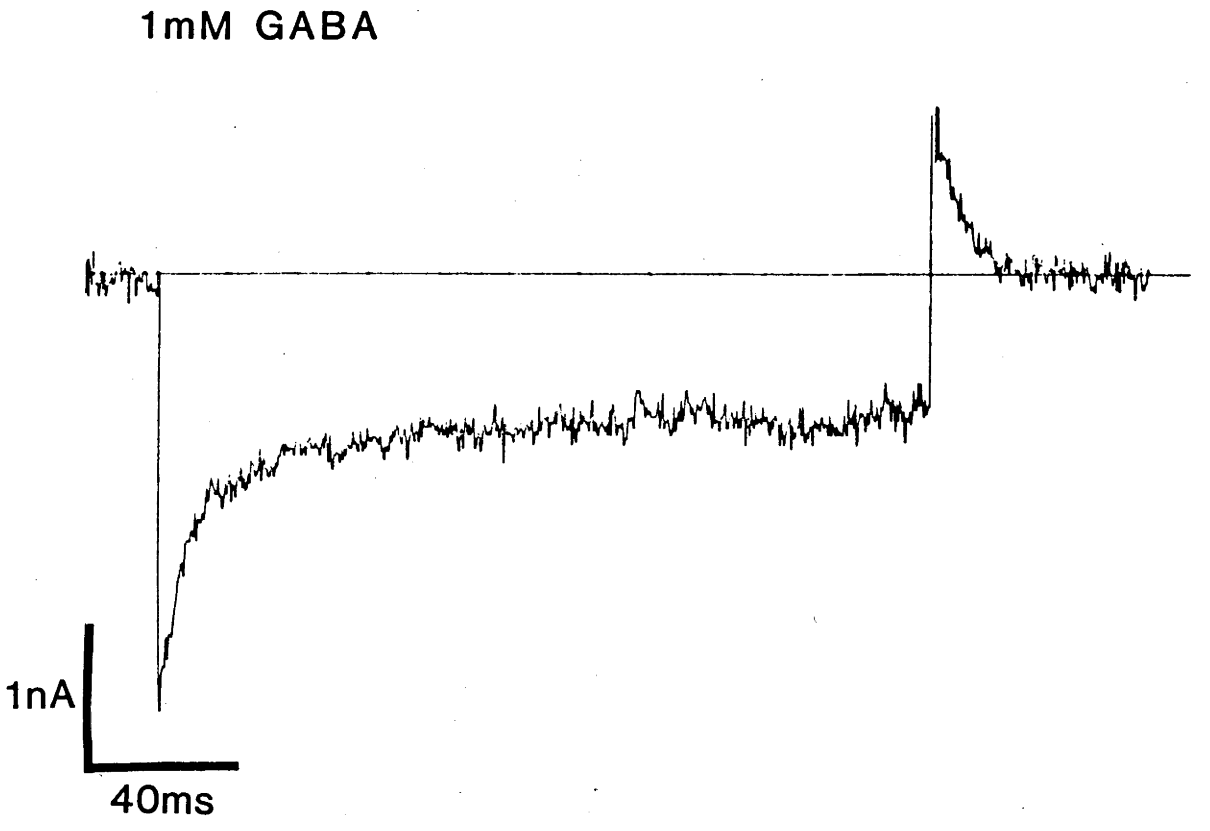


FIGURE 39: The time constant of relaxation decreases as agonist concentration is increased. In this figure, a fast on and off relaxation are observed with 1mM GABA. The step is from -40 to -120mV.

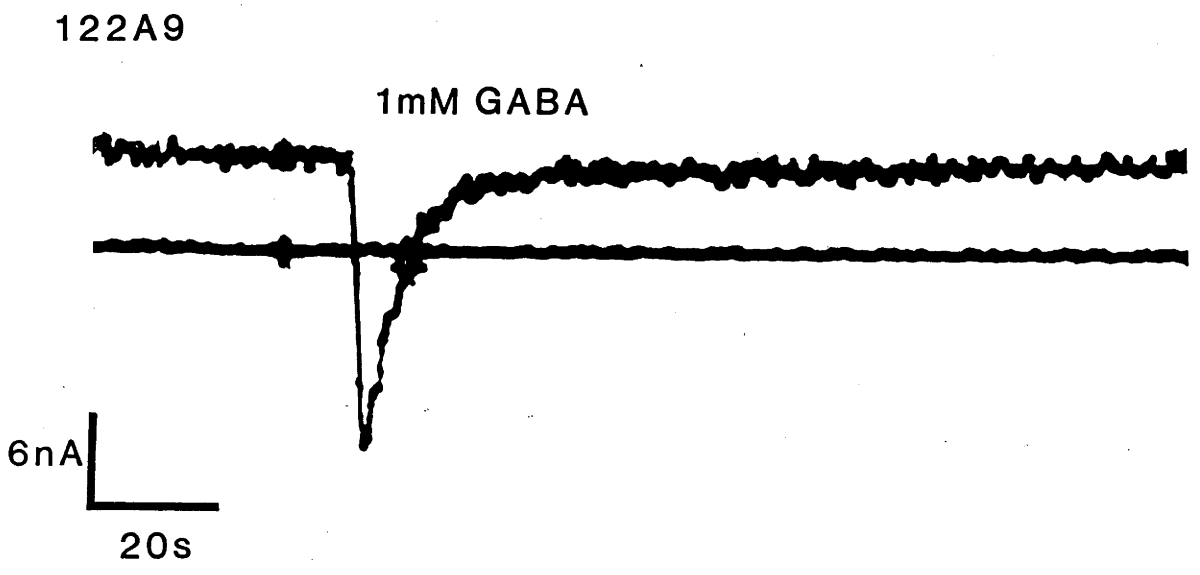


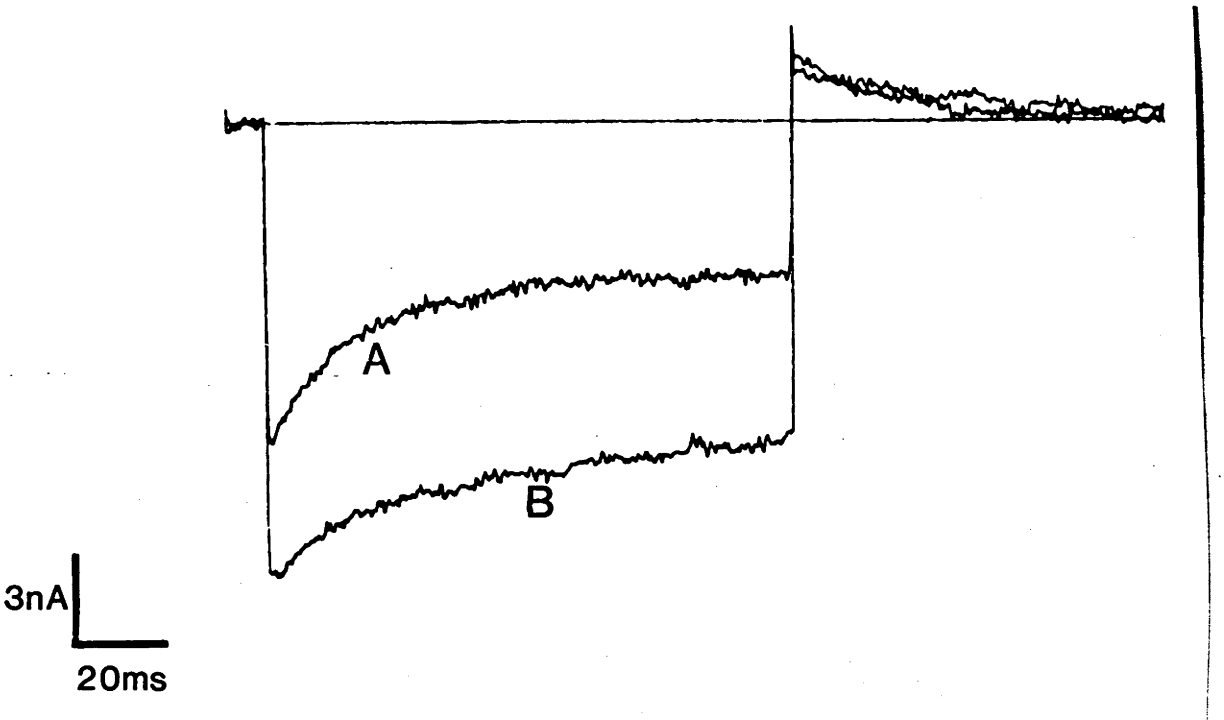
FIGURE 40: Application of 1mM GABA to a cat drg neurone results in a rapid increase in current which quickly desensitizes. No bounce is observed on washout of the agonist. The horizontal trace is the cell membrane potential (-40mV) during GABA application.

current response to a voltage step from -40 to -120mV in 1mM GABA is shown in Fig. 39.

In all the experiments with 1mM GABA (see Fig. 40), no evidence for channel block by this high concentration of agonist was apparent, since there was never any evidence of a 'bounce' in the current trace (see below) when GABA was quickly washed out, and relaxations although faster, appeared normal (Fig. 39).

Muscimol.

Muscimol is an isomer of GABA which has the carboxyl group replaced with a heterocyclic acid (see structure in Appendix A). Muscimol was bath applied at 20 to 60μM and was much more potent as an agonist than GABA in increasing membrane Cl^- conductance. In 17 experiments where GABA and muscimol, each at 60μM, were separately applied to the same cells, muscimol produced on average 2.44 ± 1.34 (x ± S.D.) more current than GABA. Desensitization was also much more significant when muscimol was used as the agonist. Relaxations during voltage jumps were much slower with muscimol than those relaxations with GABA. Fig. 41 shows the currents produced by a -60mV step from -40mV with 60μM GABA and 60μM muscimol. The time constants of the relaxations for the top figure are 19.9 and 40.5ms respectively, and 42.7 and 70.6ms for GABA and muscimol in the cell in the lower part of the figure. The steady state current at -40mV was 1.4 fold greater in muscimol for the cell in the top



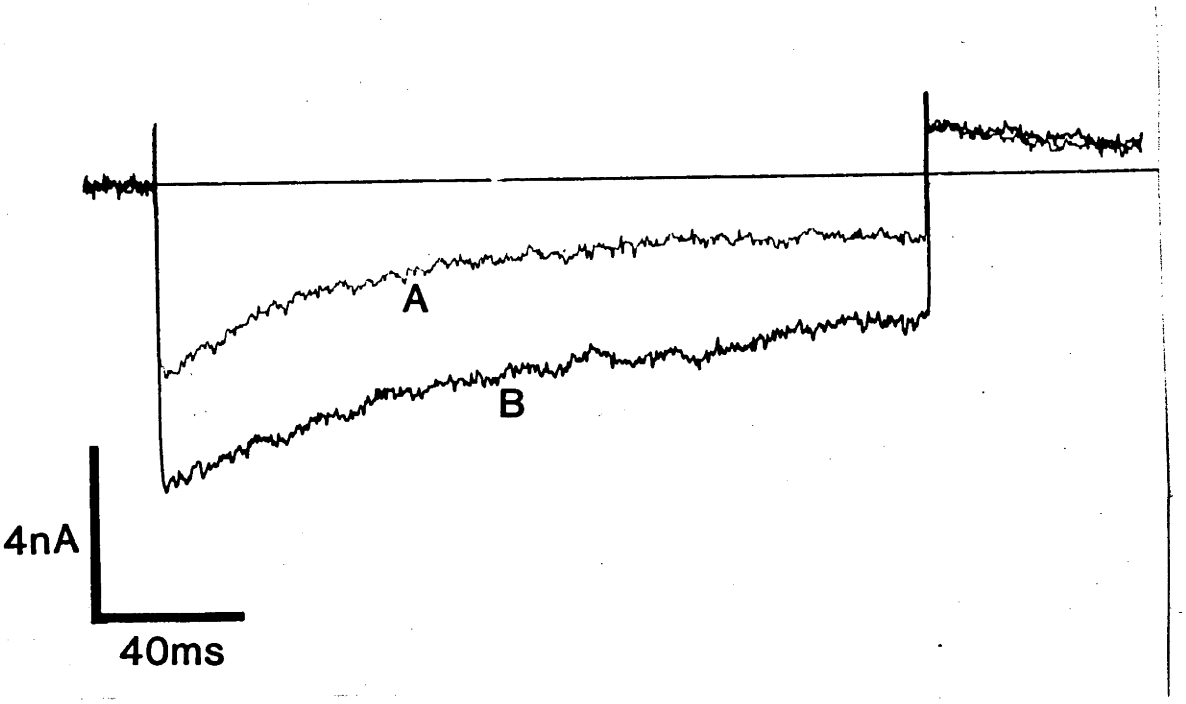


FIGURE 41: Two cells exposed to 60μM GABA (traces marked A) and 60μM muscimol (traces marked B). The current responses are the net agonist induced currents on a hyperpolarizing step from -40 to -100mV.

of the figure, and 1.7 greater in the other cell, suggesting that the increased potency of muscimol was due to longer channel open times. Muscimol washed out rapidly without showing any evidence of 'bounce' - suggesting that this agonist does not block the channel and that all of the decline of current observed during prolonged applications is likely to be due to desensitization.

PIPERIDINE-4-SULPHONIC ACID (P4S).

P4S is a GABA-like isomer which has a piperidine ring replacing the amino group of GABA and a sulphonic acid in place of the carboxyl group (see Appendix A). Results obtained by Curtis and colleagues (see Krogsgaard-Larsen, Falch, Curtis & Lodge 1984) suggest that this substance is a very potent agonist at GABA receptor channels on interneurones and Renshaw cells in the cat spinal cord. Given that muscimol potency can be correlated with longer τ_D values, it was thought worthwhile to test the action of this compound on GABA channels in the drg preparation to determine a) its potency, and b) to find if this potency could be correlated with channel mechanisms.

P4S was applied at 60 to 100μM. When both GABA and P4S (at 60μM) were applied to the same neurones, P4S induced only 0.14 ± 0.06 (x ± S.D., n = 7) of the GABA response. 100μM P4S was approximately one quarter (0.27 ± 0.07 (x ± S.D, n = 3) as effective as 60μM GABA. Some

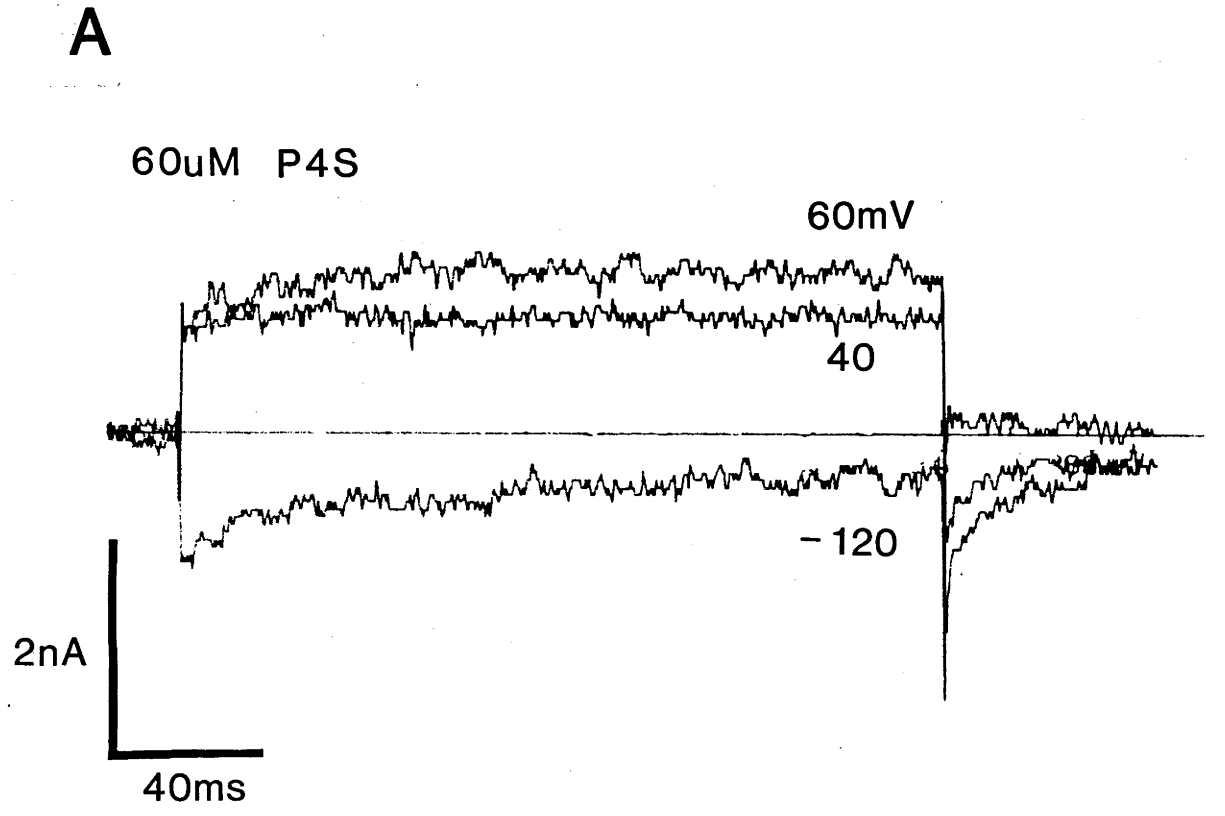
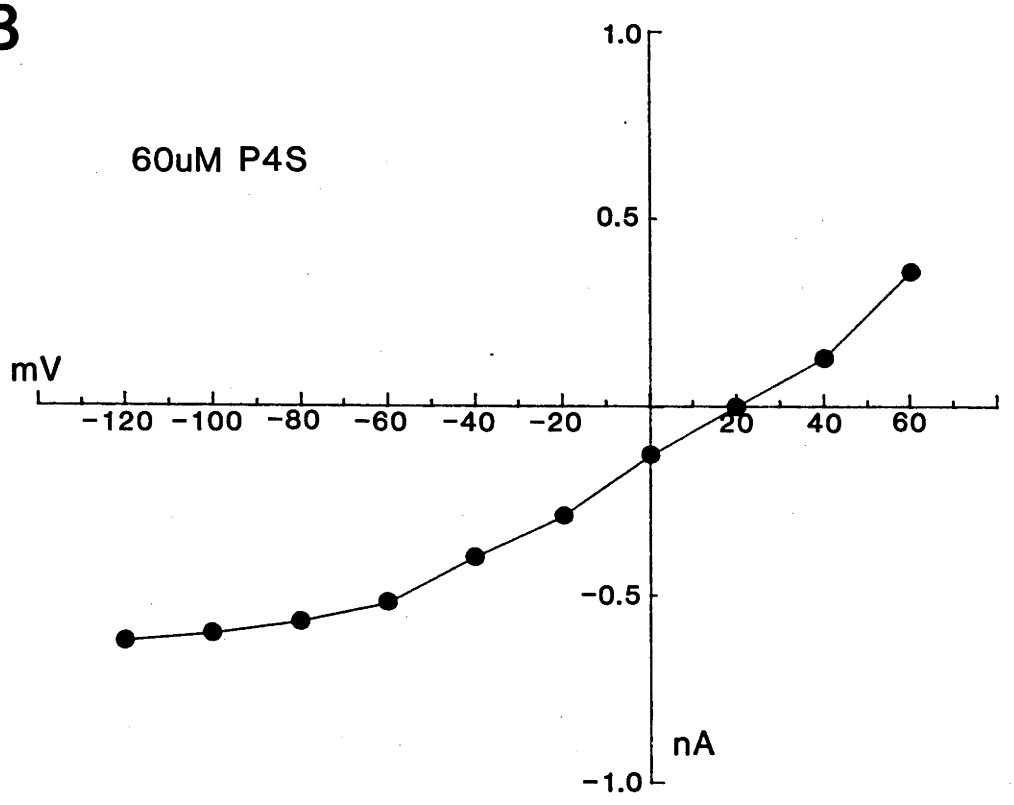
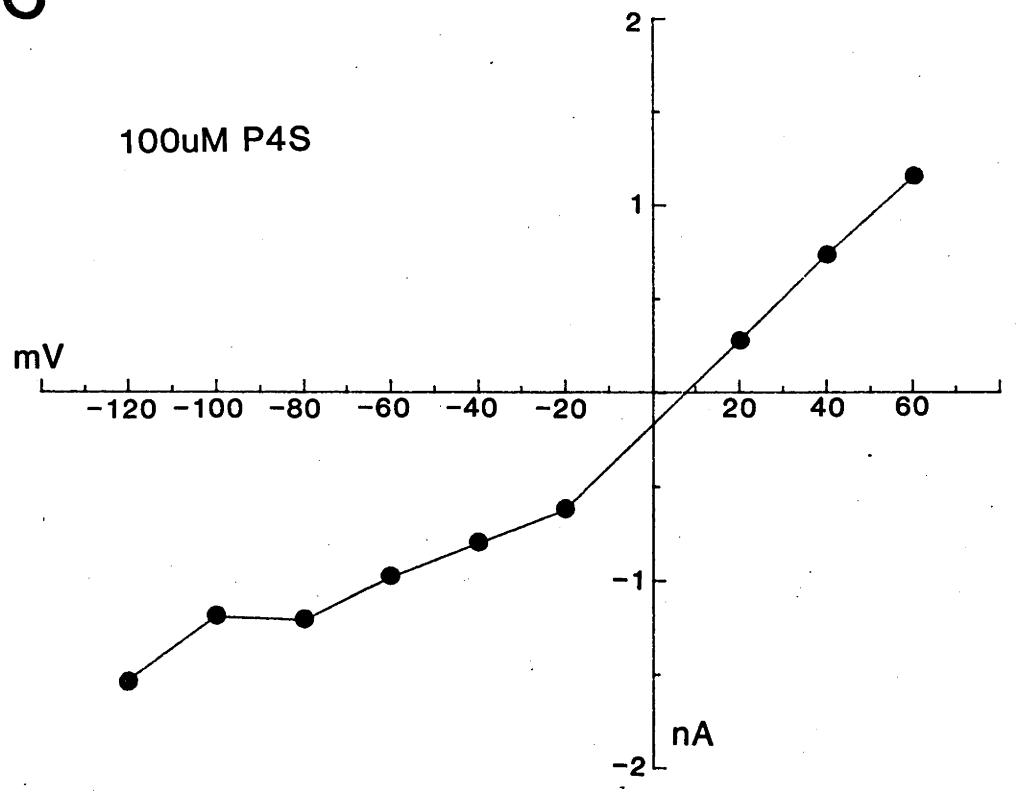


FIGURE 42: A) Voltage jump relaxations in 60μM piperidine - 4-sulphonic acid. These currents are small and noisy due to the low potency of this GABA analogue on dorsal root ganglion neurones. B) and C) [overleaf]. The steady state current voltage relations of 60μM P4S (B), and 100μM P4S (C). The currents with P4S are extremely small compared to those normally obtained with equivalent concentrations of GABA, but rectify in the same direction as GABA-activated channels.

B


60uM P4S
1.0
0.5
mV
-120 -100 -80 -60 -40 -20
20 40 60
-0.5
nA
-1.0

C


100uM P4S
2
1
mV
-120 -100 -80 -60 -40 -20
20 40 60
-1
nA
-2

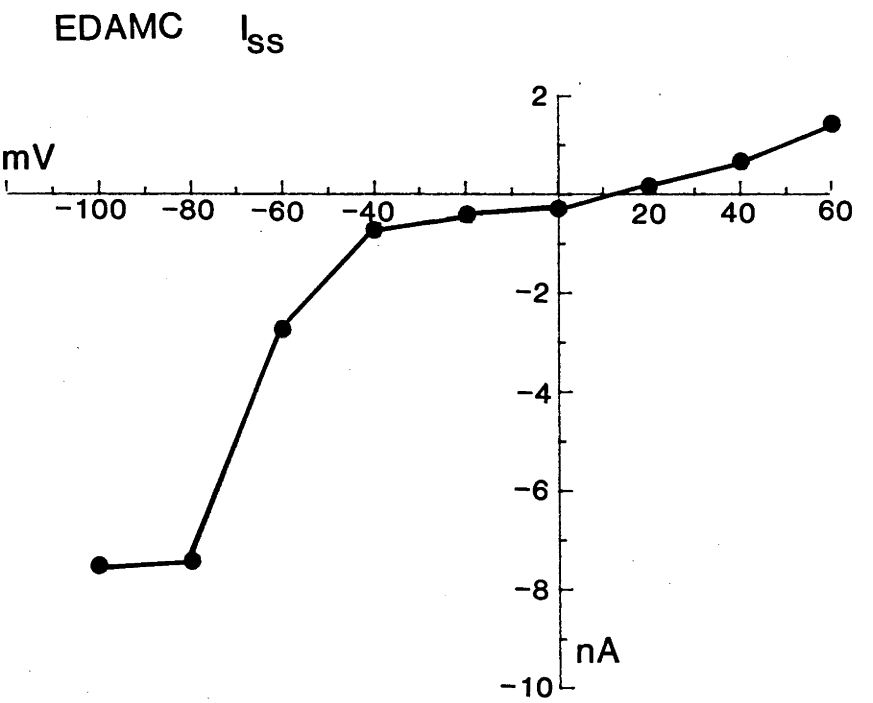
of the small amplitude relaxations obtained in P4S are shown in Fig. 42. Although the relaxations are small, a τ_D of 18.7ms was obtained from the step from -40 to 120mV. (τ_D at other potentials was 22.6ms at -40mV and 24.3ms at 60mV). The responses with 100μM P4S showed evidence of desensitization.

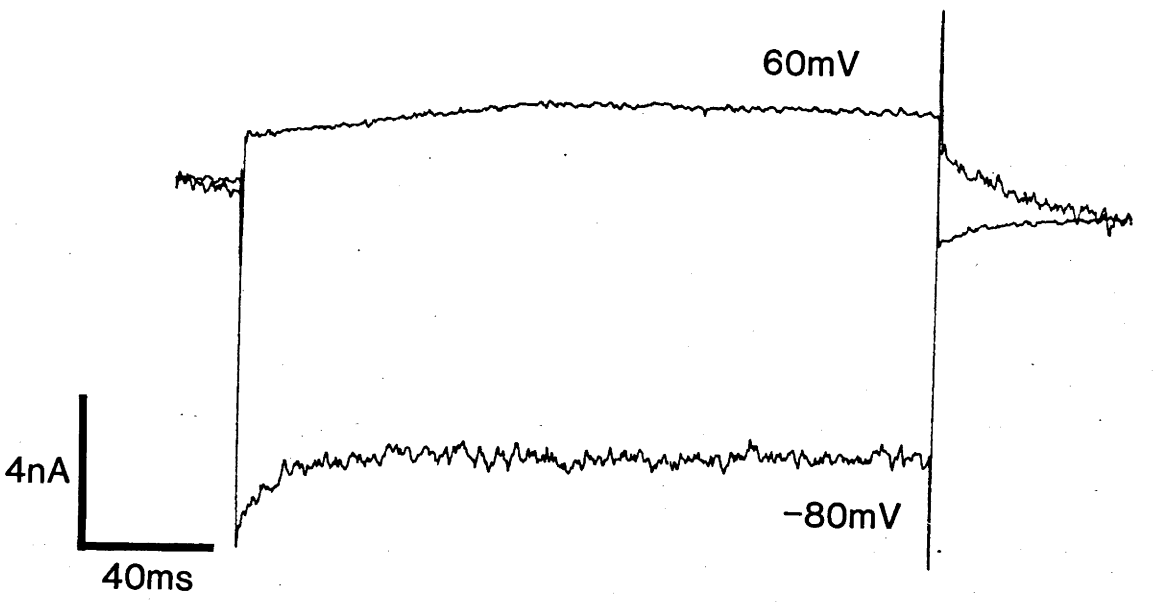
The curvature of the steady state current voltage relation in Fig. 43 suggests that P4S activated GABA channels are voltage sensitive. From these experiments, P4S is only marginally as effective as GABA in producing a chloride conductance change in drg neurones.

Ethylenediamine Monocarbamate (EDAMC).

The structure of ethylenediamine monocarbamate is shown in Appendix A. Results from other laboratories suggest that in vivo ethylenediamine is coverted to EDAMC by reacting with bicarbonate ions. From structural considerations EDAMC might be expected to posses GABA-mimetic properties. Attempts were made to convert commercially available ethylenediamine to EDAMC following the procedures of Curtis and Malik (1984), by bubbling a solution of 1mM solution of ethylenediamine with 5% carbon dioxide for one hour. This solution was then diluted to give a nominal concentration of 100 and 300μM. Application of these concentrations gave small responses in cells that responded well to GABA. In three cells

FIGURE 44: Top. The steady state current voltage relation is freshly made up ethylenediamine monocarbamate. The current responses at -100 to -60mV are disproportionately large due to rapid desensitization in the agonist and breakdown of the compound during the experiment. Voltage jump relaxation steps from -40 to -80mV and +60mV are shown in the bottom of the figure. GABA channels activated by EDAMC retain their voltage sensitivity.


EDAMC
I_{ss}
mV
-100
-80
-60
-40
20
40
60
2
-2
-4
-6
-8
-10
nA


60mV
-80mV
4nA
40ms

100μM EDAMC gave no response. In 2 other cells, 300μM EDAMC also produced insignificantly small responses in cells that responded well to GABA. EDAMC was also prepared by applying 1mM EDA with 1mM bicarbonate and gave a maximal response of 1nA, while GABA at 1mM gave 10nA. EDAMC prepared in this manner however quickly lost its effectiveness after only a short time in the solution reservoir.

Some relaxations and steady-state current voltage relations from these experiments are shown in Fig. 44. From this limited data, τ_D at -100mV was 19.6 and 15.2ms, and τ_D at +60mV was 40ms and 53.8ms in two cells where this was measurable.

Glycine.

Glycine activates chloride channels in central neurones, and it has been suggested that this amino acid can act as an agonist on GABA channels (Barker, & Dufy 1985). Glycine was tested for GABA-like agonist activity in 5 neurones, mainly to determine the characteristics of the channel when activated by another agonist, but also to determine if there were any true glycine receptors on drg neurones, and thereby compare their properties to GABA channels. Glycine was applied to drg neurones at a concentration of 60μM and 100μM on 5 cells and produced no conductance change. These cells all responded vigorously to GABA however. Application of glycine did not in any

way antagonize GABA action.

Taurine.

Taurine has both amino and carboxyl groups like GABA but has one less carbon atom in the backbone chain. Barker & Mathers (1981) have reported that this amino acid can activate GABA channels, and that the open time of these channels is much shorter than when activated by GABA. For this reason it was of interest to test if taurine acted as a GABA like agonist in drg neurones, since a shorter open time would be reflected in shorter current relaxations after a voltage step. However, in 3 experiments 1mM taurine was ineffective in activating GABA channels, although these receptor/channels were present on these particular cells.

β-p-chlorophenyl GABA (Baclofen).

Baclofen is basically the GABA molecule with a chloro phenyl ring attached to the backbone carbon chain. This compound has been described as a specific agonist for a novel type of bicuculline insensitive GABA receptors on a wide variety of neuronal and extraneuronal preparations. Other results obtained in this laboratory (Robertson & Taylor unpublished observations, see Appendix D) have shown that $GABA_B$ receptors do exist on drg neurones, but we have never found any evidence for a Cl conductance change activated by this compound. Further tests for

weak $GABA_A$ activity carried out as part of the present studies failed to reveal any response to this compound.

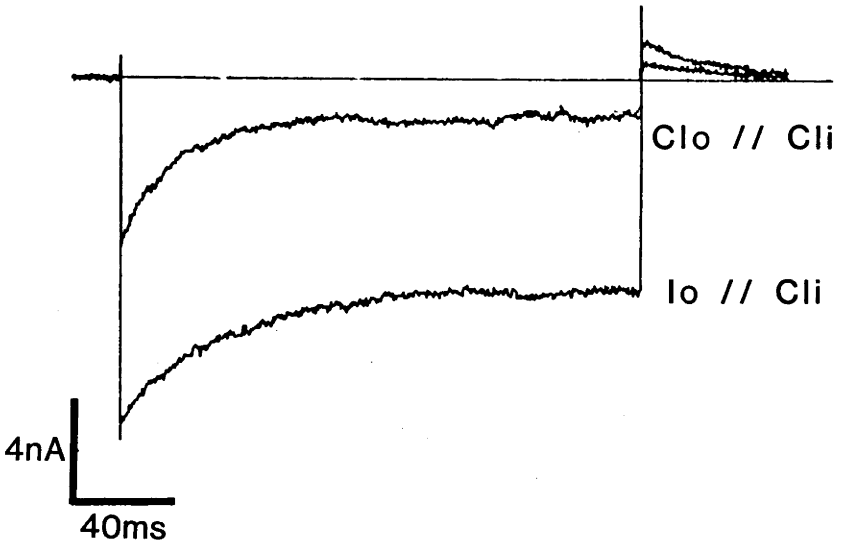
THE PERMEABILITY OF GABA-ACTIVATED CHLORIDE CHANNELS TO FOREIGN ANIONS, AND EFFECTS OF ANIONS ON CHANNEL KINETICS.

Iodide.

In order to examine the permeability of iodide ions in GABA channels in dorsal root ganglion neurones, the external solution was changed from 140mM choline chloride to 135mM choline iodide plus 5mM choline chloride. Voltage jumps in GABA were first measured in the chloride solution, GABA was washed out and the bathing solution was changed to 135mM iodide. After one to two minutes in this solution, the same concentration of GABA was applied in 135mM iodide solution and net GABA induced currents were obtained for the iodide solution. Often this procedure could be repeated more than once for each neurone.

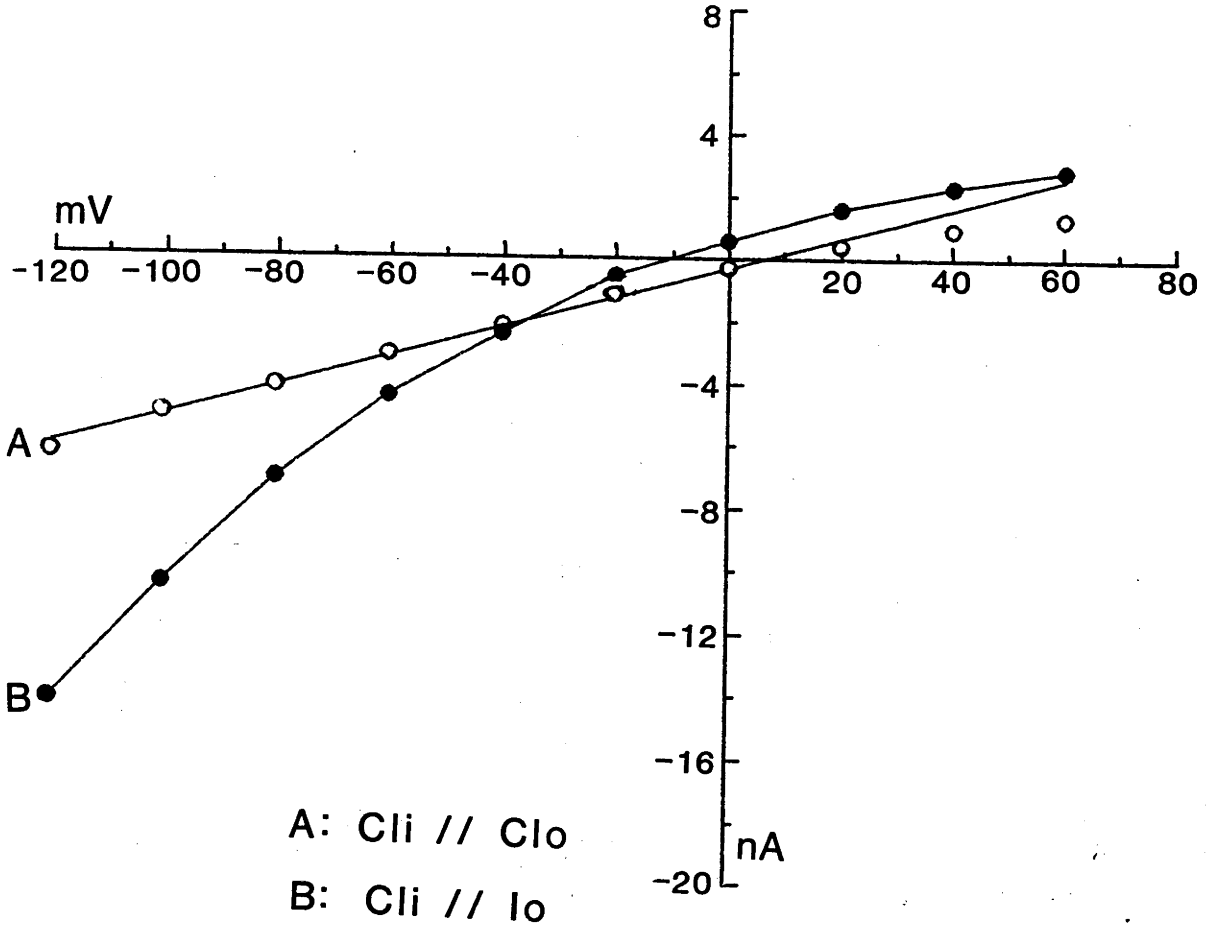
The instantaneous current-voltage relationship for GABA channels for 14 paired applications of chloride and iodide are shown in Fig. 45. The equilibrium potential with chloride externally was +5mV. When iodide replaced chl;oride externally the equilibrium potential shifted to -10mV. Assuming that the intracellular ionic composition was unaffected by the change in solution, the relative

FIGURE 45: Top: Voltage jump relaxations from -40 to -120mV with 60μM GABA, chloride internally, and chloride or iodide externally. The instantaneous current and decay time of the relaxations are increased when iodide is present externally. The steady state current at -40mV (I_{SS}) was lower in iodide than chloride for this particular cell. Bottom: The instantaneous current voltage relationship for 60μM GABA with chloride externally (line A) and iodide externally (line B). Points are the mean of 14 paired applications of GABA in chloride and iodide. Note the marked increase in inward current at negative potentials with iodide externally. The regression line through the data points in line A does not include the values obtained at +40 and +60mV, since desensitization has caused these values to be underestimated.



Iss_{-40} : −3.2nA

: −2.4nA



A: Cli // Clo

B: Cli // Io

permeability of the GABA channel to iodide and chloride can be calculated from:

$$\Delta Vnull = RT/zF.\ln (P_A[A]_o/P_B[B]_o)$$

(see e.g. Hille 1984). The shift in equilibrium potential gives a relative permeability of iodide to chloride of 1.88.

An interesting and unexpected effect of external iodide is also seen in Fig. 45. It appears that at negative potentials iodide enhances the inward current at potentials negative to -40mV. This is particularly marked at -120mV, where the instantaneous current is increased more than two fold. In this part of the potential range, the current is carried almost exclusively by chloride efflux from the neurone.

The top traces in Fig. 45 illustrates voltage jumps from -40 to -120mV in symmetrical chloride solutions, and with chloride internally and iodide externally. This shows two effects. One, the instantaneous current is increased, and two, the relaxation is considerably slower. The prolongation of the relaxation was observed in all 16 neurones examined, and this suggests that the presence of iodide externally affects GABA channel kinetics.

These effects of external iodide were also observed at concentrations of 10 and 50mM iodide. Fig. 46 illustrates relaxations obtained on voltage steps from -40 to -120mV in symmetrical chloride (0 iodide) and 10mM and 50mM external iodide. It can be seen that even at 10mM, iodide is able to increase the instantaneous current and channel open time at -120mV. The effect is more marked with 50mM iodide. These effects were observed in all 4 cells (6 applications) examined in 10mM iodide, and all 5 cells (6 applications) in 50 mM iodide. Again these actions were rapidly and fully reversible. Fig. 47 shows the pooled results from the 135, 50, 10 and 0mM iodide experiments on the

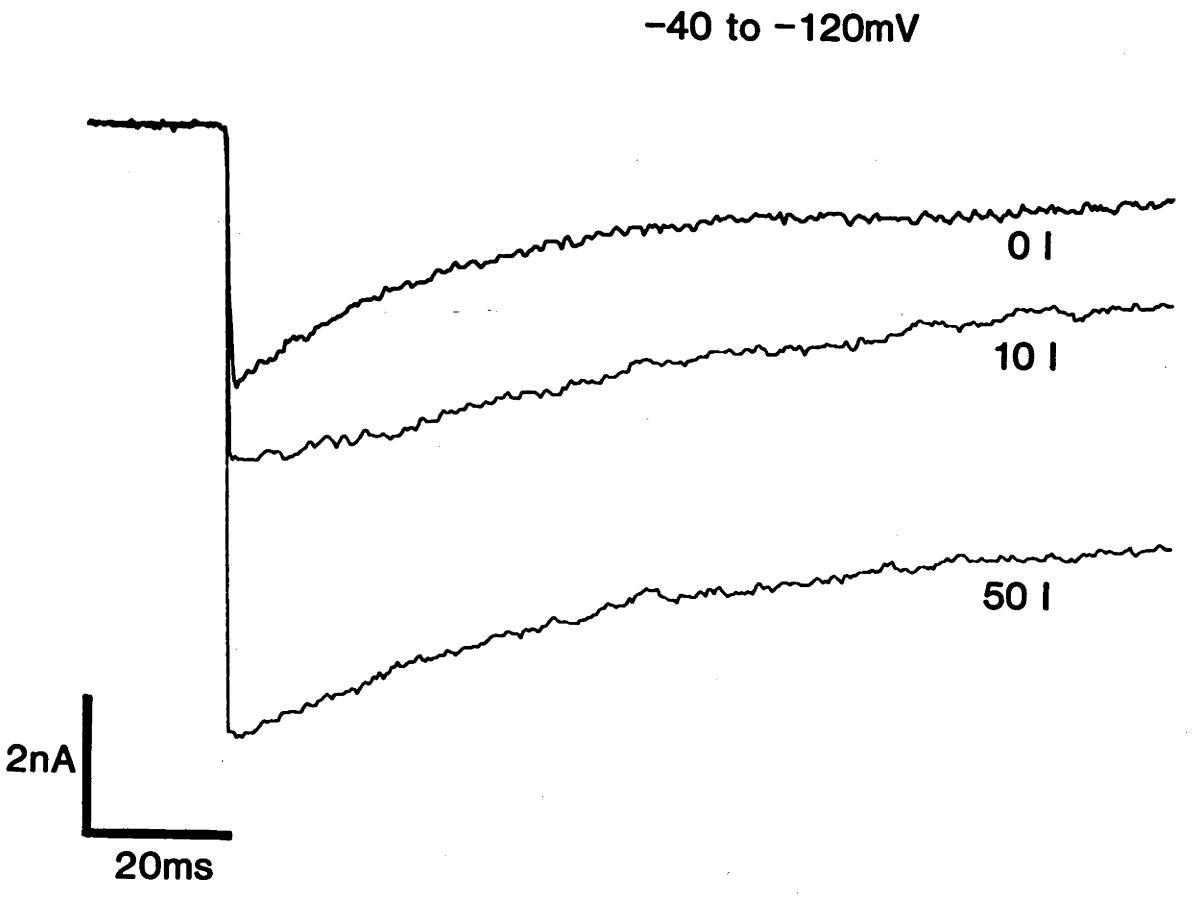


FIGURE 46: Voltage jump relaxations on stepping from -40 to -120mV in 60μM GABA. Currents were recorded in control solution (0 iodide) and with 10 and 50mM iodide. The presence of iodide externally causes an increase in the instantaneous current and a prolongation of the relaxation.

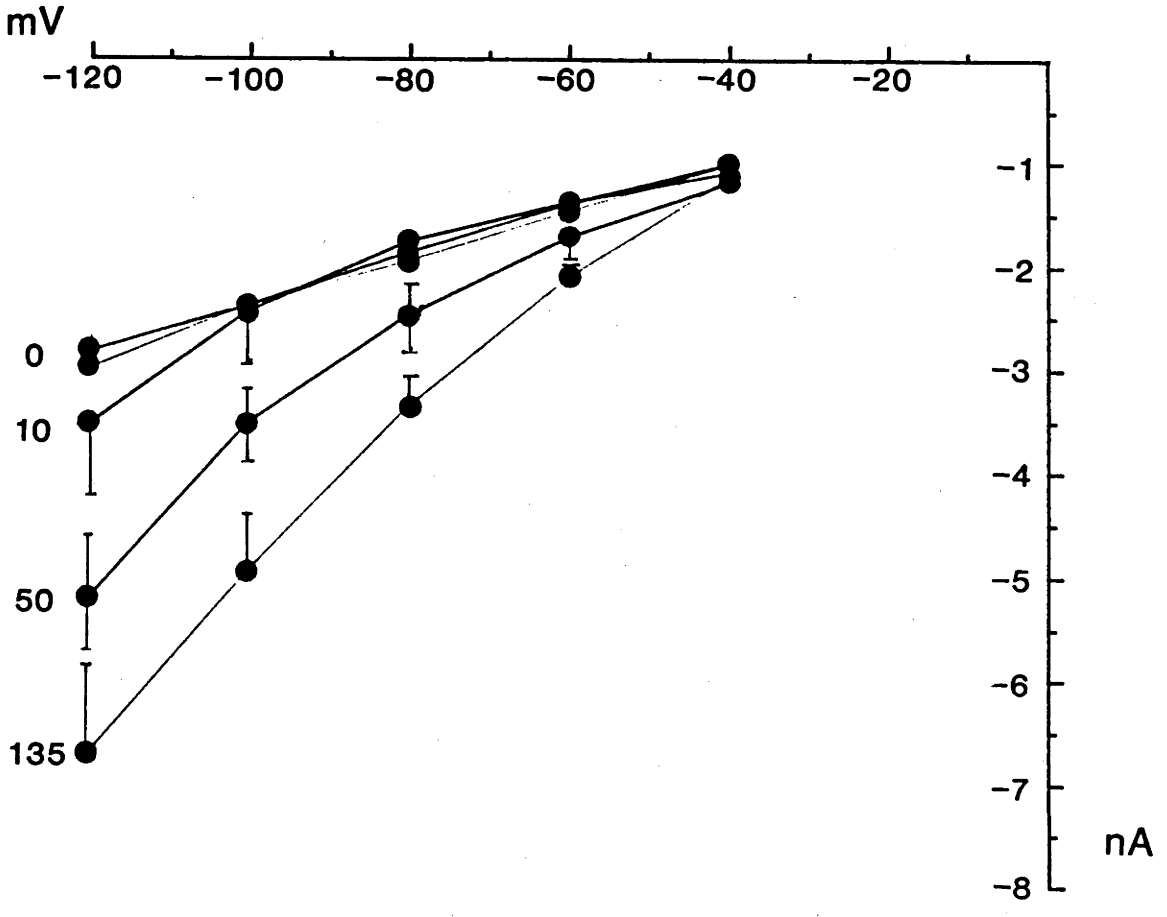


FIGURE 47: Normalized instantaneous current voltage relations for channels activated by 60μM GABA with three concentrations of iodide externally. Instantaneous currents are normalized to the value at -40mV in control (0 iodide) solution. The second (lower) set of data points in 0 iodide solution refers to the currents recorded in 135mM iodide. Points are means ± one standard error of the mean.

instantaneous current voltage relation from -40 to -120mV. There is little difference in the steady state current at -40mV over all iodide concentrations. These results suggest that changing the external anion from chloride to iodide facilitates chloride exit from the cell.

Pooled values for the time constant of decay of voltage jump relaxations in 135mM iodide at six potentials are illustrated in Fig. 48. Relaxations are longer than chloride control values at all potentials examined. It proved extremely difficult to reliably fit the smaller amplitude relaxations at -60 to +20mV. Often relaxations at +60mV were extremely short (these are not included), and the reason for these fast relaxations is not clear. Two regression lines have been fitted to the data points in Fig. 48. Line A is a fit of all the data points, and gives a value for the voltage sensitivity (H) of 204mV with $\tau_D(0)$ equal to 118ms. The line B is a fit to the hyperpolarized potentials only, and the H value obtained is greatly reduced, to ~ 700mV. Further experiments will be necessary to determine if external iodide ions do so markedly reduce the voltage sensitivity of GABA channel kinetics.

Values for prolongation of τ_D at -120mV after changing to 135mM iodide ranged from 1.77 to 3.49 fold, the mean increase over 9 cells was 2.25 ± 0.17 (x ± s.e.). Pooled values for relative increases in decay time at slightly more depolarized potentials (i.e. -80 to -100mv) ranged from 1.7 to 3.75, with a mean increase of 2.62 ± 0.26 (n = 9). Since the average $\tau_D(0)$ in Cl^- solution

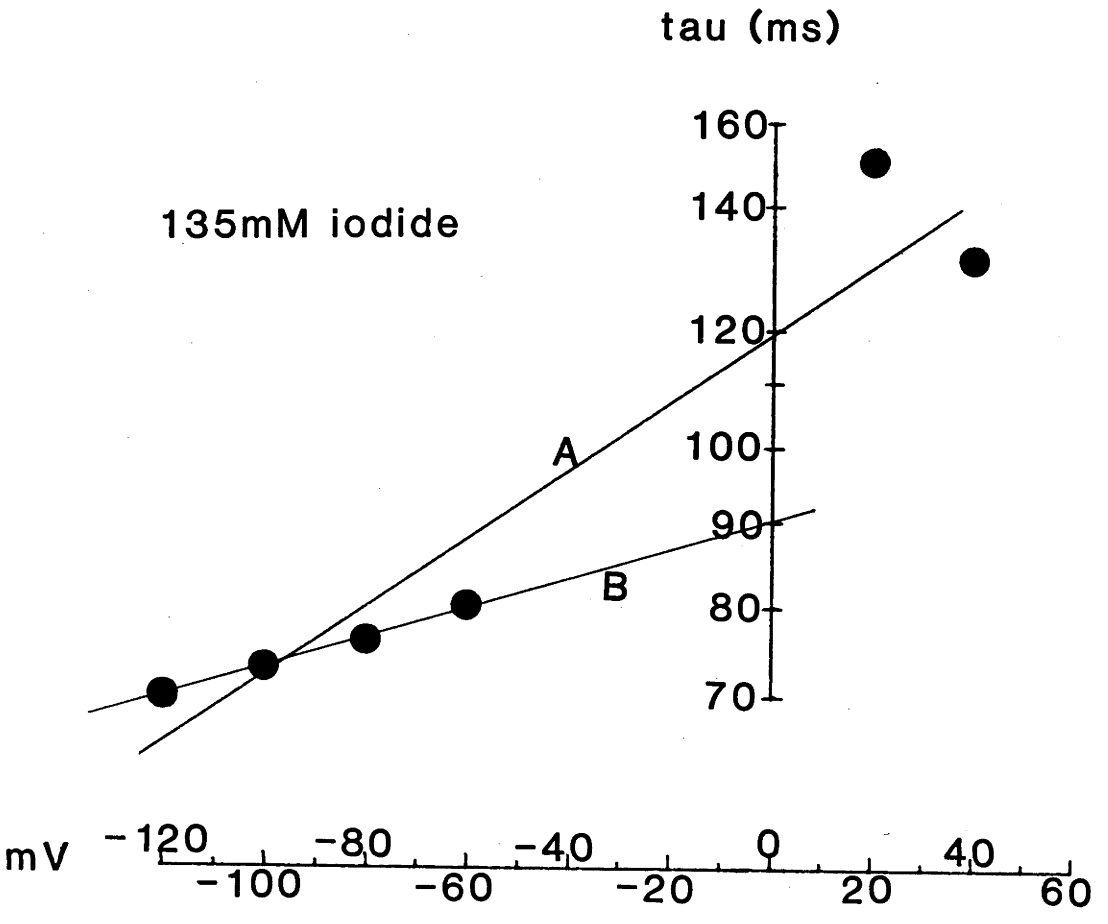


FIGURE 48: Time constant of relaxation with 135mM iodide externally, 140mM chloride internally. The relaxations are longer than those in symmetrical chloride at all potentials. Line A is a regression line for all the data points, B is a fit to the values of τ_D at negative membrane potentials only. Individual data points are the mean of 4 to 10 separate experiments.

was 57ms, if the normal voltage sensitivity of GABA channel kinetics was retained, $\tau_D(0)$ in iodide would be ~ 2.3 times $\tau_D(0)$ in chloride, i.e. 131ms. Regression line B in Fig. 48 considerably underestimates this value, while line A (with voltage sensitivity of 204mV) represents a closer approximation.

50mM iodide resulted in a mean increase of τ_D at -120mV of 1.53 ± 0.18 (x ± s.e., n = 4), while in 10mM iodide, the mean increase in time constant of decay at -120mV was 1.41 for three cells, but a 3.21 fold prolongation was obtained for one other cell. These results indicate that the effects of iodide on kinetics are concentration dependent.

In one other cell, caesium iodide electrodes were used instead of caesium chloride electrodes, to assess the effects of intracellular iodide on kinetics and permeability. With chloride externally, the instantaneous current at -120mV was 2.25 fold greater than the steady state current at -40mV. With iodide externally (symmetrical iodide) the current at -120mV was 2.5 fold greater than the current at -40mV, perhaps again suggesting that iodide externally also facilitates iodide exit from the cell. The time constant of the voltage jump relaxation at -120mV was 104ms with iodide externally. GABA induced currents with iodide as the predominant intracellular anion were still voltage sensitive, which is contrary to what was observed previously in hippocampal neurones (see Section B).

Bromide.

Bromide subsitution was made in the external solution with 135mM choline bromide and 5mM choline chloride replacing 140mM choline chloride. The same protocol was used for the bromide permeability measurements as the iodide experiments (see above). For 10 paired applications of GABA in chloride and bromide, the mean equilibrium potentials were +2 and -2mV respectively. The average instantaneous current voltage relationship is shown in Fig. 49. From this it can be seen that the steady state current values at -40mV in each solution are very close, and again the inward current is considerably enhanced at potentials between -60 and -120mV. There is a ~ 2.2 fold increase in inward current at -120mV after replacing external chloride with bromide.

Fig. 50 illustrates the current relaxations upon a voltage step from -40 to -120mV in external chloride and bromide. In addition to increasing the instantaneous current, external bromide also prolongs the relaxation time course.

Calculation of the relative permeability for bromide and chloride for these paired cells yields a value of 1.21 for P_{Br}/P_{Cl}.

It is of interest that external bromide ions also prolong GABA channel relaxations. At -120mV this lengthening varied from 1.76 to 3.43 times control values. The mean increase in time constant over 7

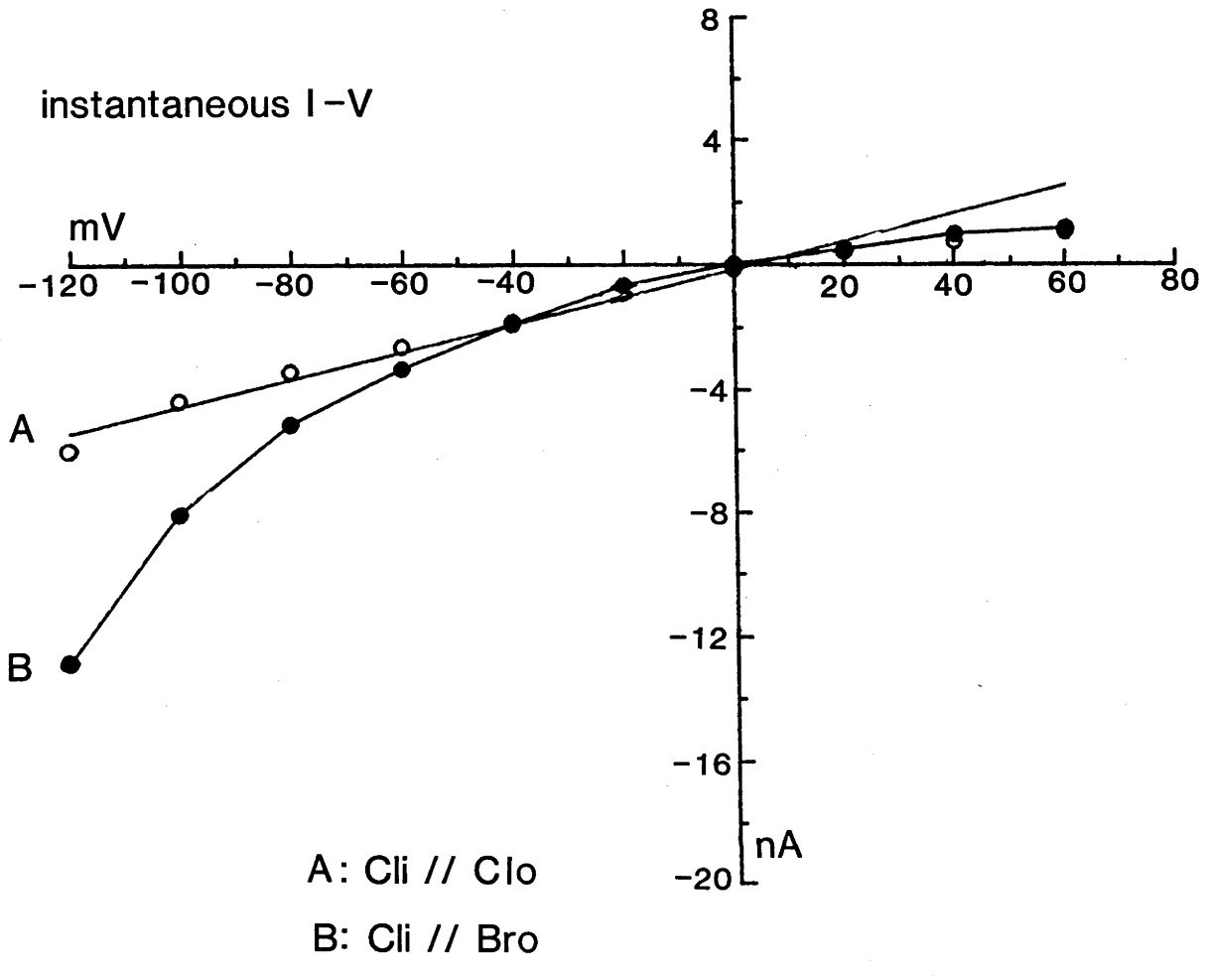


FIGURE 49: Instantaneous current voltage relation for 10 cells with chloride internally, and chloride externally (A) and bromide externally (B). The values of the current at -40mV are very similar, but the inward current progressively increases from -60 to -120mV with bromide externally. The regression line for the instantaneous current in symmetrical chloride does not include the data points at +40 and +60mV, since desensitization caused this current to be underestimated at these potential values.

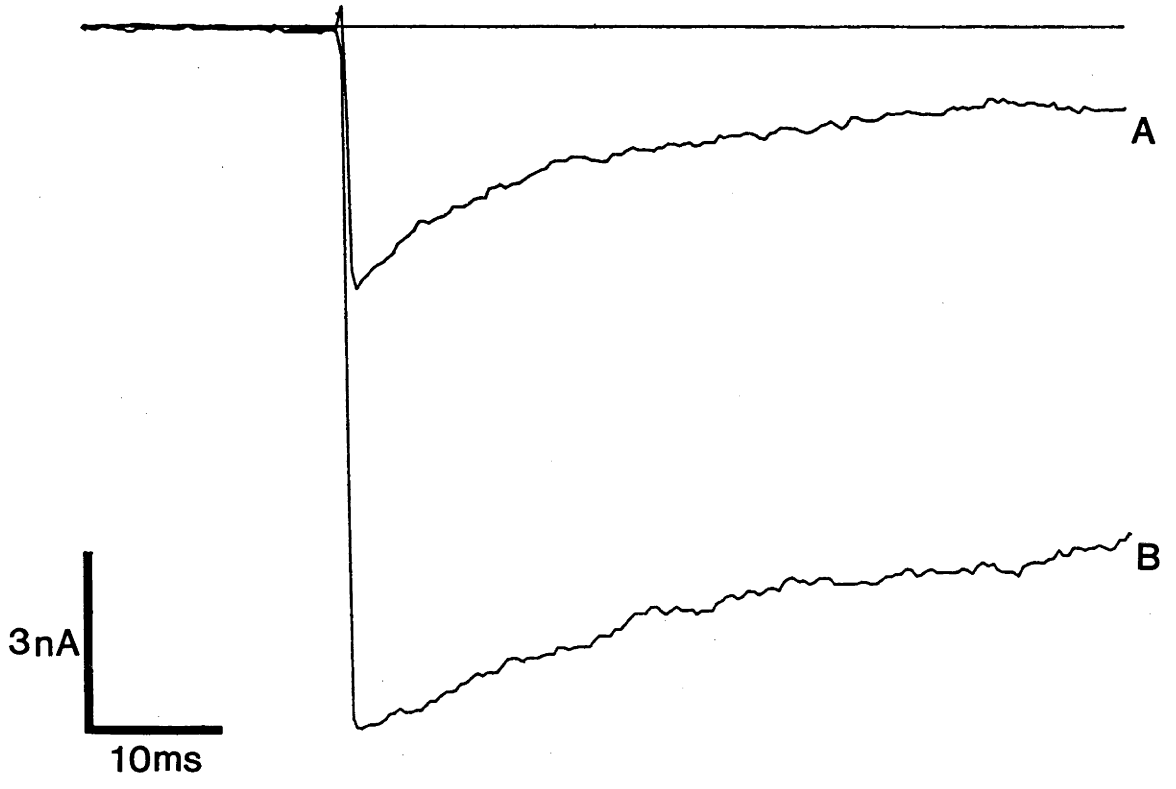


FIGURE 50: Voltage jump relaxations in response to a hyperpolarizing step from -40 to -120mV with chloride externally (A) and bromide externally (B). The GABA concentration was 60μM. Note the greater amplitude and slower decay of the relaxation in bromide.

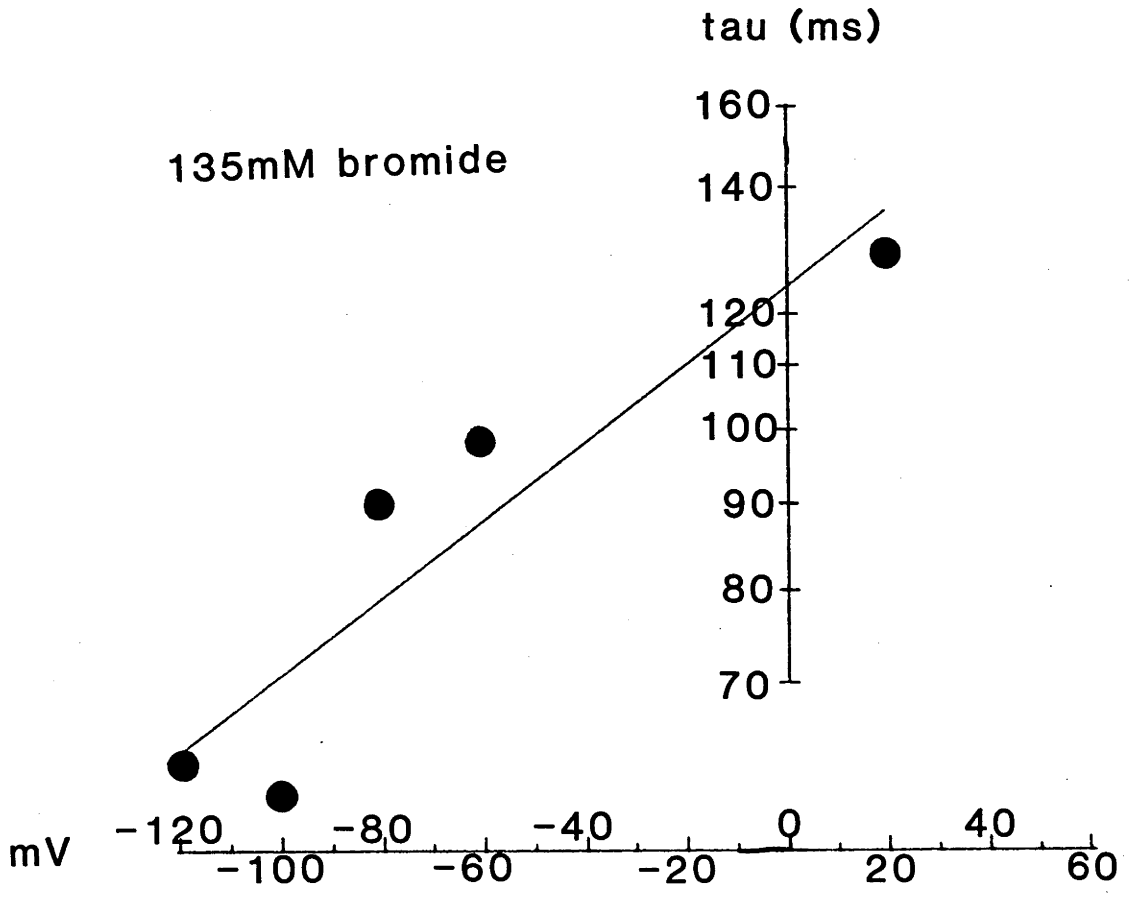


FIGURE 51: Voltage sensitivity of the time constant of relaxation with 135mM bromide ions externally. Data points are the mean values of 3 to 8 measures of τ_D at each potential.

measurements was 2.75 ± 0.25. At more depolarized values (-60 to -100mV) this lengthening was 2.4 fold. Fig. 51 shows the pooled time constants of relaxation in 135mM bromide, and their voltage sensitivity. The value of the voltage sensitivity (H) is 179mV, with $\tau_D(0)$ at 123ms. Comparing $\tau_D(0)$ in chloride solutions (57ms) with those in bromide gives a lengthening of 2.16. Although the data points are few in number, it is clear that relaxations with external bromide are approximately as voltage sensitive as those in symmetrical chloride solutions.

Fluoride.

Caesium fluoride microelectrodes were used to measure fluoride permeability of GABA-activated channels, since choline fluoride was not easily obtained. Instantaneous current-voltage relations were constructed with choline chloride externally: this meant that it was not possible to change the test ion several times on the same cell.

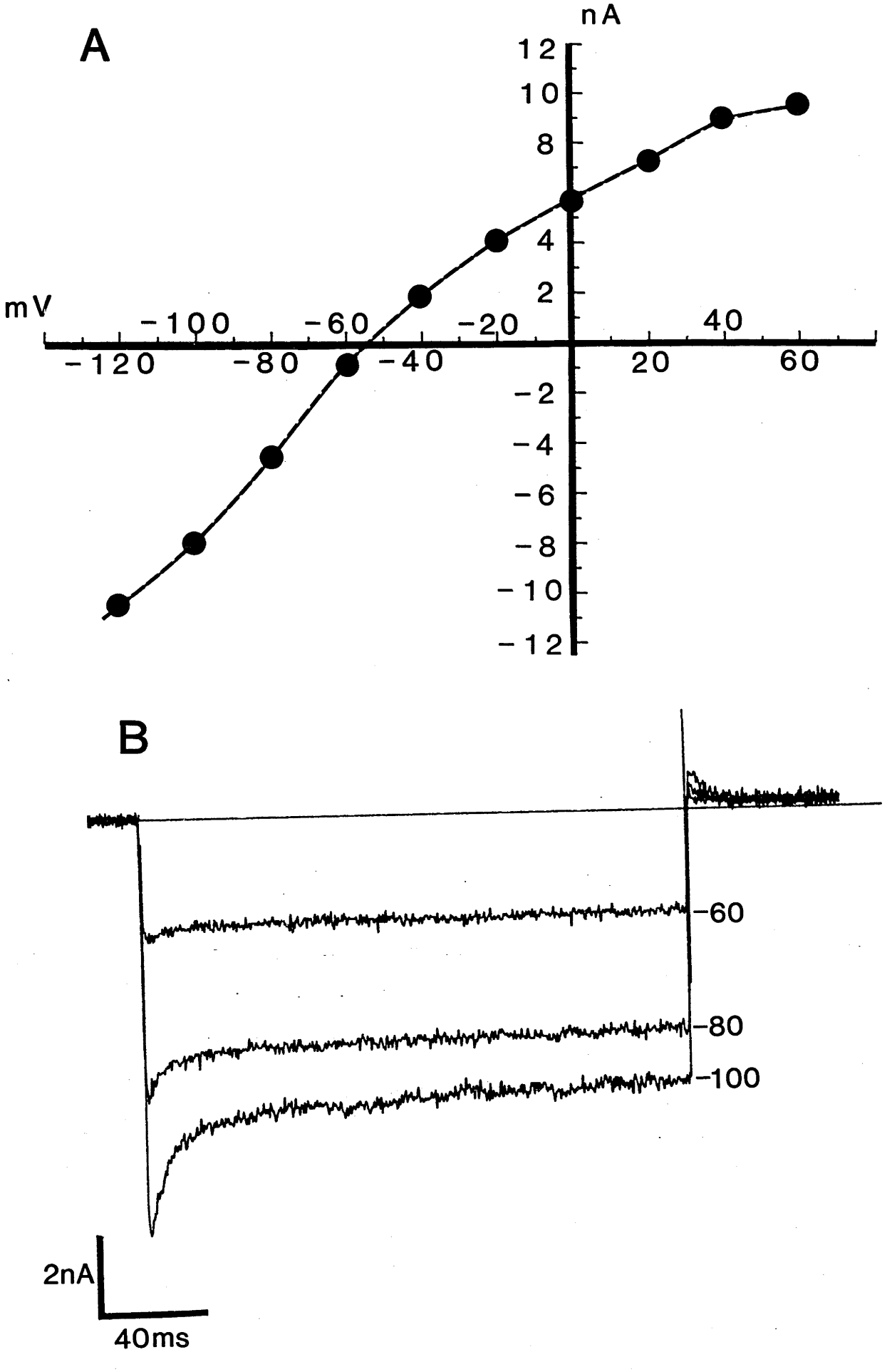
In all 13 cells tested with fluoride internally and chloride externally, GABA produced an outward current at -40mV, indicating that at this potential chloride ions were entering the cell, and that the equilibrium potential under these bi-ionic conditions was more negative than -40mV. Measurement of the equilibrium potential from instantaneous current voltage relationships gave values of -125,-113, -82 and -55mV. Fig. 52 (top) shows the instantaneous I-V from one of these fluoride containing

cells. If we take a reasonable vaue of +5mV for the equilibrium potential in symmetrical chloride solutions, then the values obtained for the equilibrium potential in fluoride/chloride give relative permeabilities of fluoride to chloride (P_F/P_{Cl}) of 0.006, 0.009, 0.03 and 0.09. The mean value of these relative permeabilities is 0.034. However, given the variability of these responses (due to the low fluoride permeability), it is probably wisest to say that the fluoride permeability is less than 0.1 of the chloride permeability.

These permeabilities were measured with 1mM GABA, since the agonist induced currents obtained with lower concentrations of agonist were too difficult to measure reliably.

Relaxations with 1mM GABA with internal fluoride solution are shown in Fig. 52 (bottom). It can be observed that the time constants of relaxation were more rapid than those previously illustrated for GABA in symmetrical chloride solution. At least part of the reason for the rapid decay is due to the fact that high concentrations (1mM) of GABA had to be used to elicit these currents. In three cells the time constant of relaxation at -120mV was 5.8, 14.6 and 12.1ms. These values are all lower than the average τ_D value at -120mV obtained for 1mM GABA in symmetrical chloride solutions (18ms), perhaps suggesting that channel closure is more rapid than usual when fluoride ions pass through the channel.

FIGURE 52: A) Instantaneous current voltage relationship for channels activated by 1mM GABA with fluoride internally and chloride extracellularly. The equilibrium potential for this cell is -55mV. B) Voltage jump relaxations from -40mV to the potentials indicated. Fluoride intracellularly, chloride extracellularly. 1mM GABA.


A
nA
12
10
8
4
2
mV
-100
-60
-20
40
-120
-80
-40
20
60
-2
-4
-6
-8
-10
-12
B
-60
-80
-100
2nA
40ms

Propionate.

In 10 applications of 1mM GABA to seven neurones with 135mM sodium propionate externally, only inward currents were observed. The instantaneous current voltage relation from one of these cells is illustrated in Fig. 53. In this cell it is clear that there is no outward current (i.e. propionate influx) even up to +60mV. Since the potential range only extends up to +60mV, propionate permeability must be less than 0.1, but may even be zero. Fig. 54 illustrates voltage jump relaxation sequences from a cell in a propionate solution. Once again, these relaxations are extremely fast at all three potentials. In this cell the time constants of relaxation are 3.0ms at -120mV, 3.4ms at -100mV and 4.9ms at -80mV. The time constants were approximately three to six times faster than those obtained with 1mM GABA with chloride ions on both sides of the membrane. The results with propionate would again indicate that the nature of the external anion affects GABA channel kinetics, in this case accelerating channel closure.

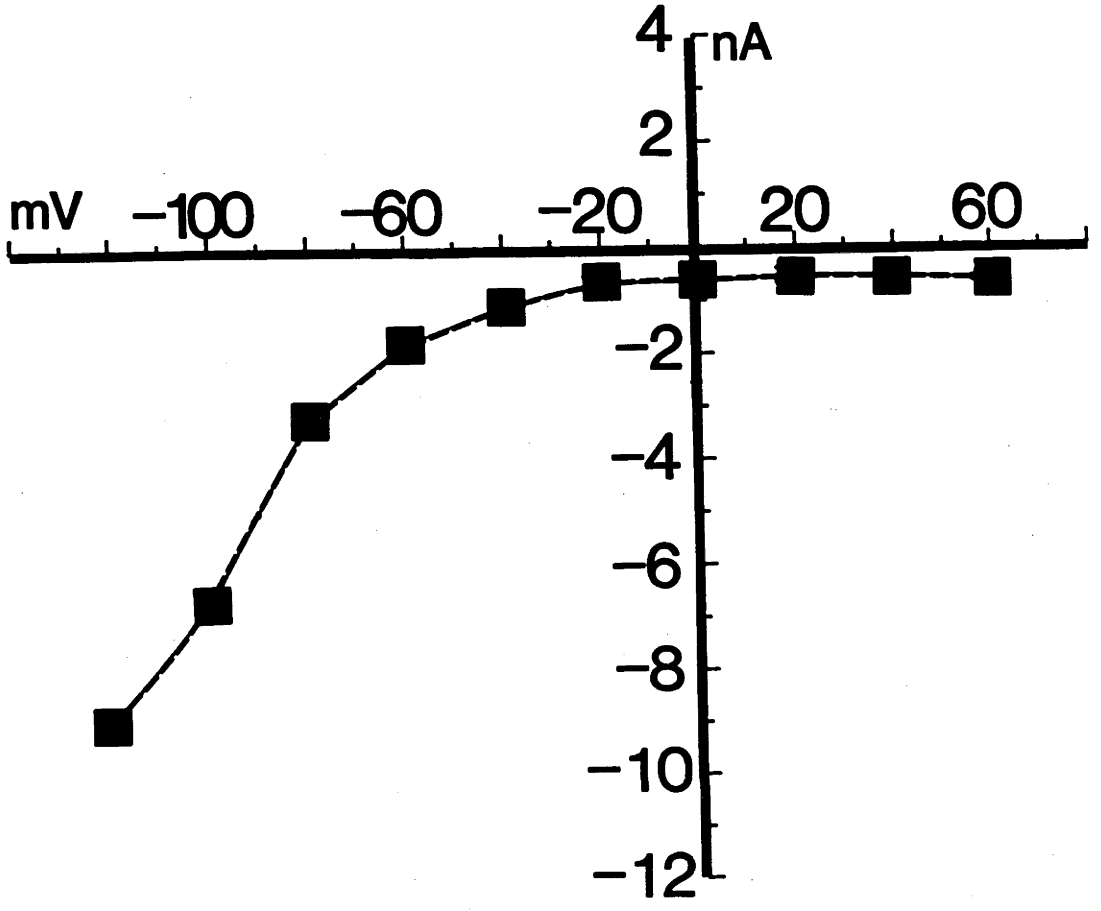


FIGURE 53: The instantaneous current voltage relationship for one cell with chloride internally and propionate ions externally. There is no reversal of the current, suggesting that propionate ions are much less permeable than chloride in these GABA channels.

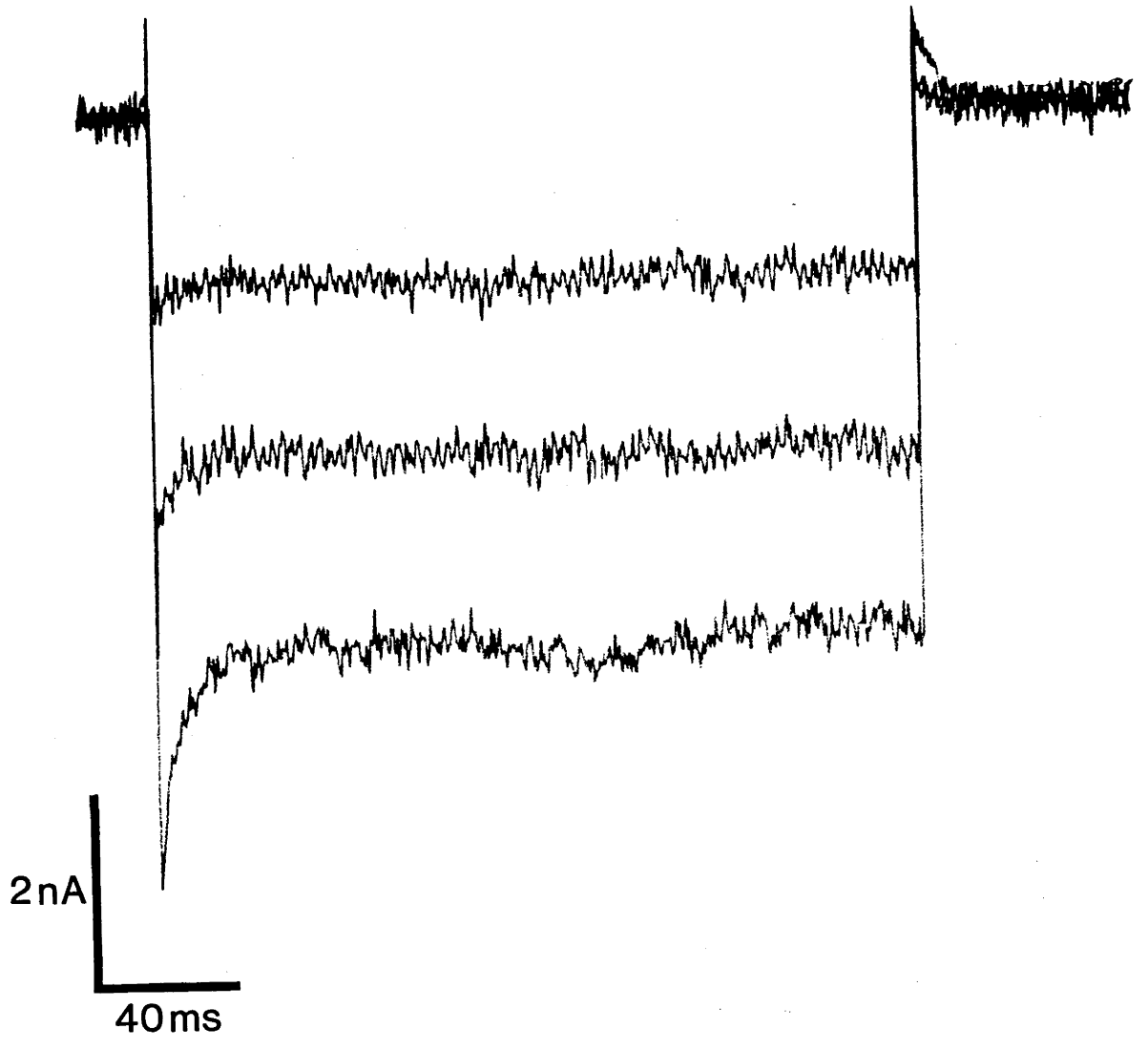


FIGURE 54: Voltage jumps with 1mM GABA, with chloride inside the cell and propionate ions in the extracellular solution. The currents are the responses to voltage jumps from -40 to -120, -100 and -80mV.

THE ACTIONS OF SOME CLINICAL ANAESTHETICS AND AVERMECTIN ON GABA CHANNELS IN DORSAL ROOT GANGLION NEURONES.

Pentobarbitone.

The clinical anaesthetic sodium pentobarbitone (which is a mixture of the (+) and (-) isomers) was bath applied to drg neurones at concentrations between 60μM and 3mM. In every cell where pentobarbitone was applied (53), GABA responses could also be elicited. Additionally, if cells did not respond to GABA, then no response to pentobarbitone could be obtained.

Where pentobarbitone was applied at the same concentration as GABA, the anaesthetic was less effective than GABA (60μM: 0.1x as potent as GABA, n = 3, 100μM: 0.6x, n = 1, 1mM: 0.4x, n= 3).

The agonist action of pentobarbitone was rapidly and reversibly blocked by 50μM of bicuculline methiodide or picrotoxinin.

In four experiments the effects of 60μM GABA and 1mM pentobarbitone applied separately and together were assessed. 60μM GABA alone evoked on average, -1.2nA of current, 1mM pentobarbitone produced -2.1nA of current, but together these agonists produced a mean of -11.1nA of current.

As pentobarbitone concentration was increased to 600μM and above, the whole cell current response first increased rapidly to a peak. This was followed by sharp

decline to a plateau level. However, an unusual response was observed when the solution multiplexer was switched back to the control solution to remove the anaesthetic from the bath. Very shortly after the switch, a marked and transient increase in current was observed. This small 'rebound' or 'bounce' of current then declined to the baseline. (Hereafter this phenomenon will be referred to as 'bounce'). The duration and magnitude of bounce depended on the position of the neurone relative to the main solution flow, and the speed of switching the multiplexer tap. If the tap was switched very quickly and the cell was was close to the solution inlet, the bounce was rapid and large. If the solution change around the cell was slow, the bounce was smaller and longer, and with very slow solution changes the bounce was not visible and there was a slow decline to the pre-agonist baseline.

The current responses to 1mM pentobarbitone at -40 and +40mV are illustrated in Fig. 55. The cells response to the anaesthetic agonist is smaller at +40mV due to the both the slightly reduced driving force for chloride at this potential, and also because of the potential dependence of pentobarbitone block (see below).

At least part of the decline in peak current in Fig. 55 is due to desensitization, but there is likely to be a contribution from channel 'block' by pentobarbitone. The ratio of the bounce to the initial peak response can give an indication of the number of channels absorbed into a non-conducting state ('blocked') by pentobarbitone.

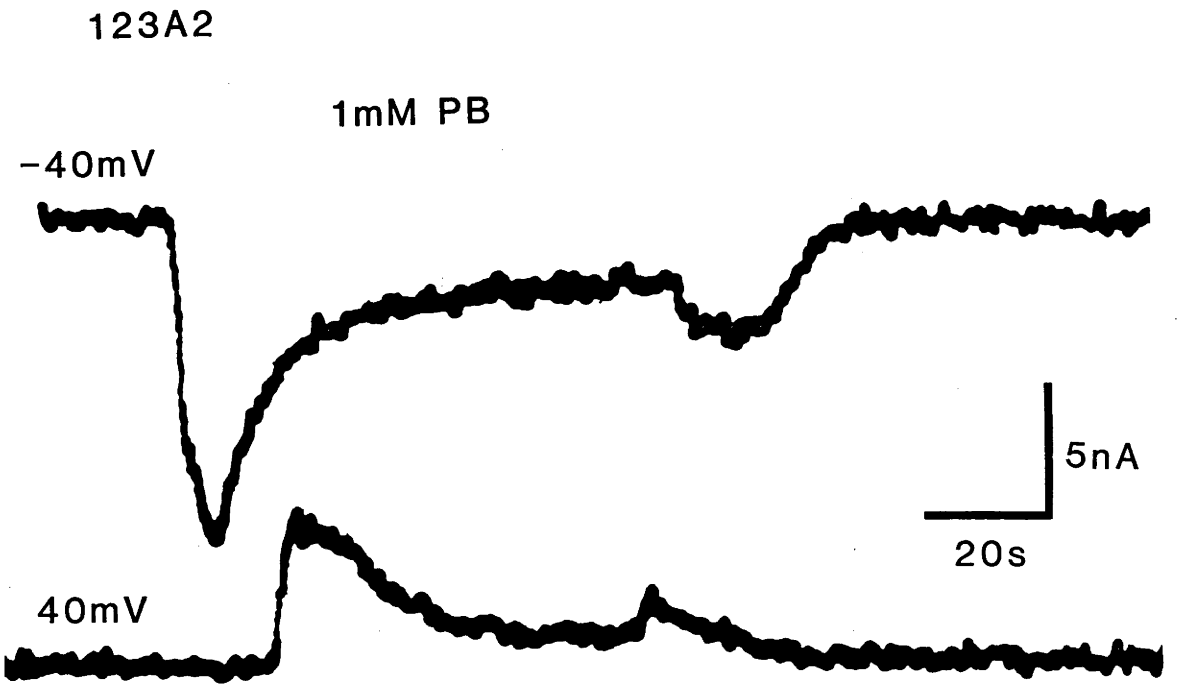


FIGURE 55: Agonist action of 1mM pentobarbitone at two potentials. Pentobarbitone produces a large increase in current, which declines to a plateau, and is followed by a secondary increase in current ('bounce') on washout of the drug.

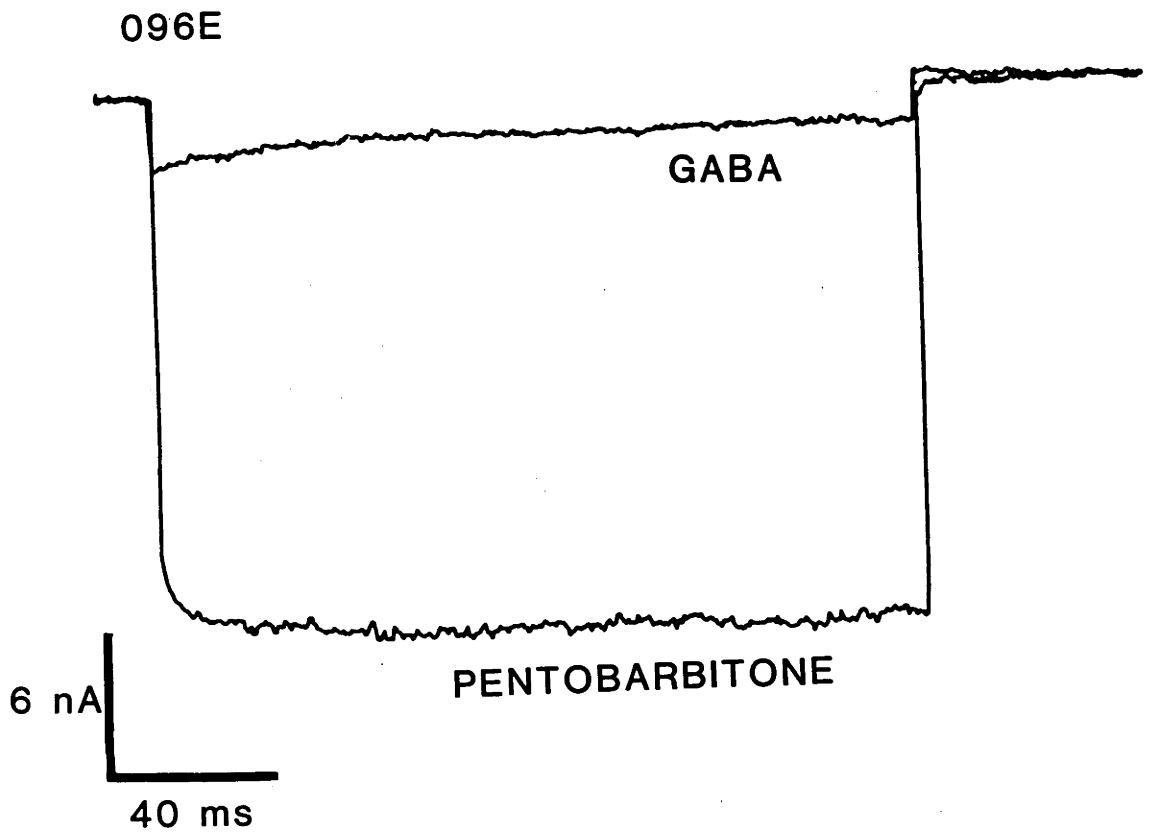


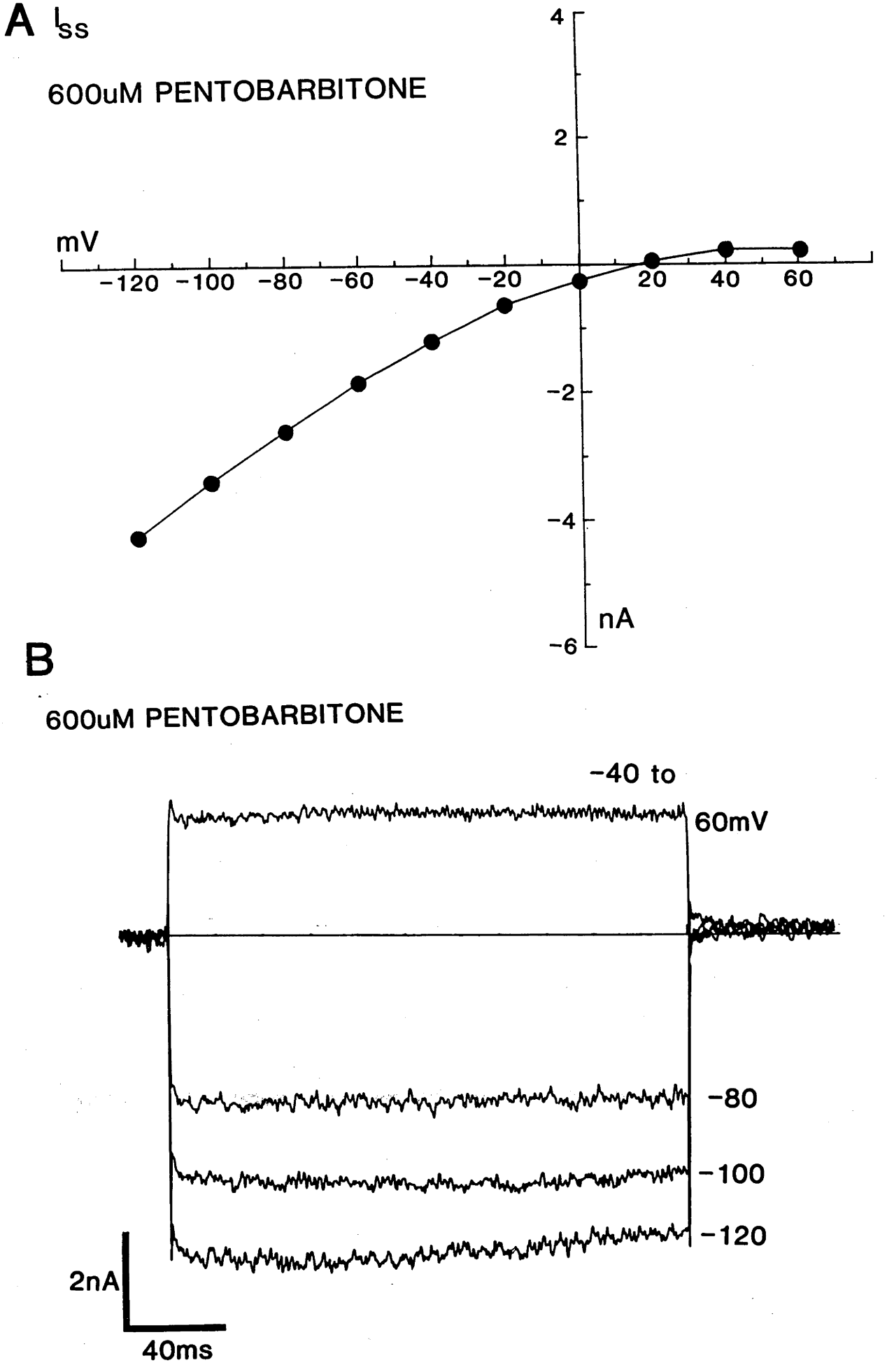
FIGURE 56: Voltage jump relaxations with 60μM GABA and 1mM pentobarbitone on a 60mV hyperpolarizing step from -40mV. Note that the relaxations are opposite in direction for the two agonists. Similarly, the off relaxations are of opposite sign.

Voltage jumps were performed before and during pentobarbitone in order to measure the voltage dependence of pentobarbitone activation of GABA channels.

Fig. 56 illustrates plots of the net relaxation produced by 60μM GABA and 1mM pentobarbitone on a potential step from -40 to -100mV. The current relaxations have very different characteristics. The GABA response shows the usual exponential decrease in current to a new steady state value, while the pentobarbitone response shows a more rapid outward relaxation, such that current increases to a new steady state value after the instantaneous jump. At the end of the hyperpolarizing voltage step, the relaxations for the two agonists are again opposite in direction, consistent with a reduction in the number of open channels during the voltage step with GABA, and an increase in the number of open channels in pentobarbitone on the hyperpolarizing step. These unusual relaxations were a typical feature with pentobarbitone at concentrations greater than 100μM, and were observed in all cells to which this agonist was applied.

Fig. 57B illustrates a family of current relaxations in response to voltage jumps with 600μM pentobarbitone. The outward relaxation is observed on all the hyperpolarizing steps, but depolarizing steps showed little change in current after a fast initial decrease in current. (The off relaxation after the depolarizing step consistent with a reduction in the number of conducting channels during this step).

FIGURE 57: A) The steady-state current voltage relationship for 600μM pentobarbitone. Mean of 14 applications. Note the marked rectification at positive potentials. B) Voltage jump relaxations in 600μM pentobarbitone.


A I_{ss}
600uM PENTOBARBITONE
mV
4
2
-2
-4
-6
-120 -100 -80 -60 -40 -20 20 40 60
nA
B
600uM PENTOBARBITONE
-40 to
60mV
-80
-100
-120
2nA
40ms

The steady state current voltage relation for cells exposed to 600μM pentobarbitone is shown in Fig. 57A. This shows that the pentobarbitone-induced current rectifies in the opposite direction for channels gated by GABA (or other GABA-like agonists). There is an increase in channel numbers as membrane potential is made more negative. At positive potentials there is considerably less current.

Fig. 58 shows expanded relaxations on depolarizing steps with 60μM GABA and 600μM pentobarbitone. The GABA current increases steadily after the instantaneous current, as observed previously, but the current induced by pentobarbitone shows a sharp initial decrease after the ohmic current, and then steadily increases. The open arrow highlights this phenomenon. Similar behaviour was observed with 10 other cells exposed to pentobarbitone.

Since pentobarbitone is 75% uncharged at physiological pH, and 25% in an anionic form, it was considered worth testing which form of the anaesthetic was responsible for reducing the current at positive potentials, and to determine whether the neutral or anionic forms were responsible for activating the GABA receptor channel.

Experiments were performed measuring the response to 1mM pentobarbitone at pH 7.4, then changing to pH 9.4 or 10.4, and measuring the response at the new alkali pH value. In two experiments at pH 10.4 and 7.4, pentobarbitone produced -4.4 and -7.0nA at 7.4, but there was zero current in response to this agonist at pH 10.4.

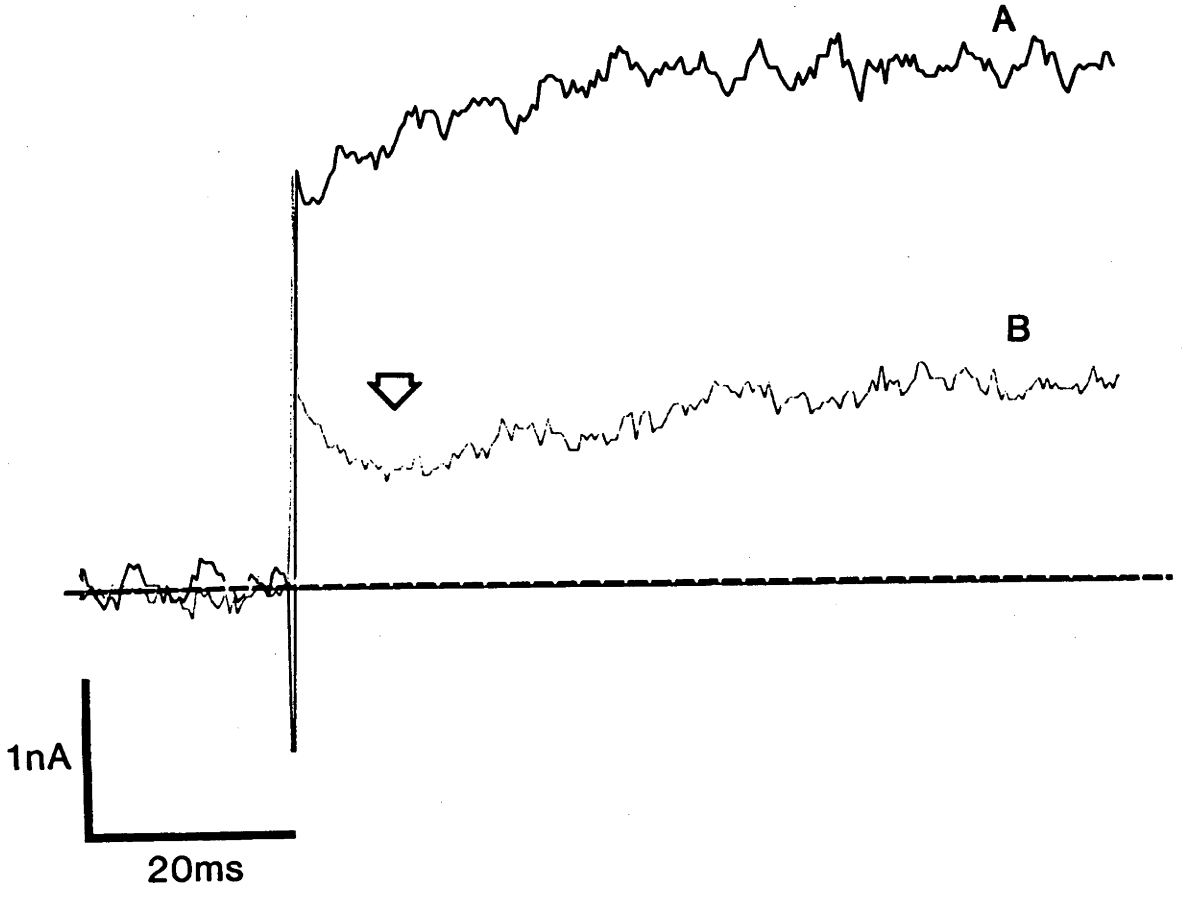


FIGURE 58: Relaxations in response to depolarizing steps to +60mV from -40mV with 60μM GABA (A) and 600μM pentobarbitone (B). Note that the GABA induced current increases steadily after the instantaneous current, while the current in pentobarbitone initially decreases, then increases to a new steady state value.

Similarly, in four successful experiments at pH 7.4 and 9.4, pentobarbitone produced a mean response of -5.5nA at 7.4, but only a mean of -0.2nA at pH 9.4. (In three of these applications pentobarbitone caused no current change at all at pH 9.4, the other cell showed a maximum change that was only 10% of the full response at pH 7.4). One of these experiments is illustrated in Fig. 59.

In order to determine if the GABA receptor channel complex itself was not disturbed by these alkali solutions, three experiments were performed to measure the effectiveness of 60μM GABA at pH 9.4. In these experiments, GABA responses at 9.4 were 88%, 85% and 79% of their value at pH 7.4, suggesting that the receptor/channel complex is substantially unaltered at pH 9.4.

Further experiments were conducted with pentobarbitone at pH 5.4. In 6 cells with 8 applications of pentobarbitone at pH 7.4 and 5.4, 1mM pentobarbitone produced 1.2 to 2.9 fold more current at acid pH values. The mean increase was 1.9 ± 0.3 (x ± s.e.) fold at -40mV. The steady state current voltage relationship from 4 cells with paired applications of 1mM pentobarbitone at both pH values is illustrated in Fig. 60. Pentobarbitone was more effective at pH 5.4 at all potentials examined. However, current responses still rectified at positive potentials. Additionally the bounce of current observed upon washout of pentobarbitone was still present at pH 5.4.

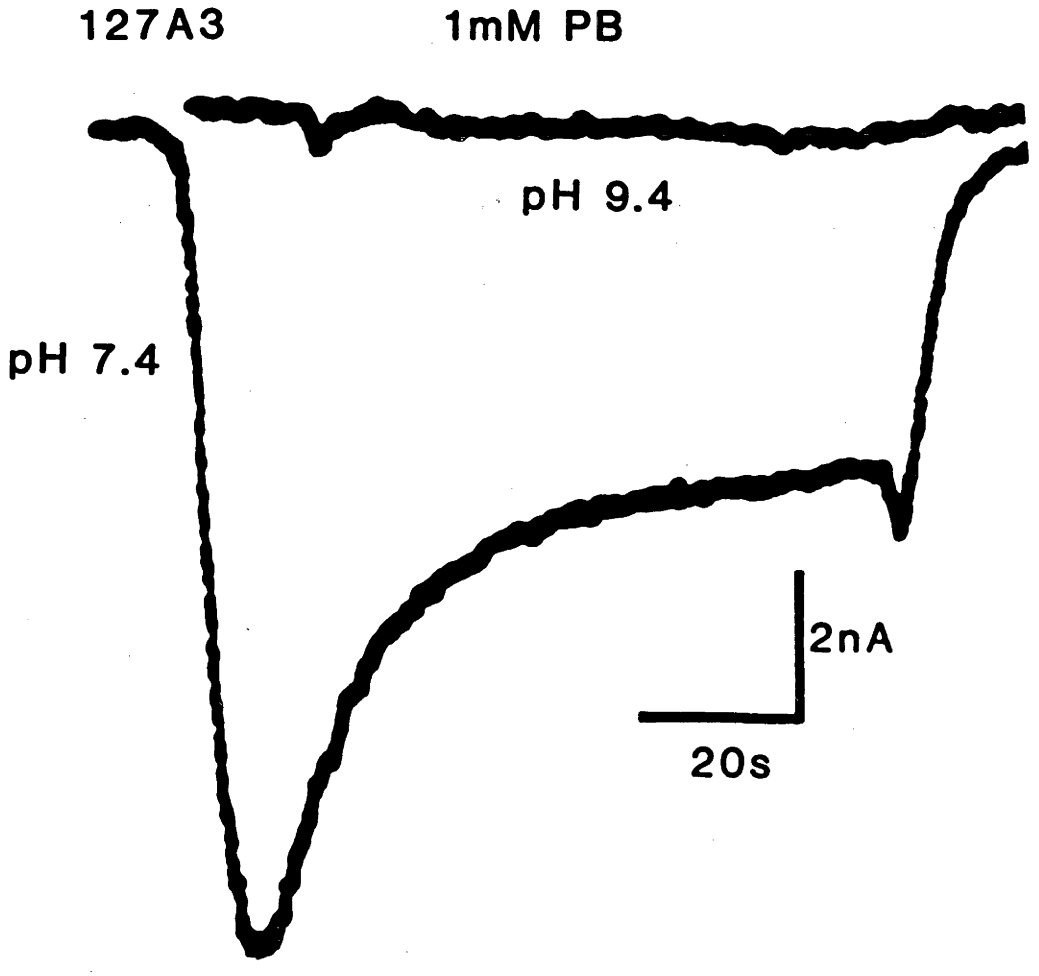


FIGURE 59: Pentobarbitone elicits little or no conductance change at pH 9.4. 1mM pentobarbitone produced a large inward current at -40mV at pH 7.4, but had an insignificant action at pH 9.4. There is a small rapid bounce at pH 7.4 on washing the anaesthetic agonist from the bath.

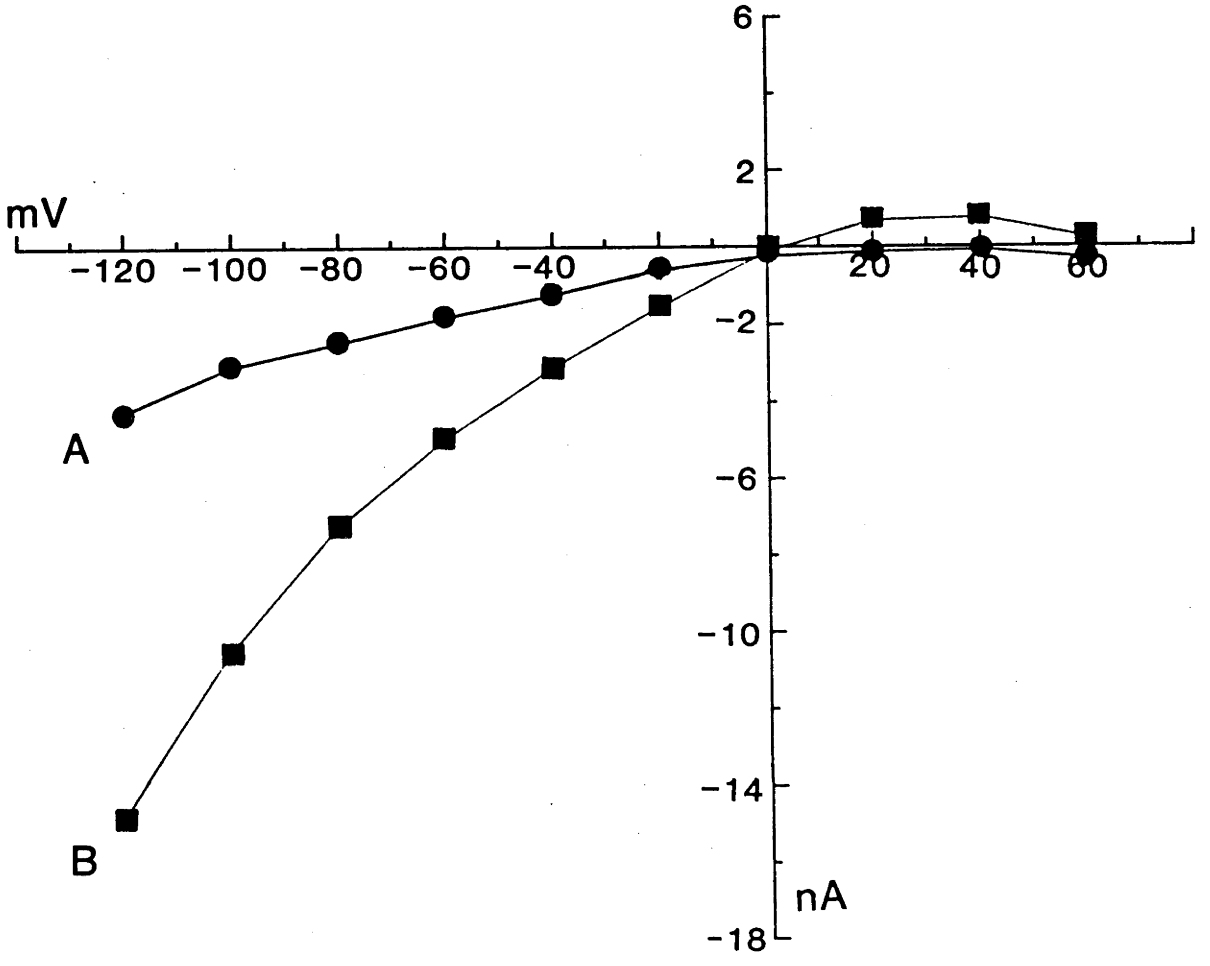


FIGURE 60: Steady state current voltage relationship for 1mM pentobarbitone at pH 7.4 (line A) and pH 5.4 (line B). Pentobarbitone induced current is reduced at positive potentials in both cases.

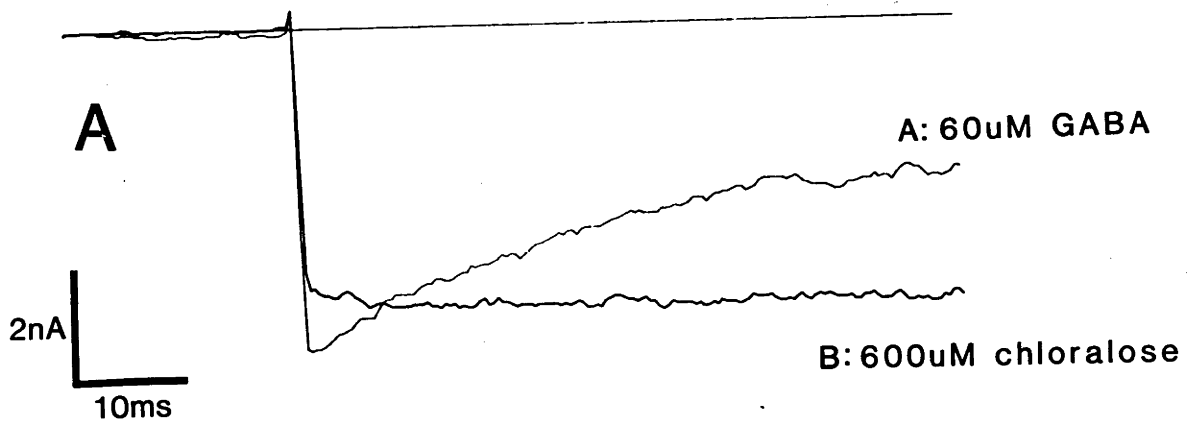
Chloralose.

α Chloralose was effective at activating GABA receptor/channels at concentrations of 100μM and above. Current responses to 600μM chloralose were rapidly and reversibly blocked by 50μM picrotoxinin and 50μM bicuculline methiodide. In 7 cells where the potency of 60μM GABA and 600μM chloralose were compared, GABA produced a mean inward current of -2.25 ± 0.47nA, and 600μM chloralose produced -2.51 ± 0.4nA on these same cells.

There is was no evidence of 'bounce' or desensitization at 600μM chloralose.

Voltage jump relaxations (Figs. 61 A & B) in 600μM chloralose did not show the form of relaxations as observed when GABA activates these channels. Again, if any relaxations were observed, they were of the opposite direction to those in GABA (i.e. outward on hyperpolarizing steps). The steady state current voltage realation for 13 experiments on 11 cells activated by 600μM chloralose is presented in Fig. 62. The slight curvature in this relation is opposite in direction to the steady state I-V observed with GABA and its stuctural analogues.

With higher concentrations of chloralose (1mM), current responses showed a decline after an initial peak, and a slight bounce after removal of the agonist (see Fig. 63A). This would suggest that at higher concentrations



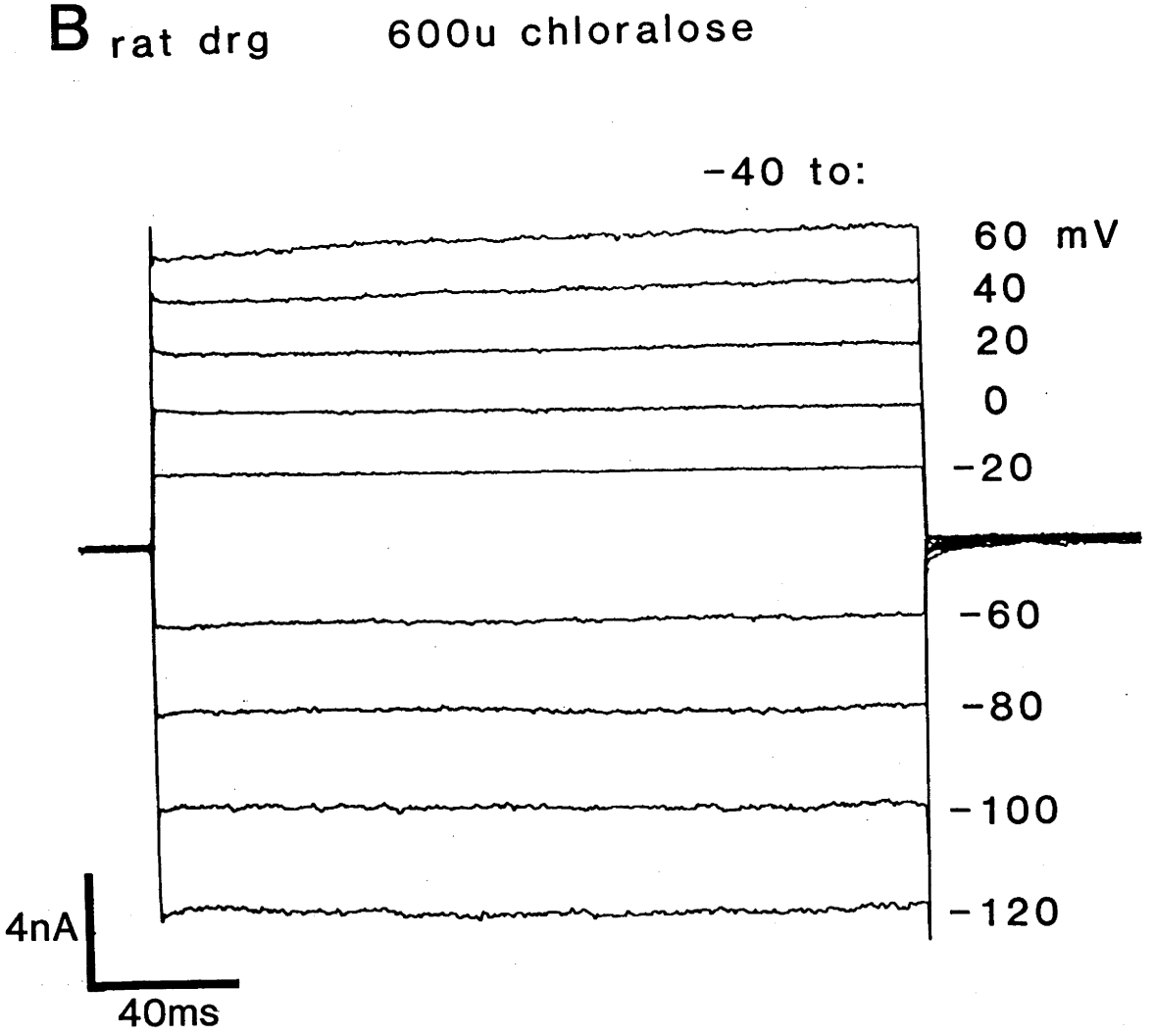


FIGURE 61: A) Voltage jump relaxations on a -80mV step from -40mV with 60μM GABA and 600μM chloralose. B) A family of relaxations with 600μM chloralose, stepping from -40mV to the potentials indicated at the right hand side of the currents.

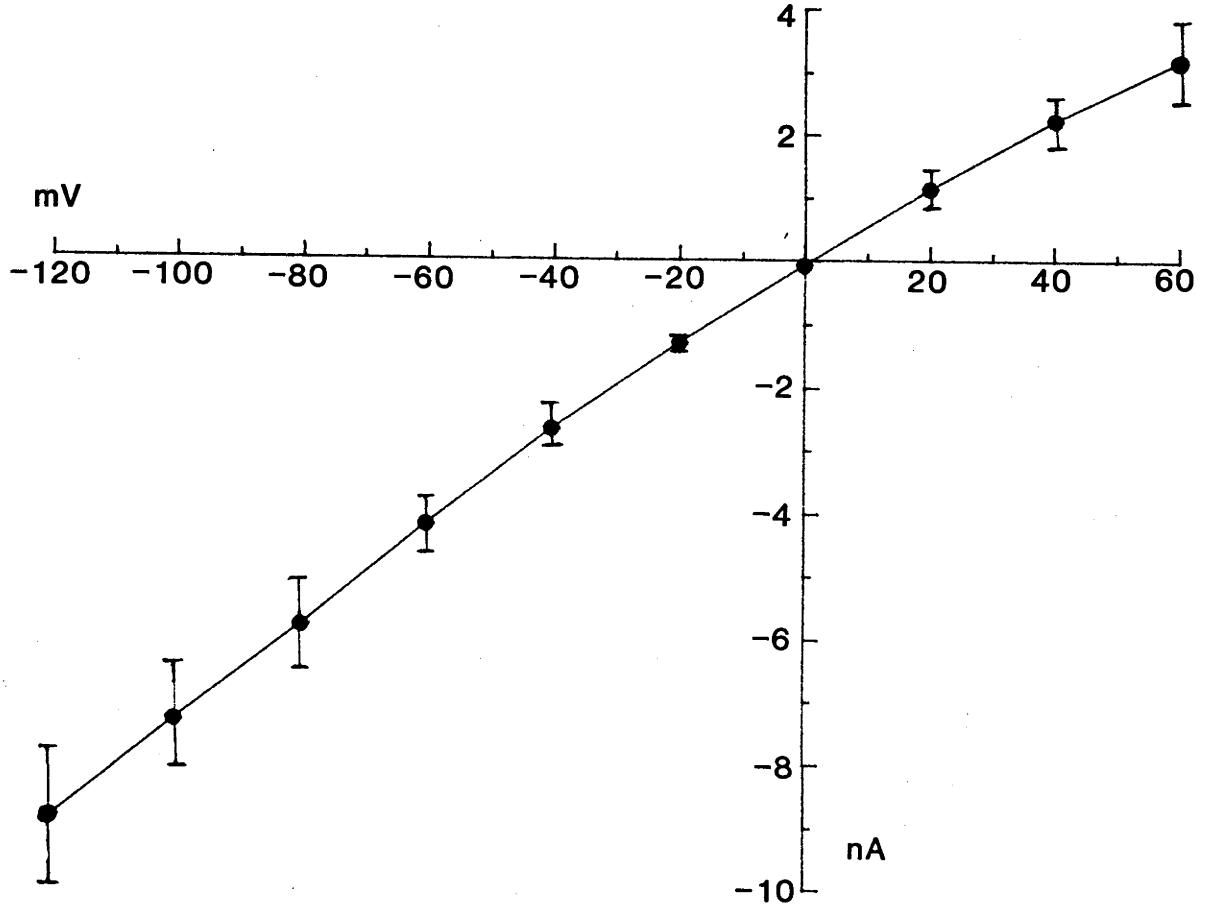


FIGURE 62: The steady state current voltage relationship for 13 applications of 600μM α chloralose to 11 drg neurones. Points are the mean current values ± one standard error of the mean. This curve rectifies in the opposite direction from the steady state current relation in GABA.

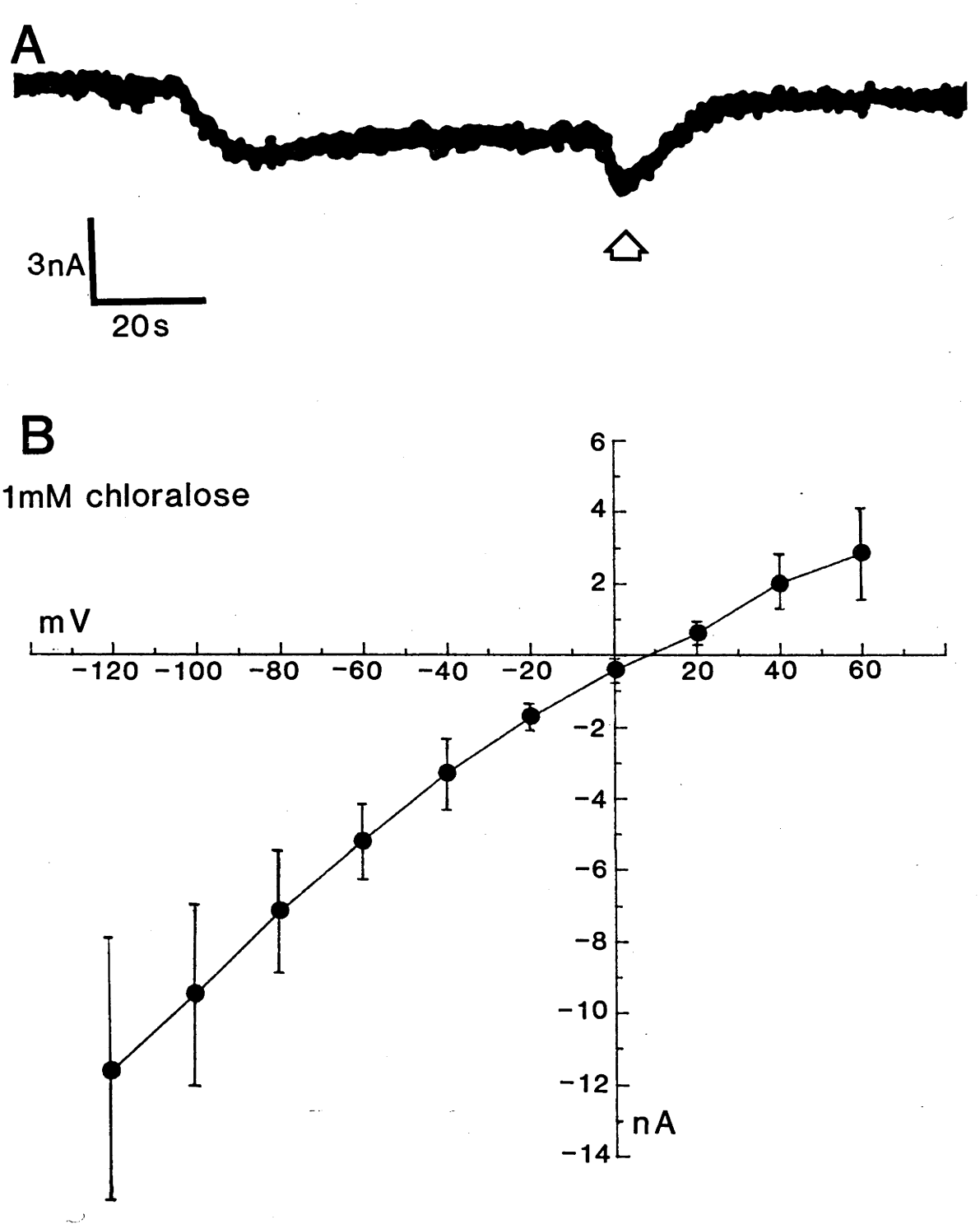


FIGURE 63: A) The response of a drg neurone to 1mM chloralose. Clamp poptential -40mV. Note the decline after the initial peak and the bounce (arrow) on washout of the anaesthetic agonist. B) Steady-state current voltage relation for 1mM chloralose.

chloralose has greater blocking activity. The steady state I-V for 1mM chloralose on 3 cells is shown in Fig. 63B. There is a greater degree of curvature with this concentration of the anaesthètic agonist.

Etomidate.

The non-barbiturate anaesthetic etomidate was effective in eliciting conductance changes on drg neurones at concentrations of 30, 60 and 100μM. These responses reversed at the chloride equilibrium potential and were blocked by the GABA antagonists bicuculline methiodide and picrotoxinin (50μM). These results suggest that the anaesthetic was activating GABA receptor/channels. In 11 cells where 60μM etomidate was compared to 60μM GABA, the average response in etomidate was -1.27 ± 0.16nA (x ± s.e.) and -2.24 ± 0.39nA (x ± s.e.) in GABA. However, these figures only represent an approximate index of potency. As Fig. 64 shows, when etomidate was washed out from the bath, a large and rapid bounce was observed. The bounce could be as large as 4 times the steady state (plateau) response. In the cell illustrated in Fig. 64, 60μM GABA produced -4.6nA inward current at -40mV, but 60μM etomidate gave a plateau current of -1.5nA, followed by a bounce reaching -6nA. Therefore if one considers the amplitude of the bounce, 60μM etomidate was almost as potent as the same concentration of GABA in activating the GABA receptor/chloride channels. If etomidate was

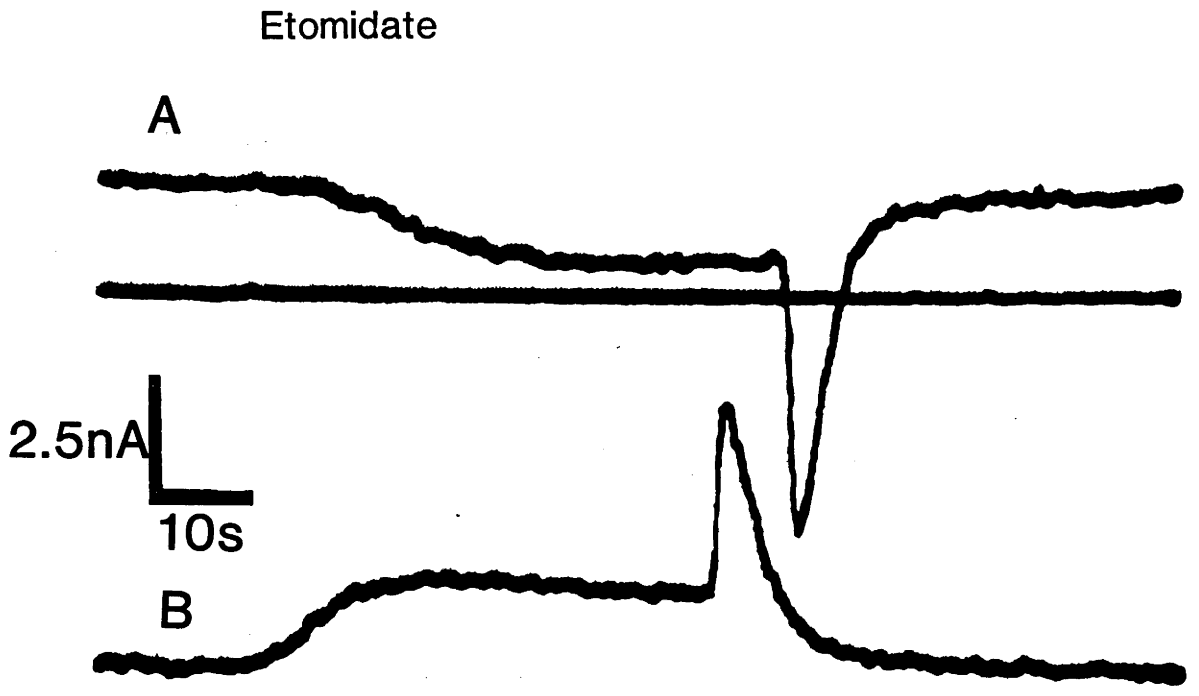
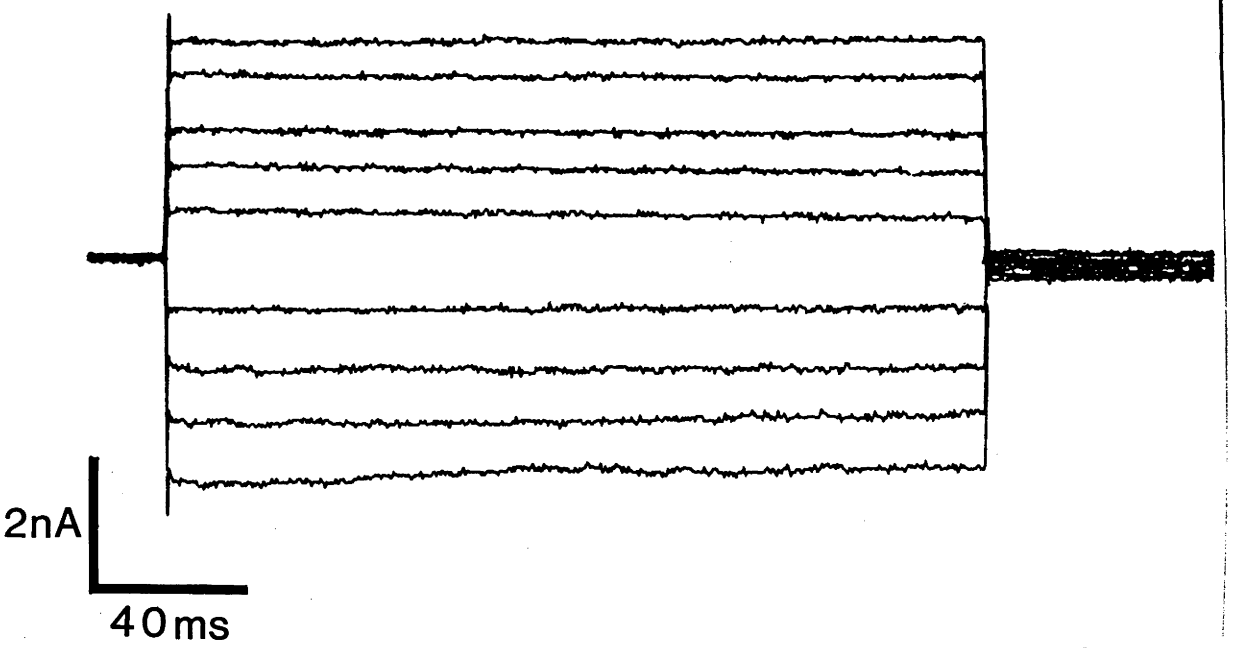
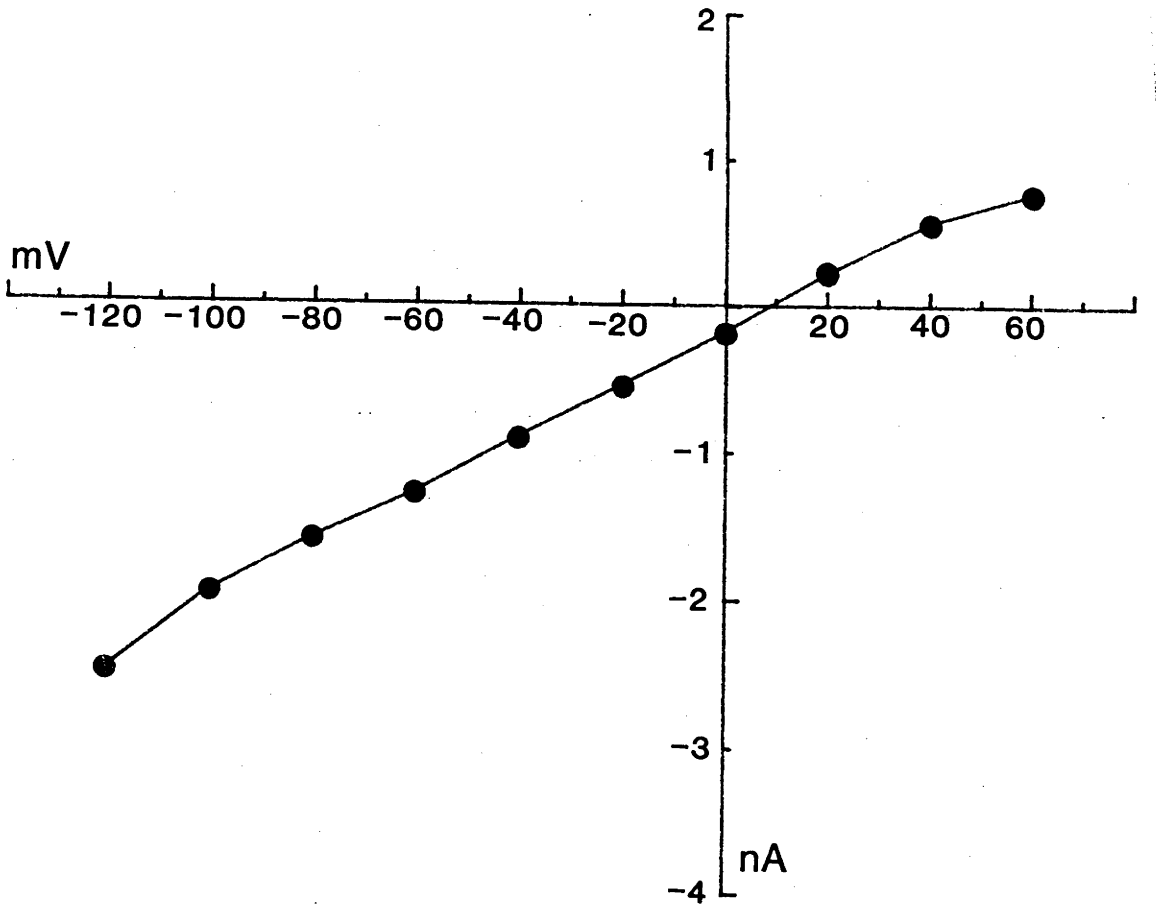


FIGURE 64: Responses to 60μM etomidate at -40mV (trace A) and -40mV (trace B). The current rises slowly to a plateau, and there is a large transient increase in current when etomidate is washed out of the bath. The middle trace is the clamp potential during exposure to the anaesthetic.

FIGURE 65: Typical voltage jump relaxations (top) and the steady state current voltage relation in 60μM etomidate (mean of 7 applications). Note that there is no obvious 'GABA-type' current relaxation, and the current voltage relationship is almost linear.

60u etomidate





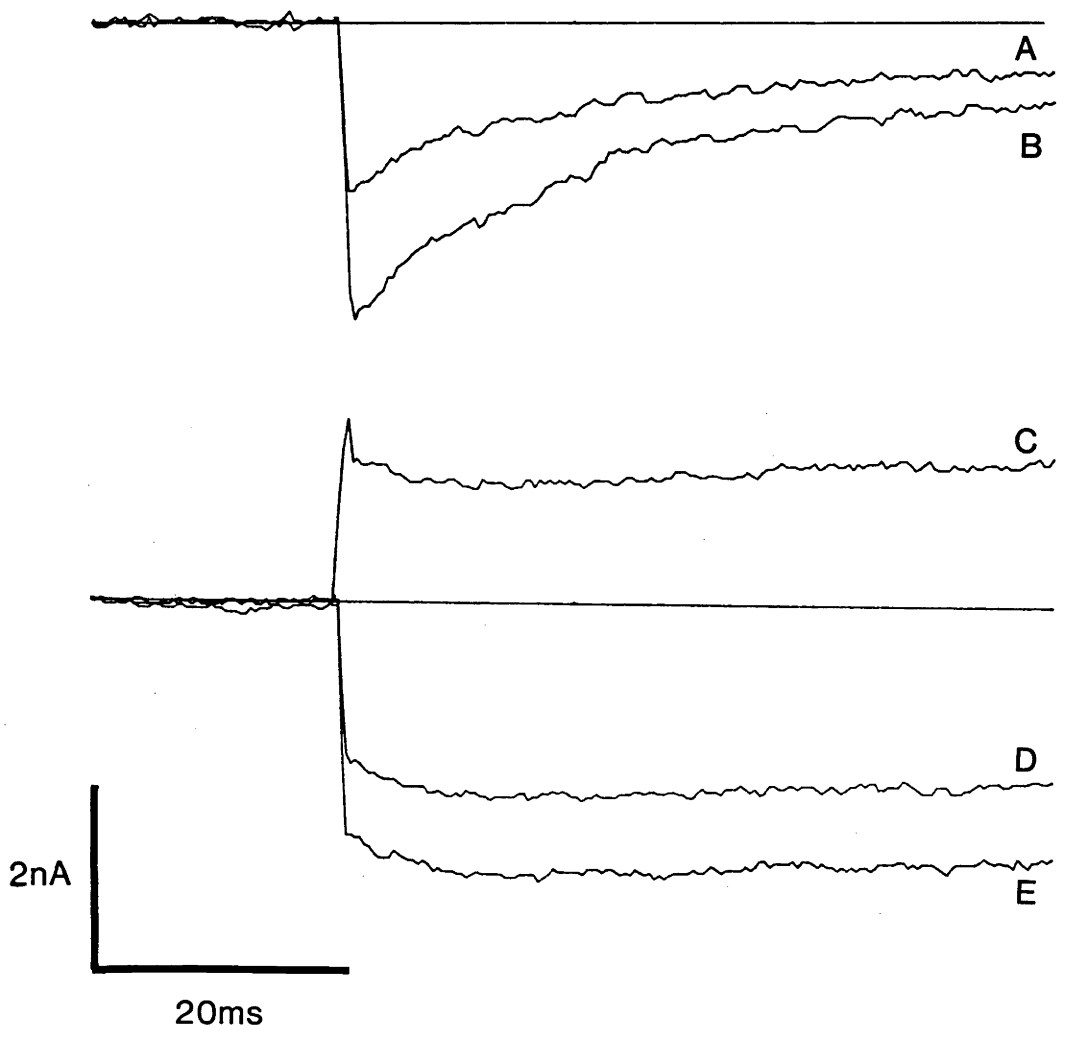


FIGURE 65a: Voltage jump relaxations in response to hyperpolarizing steps from -40mV in 60μM GABA (A: step to -120mV, B: step to -100mV) and 100μM etomidate in the same cell. Jumps in etomidate are to +60mV (C), -100mV (D) and -120mV (E). The outward relaxations on hyperpolarizing steps in etomidate are quite dissimilar to those responses in GABA, as is the reduction in current after the instantaneous curent in trace C. Rat drg neurone.

applied for long periods (greater than 2 minutes) the peak response declined slightly, but a large bounce was still observed on washout.

In three experiments where GABA and etomidate were applied separately, then additively, the response to the two agonists together was greater than the algebraic sum of the individual responses.

Current responses to voltage jumps in 60μM and 100μM etomidate are illustrated in Figs. 65 and 65a. As was observed for pentobarbitone and chloralose, these currents did not appear to 'relax' in the manner of GABA-like agonist induced responses. The voltage jump responses in etomidate were essentially flat, with a small increase in current being observed on hyperpolarizing jumps (Fig. 65). The steady state current voltage relation in 60μM etomidate is shown in Fig. 65. Overall, there appears to be little voltage dependence of etomidate responses, and any voltage dependence is opposite in direction to GABA induced responses.

The response of primate drg neurones to etomidate will be presented below.

Alphaxalone.

The steroid anaesthetic alphaxalone (10-60μM) activated a chloride conductance in 20 drg neurones, and these responses were blocked by 10 or 50μM picrotoxinin. Cells that were not responsive to GABA did not produce current in response to application of alphaxalone.

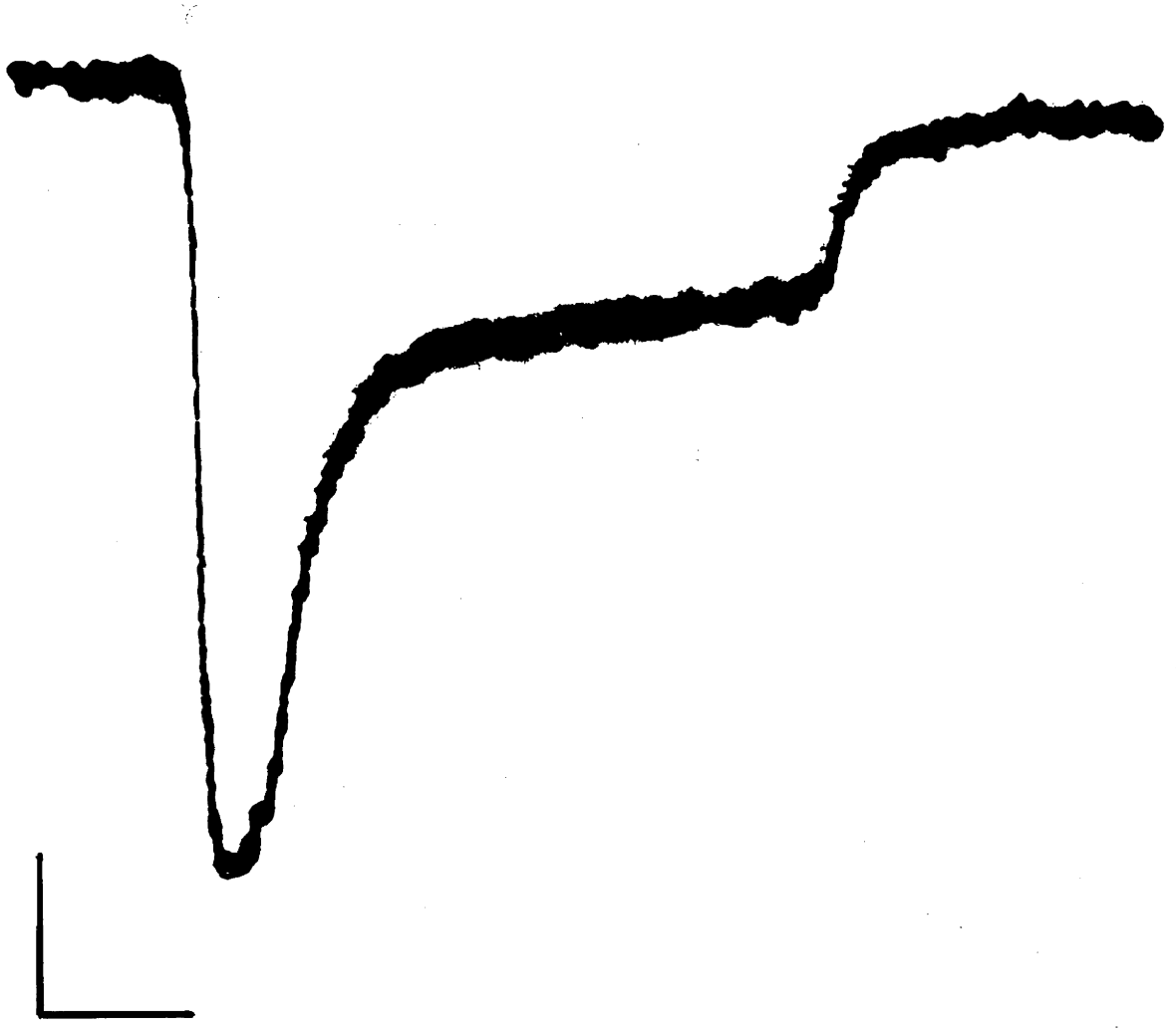


FIGURE 66: Alphaxalone acts as a GABA agonist. The figure shows the current induced by 10uM alphaxolone. Note there is no evidence of bounce on washout of this drug. Calibration bars are 1nA and 10 seconds.

Current responses to bath application of 10μM alphaxalone are shown in Fig. 66. Only occasionally was there any evidence of bounce on removal of the agonist.

In 4 cells the currents in response to 10μM alphaxalone and 60μM GABA were compared. This concentration of alphaxalone was approximately half as effective as 60μM GABA (-2.3 ± 0.7nA and 5.6 ± 2.1nA respectively). When it was possible to compare 10μM and 60μM alphaxalone on the same cells, the lower concentration of the steriod anaesthetic produced a greater response in 4 out of 5 experiments. In the fifth experiment responses were equal. (10μM alphaxalone: -3.28 ± 1.38nA, 60μM alphaxalone: -1.2 ± 0.97nA).

Voltage jumps in alphaxalone were again mainly flat, with small fast relaxations in the opposite direction to GABA currents.

Avermectin.

Avermectin B_1a is an extremely large molecule, and structurally is quite disimilar to any of the other agonist-like substances tested (see Appendix A). Avermectin (AVM) at 10μM activated picrotoxinin sensitve (100μM) chloride selective channels in all 5 cells examined. Responses to AVM were typically much slower than current responses to the other agonists examined, often taking minutes to produce a peak response. 10μM

AVM elicited currents between -1 and -5nA, with the mean response being -3.1 ± 0.88nA (x ± s.e.), compared to -8.3 ± 2.24nA to 60μM GABA on these 4 cells. Despite the slow rise and decline of the AVM induced current, this was very rapidly blocked by including the GABA antagonist picrotoxinin in the perfusate. The block by the antagonist was fully reversible.

Current responses to voltage jumps in AVM are illustrated in Fig. 67, and the steady state current voltage relationship (Fig. 68) is approximately linear. AVM currents displayed small outward relaxations on hyperpolarizing potential steps, and inward relaxations on steps of the opposite polarity. At the end of the step, a small relaxation was also observed. These results are consistent with an increase in the number of conducting channels at more negative potentials.

No obvious bounce was detected upon washing AVM out of the bath, instead the current declined slowly back to control values. In one experiment, 10μM AVM was applied with 60μM GABA (Fig. 69). AVM appeared to slow the normal GABA relaxations at hyperpolarized potentials down considerably. This can be seen with the slow change in current amplitude during the hyperpolarizing voltage step.

Due to the poor solubility of AVM this substance had to be dissolved in a solution of dimethyl sulphoxide (DMSO), giving a final concentration of 0.1% DMSO in the bath. In order to ascertain whether DMSO itself was producing inward current, or altering GABA channel kinetics, 0.1% DMSO was appied to 3 voltage clamped drg

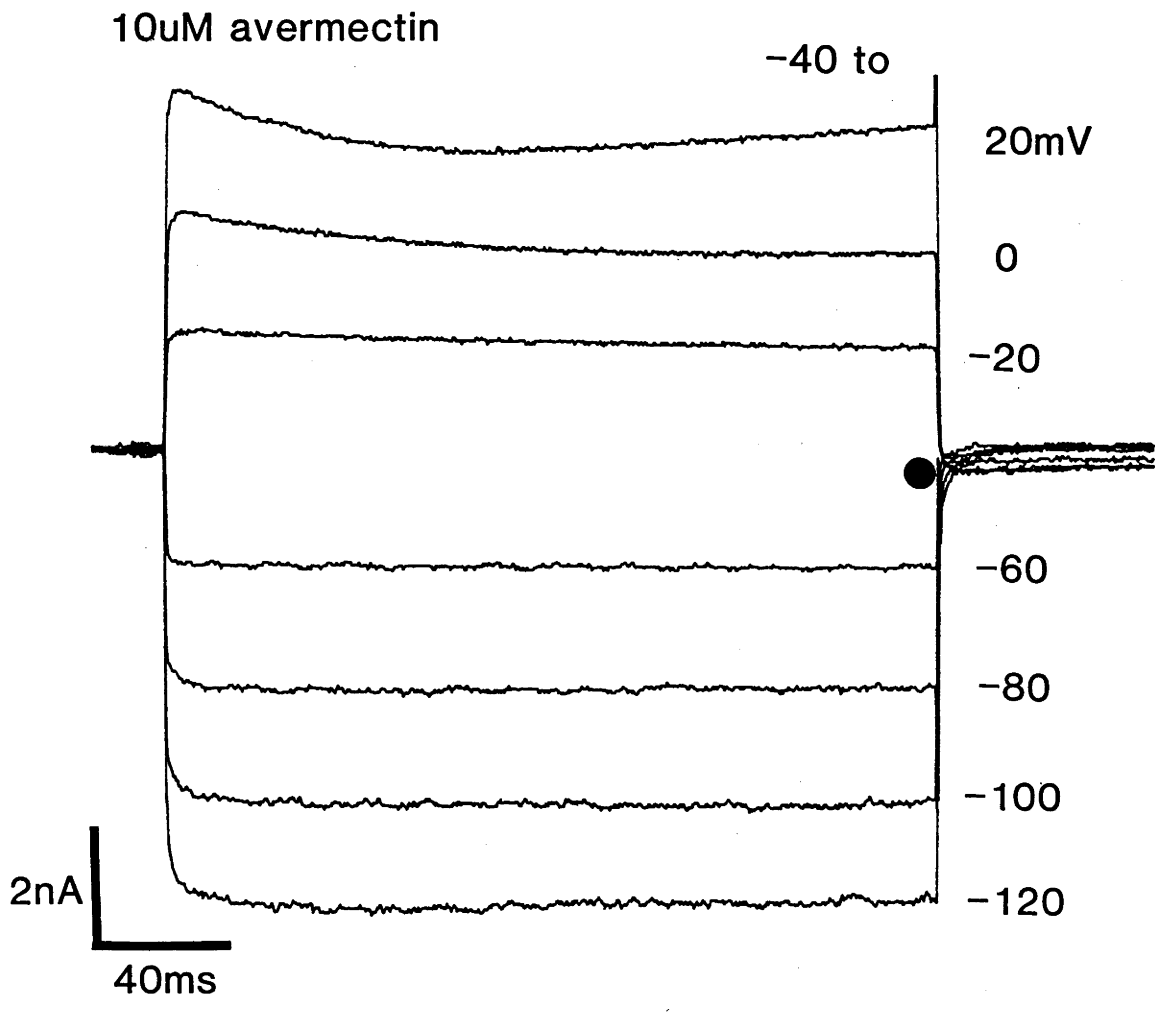


FIGURE 67: Relaxations in response to voltage jumps in 10 μM avermectin are very different from those obtained in GABA. Currents are those resulting from potential steps from -40mV to the potentials indicated on the right hand side of the figure. Note that the current increases at the beginning of the hyperpolarizing step, and then relaxes back to the baseline again at the end of the step (indicated by the solid circle).

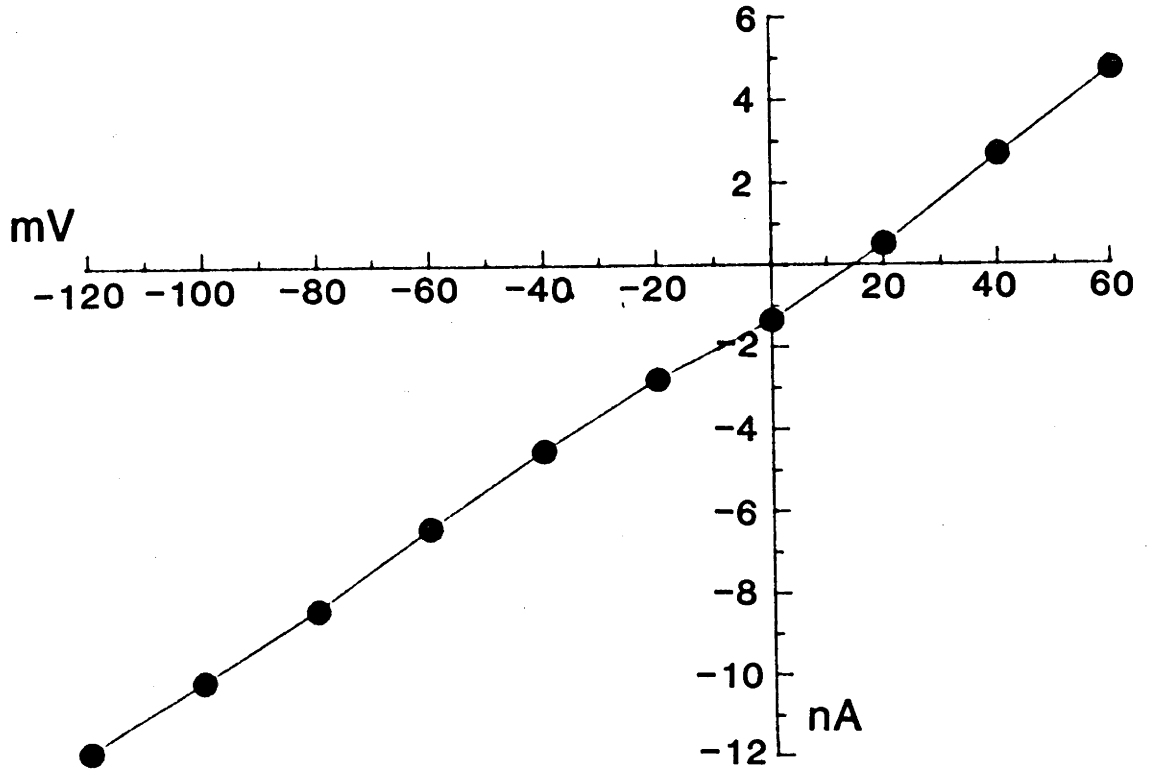


FIGURE68: The steady state current voltage realtionship for 10μM avermectin in one cell. This relationship is approximately linear.

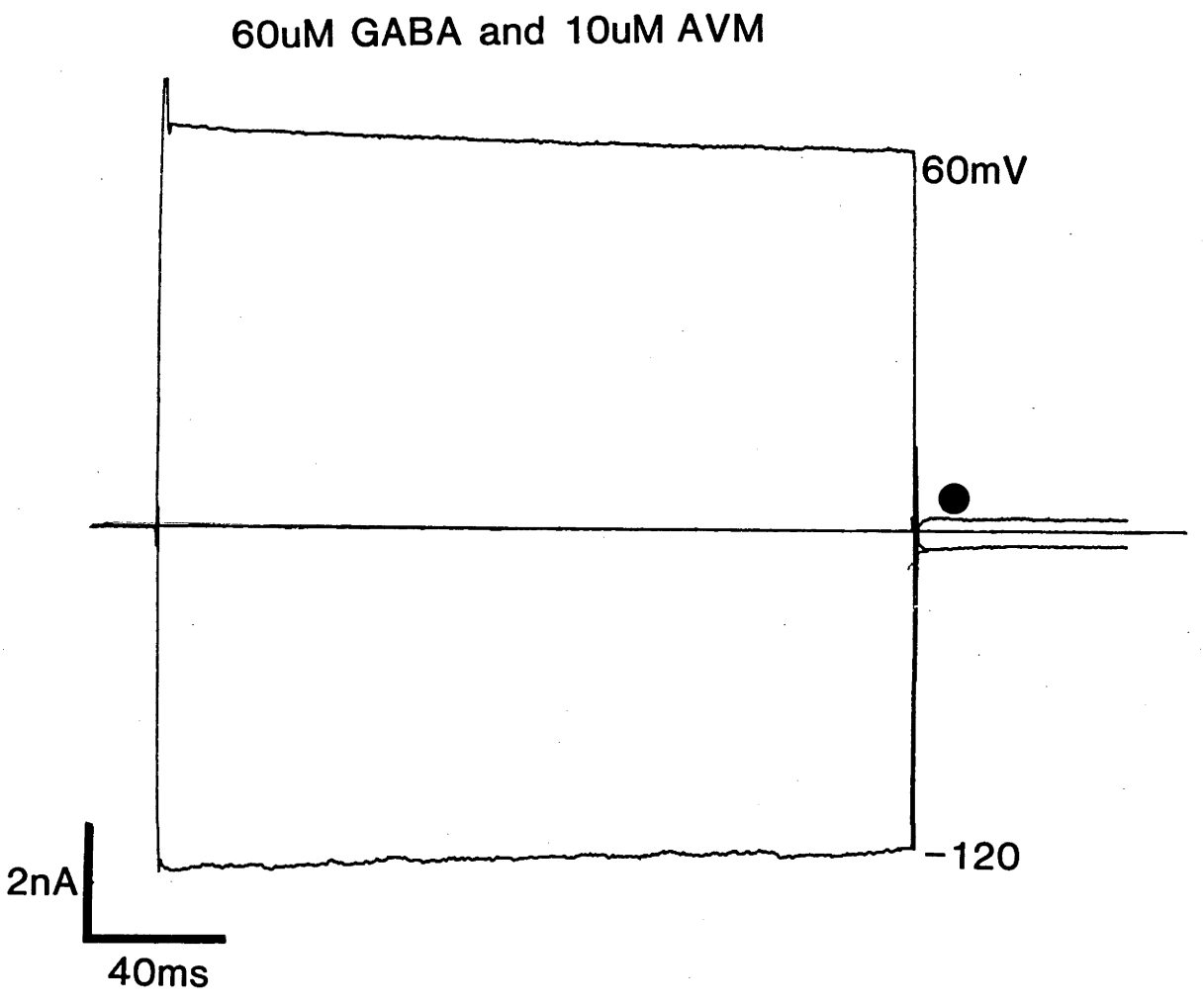


FIGURE 69: Simultaneous application of 10μM avermectin and 60μM GABA results in altered current relaxations in response to potential steps. In the relaxation obtained on a step from -40 to -120mV, AVM markedly slows the inward relaxation due to GABA channel activation, and slows the off relaxation at the end of the voltage step.

neurones for a period of over 2 minutes each. No significant change in holding current was observed, and GABA responses were typical sizes and were voltage sensitive. These results suggest that DMSO was not involved in opening or modulating GABA channels, and that the agonist action was solely due to AVM.

Halothane, Ketamine and Diazepam.

No conductance change whatsoever was obtained with 1mM halothane (n = 3), 1mM ketamine (n = 3) or 100nM or 20μM diazepam (n = 6), although all of these neurones responded vigorously to 60μM GABA. The implications of these results will be discussed below.

Effects of GABA, Iodide and Etomidate on Monkey Dorsal Root Ganglion Neurones.

Only 4 out of the 20 monkey neurones tested responded to GABA. Three ganglia were obtained from two monkeys, and of 13 neurones from the first animal tested with 60μM GABA, only 3 responded with currents of -0.8, -1.6 and -1.9nA at -40mV. In cells from the second animal, only 1 out of 7 neurones responded to 100μM GABA or 100μM etomidate, and the peak response to GABA was only -1.1nA.

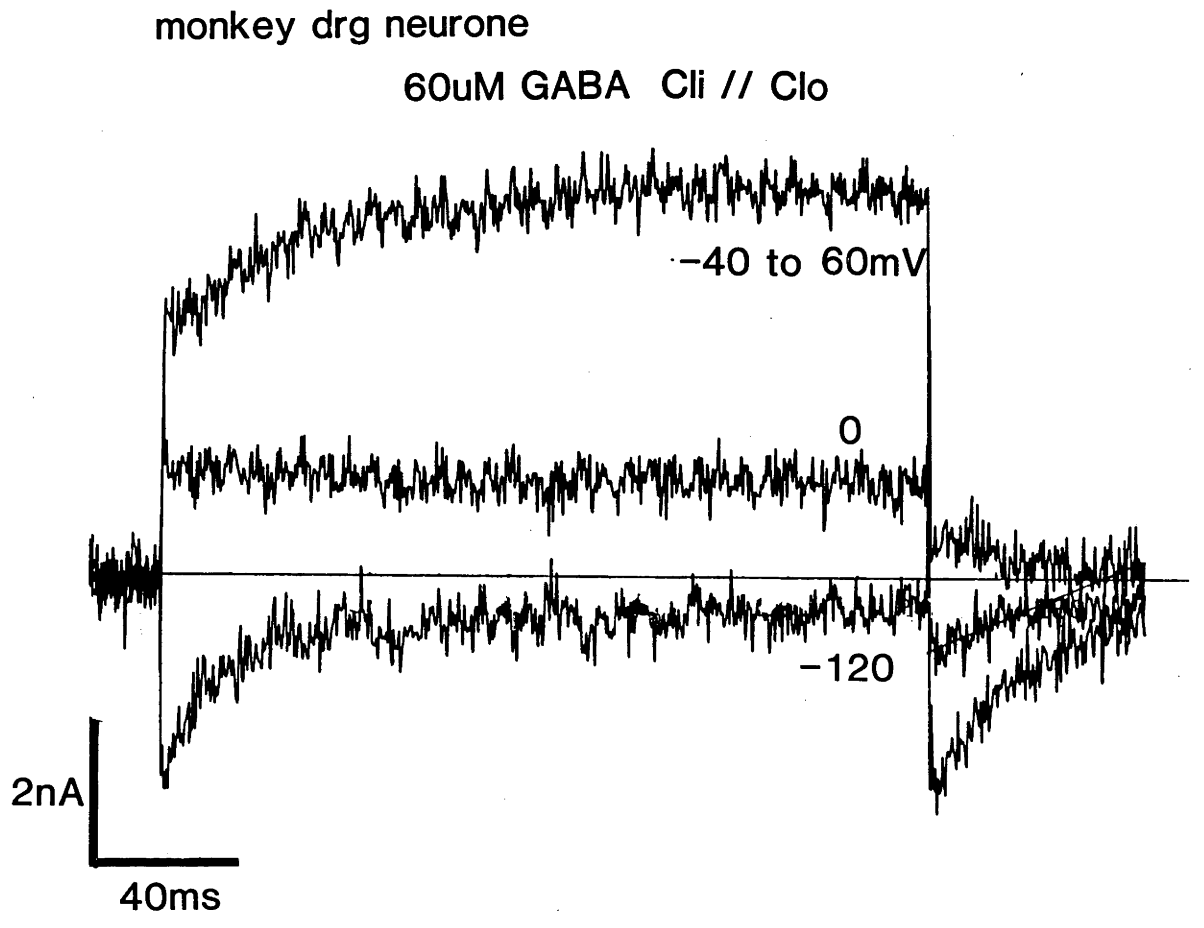


FIGURE 70: Voltage jump relaxations in 60μM GABA for a monkey drg neurone with symmetrical chloride concentrations. Step potentials are indicated alongside each relaxation. (Compare to Fig. which is from the same cell but with iodide externally).

Fig. 70 illustrates voltage jump relaxations in response to 60μM GABA. The time constant of decay was voltage dependent, increasing from 16.9ms at -120mV to 40.8ms at +60mV. The off relaxation at -40mV had a time constant of 25.4ms. As expected for GABA channels with voltage sensitive kinetics, the steady state current voltage relation is curvilinear (see Fig. 71). The instantaneous I-V is linear, as was previously observed for cat and rat drg neurones.

The time constant of decay at -120mV is approximately half of that obtained for cat and rat neurones with the same concentration of GABA, but it would be difficult to make definate statements about species differences in GABA channel kinetics until a greater sample of primate neurones could be obtained.

In one cell it was possible to measure the effects of a change from a chloride external solution to one containing iodide ions (see above). The instantaneous current voltage relation for this cell is shown in Fig. 72. Again iodide ions increase chloride efflux at potentials more negative than -60mV, with a 1.6 fold increase in instantaneous current at -120mV. The equilibrium potential in symmetrical chloride solutions was +11mV. In 135mM iodide, the equilibrium potential shifted to -2mV. Calculation of the relative permeability of iodide to chloride ions (see above) yields a value of P_I/P_{Cl} of 1.73, which is similar to the value previously obtained.

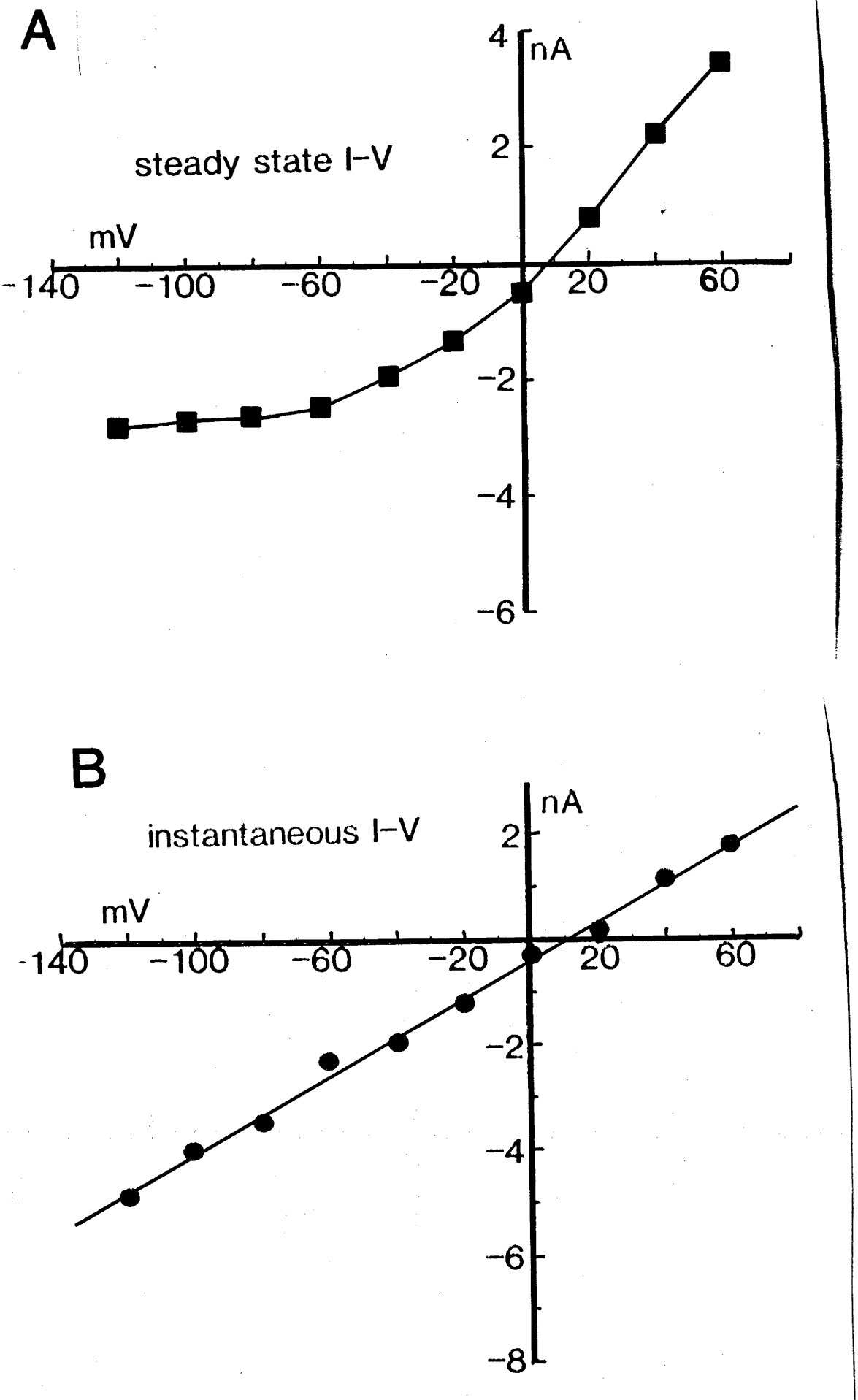
External iodide ions also exerted effects on the

kinetics of GABA channels in monkey sensory neurones. Fig. 73 shows relaxations in chloride and iodide solutions. Replacing chloride with iodide externally prolonged the time constant of relaxation at -120mV from 16.9 to 63.7msecs.

It was only possible to obtain responses from one primate neurone from the second monkey. Fortunately, it was also possible to record the actions of both 100uM GABA and 100uM etomidate on this cell. The anaesthetic gave -1.4nA of inward current at -40mV, while the natural agonist gave -1.1nA at the same potential. A family of current responses to voltage jumps in 100uM etomidate is shown in Fig. 74. The outward relaxations at negative potentials and brief inward current at positive steps can be clearly seen.

FIGURE 71: A) The steady state current voltage relationship for channels activated by 60uM GABA in a monkey drg neurone.

B) Instantaneous current voltage relation for the same cell as in A, and in Figure 70.


A
steady state I–V
nA
4
2
mV
-140
-100
-60
-20
20
60
-2
-4
-6
B
instantaneous I–V
nA
2
mV
-140
-100
-60
-20
20
60
-2
-4
-6
-8

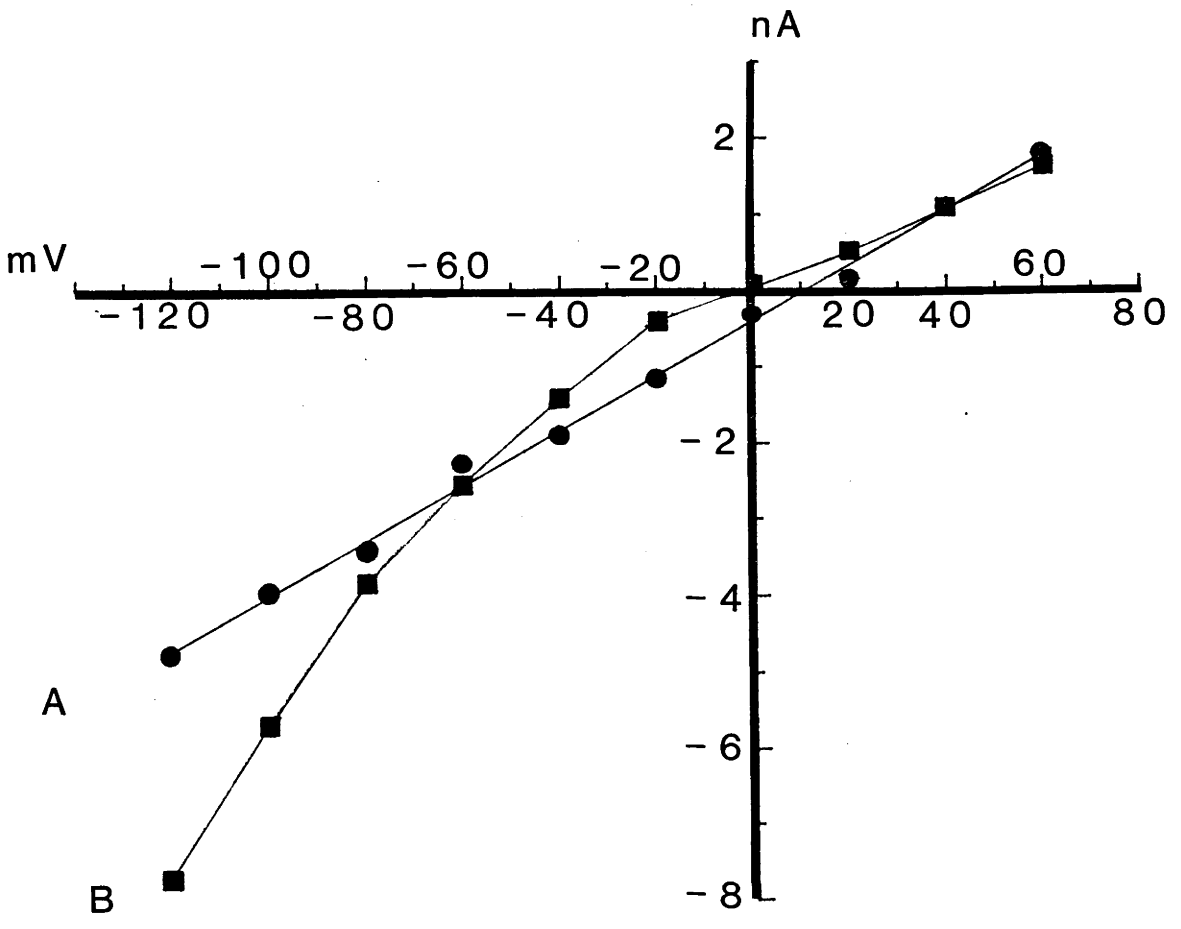
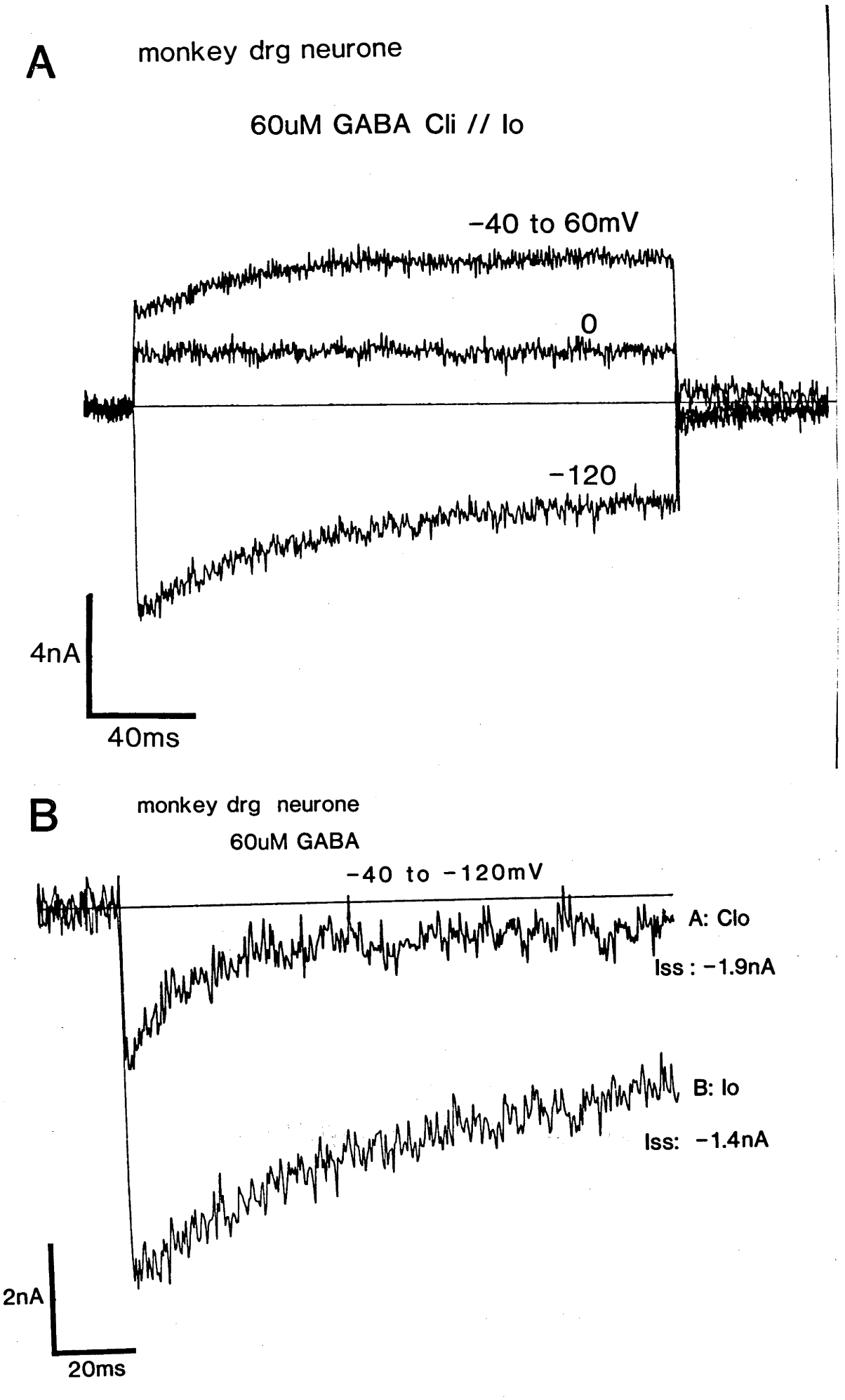


FIGURE 72: Monkey drg neurone. The effects of changing external chloride for iodide on the instantaneous current voltage relation in 60μM GABA. Line A is a regression line for instantaneous current in symmetrical chloride, curve B is the instantaneous current with iodide on the outside of the cell. Note that the current at -40mV in iodide is less than that in chloride, and iodide enhances chloride efflux at potentials more negative than this.

FIGURE 73: Monkey dorsal root ganglion neurone. A) Voltage jumps with 60μM GABA with chloride inside the cell and iodide outside. (Compare this figue to those traces obtained in symmetrical chloride for this cell, Fig.70). B) Iodide externally increases the instantaneous current at -120mV and prolongs the decay of the relaxation.


A
monkey drg neurone
60uM GABA Cli // Io
-40 to 60mV
0
-120
4nA
40ms
B
monkey drg neurone
60uM GABA
-40 to -120mV
A: Clo
Iss : -1.9nA
B: Io
Iss: -1.4nA
2nA
20ms

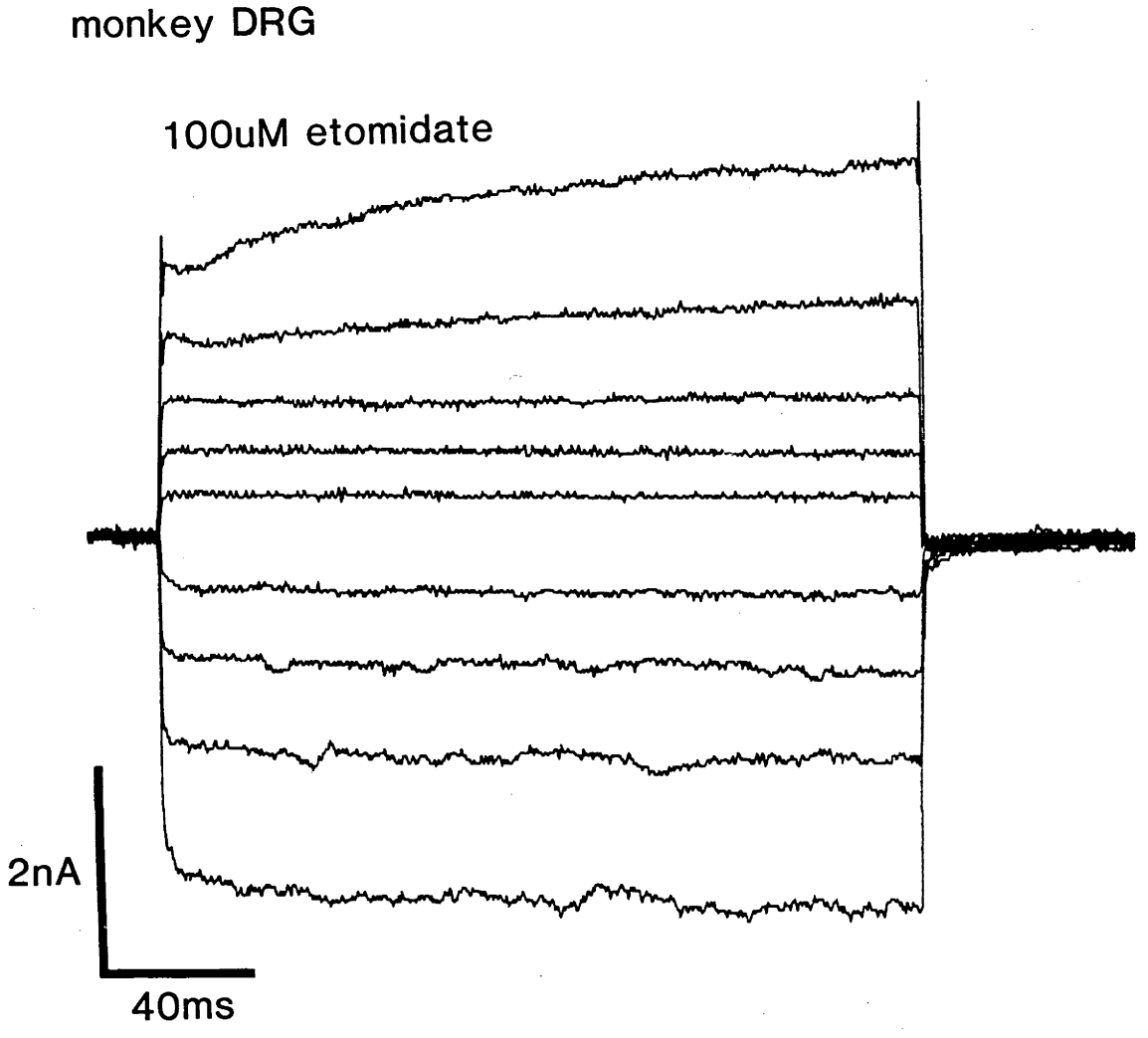


FIGURE 74: Monkey drg neurone. Family of voltage jump relaxations in 100μM etomidate. The current relaxes outwards on the hyperpolarizing steps (-120 to -60mV), and there is a brief inward relaxation on the two largest depolarizing steps (+40 and +60mV).

SECTION C: DISCUSSION.

GABA RESPONSES ON PRIMARY AFFERENT NEURONES.

Approximately 90% of the dorsal root ganglion neurones obtained from adult rats and cats responded to bath applied GABA. However, drg contain a variety of different neuronal types, such as Aα, Aβ, Aδ and C -types (see e.g. review by Lieberman 1976), which convey different sensory modalities to the spinal cord (e.g. Fyffe 1983). It is not known from the present study which type of neurone responded best to GABA, and which did not. Subtypes of drg neurones have been determined on the basis of conduction velocity, size and morpholgy (e.g. Harper & Lawson 1985a,b), but it would have been extremely difficult to classify cells and determine if there were any correlation between type and density of GABA receptor/channels in the present experiments, since in order to examine GABA activated chloride currents in isolation, it was necessary to block all of the other ionic conductances. However, Desarmenien, Santangelo, Loeffler & Feltz (1984) have suggested that Aδ and Aβ cells in rat drg responded more strongly to GABA than the smaller, unmyelinated C cells. In the present study however, small drg neurones appeared to respond to GABA as well as larger neurones. Further work in this area would be useful, especially if the appearance of GABA receptors

on the somata were an indication of the density of these receptors on the central terminals of these neurones, since this may have implications for presynaptic inhibition of sensory traffic.

It was of interest that drg neurones from neonatal rats did not respond to GABA. This would suggest that the channels are expressed at some point in the development of the rat peripheral nervous system. It was noted earlier (see Section B, Discussion) that GABA channels are expressed in the hippocampus of neonatal rats, but that the kinetics of these channels were slower than those observed in adults. Further experiments to determine the time of expression (and the properties) of GABA channels in drg neurones would be useful, and it would be interesting to know if presynaptic inhibition occured at the central terminals in neonatal rats.

Studies of drg from animals other than rats and cats suggested that there were also species differences in the densities of GABA channels on these neurones. Only 20% of monkey neurones responded to GABA, and weak GABA responses were found infrequently in guinea-pig and rabbit ganglia. However, when GABA did activate a sufficient number of channels to be useful experimentally (on primate neurones for instance), these channels had similar kinetics and permeability to those observed on rats and cats. Since these properties are so similar, this would suggest that the GABA receptor/channel complex is highly conserved, and may have similar properties in man. This might suggest that the results obtained in the present study have relevance to the effects of drugs, e.g.

etomidate, in clinical practice.

The mean open time of GABA channels in drg neurones (extrapolated from the time constant of relaxation at zero agonist concentration) was ~ 50ms at -120mV. This compares to a value of ~ 8ms for GABA channel lifetime at the same potential in CA1 pyramidal neurones, (assuming that GABA concentration has fallen to zero by the peak of the IPSC). The reason for the longer open time of GABA channels in peripheral neurones is not known. However, extrasynaptic Ach channels have longer open times than those in the synaptic region (see e.g. Gage & Hamill 1980).

It is possible that differences in lifetime between central and peripheral GABA channels are due to dissimilar membrane environments. Sakmann & Boheim (1979) have found that the open lifetime of the 'model' channel alamethicin was markedly different when this protein complex was incorporated into rat or frog skeletal muscle, and these authors suggested that the local membrane environment was able to influence channel kinetics. Alternatively, differences in the 'packing density' of GABA channels between central and peripheral neurones may account for the difference in lifetime. The density of GABA channels under an inhibitory synapse is likely to be quite high (of the order of hundreds to possibly thousands / μm^2), while estimates of channel density on drg neurones suggest that there is less than one GABA channel / μm^2. Young & Poo (1983) have found that changing the packing of Ach channels in Xenopus muscle cells alters channel open time. (But in this preparation increases in

packing density cause a slowing of channel lifetime).

The difference in open lifetime between central 'synaptic' and peripheral 'extrasynaptic' GABA channels may also be due to differences in the amino acid composition of the channels themselves. As yet we know very little about the structure of any GABA receptor/channel, or the amino acid sequence (but it may not be long before we have this data, see e.g. Barnard et al. 1983). It would be very useful if we could compare the detailed structures of central and peripheral GABA channels. It is interesting to note that Landau, Gavish, Nachsen & Lotan (1981) have proposed from a theoretical model that the differences in the channel open time for Ach receptor/channels on two different species of frogs could be adequately explained by a change in a single amino acid (and a single nucleic acid codon in the DNA coding for these amino acids) in the channel complex. It is tempting to speculate that such an apparently small change can also account for the differences in GABA channel open time between different neurones in the same animal.

In summary, there are a variety of mechanisms that could account for the differences in lifetime between central and peripheral GABA receptor/channels, but further work will be required to determine which, if any of the possibilities proposed above is the cause.

The voltage sensitivity of open time that is expressed in hippocampal neurones (Segal & Barker 1984b, see also Section B) is also shown by GABA channels in drg

neurones. The direction of the voltage sensitivity is the same, and the magnitudes are roughly equivalent. This suggests that the part(s) of the channel responsible for voltage sensitivity are conserved in the central and peripheral nervous system. Voltage sensitivity of GABA channel lifetime is expressed in animals which evolved earlier than mammals, but the rationale for this voltage sensitivity is unclear (see Section E).

The voltage sensitivity of channel open time can adequately account for the curvature of the steady state current voltage relationship in GABA, and the channel opening reaction (β) has little voltage sensitivity, as is also observed for Ach receptor/channels (e.g. Neher & Sakmann 1975). It is not yet known why the forward rate for channel opening has little voltage sensitivity, while the backward rate α, is more voltage sensitive.

The relation between the time constant of relaxation (expressed as a rate constant) and GABA concentration is sigmoidal, with a levelling off at both low and high concentrations. The saturation of the rate constant at high concentrations indicates that the conformational changes associated with channel opening and closing are rate limiting, since if the agonist binding was the rate limiting step in channel opening, then the relaxation rate would increase indefinately as agonist concentration was increased (see e.g. Sakmann & Adams 1979, Colquhoun 1981). An approach to a plateau for the relation between the rate constant of relaxation and agonist concentration was

observed by Sakmann & Adams (1979) for carbachol, but it is much more difficult to obtain reliable rate constants at high concentrations of Ach, due to channel block by the agonist and rapid desensitization (e.g. Sakmann & Adams 1979, Colquhoun 1981, Chabala, Gurney & Lester 1986).

The 'foot' of the rate/concentration relation implied in the present experiments with GABA indicates that at low concentrations GABA channel kinetics show only a weak concentration dependence. This is similar to the sigmoidal relationship between agonist concentration and conductance, and would imply that at least two molecules of agonist are required to trigger channel opening (Sakmann & Adams 1979, Colquhoun 1981).

The instantaneous current voltage relationship was well described by a straight line in symmetrical chloride solutions. This would suggest that in chloride at least, the channel is symmetrical in nature. Similar results have been obtained by Hamill et al. (1983) and Sakmann et al. (1983). However, Gray & Johnston (1985) found that the GABA channel elementary conductance increases at postive potentials in freshly dissociated hippocampal neurones. Unfortunately it was not possible to obtain equivalent data in the hippocampal experiments reported in Section B. Further discussion of the instantaneous I-V relationship is presented in Section E.

Permeability of anions and kinetic effects of foreign anions.

Dorsal root ganglion neurones were permeable to iodide (P_I/P_{Cl} = 1.88) and bromide (P_{Br}/P_{Cl} = 1.21), as was observed for hippocampal pyramidal cells. However, fluoride and propionate ions were much less permeable ($P_F/P_{Cl} \leq 0.1$, $P_{pr}/P_{Cl} < 0.1$). It is significant that the presence of these ions also altered GABA channel kinetics, as was observed in the hippocampus (see Section B). Iodide was effective at prolonging GABA channel open time in both preparations. Iodide was able to prolong GABA relaxations in drg neurones even at concentrations as low as 10mM. However, external bromide was more effective than iodide at prolonging relaxations in drg neurones, yet hippocampal IPSC decay times were roughly similar for bromide and chloride. These results suggest that GABA channels on central and peripheral neurones may have different sensitivities to anions, or that bromide is only effective at altering kinetics from the outside of the cell. Relaxations in iodide and bromide solutions were still voltage sensitive, but in the hippocampus internal application of these halides reduced voltage sensitivity. GABA induced relaxations in ganglion neurones with iodide internally were still voltage sensitive. Further experiments will be required to elucidate why bromide and iodide abolish voltage sensitivity in the hippocampus but not in drg neurones. The reason may be due to some structural/electrostatic difference between central and peripheral GABA channels.

Fluoride and propionate ions may also have produced an acceleration of GABA channel closure in drg neurones. Perhaps there is some cationic site on the GABA channel that binds the predominant anion and influences channel closing rate. Further discussion and speculation as to the basis of this effect will be presented later (see Section E).

The increase in instantaneous conductance at negative potentials with iodide and bromide externally was completely unexpected. It is unlikely that this increased current was due to a greater number of open channels at -120mV, since although iodide and bromide prolonged channel lifetime at all potentials, the steady state currents at -40mV were very similar (in some cases the current at -40mV in the foreign anion was less than that in chloride). If more channels were open, then the current at -40mV would be greater by a factor equivalent to the prolongation of channel open time, less the reduced driving force for iodide and bromide at -40mV. (In particular, the steady state current at -40mV for bromide should have been over 2.5 fold greater than with symmetrical chloride, because this anion was more effective at prolonging τ_D, and there was only 4mV difference in driving force).

The results would therefore imply that substitution of external chloride by iodide or bromide enhances the exit rate of internal chloride ions. Such an effect has not been reported in the literature before, and will be discussed in more detail in Section E. It is worth

noting here however that Hutter & Warner (1967) observed that low concentrations of external iodide reduced the efflux of isotopic chloride from frog skeletal muscle, which is the opposite result to iodides action on chloride flux in drg neurones.

The action of GABA-like agonists on dorsal root ganglion neurones.

The GABA analogue muscimol elicited over two times more membrane current than the same concentrations of GABA, and the voltage jump relaxations were longer when muscimol was the agonist. These results are simlar to those obtained by Mathers & Barker (1981) and suggest that the greater potency of muscimol is principally due to a longer channel open time of the GABA channels gated by this agonist. However the analogue P4S was only about one-seventh as effective as GABA at the same concentration, but the channels were open for about half as long as GABA-gated channels. It is possible that the reduced potency of this compound on drg neurones is not due solely to shorter channel open times, but perhaps P4S has a lower affinity for the GABA receptor and consequently opens less channels.

It was difficult to accurately assess the potency of EDAMC relative to GABA, since the true concentration of this agonist in the bath was not known because of difficulties in making known quantities of this analogue

and its instability in the recording bath (Dr. David Kerr, personal communication). It can only be said that high concentrations of EDAMC were necessary to elicit very small current changes (while GABA was very effective on these same neurones), and the time constants of relaxation were slightly shorter than those obtained with GABA. Further experiments should be done to measure the true potency of this compound, and determine its affinity for the receptor site and the kinetic properties of the channels gated by this agonist.

Glycine and taurine were not effective in activating GABA channels in the freshly excised drg preparation.

The agonist actions of some clinical anaesthetics.

Pentobarbitone activated GABA channels at concentrations as low as 60μM, and up to 3mM. The anaesthetic range for pentobarbitone is approximately 100-200μM (see Richards 1972), and it is therefore possible that the GABA-mimetic action of this drug plays a role during surgical anaesthesia. The potency of pentobarbitone relative to GABA was fairly low, being less than one tenth to one half as effective as GABA at identical concentrations. Addition of pentobarbitone and GABA together produced currents that were 3-4 times greater than the algebraic sum of individual GABA and pentobarbitone responses. However, this would be due both to the potentiation of GABA responses as well as the agonist action of pentobarbitone. Similar observations of the agonist GABA-agonist like effects of pentobarbitone

have previously been made by Nicoll (1975), Nicoll & Wojtowicz (1978), Barker & Ransom (1978), Connors (1981) and Akaike, Hattori, Inomata & Oomura (1985).

As well as exerting agonist like actions, the presence of a bounce on washout of the anaesthetic suggested that this compound may also be 'blocking' the GABA receptor/channels. The phenomenon of bounce has also been observed for pentobarbitone by Akaike et al. (1985), (see Fig. 3 of their paper), but was not commented on by these authors. Similarly Adams (1975) observed bounce (or 'humps' in his terminology) on washout of high concentrations of the nicotinic agonists hexyl and heptyl trimethylammonium from frog muscle. Adams (1975) suggested that these humps were due to the agonist binding to 'hypothetical non-receptor sites', and the agonists were slowly released from these reservoirs during washout. This possibility was excluded for pentobarbitone during the present experiments by changing the solution very rapidly round a single drg neurone. This was accomplished by placing the cell directly in the solution flow, and far enough away from the rest of the ganglia, so that release of pentobarbitone from other sites on the ganglia would not reach the cell under study.

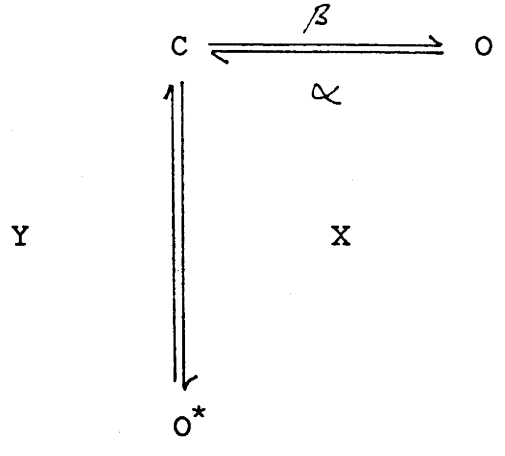
The results obtained here were more consistent with a rapid blocking of the GABA receptor/channel by pentobarbitone, and these channels becoming unblocked during washout of the agonist. It must be stressed that we have no evidence for the nature of this blocked state. Block of Ach responses by anaesthetics (including pentobarbitone) has been suggested to occur by occlusion

of the ionophore itself, thereby preventing current flow. However block may be occuring by some allosteric mechanism, i.e. binding of the anaesthetic to some region of the receptor/channel resulting in a closure of the channel. We can only suggest that the results obtained here are consistent with the anasethetic producing the absorption of significant numbers of channels into a non-conducting state. This state is simply called a 'blocked' state as a matter of convenience, and is not meant to imply any physical mechanism of drug action.

The steady state current voltage relationships with pentobarbitone showed that current was reduced at positive potentials, suggesting that there was a greater degree of block by the agonist at positive potentials. Since pentobarbitone exists in both anionic and neutral forms at pH 7.4 (the pK_a is 8), it was possible that the anionic form might be entering the channel and transiently reducing ionic conductance at positive potentials. To test this possibility, the agonist and blocking action of pentobarbitone was examined at pH 10.4, 9.4, 7.4 and 5.4. In experiments at alkali pH pentobarbitone produced little or no conductance change. At this pH most of the anaesthetic is in the anionic form, and may have been completely blocking the channels. However at pH 5.4, where over 99% of pentobarbitone would be uncharged, the steady state I-V still rectified at positive potentials and bounce was still observed on washout of the agonist. The simplest interpretation of these results is that pentobarbitone can block the channels in a neutral form,

and the neutral and not the anionic form of the anaesthetic is the agonist. The Ach receptor is also 'blocked' by uncharged anaesthetic compounds (benzocaine: Ogden, Siegelbaum & Colqhoun 1981; pentobarbitone: Adams 1976).

The voltage jump relaxations in pentobarbitone may be interpreted by unblocking of the channel being favoured by hyperpolarization, while depolarization enhances blocking (see Appendix 2). Alternatively, the inverse relaxations (i.e. relaxations in the opposite direction from those observed with GABA or GABA-like agonists) may be due to an entirely different reaction scheme where the rate constants have the opposite voltage sensitivity from those observed in GABA, e.g.:



This scheme would imply that the potential sensing mechanism in the agonist gated channels leading to state O^* would have a completely different electrostatic configuration from 'normal' GABA-activated channels, such that hyperpolarization increases the lifetime of the open state (O^*). It is difficult to easily

reconcile such a scheme with our current views on the processing controlling the voltage dependence of ionic channels (see Section E). However, such a mechansim may not be impossible.

A simple, more economical method of explaining the direction of the voltage jump relaxations is in terms of a voltage sensitive bolcking/unblocking reaction (see Appendix 2). In this case the exit from the blocked state is favoured by hyperpolarization. The association of the anaesthetic agonist with the 'blocking site' need not be dependent on the presence of a charge on the compound to be voltage sensitive, since the association/dissociation of the agonist with the channel could be influenced by secondary allosteric effects which are dependent on the membrane field. The 'blocking' by the anaesthetic agonists can therefore be potential dependent without the necessity of the anaesthetic itself being charged.

It was a common observation that 200uM pentobarbitone reduced the amplitude of IPSCs in the hippocampus, in addition to its action of prolonging the decay phase. Since pentobarbitone has agonist-like activity at this concentration in drg neurones, it is conceivable that pentobarbitone may also be activating central GABA channels. If this did occur, then the anaesthetic may be activating channels and sending them into a desensitized state, making fewer channels available for activation by the neurally released transmitter. The agonist-like action of pentobarbitone could have been missed if the anaesthetic was applied slowly (as was the case in the hippocampal experiments), and any slight increase in holding current upon activation could be confused with deterioration of the neurone.

Further experiments will be necessary to determine the mechanism of block by pentobarbitone, and its potential

dependence. This would best be accomplished with patch clamp techniques and measurements of single channel closed times, open times and the number and duration of gaps during openings. These measurements would provide estimates of the numbers of open and closed states, and one could obtain data concerning the potential dependence of the reaction rates between these states. Such a study would provide a clearer picture of both the activation and the block by pentobarbitone, and may be expanded to other anaesthetics.

Additionally, since the pentobarbitone used in the present study was a racemic mixture of both the (-) and (+) isomers, it would be interesting to examine the effectiveness of the agonist and blocking actions of each isomer.

Etomidate was the most potent anaesthetic in activating GABA receptor/channels, indeed this substance had a potency similar to GABA when the blocking action of this compound was considered. To account for the pronounced bounce produced by this anaesthetic agonist, model simulations (see Appendix 2) suggest that activation of the channel is followed rapidly by a closed, 'blocked' state. This blocked state can quickly absorb approximately 80% of the total number of the agonist activated channels. Once again, it must be stressed that this blocking model is not a unique solution, but represents the simplest model and is consistent with current receptor/channel theory. Voltage jump relaxations in etomidate were also consistent with etomidate being a voltage sensitive blocker.

Further discussion of the action of etomidate will be presented in Section E.

Diazepam.

The benzodiazepine diazepam was tested for agonist activity since the structure (see Appendix 1) is quite different from that of GABA and the other agonists tested, and Barker, Gratz, Owen and Study (1984) have reported that diazepam is an extremely potent agonist on GABA receptor channels (see e.g. Fig. 4 of their article, which shows that 10uM diazepam elicits approximately two times more current than 25uM GABA). It has also been suggested that the conductance and lifetime of these diazepam-activated channels are identical to GABA-activated channels, and these channels are blocked by the agonists bicuculline and picrotoxin (Barker et al., 1984). Barker (1983) has also reported that GABA channels can be activated by minute, (5 picomolar), quantities of flurazepam. In the present study, 100nM and 20uM diazepam were completely ineffective in eliciting a conductance change, even though GABA responses were quite marked in the same cells. It may be that in the cultured neurones used by Barker benzodiazepine binding does activate GABA channels and more data should be obtained to confirm this intriguing result. However, diazepam may be simply enhancing the action of endogenous GABA in these cultured preparations, and this possibly has not been tested yet.

In summary, GABA receptor channels on mammalian dorsal root ganglion neurones have similar properties to those on hippocampal neurones. The time constant of relaxation is dependent on both the membrane potential and agonist concentration. The channels arec also activated by the GABA-like agonists muscimol, P4S and

EDAMC, and the potency of these agonists can be correlated with the length of time channels are open, and possibly the binding affinity of these agonists. The GABA channels on drg neurones are also activated by the clinical anaesthetics pentobarbitone, chloralose, etomidate, alphaxolone, and the antehelminthic avermectin. The results presented here also suggest that these agonists can block the channel in addition to their gating action. GABA channels in drg are more permeable to iodide and bromide ions than chloride, but are much less permeable to fluoride and propionate. The anions iodide and bromide also favour chloride efflux from the cell at negative potentials. All of the foreign anions appeared to have an action on GABA channel kinetics, with the more permeable anions lengthening channel lifetime, while the less permeable ones accelerated channel closure.

SECTION D :

OTHER CHLORIDE CHANNELS IN DORSAL ROOT GANGLION NEURONES

SECTION D: PATCH CLAMP OF DORSAL ROOT GANGLION NEURONES AND OTHER CHLORIDE CURRENTS PRESENT IN THESE NEURONES.

PART A: PATCH CLAMP STUDIES.

Introduction

Patch clamping of dorsal root ganglion neurones was attempted in order to record single channels activated by GABA and the anaesthetics pentobarbitone and etomidate. With this technique, both the conductance and kinetics of GABA channels can be determined. The conductance measurements would reveal whether 'subconductance' states of this channel occur in freshly isolated neurones, and the kinetic information would reveal the minimal number of closed and open states of GABA channels. The kinetics of activation and block would have been of particular interest, since voltage jumps could not provide this data.

Unfortunately only two weeks could be spent on the patch clamp studies. Isolation of single GABA channels proved impossible in this short space of time, due to the presence of a noisy large conductance channel.

Methods.

Single DRG neurones from cat and rat ganglia were isolated in the following manner. Ganglia were dissected and enzyme treated as normal (see page 89), but isolation of individual neurones and electrode seals was facilitated when 0.75-1.0% of both trypsin and collagenase were used. After one hour in these enzymes at 30^{0}C (in standard solution), the ganglia were placed in a 5cm diameter Petri dish. Ganglia were then triturated with a fire polished Pasteur pipette for about one minute. This procedure was sufficient to isolate large numbers of individual neurones, most of which had only a small length of axon (<80 µm) attached to the cell soma. Individual neurones could be seen under the 200 x power of an Olympus inverting microscope. Patch electrodes were moved up to suitable cells using a 3-dimensional microdrive . The development of the electrode-cell membrane seal was monitored by the decrease in both current noise and a calibration pulse on an oscilloscope. Good seals (~ 20GΩ) were characterised by a rapid decrease in the current pulse when the patch electrode was placed on the cell membrane. A further improvement in the noise level could then be obtained by applying negative pressure to the patch pipette holder. After a little practice in manufacturing patch electrodes it was possible to obtain giga-ohm seals on isolated DRG neurones in approximately 80% of attempts. Membrane patches were stable for up to

40 minutes, in cell attached, ripped off and outside out configurations.

Patch electrodes were fabricated from Vitrex Modulohm haematocrit glass (which was also used to make suction electrodes) in a two stage process (e.g. Hamill et al. 1981). The first pull was used to give the 'hourglass' electrode shape, and the second pull gave a steep taper. These electrodes were then heat polished under high magnification on a microforge assembly. Electrodes were used for experiments straightaway. No hydrophobic coating was used for these particular experiments.

An EPC-5 patch clamp amplifier was used (List Electronics) and channel currents were recorded both on magnetic tape and on an 11/23 microcomputer.

Recordings were made in either the standard solution or the solution given previously for voltage jump experiments. Patch electrodes were filled with the same solution as in the Petri dish.

RESULTS

Silent patches, i.e. those with no detectable channel activity were detected in approximately 20% of giga-ohm seal membrane patches. In the remaining patches, a high conductance (~ 100-300pS maximal conductance) channel was observed. This channel reversed at ~ 0mV in symmetrical chloride solutions. The channel increased in frequency and amplitude and became more noisy as patches were polarized away from

0mV to both positive and negative potentials. It is unlikely that this channel was an artefact due to patch breakdown since often full closures were observed between large and small bursts of activity and it was often observed at potentials close to zero. Channel activity was not altered by small changes in pressure (positive and negative) applied to the pipette. This channel was observed in cell-attached, outside out and inside out type patches. The channel was similar in both normal solution (containing Na^+, Ca^{2+}, K^+ and Cl^-) and in the solution containing choline, Cs^+, Mg^{2+} and Cl^-. It was probably either a non-selective cation channel (presumably passing cations as large as choline) or a chloride channel. Illustrations of one of these channels are shown in Figs. 75 & 76. This channel (or channels) was observed in both cat and rat DRG neurones. Figs. 75 & 76 are from a ripped off (inside out) cat neurone.

The presence of this large noisy channel made it almost impossible to resolve GABA or anaesthetic gated channels, since these would presumably have a much lower conductance (~ 20-40pS). In one patch with GABA in the pipette, a smaller channel was observed, but this membrane patch became unstable and was lost before recording could be started.

DISCUSSION.

The high conductance channel observed in over twenty patches here appears similar to the high conductance

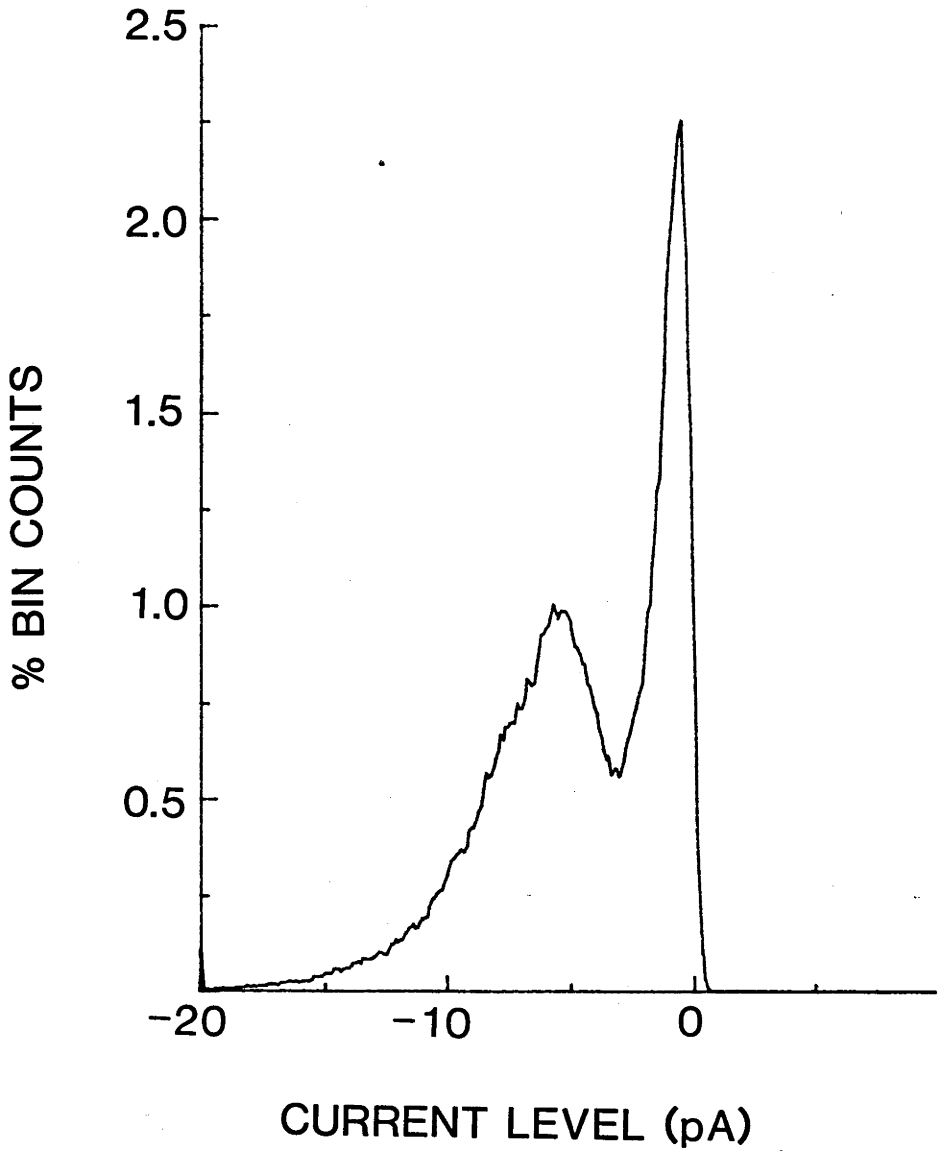
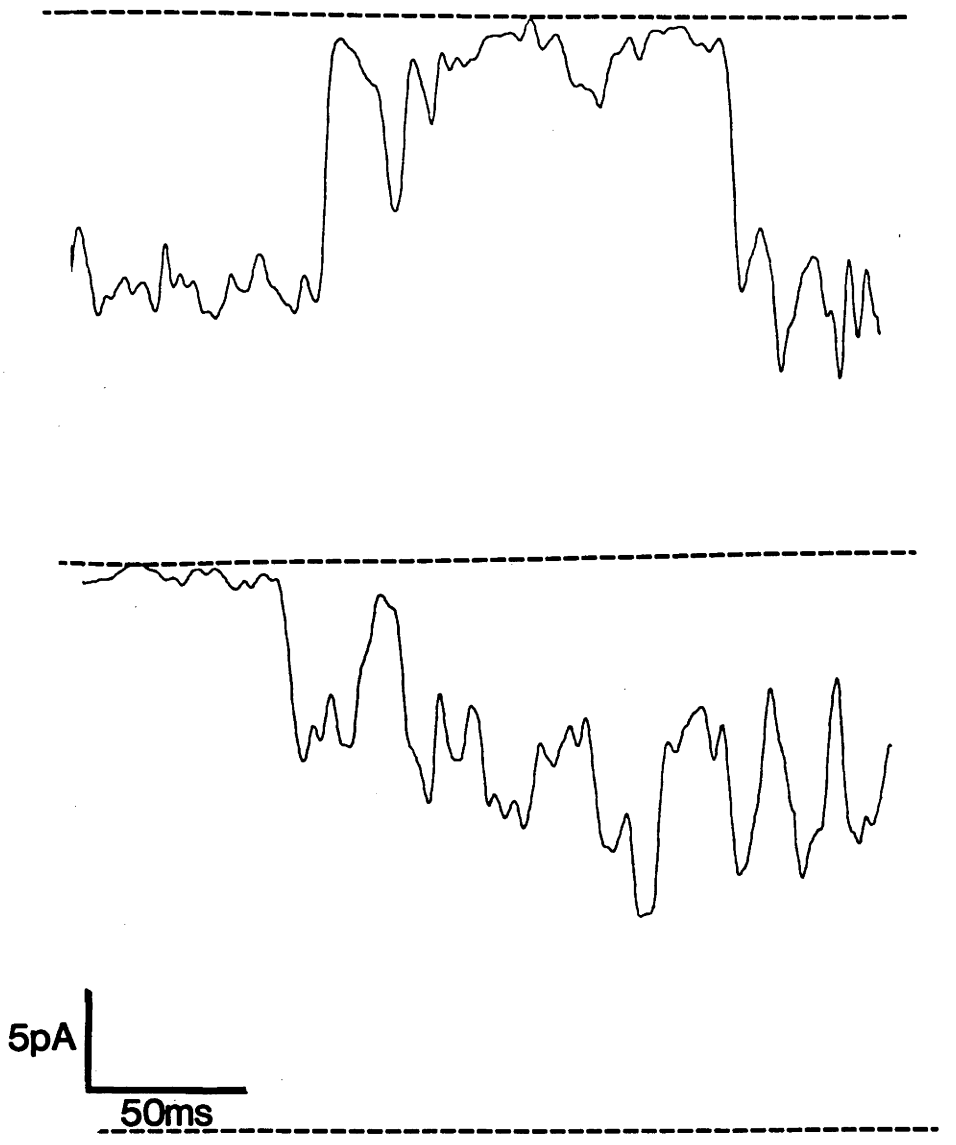
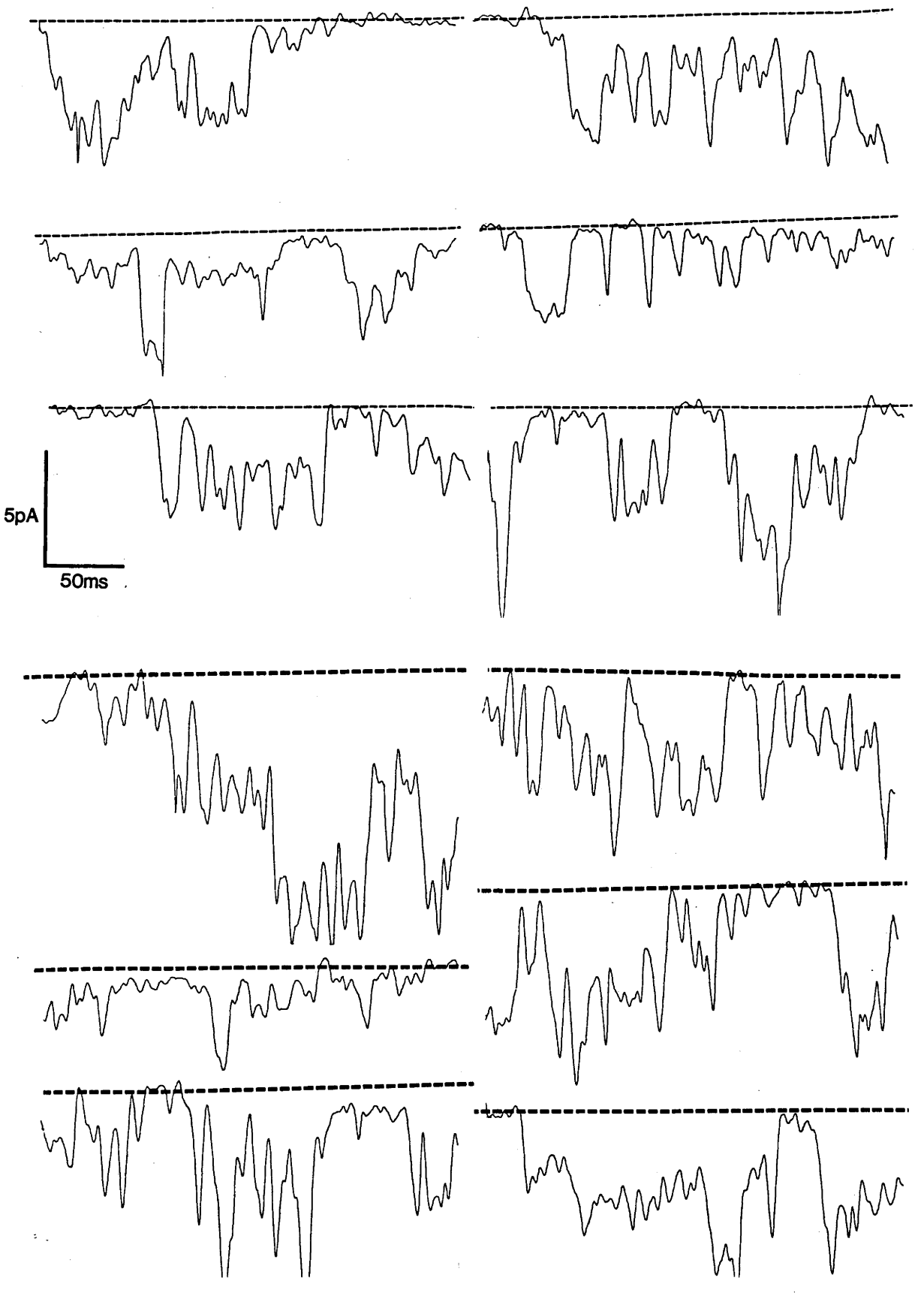


FIGURE 75: Amplitude histogram for the high conductance 'noisy' channel illustrated in the following figures.

FIGURE 76: Sample records (see also next page) of the high conductance channel in cat drg neurones. It appears that the channel switches between different conductance levels. Records were filtered at 300Hz prior to plotting. The dashed line is zero current. The potential across the patch was -12mV, and the channel was recorded in symmetrical solutions.


5pA
50ms


5pA
50ms

chloride channel observed by other workers using neurones (Geletyuk & Kazachanko 1985 in Lymnaea parietal ganglion neurones, and Gibb (1985) in hippocampal pyramidal cells) and epithelial cells (Krouse, Schneider & Gage 1986). One interpretation of the noisy, multistate behaviour observed here is that this channel displays multiple conductance states, and the channel can flip between these different states. It has been suggested that there are 16 conductance sublevels for Cl^- channels in molluscan neurones (Geletyuk & Kazachenko 1985). The channel reported here was often much noisier than those in other reports of high conductance Cl^- channels. This may have been due to rapid switching to other sublevels, or blocking by some other ion. Noisy behaviour of a high conductance Cl^- channel in pulmonary epithelia has recently been observed in this laboratory by M. E. Krouse (personal communication). Although there is only a limited amount of evidence presented here, the behaviour of this large channel is consistent with those high conductance Cl^- channels previously reported in the literature.

We may ask the question, what are these Cl^- channels for? It has been suggested that the equivalent Cl^- channels in epithelia are involved in water transport (e.g. Krouse et al. 1985) while those occuring in Schwann cells have been implicated in transport of materials from glia to neurones (Gray, Bevan & Ritchie 1984). Given the fairly high

frequency (~ 80%) with which these channels were obtained in high resistance seal patches, they may exist with high density on the DRG neurones. Since openings were qualitatively more frequent at positive and negative potentials these channels could contribute significantly to the resting properties of DRG neurones unless there were some intracellular regulator normally keeping these channels closed. It is perhaps reasonable to assume that most of these high conductance channels are closed under normal physiological conditions, and their appearance under the disruptive conditions employed here reflects more the pathology of these neurones rather than their physiology.

It would be interesting and useful if these preliminary observations could be extended, and we could resolve the kinetic and conductance properties of these unusual channels more fully.

It was unfortunate that the frequency and amplitude of these channels did not permit clear resolution of the smaller conductance GABA channels in this preparation, since it was the primary intention of the patch clamp experiments to explore the kinetic propeties of GABA channels in more detail. These studies should certainly be carried out, but it will be necessary to find a way of eliminating or blocking the high conductance channel without disturbing the GABA receptor channel complex.

(2). OTHER CHLORIDE CURRENTS.

A) The calcium-activated chloride current.

During the present experiments a prolonged depolarization followed calcium spikes on many occasions, and this was found more frequently in rat than in cat neurones. In voltage clamp experiments, a depolarizing pulse elicited a calcium current followed by a prolonged 'tail' current, which often took a few seconds to completely inactivate (see Fig. 77). In rat neurones, this current was often larger than the calcium current. It is probable that this is the calcium-activated chloride current that has been reported in Xenopus oocytes (Barish 1983, Miledi & Parker 1984), cultured motoneurones (Owen, Segal & Barker 1984) and cultured rat DRG cells (Dunlap & Fischbach 1981, Mayer 1985). This current followed the activation curve of, and ran down in parallel with the calcium current (see Fig. 78). This current was not observed when Ca^{2+} channel blockers (cadmium ions or replacement of calcium with manganese ions) were applied.

It is not clear what function this current might have in primary afferent neurones. If it is present in the central terminals of these cells it would probably be much smaller in amplitude, since the chloride equilibrium potential is closer to the resting potential of DRG neurones *in vivo*. However, the small residual depolarization at the end of an action potential could be involved in decreasing the probability of high frequency repetitive firing, or could enhance transmitter release.

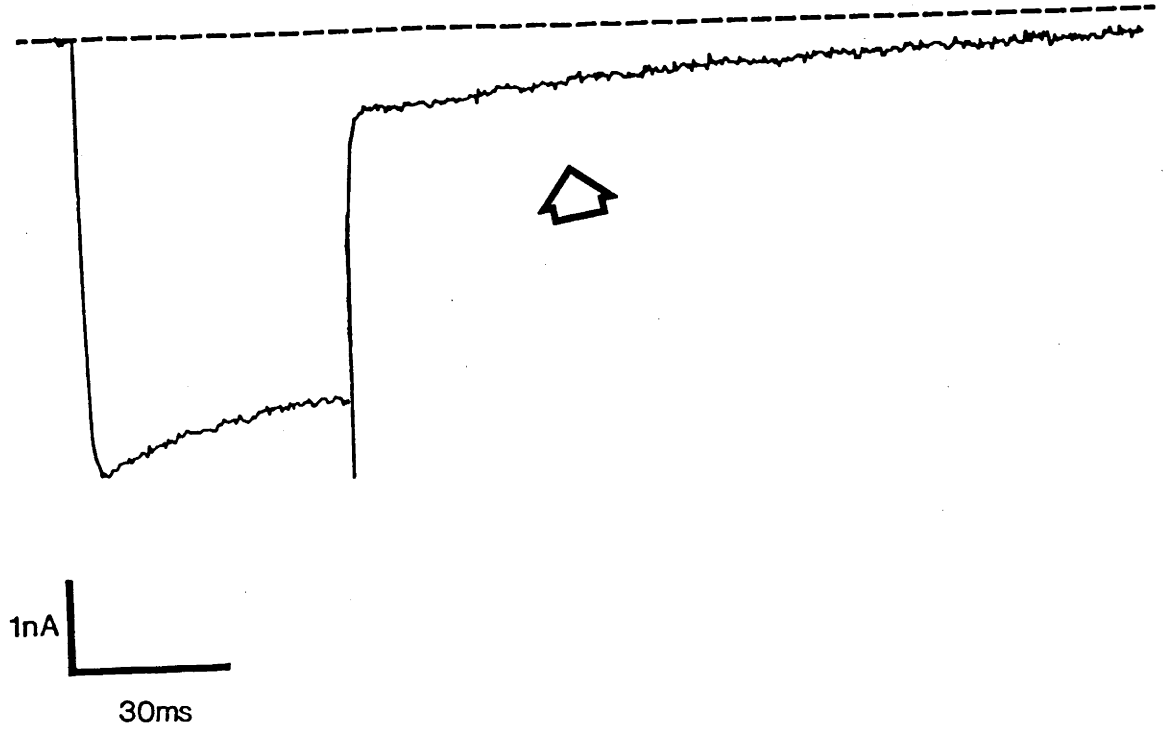


FIGURE 77: Calcium activated chloride current: Inward calcium current in a cat drg neurone evoked by a step from -50 to +20mV. Note the decay after the peak calcium current (presumed due to outward chloride current at this potential) and the long inward 'tail' current following the calcium current (open arrow).

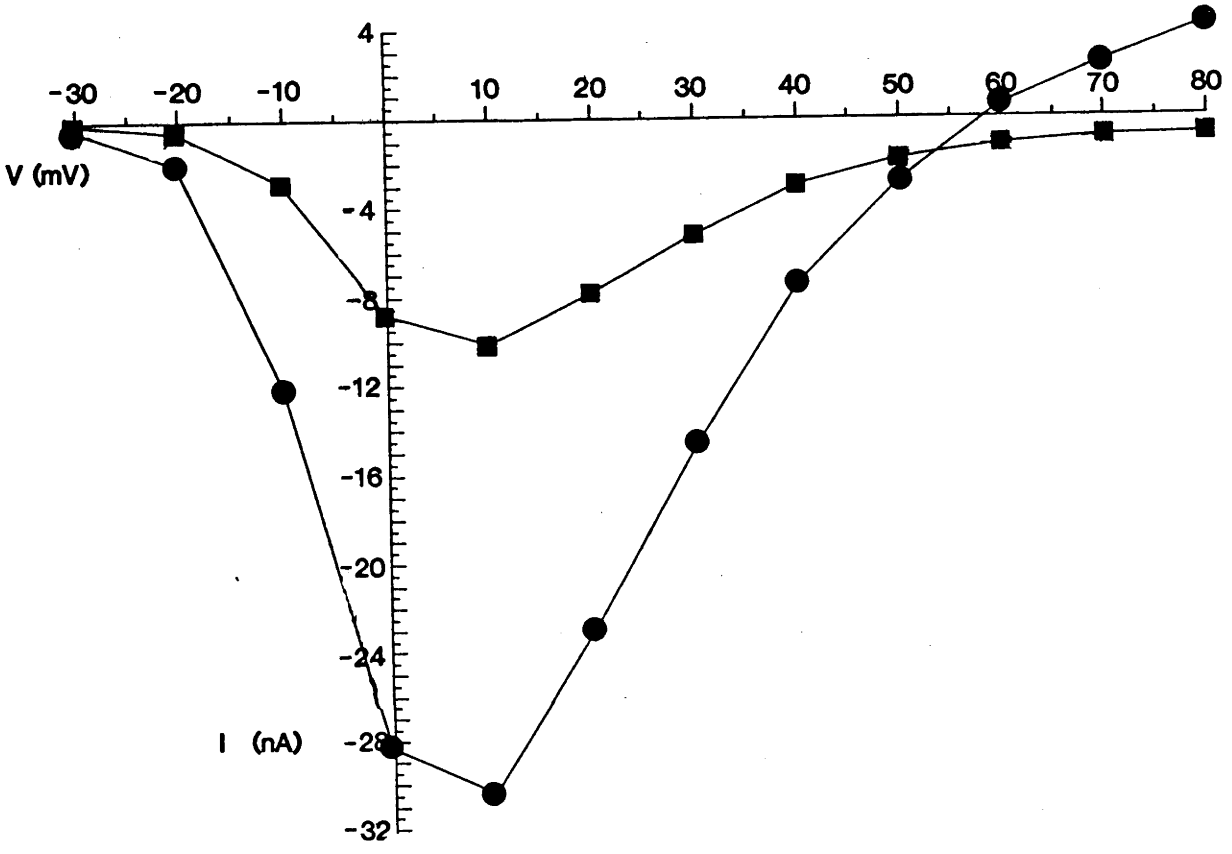


FIGURE 78: Calcium-activated chloride current. The peak calcium current on steps from -50mV is shown by filled circles. The amplitude of the calcium activated chloride current on returning to -50mV after the depolarizing step is shown as with squares. There is a close correlation between the amplitudes of the two currents. Cat drg neurone.

These are teleological speculations though, and further experiments will be necessary to determine the function of this conductance.

It is unlikely that this chloride current was responsible for the large noisy Cl^- channels observed in patch recordings, since the single channels were also obtained without Ca^{2+} ions. Although there have been no reports of the single channel conductance of calcium-activated chloride channels in neurones, a similar channel in lacrimal gland cells has a conductance of only 1-2pS (Marty, Tan & Trautmann 1984), which would be an order of magnitude smaller than the minimum discrete conductance of the single channels observed here.

B) Caesium-activated chloride current.

McBurney, Smith & Zorec (1986) have recently reported that increases in extracellular caesium concentration produce an inward current carried by chloride ions in cultured spinal cord neurones. This current was not blocked by 100µM Cd^{2+}, TTX or TEA, and did not appear to be dependent on external Ca^{2+} influx. Similarly, Kislov & Kazachanko (1975) have reported that external Cs^+ (and Rb^+ and K^+) ions can activate Cl^- channels in snail neurones.

In the present work, increasing extracellular Cs^+ concentration from 5 to 35mM also produced an inward Cl^- dependent current. This current was seen in all 8 rat cells tested, and amplitude varied between -0.2 and -1.15nA (-0.56 ± 0.13nA, x ± SEM). It is unlikely that

increases in Cs^+ were causing activation of GABA Cl^- currents since the inward current was not affected by 50μM picrotoxinin. However, this current was decreased if 1mM Cd^{2+} was included. Cd^{2+} antagonized between 66 and 100% of the inward current. The Cs^+ activated current reversed close to the Cl^- equilibrium potential, and subtraction of hyperpolarizing voltage steps in control and Cs^+ solutions revealed a small inward relaxation, which was in some respects similar to those relaxations seen with GABA (see Fig. 79).

It appears then that extracellular Cs^+ ions activate an inward Cl^- current. This is not caused by activation of GABA channels. McBurney et al. (1986) also concluded that extracellular Cs^+ was not having its action via release of, or contamination with inhibitory amino acids, but these authors did not use GABA antagonists. However, McBurney et al. reported that the current was unaltered by 100μM Cd^{2+}, while in the present study 1mM Cd^{2+} was fairly effective in blocking the chloride current.

The activation mechanism of this chloride current is obscure. It is unlikely that Cs^+ ions are moving through Ca^{2+} channels and activating a chloride conductance, since the voltage activated Ca^{2+} channel is probably not open in membranes resting at -40mV (although this cannot be ruled out on depolarizing voltage steps, any inward Cs^+ current through the calcium channels would be very small) and activation of the calcium-activated chloride current seems to be fairly specific for Ca^{2+} ions, at least in cultured rat DRG neurones (Mayer 1985). Since the Cl^-

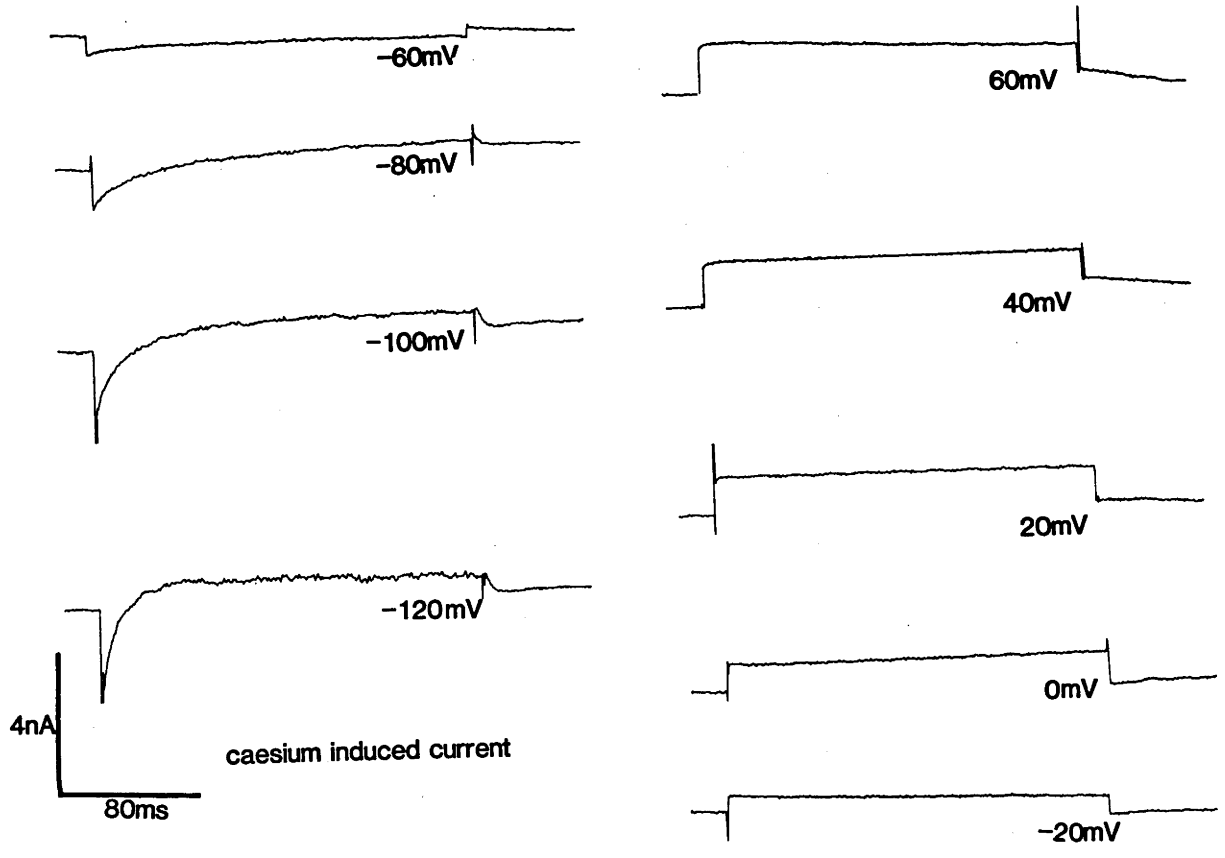


FIGURE 79: Relaxations on voltage steps from -40mV after changing from 5mM caesium externally to 40mM caesium. At hyperpolarized potentials, the 'caesium-activated chloride current' shows relaxations similar to, but much faster than those obtained with GABA. Rat drg neurone.

current activated by Cs^+ is voltage sensitive (since relaxations were observed), it is possible that there is some voltage activated chloride conductance in these neurones. A variety of voltage-activated chloride currents have been observed in other neurones. Chesnoy-Marchais (1983) has reported a chloride current that is activated by hyperpolarization in Aplysia neurones, and a voltage insensitive chloride current (with a larger single channel conductance) which is also slightly permeable to cations (Chesnoy-Marchais & Evans 1985). Brown & Selyanko (1985) have described a voltage insensitive Cl^- current in rat sympathetic neurones (which they call I_X) which can be inhibited by muscarinic receptor activation. Brown & Selyanko also suggest that I_X makes a substantial contribution to the resting membrane conductance of these sympathetic neurones. Selyanko (1984) has observed a slow voltage dependent Cl^- current in rat sympathetic neurones which is similar to that observed by Chesnoy-Marchais in Aplysia neurones, and this current was inhibited by 200μM Cd^{2+}. In this regard the current is similar to that observed in the present study, but in DRG neurones the chloride current turns off with hyperpolarizing steps; the current observed by Selayanko turned on with hyperpolarization.

Blockage of chloride currents by Cd^{2+} ions has been observed in some other preparations (see Selyanko op cit.) and in turtle retinal cones 10μM Cd^{2+} ions decreased GABA activated chloride responses to ~ 26% of control values (Kaneko & Tachibana 1986).

The mechanism of Cs^+ activation of the voltage dependent chloride current and assessment of the role of this current in determining cell properties should be futher investigated. Additionally, it will be of interest to examine the mechanism of Cd^{2+} block, and to determine if Cd^{2+} ions also block GABA induced chloride currents in these cells. As pointed out by Kaneko & Tachibana (1986), if Cd^{2+} ions directly block chloride responses, then this will have implications for the validity of studies using this ion as a 'specific' calcium channel blocker, e.g. the calcium activated chloride current or presynaptic release of inhibitory transmitters. [It is thought unlikely that the reduction in IPSC amplitude in Cd^{2+} solutions was due to a postsynaptic rather than a presynaptic effect (e.g. to reduce calcium dependent transmitter release), since quite large IPSCs were still present in Cd^{2+} and their kinetics were similar to those in control solution. However, a possible postsynaptic action of Cd^{2+} in the hippocampus should be investigated in light of the results of Kaneko & Tachibana (1986). (However, six of the preliminary experiments on GABA responses in drg neurones were conducted in 1mM Cd^{2+} solution, which was used to block the calcium current. GABA responses were in all respects normal in these experiments).

Conclusion.

There are at least three other types of chloride channel in mammalian DRG neurones in addition to those activated by GABA. It is suggested that there is a high conductance chloride channel revealed in patch clamp recordings, which becomes more active as the membrane is hyperpolarized or depolarized. Secondly, there is a calcium activated chloride conductance, which produces a slow depolarization following the action potential. Finally there is a caesium-activated chloride current which is turned off by hyperpolarization. Further experiments will be necessary to examine the gating and kinetics of these channels, and to determinne what role they may play in the intact animal.

The presence of these other chloride currents will complicate the examination of GABA activated chloride currents in DRG cells, and the care will have to be taken when designing extracellular solutions to examine GABA channels in isolation.

SECTION E :

GENERAL DISCUSSION

SECTION E: GENERAL DISCUSSION

In this section, some of the more general aspects of the results obtained with GABA-activated channels will be discussed, and the literature is reviewed more widely. Much of the discussion will be of a purely speculative nature, and draws heavily on the biophysics and pharmacology of other receptors and channels (in particular the acetylcholine receptor channel at the muscle end-plate). The hope is that some of the priciples derived from work on these better characterized systems will be applicable to GABA channels.

Do the channels and receptors on the somatic membrane of drg cells have any role?

The present work has focussed on chloride channels coupled to GABA receptors in drg neurones, but there is also evidence indicating that a variety of other channels and receptors are also present on the cell bodies of these neurones. These include: a TTX sensitive and TTX insensitive sodium current, a 'delayed rectifier' potassium current, a 'sustained' and 'transient' calcium current, a calcium-activated chloride current, a caesium activated chloride current, $GABA_B$ (baclofen) receptors (B. Robertson and W.R. Taylor, personal observations). We may ask the question: Why are these channels and receptors

there?

The cell body of primary afferent fibres may serve a purely trophic function, that is maintaining both of the peripheral and central processes of these neurones. Indeed, this was the conclusion of Steinbach (1899). Steinbach cut off the blood supply to the dorsl root ganglion of the frog, and could still elicit reflex activity after one or two days. He believed that the cell bodies were purely trophic, and played no part in conduction of impulses to the spinal cord. However, there is evidence that there is a small delay in the T junction at the connection between the cell body and the axon (e.g. Dunn, 1955) and the action potential always invades the cell soma (e.g. Sato & Austin, 1961; Harper & Lawson, 1985). It may be possible then that in some conditions the active currents present in the somatic membrane are necessary for impulse propagation along the dorsal roots.

The neuronal cell body is the main site of protein synthesis, so presumably channels and receptors are manufactured in the soma and transported to the nerve endings via axoplasmic transport, or in the membrane lipids themselves (see e.g. Lombet, Laduron, Mourre, Jaconet and Lazdunski, 1985).

It may be possible that the receptors and channels neccessary for control of information transmitted to the spinal cord (e.g. $GABA_A$ and $GABA_B$ receptors) are manufactured in the soma and transported down the membrane to the central terminals of these neurones. It should be easy to test this hypothesis by attaching radioactive label to receptors or channels or the cell soma and measuring any migration. Alternatively, the appearance of channels and receptor proteins on the cell soma may be due to overspill, and have no physiological relevance.

NO PAGE 167
TEXT O.K.

Voltage sensitivity of GABA channel open time.

Results have been presented here which indicate that the mean open time of GABA channels is shortened by membrane hyperpolarization in both central (hippocampal pyramidal cells) and peripheral (dorsal root ganglion) mammalian neurones. This phenomenon has been reported perviously for GABA activated chloride channels in invertebrate muscle (Onodera & Takeuchi 1979, Dudel 1977, Cull-Candy 1986) and neurones (Adams, Constanti & Banks 1981). Agonist activated chloride channels in other preparations also display a positive volt constant, e.g. Ach gated channels in Aplysia (Adams et al. 1982) and in Helix neurones (Finkel 1983). There are few other studies which have examined the voltage sensitivity of GABA channel lifetime in vertebrate and mammalian neurones. Segal & Barker (1984b) have reported that evoked IPSCs in cultured hippocampal neurones were shortened by membrane hyperpolarization, although only a few potentials were examined. Barker, McBurney & MacDonald (1982) examined channel open time from noise analysis over a small voltage range in cultured spinal cord neurones, and reported no significant voltage sensitivity of open time. In other studies of GABA channel kinetics under patch clamp conditions have no comment has yet been made on the direction or magnitude of voltage sensitivity of channel open time (e.g. Hamill et al. 1983). However the rectification of the steady state current voltage relationship for GABA induced Cl^-

currents in other preparations is consistent with the direction and magnitude of voltage sensitivity observed in the present study (e.g. Akaike et al 1985). There is a paucity of data concerning the voltage sensitivity of kinetics of glycine activated chloride channels, but recently Gundersen, Miledi & Parker (1986) have reported that the curvature of the steady state current voltage relation for glycine can be attributed to shorter channel lifetimes at hyperpolarized potentials.

We still do not understand the mechanism by which changes in membrane field influence channel kinetics. Magleby & Stevens (1972b) suggested that the voltage dependence of channel open time could be modelled in a manner similar to that proposed for voltage-gated channels. That is, during channel opening or closing there is a redistribution of charges or dipoles associated with the channel. When the channel moves from a closed (C) to an open (O) state:

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O$$

there is some conformational change involving displacement of some charge of valence z and magnitude e. From a rate theory type model, a single energy barrier exists between the C and O states of Free Energy difference U, this will be related to the channel closing reaction α by:

$$\alpha = \nu \exp(-U_{CO}/kT)$$

where ν is a constant (the effective vibration frequency), k is Boltzmann's constant and T is absolute temperature.

Magleby & Stevens (1972b) modelled the potential dependence of the α reaction by including a change in the dipole moment in the channel. The Free Energy difference for a changing dipole moment in the presence of an electric field is:

$$U = U_{co} - E\ \delta\mu_{co}$$

where U_{co} is the energy in the absence of the field (0mV), E is the field strength [E = V(membrane potential)/M (thickness of the membrane)], and $\delta\mu_{co}$ is the difference in the dipole moment of the channel between the open and the transition state. Including this relation in the expression for α:

$$\alpha = \nu \exp(-U_{co}/kT).\exp(\delta\mu_{co}V/MkT)$$

If we let $A = \mu/MkT$ and $B = \nu \exp(-U_{co}/kT)$ then $\alpha = B \exp^{AV}$ where $A = 1/H$. This equation describes the voltage dependence of the closing rate α on membrane potential V in terms of constants B and H (the quantity used throughout the present work). Effectively, B is a constant being a combination of the free energy of the transition and the attempt frequency, and H is the change of dipole moment. The majority of the results presented here are consistent with a single exponential function describing the voltage sensitivity of α. However some

cells displayed deviations from a single exponential, Similar behaviour has been observed at the neuromuscular junction and nerves of certain species of frogs (e.g. Neher & Stevens 1979, Levitan & Palti 1975) and at other Ach receptors (submandibular ganglion Rang 1981, Aplysia neurones, Marchais & Marty 1979). This may be due to an effect of membrane electric field on the 'polarizability' of the channel protein. Intuitively, this could be brought about by dissociation of an ionic bond at large potentials. The difference in saturation of voltage dependence between preparations could be due to slight differences in the pK_a of the groups contributing to this ionic bond. It is considered that the field can influence the dipole by distortion polarization, the field inducing rotation of the structure or dissociation of an ionic bond or a combination of all of these factors. Theoretically these effects can be included by expanding the term for $\delta\mu$ into a Taylor series ($\delta\mu = \delta\mu + V(d\delta\mu/dV) + (etc)$.

Taking an average H value of 140mV the 'equivalent gating charge' (z) to reach the transition state in channel closing is ~ 0.17 electronic charges (since α changes e-fold for kT/ze mV, and kT/e is ~ 25mV). This is only a small fraction of the equivalent charges involved in the opening of sodium channels (where z ~ 6), which have a considerably steeper voltage dependence (Hodgkin & Huxley 1952).

It must be emphasized that the degree of voltage sensitivity is only equivalent to 0.17 electronic charges moving through the whole membrane field. It is likely that charged groups would move only over a very small region of the membrane field, and these groups could sense any proportion of that field depending on their orientation and their local electrical environment. Additionally, the charge movement may be distributed amongst more than one open state, in which case the equivalent charge displacement reflects the total charge moved between the open states (see e.g. Almers 1978).

It is probably unlikely that the dipole is in the lipid itself and lying perpendicular to the membrane field. The dipole is more likely to be inside the protein complex; Fernandez, Bezanilla & Taylor (1982) have suggested from the different actions of chloroform on charge movement in Na^+ channels and a charged membrane transport molecule that the voltage sensor is not exposed to the membrane lipids but is embedded in the Na^+ channel complex.

It is not yet known which part of the GABA channel complex acts as the voltage sensor and is responsible for relating the electric field strength to the channel closing reaction. Advances in this direction are being made for the Ach channel. Sakmann, Methfessel, Mishina, Takahashi, Takai, Kurasaki, Fukuda & Numa (1985) have successfully produced 'hybrid' Ach receptor/channels from Torpedo and calf DNA in Xenopus oocytes and have found that the voltage dependence of channel lifetime was

dependent on the composition of the δ subunit of the oligomer. Torpedo channels are normally voltage insensitive, but substitution of the Torpedo δ subunit with a δ subunit form calf imparted voltage sensitive kinetics to the hybrid. It should be possible to apply this technique to GABA channels, since cloning of these complexes is already underway (Barnard et al. 1983), and we may soon be able to isolate and identify the specific regions of the channel which act as the electric field sensor. It is possible that the differences observed in the degree of voltage sensitivity of GABA channels may reflect differences in the environment (e.g. lipid) or the amino acid composition of the voltage sensor. Miller & Rosenberg (1979) have approached the problem of identification of the voltage sensor by chemically modifying the channel protein in situ, and have observed that a specific endopeptidase attacks the sarcoplasmic reticulum K^+ channel, cleaving the voltage sensor from the trans face of the membrane. This procedure almost completely abolishes the voltage sensitivity of the channel. Similarly, Doring & Colombini (1984) have found that succinic anhydride (which conjugates accessible NH_3^+ groups) reduces the voltage dependence of the mitochondrial voltage dependent anion channel (VDAC). Hopefully this approach may provide useful clues as to the nature of the voltage sensing apparatus, and it would certainly be worth applying these methods to GABA channels.

As discussed previously, it is unlikely that the voltage sensitivity of GABA channel lifetime plays an important physiological role, particularly since the temperature dependence of H is fairly high (since $H = MkT/\mu$), and causes H to become smaller at lower temperatures*. It is conceivable that the small hyperpolarization associated with an IPSP may assist in the removal of agonist as channel lifetime becomes shorter, but given the magnitude of IPSPs this effect is probably minimal. It may be that the mammalian GABA receptor voltage sensor is a remainder from our past evolution, where it did confer some physiological advantage for inhibitory neurotransmission. Perhaps it is sensible that the neuronal membrane is not loaded with all varieties of potential sensing dipoles, since as Hodgkin (1975) suggested, this would tend to increase the overall membrane capacity, making for instance nerve impulse propagation more difficult. (This may be of particular importance in the axon initial segment of hippocampal pyramidal cells, since there is a dense inhibitory innervation in this region, and if GABA channels were to have a large number of dipoles the generation of action potentials could be compromised).

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* The temperature dependence of H measured in the hippocampus was similar to that obtained at the end-plate i.e. H increased at higher temperatures. This is

consistent with the Magleby & Stevens model. However, Onodera & Takeuchi (1979) observed no temperature dependence of H (but see the figure presented in their paper). Similar results were obtained by Dudel (1977) for the GABA channel in lobster muscle. Anderson, Cull-Candy & Miledi (1978) have found that voltage sensitivity was reduced at lower temperatures. These results are inconsistent with the Magleby & Stevens model, and may suggest that in these synapses at least that another model would be more appropriate. It would be useful if further temperature experiments could be performed on glutamate and GABA channels to confirm and expand these results.

The elementary conductance of mammalian GABA channels: are there subconductance states?

The advent of the patch clamp technique (Neher & Sakmann 1975, Hamill et al. 1983) allowed for the first time direct examination of the kinetics and conductance of single membrane channels. The Ach receptor of skeletal muscle is still the best characterised excitable channel. Previously, noise analysis tecchniques were used to determine the elementary conductance of Ach channels: for instance, Anderson & Stevens (1973) obtained a value of ~ 30pS. However Hamill & Sakmann (1981) using the patch clamp technique, observed 'sublevels' of conductance, that is current transitions that were of a smaller amplitude than the usual fully open channel. Similar results have been obtained by other groups working on Ach activated channels (e.g. Sine & Steinbach 1986), and chloride (e.g. Krouse, Schneider & Gage 1986).

Hamill et al. (1983) have reported that glycine and GABA activated channels in cultured mouse spinal cord neurones display three conductance levels - for GABA these levels were 20, 31 and 46pS. Before this report, Barker, MacDonald & McBurney (1981) had obtained a value of 18pS from noise analysis of GABA channels in cultured spinal cord and dorsal root ganglion neurones. Various other groups using patch clamp techniques have also reported multiple conductance states for GABA channels on cultured

neurones: (Bormann & Clapham 1985, cultured chromaffin cells: 18, 31 & 45pS, Ozawa & Yuzaki 1984, cultured hippocampal neurones: 9, 19 & 29pS, McBurney, Smith & Zorec 1985, cultured spinal cord neurones: 20, 30 & 44pS, Cottrell, Lambert & Peters 1985, cultured chromaffin cells: 17, 29 & 37pS, Weddle & MacDonald 1985, cultured spinal cord and cortical neurones 21-25, 35-40 & 47-52pS). However the occurrence of multiple conductance states for GABA channels has not been observed in all preparations. For instance, Gray, Kellaway & Johnston (1983) have reported a single conductance level with a value of between 19 and 45pS in dissociated hippocampal neurones, although these authors have more recently reported a marked potential dependence of single channel conductance, ranging from ~ 20pS at hyperpolarized potentials to ~ 40pS at +40mV. Miledi, Parker & Sumikawa (1983) have successfully produced chick optic lobe GABA receptors in Xenopus oocytes, and with patch clamp have obtained a conductance of 29pS, but had evidence for another discrete population of GABA channels with a different conductance. Yasui, Ishizuka & Akaike (1985) also found evidence for two different populations of GABA activated chloride channels in frog DRG neurones, one with a single channel conductance ranging from 9-25pS, the other displaying a somewhat higher conductance of 18-25pS. Cull-Candy & Ogden (1985) have determined from noise analysis that GABA channels in cultured cerebellar neurones have a conductance of ~ 16pS. Preliminary results obtained in this laboratory by A. J. Gibb (personal communication)

suggest that GABA channels in dissociated hippocampal neurones have a single channel conductance of ~ 25pS, with no evidence for substates.

It is noteworthy that all of the demonstrations of subconductance states (in the strict sense where a channel flips between two levels) for GABA channels have been in cultured cells only. From the evidence so far obtained on GABA channels on freshly isolated neurones, only one level is displayed, or in some cases separate channels with different levels are seen. Further experiments will be necessary to demonstrate if this is really the case or not, but if so then these results raise interesting possibilities for the regulation of channels in vivo and in culture. Consider the case where GABA channels are capable of three different conducting states, but is normally stabilized at one of these levels in vivo. If this stabilizing or regulatory mechanism is removed or is somehow lost in cultured cells, the channel can then flip between each of the levels. If this purely hypothetical 'regulatory unit' could be identified, determination of its nature and mechanism may provide clues as to how other channels displaying substates (e.g. Krouse, Schneider & Gage 1986) are controlled.

ANION SELECTIVITY.

The results of experiments with foreign anions in hippocampal GABA channels suggest that sulphate, acetate, citrate and methylsulphate ions are impermeant, while iodide, bromide, chloride, thiocyanate, nitrate, cyanate, azide and chlorate ions are significantly permeable. The more restricted studies of GABA-activated channels on dorsal root ganglion neurones gave a permeability ratio of $P_I/P_{Cl} \cong 1.8$, $P_{Br}/P_{Cl} \cong 1.2$, and neither fluoride nor propionate ions were significantly permeant.

As discussed previously, the anion permeability for the GABA channel from the CNS is similar to that obtained by Eccles et al.(1977) and more recently by Biscoe & Duchen (1985). In this respect, the GABA channel here has permeability to anions similar to that of glycine-activated chloride channel of cat motoneurones (Coombs et al. 1955, Araki et al. 1961, Ito et al.1962).

There have been other studies of the permeability of GABA-activated channels in a variety of preparations. Asada (1963) examined the permeability of the chloride channel of the goldfish Mauthner neurone, and found similar results to those obtained for cat spinal motoneurones. Takeuchi & Takeuchi (1971b) have reported that the permeability sequence of anions depended on the GABA concentration used to activate the channels. With high concentrations of GABA, nitrate and iodide were less permeable than chloride, but with low concentrations of

the transmitter, the permeability sequence is bromide > chloride > iodide > nitrate. Takeuchi & Takeuchi suggested that GABA binding somehow modulates the selectivity filter of the channel.

Motokizawa, Reuben & Grundfest (1969) have also examined the anionic permeability of the postsynaptic chloride channels of the lobster muscle fibre, and obtained the permeability sequence nitrate > thiocyanate > bromide > chloride, with additional small permeability to acetate and propionate (in contrast to the present study).

Adams & Brown (1975) concluded from their studies of GABA action on rat sympathetic ganglion cells that the 'large' anions isethionate, sulphate and glutamate are impermeant, while bromide was permeable. Gallagher et al (1978) investigated the ionic basis of GABA depolarizations of cat primary afferent neurones, using both intracellular and extracellular substitution of anions and found that anions with a hydrated radius less than chlorate ions were permeant, e.g. bromide, iodide, nitrate, thiocyanate and chlorate could carry current while fluoride, acetate and citrate were excluded. Unfortunately, these authors did not measure the relative permeabilities of these anions, which would have been useful to compare to the results presented here.

One of the most detailed studies of the permeability of the GABA channel to date is the work of Hamill, Bormann & Sakmann (1983). These authors examined the permeability and conductance properties of channels activated by GABA and glycine in cultured spinal cord

neurones. The permeability sequence bromide > iodide > chloride > fluoride was obtained, with permeability ratios 1.8 : 1.4 : 1.0 : 0.4 for GABA channels. That Hamill et al obtained such a high permeability for fluoride is surprising since most other studies of GABA activated channels in mammalian preparation find that fluoride is impermeable (e.g. Gallagher et al (1978), Eccles et al (1977), Ito et al (1962). The results presented here suggest that fluoride permeability of GABA channels in dorsal root ganglion neurones is less than one tenth that of chloride. Bormann, Hamill & Sakmann (1986) have recently reported that acetate is permeable, indicating a minimal pore diameter of 5-6 angstroms for this chloride channel - if this were the case then large anions such as methylsulphate also ought to be permeant. It may be likely then that there is a real difference between the size of the selectivity filter in GABA receptor channels in cultured and freshly isolated preparations. If this is the case, this would certainly warrant further investigation, since it should then be possible to identify that part of the channel structure which constitutes the selectivity filter. Approaches of this kind would gradually lead to an understanding of the mechanisms associated with ion permeability and selectivity.

The permeability of a few other transmitter-activated chloride channels has been determined. Yarowsky & Carpenter (1978) have reported that chloride channels activated by Ach and GABA in the giant R2 neurone of

Aplysia are partially permeant to propionate and isethionate, but impermeant to sulphate, methylsulphate and acetate. Adams, Gage & Hamill (1982) investigated the anionic permeability of Ach-mediated IPSCs in the buccal ganglion of Aplysia, and found that the permeability sequence was chloride > bromide > iodide, suggesting that these channels are highly chloride selective. The voltage gated chloride channel from Torpedo electroplaque also shows a strong preference for chloride over other anions, with bromide the only ion other than chloride that is appreciably permeant (P_{Br}/P_{Cl} = 0.7) while thiocyanate and iodide reversibly block this channel at low concentrations. The function of this extreme selectivity for chloride ions is as yet unclear, but this chloride channel must have a different selectivity mechanism for anions from the GABA-activated chloride channel. The 'high conductance' anion channel observed by Gray, Bevan & Ritchie (1984) in cultured Schwann cells has a permeabililty sequence iodide > bromide > chloride (1.4 : 1.2 : 1.0) but is also significantly permeable to methylsulphate, sulphate, isethionate and acetate (0.7 : 0.6 : 0.4 : 0.4). The larger pore size necessary to permit flux of the larger anions may account for at least part of the high conductance (~ 450pS) of these channels.

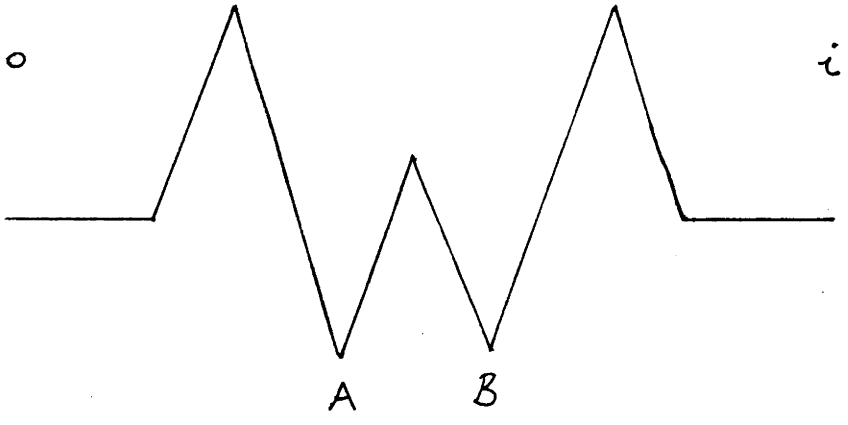
ANION CURRENT VOLTAGE RELATIONS.

The instantaneous current-voltage curve for GABA channels in symmetrical chloride solutions in DRG neurones

was linear over the range -120 to +60mV, suggesting that in chloride solutions at least, this channel is symmetrical in nature. However, when some or all of the external chloride was replaced with iodide or bromide, there was a marked increase in instantaneous current at negative potentials. In this region, the majority of the current is caused by chloride efflux from the cell. In simple terms, the presence of iodide and bromide ions externally enhances the outward flux of chloride. Possible mechanistic explanations for this effect may be advanced. For instance, an energy barrier at the outer end of the chloride channel could be lowered, favouring chloride exit. This energy barrier could be some cationic site to which external anions bind. If this site had a lower affinity for bromide and iodide ions, the 'unmasked' positive charge at the mouth of the channel would favour chloride exit and entry, increasing single channel conductance. This model is qualitatively similar to that proposed by Sigworth & Spalding (1980) and Spalding (1980) for trimethyloxonium ion ($(CH_3)_3O^+$) modification of sodium channels in frog skeletal muscle and nerve. These authors suggested that TMO removed a fixed negative charge at the mouth of the channel (TMO methylates carboxylic acids), thereby increasing the local concentration of cations and consequently single channel conductance. Iijima, Ciani & Hagiwara (1986) have proposed a similar kind of model for pH effects on clcium channels, where hydrogen ions alter innward, but not outward Ca^{2+} flux by binding to and altering the local

surface potential that the channel senses.

Another possible model could be that iodide and bromide ions bind less well to a site inside the channel e.g.



The efflux of chloride ions (i to o) would be enhanced if this site were vacant, that is movement of the anion from site B to A is facilitated if site A is vacant. The increase in outward chloride flux would be most marked at negative potentials because it would be more difficult for the external anion to reach this site through the negative membrane field. This model would predict that iodide ions would have a greater single channel conductance than chloride ions, since there would be less tight binding of iodide ions inside the channel, which tends to slow permeation (see Bezanilla & Armstrong 1972).

There have only been a few other reports of anomalous changes in channel permeability upon changing ions. For instance, Fukushima (1982) has observed that external

sodium ions increased the single channel channel conductance of anomalous rectifier K^+ channels, although sodium ions were not themselves permeant. Also DiFrancesco (1982) noted a peculiar effect of external caesium ions in enhancing outward current through the I_f, or 'pacemaker' channel in calf Purkinje fibres. DiFrancesco suggested that there was some external 'activatory' site for cations on these potassium channels. It is not yet clear however if the increase in current with external caesium is due to a change in channel kinetics or single channel conductance.

HOW MANY ENERGY BARRIERS DOES THE GABA-ACTIVATED CHLORIDE CHANNEL HAVE ?

The discussion above on the effects on the effects of foreign anions on the instantaneous current voltage relation suggests that there would be at least three energy barriers (two wells) for this chloride channel. Although the results presented here are not sufficient to fully test this model, we may make some predictions as to the minimum number of energy barriers/wells necessary for GABA channels.

Lauger's (1973) rate theory predictions for a symmetrical two barrier channel predicts that the maximal channel conductance voltage relation (i.e. that measured in saturating concentrations of permeant anions) will be given by (adopting the conventions of Coronado, Rosenberg & Miller 1980):

$$\gamma^m/\gamma^m_o = (\sinh[\Psi/2])/(\Psi/2)$$

where γ^m is the maximal channel conductance and γ^m_o is the zero voltage value of maximal channel conductance, $\Psi = FV/RT$ and V is the membrane voltage. This equation predicts that the maximal conductance-voltage relation will be markedly nonlinear. Linearity can only be increased by increasing the number of energy

barriers. A three barrier model would be given by the equation:

$$\frac{\gamma^m}{\gamma^m{}_o} = \frac{(2 + \exp[p_c - p_o]\sinh\Psi)}{\Psi\{\cosh([\delta - 1]\Psi) + \cosh([3\delta - 1]\Psi + \exp(p_c - p_o)\cosh(2\delta\Psi)\}}$$

where p_c and p_o are the peak heights of the central and outer barriers respectively, and δ is the position of the energy wells with respect to the ouside of the membrane. With this equation, linearity in the current voltage relation in saturated solution can be produced by adjusting barrier heights or well locations. The conductance voltage relation in symmetrical 140mM Cl^- solutions obtained in the present experiments was very nearly linear. However, only one concentration of Cl^- was examined, and it would be necessary to determine the conductance of the GABA activated channel in various Cl^- concentrations and to determine the conductance voltage relation for saturating solutions to determine the degree of linearity, and hence how many energy barriers would be needed to fit the data. The results of Sakmann, Bormann & Hamill (1983) suggest that the half saturating Cl^- concentration for the GABA channel in spinal cord and hippocampal cells is 178mM and the maximal conductance is 73pS. This would indicate that in the present experiments, where the Cl^- concentration was 140mM, the channel was quite far from saturation, so we cannot realistically use the conductance voltage relations obtained in the present experiments to determine the number

of energy barriers in the GABA channel.

It is of interest to note that the electrodiffusion theory of Barry (Barry, Gage & Van Helden 1979, Barry & Gage 1984) also predicts linear conductance voltage relations for symmetrical solutions of ions, and is equivalent to rate theory predictions when multilpe barriers and sites are introduced in the latter, permitting multiple occupancy of the channel. To summarize, the results obtained here suggest that a multiple barrier model of GABA channels is necessary to explain the results obtained, but further experiments would be required to confirm this hypothesis.

Effects of anions on channel kinetics and voltage dependence.

The alteration in GABA channel lifetime by changes in anion composition on either side of the membrane has many precedents. Lillie (1910) and Hill & MacPherson (1954) noted changes in 'excitability' with various sodium salts, and Hill & MacPherson observed changes in the 'active state' of skeletal muscle when citrate, iodide and bromide ions replaced cholride.

Kolb & Bamberg (1977) observed that permeant ions altered the kinetic properties of the gramicidin channel, and Van Helden et al. (1977) reported that substitution of caesium, potassium and lithium for sodium resulted in an alteration of the open time of Ach activated channels.

Other authors have also observed changes in channel lifetime with changes in permeant ions (e.g. Hagiwara, Miyazaki & Rosenberg 1976 for the anomalous rectifier channel, Swenson & Armstrong 1981 for the K^+ channel in squid axon, Chesnoy-Marchais 1984 for hyperpolarization activated Cl^- channels, and Nelson, French & Kreuger 1984 and Chesnoy-Marchais 1985 for Ca^{2+} channels). Gage & Van Helden (1979) found a reciprocal relationship between average Ach channel lifetime and conductance of the cation, and suggested that the permeant ion was binding to some site(s) inside the channel and the channel lifetime is determined by the rate of dissociation of the ion from this site. Marchais & Marty (1979) similarly proposed that the lifetime of Ach activated cationic channels in Aplysia neurones was controlled by binding of the permeant ion to some intra-channel site, and that the channels could not close while an ion is bound to this site. In this model the less permeant ions decrease the conductance of the channel by binding inside the channel, impeding the flow of permeant ions and decreasing the closing rate of the channels. (This model was also used to explain the saturation of the dependence of channel lifetime on membrane potential. Since the permeant ions sense a fraction of the membrane field as they bind with the hypothetical site, then the binding equilibrium constant will be sensitive to the membrane potential). However this model would predict that the single channel conductance should also be influenced by membrane potential, since the equilibrium of binding determines

both conductance and open time.

In the present study, GABA-mediated IPSC decay in the hippocampus was considerably longer if the current was carried by iodide or thiocyanate ions rather than chloride ions. As discussed previosly, it was not possible to measure the relative permeabilities for these and the other anions tested in the hippocampus, since changing the nature or the concentration of the intracellular ion would have been very difficult. However, if we assume that iodide and thiocyanate are more permeable than chloride (which seems to be justified from the experiments carried out on DRG neurones and the permeability ratios of other GABA-chloride channels) then we would expect from the models of Barry et al. (1979) and Marchais & Marty (1979) that the GABA channels should close more rapidly when iodide and thiocyanate are the main charge carriers. It may be suggested then that the internal anions influence lifetime in some other way rather than simply 'plugging' the ionophore.

The experiments on DRG neurones were performed in order to examine more rigorously the influence of foreign anions on the kinetics and permeability properties of GABA activated Cl^- channels, since in this preparation it was possible to control the concentration of the intracellular anion and to change the extracellular anion repeatedly and rapidly. The results showed that changing the external anion also altered the closing rate, since in solutions containing iodide and bromide ions channel lifetime was lengthened, and when extracellular chloride was replaced

by propionate channel open time was shorter. External iodide was effective at lengthening the currents even at concentrations as low as 10mM. These results would tend to suggest that there is some site on the external aspect of the ionophore which can also influence channel kinetics. There are precedents for this effect in the chloride channel literature. Ochi(1969) observed that replacement of chloride externally with iodide and thiocyanate prolonged GABA-mediated IPSPs in the giant motor nerve of the crayfish. Gallagher et al.(1978) also noted that substituting external chloride with various anions (inclu ding bromide, iodide and nitrate) caused a prolongation of the time course of GABA depolarizations (bromide also acted to enhance the amplitude of the depolarization). Onodera & Takeuchi (1979) found that replacing chloride with iodide or bromide lengthened IPSC decay in crayfish muscle fibres - even inward IPSCs which they argued were still carried predominantly by outward chloride flux. These authors suggested that "the presence of I^- in the bath solution is more important for determining the time course of the IPSC rather than I^- ions which carry the synaptic current". A similar result was found by Adams, Gage & Hamill (1982) at the cholinergic synapse of Aplysia, where replacement of iodide and bromide for chloride externally prolonged IPSC decay at hyperpolarized (where Cl^- ions were the principal charge carrier) as well as depolarized potentials.

On the basis of the results presented here and on the findings of others, it may be suggested that there is some

key site on or near the external side of the chloride channel which can bind ions and thereby modulate channel kinetics. The nature of this site is not yet known. Binding studies have also suggested that anions are important in the binding of agonists and antagonists to this channel. Enna & Snyder (1977) demonstrated that anions influence the affinity of the 'bicuculline receptor' of the Cl^- channel. Anions are important in determining the binding of benzodiazepines (see e.g. review by Olsen 1981), and barbiturates (e.g. Olsen & Snowman 1982) to the GABA receptor channel complex. The most effective anions are those which are permeable through the $GABA_A$ ionophore. It will be of interest to localize the site(s) at which these interactions take place, since it is possible that anions can allosterically modify the receptor/channel complex to enhance agonist or modulator binding.

One simple hypothesis to explain the longer open-times of GABA channels in foreign anion solutions is that the anions act to stabilize the transmitter/receptor complex, thereby keeping the agonist attached for longer and preventing channel closure (if dissociation of agonist is the rate limiting step in control of channel lifetime). However, Enna & Snyder (1977) reported that the affinity of the receptor for GABA agonists was unaffected by the nature of the anion. Clearly further experiments, both binding and electrophysiological, are desirable to identify the external 'anion site' proposed here. It is conceivable that this site could be vital for the action of useful drugs and other modulators of GABA channel function.

In addition to modulating chloride channel properties, anions also affect a variety of cationic channels. For instance, fluoride ions (amongst others) 'stabilize' the excitability of squid giant axons (e.g. Baker et al. 1962) and modulate potassium channels in this preparation, interacting with a site on the intracellular surface to reduce the probability of channel opening (Adams & Oxford 1983). Fluoride ions, even at concentrations as low as 5mM, alter the open time of Ach channels (Kaibara, Kuba, Koketsu & Karczmar 1978). Kostyuk et al. 1975 have noted that internal fluoride abolishes calcium currents in molluscan neurones. (However, calcium currents can still be recorded with 140mM internal fluoride in drg neurones, personal observations). Dani, Sanchez & Hille (1983) found that lyotropic anionsx shifted the voltage dependence of sodium channel activation and inactivation in both muscle and nerves of frogs. These authors suggested that this effect could be due to the anions adsorbing electrostatically to either the channel protein itself or the nearby membrane lipid. It is clear that anions can affect a wide variety of channels in excitable membranes. The actions of the few anions tested here on GABA receptor channels have still to be clearly explained mechanistically, and further experiments will be necessary. Some hypotheses based on physicochemical studies with anions may be advanced however.

Anions may directly alter the conformation of the channel protein, and cause a slowing of the kinetics.

The pK_a of serum albumin is dependent on the nature of the predominant anion. As iodide replaces chloride, the solution pH increases as protons are drawn from the solution to the protein, since iodide binds more readily than chloride to sites on serum albumin, increasing its negative charge. The results were consistent with there being over twenty anion binding sites on bovine serum albumin with varying affinities (see e.g. Record, Anderson & Lohman 1978).

Alteration of the anion composition at lipid bilayers could cause changes in the electric profile across the channel protein by altering the surface charge. However, this mechanism may be unlikely since most mammalian cells including dorsal root ganglion neurones (Mironov & Dolgaya 1985) possess a net negative surface potential which would probably prevent binding or screening actions of the anions. It should be stressed however that we have no real evidence for the nature of lipids at the channel region, and positive charges near channel proteins have been suggested previously (e.g. Hille et al.1975).

It is perhaps more attractive to suppose that anions act at charged sites on the channel protein complex itself, or at sites closely associated with it, and binding to these sites alters channel gating. Further characterization of these hypothetical sites could be partly accomplished by pH titration (since both the number and affinities of these sites will be dependent on pH). Additionally chemical modification techniques could be employed (e.g. Sigworth & Spalding 1980).

In hippocampal neurones, the normal voltage dependence of channel open time was reduced when IPSCs were carried by iodide, bromide and nitrate ions. This effect was particularly striking for iodide, and is similar to the depression of voltage sensitivity observed by Adams et al. (1982) for Ach mediated chloride currents in Aplysia neurones. Modification of voltage sensitivity by permeant ions has been observed for Ach activated cationic channels in amphibian muscle (Gage & Van Helden 1979) and Aplysia neuyrones (Marchais & Marty 1979).

In contrast to the present observations on hippocampal neurones, GABA responses in DRG neurones with internal and external iodide displayed normal voltage sensitivity. Onodera & Takeuchi (1979) also observed similar degrees of voltage dependence in iodide and chloride solutions in GABA mediated IPSCs in crayfish muscle. Since the internal anion differentially alters voltage dependence of channel kinetics, these results suggest that there may be some difference in the structure and sensitivty of GABA channels in hippocampal and DRG neurones. As discussed previously, anions can absorb to protein complexes and exert electrostatic effects on the macromolecule. It is conceivable that anions could bind to cationic groups near that part of the channel which 'senses' the electric field across the membrane, and neutralize or weaken the effect of the field in that region.

Implications of the kinetic effects of anions for demonstration of anomalous mole fraction effects.

The effects of externally applied foreign anions on the kinetic properties of GABA activated chloride channels has implications for previous studies where anomalous mole fraction behaviour has been observed for GABA channels, which has been deduced from conductance measurements of a large population of channels in steadt state. Takeuchi & Takeuchi (1971) observed that GABA induced membrane conductance decreased when 25-50% of external chloride was replaced with thiocyanate, nitrate or iodide ions, but increased in higher concentrations of these relacement anions. This anomalous mole fraction behaviour has been interpreted as evidence that ion channels can contain more than two ions at a time (e.g. Hagiwara, Miyazaki, Krasne & Ciani 1977, Hess & Tsien 1984). However, as even small concentrations of anions can affect the open times of GABA activated channels, the total membrane current could reflect principally the new number of open channels rather than the permeability properties of a constant number of channels. These kinetic effects must be taken into consideration and corrected for when measuring the permeability sequences of ions from macroscopic current amplitudes rather than instantaneous currents or single channel currents. Ideally anomalous mole fraction behaviour should be demonstrated with single channels under patch clamp conditions, where a direct measure of conductance in foreign ions can be obtained. Even the

non-stationary fluctuation analysis used by Hess & Tsien (1984) to demonstrate this behaviour is prone to error since the variance of the current depends both on the magnitude of the single channel current and the probability of channel opening.

Properties of channels activated by GABA-like agonists.

One of the outstanding problems in understanding channel gating is knowledge of the process by which agonists open the channel by interacting with the receptor channel complex. One way to approach this problem is to employ different agonists with varying chemical structures and specificities, and to find out how they gate their channels. This should also help us to determine which processes in the activation sequence are influenced by the agonist, and those steps which are not.

Katz & Miledi (1972,1973) compared the open times of channels activated by carbachol, acetylcholine, decamethonium, acetylthiocholine and suberyldicholine at the frog motor end-plate, and found that these agonists produced varying channel open times. For instance, the lifetime of channels opened by suberyldicholine was about double that of Ach gated channels, while channels activated by carbachol were only open for one third as long as Ach channels. These early studies suggested that the more 'powerful' agonists held channels in the

conducting state for longer times. Subsequently there have been a few detailed studies investigating differences in channel properties when activated by different agonists. For instance, Auerbach, del Castillo, Specht & Titmus (1983) examined the effects of several N - substituted trimethylammonium derivatives on Ach channel kinetics, and concluded that it is the ester portion of the Ach molecule which stabilizes the channel in the open conformation and this group may also decrease the probability of blockade of the channel by the agonist.

At the nicotinic Ach receptor, different agonists have been found to produce longer or shorter open times, but the single channel conductance remains unaltered (Gardner, Ogden & Colquhoun 1984). Similar results have been obtained for glutamate activated channels on the locust muscle membrane (Anderson, Cull-Candy & Miledi 1978) and the GABA receptor of cultured neurones (Barker & Mathers 1981). These results indicate that the effectiveness of an agonist does not depend on the magnitude, but rather on the duration of the conductance change activated by the compound.

In principle, it should be possible to vary the chemical groups on the GABA molecule, and determine the effects of these changes on channel kinetics.

In the present study and others (Mathers & Barker 1981, Jackson, Lecar, Mathers & Barker 1982) it was found that muscimol activated Cl^- channels with about twice the average duration of GABA activated channels. This kinetic change probably accounts for the greater potency

of muscimol. Similarly, dihydromuscimol opens channels of a longer duration than the 'normal' agonist (Mathers & Barker 1981). Chemically, muscimol is a heterocyclic analogue of GABA, in which the carboxyl group is replaced with a flattened acid ring. This has the effect of conformationally freezing the acid group, and this substitution also increases the binding of these agonists to the GABA receptor (Falch & Krogsgaard-Larsen 1982). It may be the case that replacement of the carboxyl group of GABA with an organic ring structure acts in the same way as the ester group on Ach agonists, namely stabilizing the open conformation of the channel. In contrast, Barker & Mathers (1981) found that 5-methylmuscimol (which isomer was used was not indicated) opens GABA channels for only half as long as GABA.

Replacing the carboxyl group of GABA with a sulphonate group to give 3-aminopropanesulphonic acid (3-APS) produces a compound that is more potent than GABA in depressing neuronal firing (e.g. Krogsgaard-Larsen, Falch Curtis & Lodge 1984). However the noise analysis of Barker & Mathers (1981) suggests that 3-APS opens channels only one third as long as GABA.

The amino-sulphonic acid P4S has been reported to be a potent and specific bicuculline sensitive GABA agonist (Krogsgaard-Larsen, Falch, Curtis & Lodge 1984). In this compound, the amine group of 3-APS is changed to a piperidine ring, and this enhances the binding of P4S over 3-APS to GABA receptors (see e.g. Krogsgaard-Larsen et al. 1984). Barker & Mathers (1981) found that GABA channels

gated by this compound had shorter open times than GABA channels. The experimental results presented here from application of P4S to DRG neurones indicates that it is ~ one seventh as potent as equivalent concentrations of GABA at activating macroscopic chloride currents. The voltage jump relaxation experiments suggest that P4S opens channels of shorter duration than GABA.

Barker and colleagues (see e.g. Barker, Dufy & McBurney 1986) have suggested that taurine and glycine may act as weak agonists at the GABA receptor. These findings were not confirmed in the present study, since neither of these amino acids activated GABA receptors even at concentrations as high as 1mM. Similar results have been obtained by Gallagher, Higashi & Nishi (1978), who also applied glycine and taurine at 1mM. Gallagher et al. (1978) suggested from their studies on the structure/activity relationship of GABA-like compounds that: A) the correct distance between the terminal amino group and the carboxyl group is important -increasing or decreasing the distance results in a reduction of activity. B) The terminal amino group cannot be replaced by a hydroxyl group (as in γ-hydroxybutyric acid). C) Substitution of the caboxyl terminal with a sulphonic acid results in an elimination of activity. (However, this is not the case, since 3-APS is a potent GABA agonist). It is more likely that their interpretation of their result

with taurine was due to the removal of the $-CH_2-$ group from the molecule, and finally, D) that substitution on the carbon chain of GABA reduced agonist potency.

Curtis & Malik (1984) found that ionophoretically ejected ethylenediamine monocarbamate (EDAMC) was as potent as GABA in depressing the firing of interneurones in the dorsal horn, and Hill (1985) has demonstrated that ethylenediamine may be converted to EDAMC in the presence of bicarbonate ions and thereby act at $GABA_A$ receptors. EDAMC was found to act at GABA channels in the present study, but uncertainties about the final concentration of EDAMC in the bathing solution make it difficult to determine the potency of this agonist compared to GABA. EDAMC, with the presence of an amino acid and acid group at an appropriate distance, fulfills the criteria originally proposed by Curtis & Watkins (1960) for the structural requirements for GABA receptor activation.

Aside from strucural and conformational factors influencing the response of receptors to GABA-like agonists, electronic factors (i.e. the charge distribution about the molecule) will also be important in determi ning the potency of agonists. It is difficult to accurately define the charge distribution of zwitterionic agonists, and as yet impossible to determine the electronic structure of the agonists when they are combined with the receptor. The sulphonate group on P4S will have quite a different charge distribution than the carboxyl group of GABA, and this may account for part of the potency of these aminosulphonic agonists.

Problems in comparing results for agonist potency.

As discussed above, some agonists appear to have different potencies relative to GABA when tested on mammalian neurones *in vivo* and in cultured or freshly isolated neurones. One possible reason for this is that there are true differences between central and peripheral GABA receptors. Such differences have been suggested before (see e.g. Nistri & Constanti 1979). However, the difference in effectiveness of glycine and taurine in activating GABA channels in cultured (Barker & Dufy 1985) and freshly isolated neurones (the present work and Gallagher et al. 1979) gives rise to greater concern. It may be possible that in cell culture the GABA receptor changes its conformation so that it is more likely to accept taurine as an agonist. Alternatively, taurine and glycine may be activating their own, specific receptors but these are now antagonized by bicuculline.

A further difference in potency orders in *in vitro* and *in vivo* preparations is the problem of uptake of transmitter. For instance, the differences in the transport of the S and R isomers of methylmuscimol may mask the true difference in potency of these optical isomers on the GABA receptor (see e.g. Krogsgaard-Larsen, Falch, Peet, Leah & Curtis 1983). The problem of GABA-like agonist uptake can be eliminated in isolated preparations, but is practically impossible to control in the living animal.

Other problems arise when comparing the efficacy of GABA agonists between isolated preparations. The relative activity of agonists determined by Gallagher et al. (1978) and Gallagher, Nakamura and Shinnick-Gallagher (1983) was performed using the same concentration (1mM) of agonists. However, the only other *in vitro* assessment of potency is that of Mathers & Barker (1983) (see also Barker 1983). These authors used varying concentrations of agonist, ranging from 20μM muscimol to 1mM taurine, and differences in channel open time were determined from noise analysis. It is likely that at high concentrations the kinetics of these agonist gated channels will be

accelerated. A further problem could be that different compounds will have different rates of desensitization, giving changes in the variance to current ratio. The assumptions used in noise analysis require that only a small fraction of channels are activated, and variance should be directly related to membrane current. If this condition is not met and the fraction of open channels changes significantly during noise sampling then spurious values for channel open time could be obtained.

It would be interesting to re-examine the potency series derived by Barker & Mathers (1981) using lower, but consistent concentrations of agonist. Ideally this should be done with patch clamp techniques, which would have the advantage of providing a wealth of kinetic data.

Differences in agonist pharmacology between neurones and different regions of the same neurone.

Studies examining responses to GABA-like agonists have revealed that not only different neurones have different sensitivities to agonists, but the order of potency may be dissimilar on various regions of the same cell. There is not space here to deal fully with this topic, but two reviews are available (see Nistri & Constanti 1979, Gallagher & Shinnick-Gallagher 1983).

It is possible that the GABA receptors at the terminals of primary afferent neurones in amphibia are different from those on the cell body, since glutamate, glycine and taurine are ineffective at the soma

(Nishi et al. 1974) but are apparently active at frog primary afferent terminals (Curtis, Phillis & Watkins 1961). In mammals, there are differences in the sensitivity to GABA-agonists on primary afferent terminals (Curtis, Lodge, Bornstein, Peet & Leah 1982, Curtis & Lodge 1982) and the cell bodies of these neurones (Gallagher et al. 1983b). (However, at least part of this difference may be due to problems with uptake, and the experimental methods used to assay potency). Allan, Evans & Johnston (1980) found that the relative molar potencies of GABA-agonists in depolarizing spinal root fibres in the rat was not paralleled by the molar potency of these agonists in depressing synaptic activity in the hemisected spinal cord of the rat. These authors suggested that fibre depolarization and depression are probably mediated by different kinds of $GABA_A$ receptor. This difference was most marked with the agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), which was twenty times weaker than GABA in depolarizing fibres, yet was twenty times stronger than GABA at synaptic depression. (P4S was ~ one third as potent as GABA on dorsal root responses, but 400 times more potent at depressing synaptic activity). Allen et al. suggested that there are at least two kinds of GABA receptors in the spinal cord of the neonatal rat - 'extrasynaptic receptors' (found on ventral roots) which are more effectively activated by GABA than THIP, while at 'synaptic' receptors THIP is more potent than GABA. Similar results have been found by Alger & Nicoll (1982).

These authors
found that that THIP is more potent than GABA in activating dendritic 'extrasynaptic' responses. Brown, Higgins, Marsh & Smart (1981) have also suggested that two different kinds of GABA receptor exist on innervated and noninnervated regions of neurones.

In conclusion, it appears that there is a wide variety of bicuculline sensitive binding sites for GABA, as determined by differences in the rank order of potency for GABA agonists. To be able to elucidate the molecular basis for differences in agonist potency (see below) it will be necessary to compare the agonists at least at equivalent concentrations (and ideally over a range of concentrations) in an isolated preparation where problems with uptake of transmitter can be eliminated. The agonists should be bath applied in order to avoid differences in transport number which can be a problem with ionophoretic ejection of agonists. The conductance and open times of the channels should also be determined over a range of concentrations to dissociate concentration-dependent changes in lifetime, desensitization and possibly also channel block. It will only be by performing these kinds of experiments with subtle changes in the stereochemical, electronic and conformational properties of the agonists that we will be able to understand how and why particular agonists alter GABA channel kinetic behaviour. These studies will then have to be repeated on a variety of GABA receptor channels from several anatomical locations, to determine

differences in
GABA receptor properties throughout the nervous system.

A note on the meaning of transmitter potency and its basis.

In synaptic transmission it is the 'potency' of the transmitter in opening the channel which is the important determinant of satisfactory transmission at the postsynaptic membrane. Biochemical binding studies measure the affinity of the agonist for the receptor, and this may be quite different from the 'efficacy' of the molecule in gating the channel. In building physiologically useful structure-activity relationships it is desirable to separate the contributions from receptor affinity and efficacy (see e.g. Stephenson 1956).

Colquhoun & Sakmann (1985) have considered potency to be a product of affinity and efficacy. Assuming a conventional association rate of transmitter and receptor of $10^{8} M^{-1}.sec^{-1}$, potency can be defined as:

$$\frac{1}{k_{-1}} \cdot \frac{\beta}{\alpha}$$

(modified for single binding of agonist to receptor). Ach has a lower affinity ($1/k_{-1}$) for its receptors than suberyldicholine, but the efficacy (β/α) for suberyldicholine is lower than that for Ach. The product of receptor affinity and efficacy means that Ach is about

one third as potent as suberyldicholine, principally due to its lower affinity.

At high concentrations of the agonist, open probability no longer depends on concentration but the ratio $\beta/(\alpha + \beta)$. For high concentrations of Ach, approximately 90% of the Ach channels would be activated. The same concentration of suberyldicholine would activate about 97% of the channels (Ogden & Colquhoun 1983). We can apply these kinds of measurements of potency (affinity and efficacy) to GABA activated channels. Using the values of Sakmann et al. (1983) for β and α ($330sec^{-1}$ and $50sec^{-1}$ respectively, the present study would provide a value of α of $\sim 30sec^{-1}$ at hyperpolarized potentials), efficacy would be $\beta/\alpha = 7$, or approximately one sixth that of Ach measured under similar conditions (Colquhoun & Sakmann 1985, Ogden & Colquhoun 1983, Sakmann et al. 1983) However, assuming that GABA has a similar association rate constant as Ach (and most other transmitter-receptor interactions, i.e. $10^8 M^{-1}.sec^{-1}$), and again using Sakmann et als. (1983) value for k_{-1} of $50sec^{-1}$, GABA has a K_D (k_{-1}/k_{+1}) of 0.5μM, or approximately a two hundred fold higher affinity for its receptor than Ach. This high affinity means that overall GABA has a potency $\sim$ 76-fold greater than Ach at its receptor channel complex. The very high affinity overcomes the lower efficacy of GABA channels to make this an extremely potent agonist relative to Ach at the neuromuscular junction.

At high concentrations, GABA will open approximately 86% of its channels. As yet we have no idea of the

concentrations achieved during synaptic transmission in central neurones, but by analogy to the end-plate, high concentrations that may be achieved transiently are sufficient to saturate the postsynaptic receptors.

The rate constant α is decreased by muscimol, and if β is unaffected this agonist will have a greater efficacy than GABA. Dissociation constants have not yet been measured by electrophysiological methods for muscimol, but binding studies suggest that this agonist has a higher affinity for the receptor than GABA (as measured by inhibition of high and low affinity GABA receptor binding, see e.g. Krogsgaard-Larsen & Falch 1981). This would mean that both affinity and efficacy would be increased to make muscimol considerably more potent than GABA.

It will be interesting to determine the effects of GABA-like agonists on the rate constants α, β, and k_{-1} of GABA receptor channels. This can be most easily accomplished by using patch clamp methods and analysis of burst length and the number of gaps per burst. It may then be possible to determine if slight alterations in the structure and electronic charge distribution of GABA agonists affect binding or channel opening/closing rates. We may then obtain clues as to the nature of the processes controlling each reaction.

Structure-activity series and the design of compounds for clinical use.

As we understand more about the steric requirements of GABA agonists to increase their affinity at the receptor and efficacy at the level of channel kinetics, it should be

possible to design more potent compounds. However, although this may prove highly satisfactory and useful for biophysical and pharmacological studies of the GABA channel, such compounds may not be of use clinically. The potency of GABA agonists depends on their ability to cross the blood brain barrier, and so far only a few agonists have been successful in this regard. Muscimol is one of the few potent agonists that cross the blood brain barrier, but unfortunately is highly toxic, possibly as a result of the formation of active metabolites in nerve terminals. However, Krogsgaard-Larsen and his colleagues used muscimol as the model compound to synthesize various isoxazole analogues in an attempt to find a safer agent that could be used therapeutically. The potent agonist THIP (see above) came from these studies.

GABA receptor agonists may prove useful for the treatment of those disorders believed to be at least partially caused by deficiences in GABAergic neurotransmission, such as Huntingtons disease, epilepsy, depression and Parkinsonism.

Bicuculline insensitive GABA receptors: The $GABA_B$ receptor.

A great deal of evidence has accumulated to suggest that in mammalian neurones GABA may modulate membrane channels other than the 'classical' chloride channel. These GABA receptors are resistant to the GABA receptor

antagonist bicuculline, and are selectively activated by the GABA analogue β-p-chlorophenyl γ-aminobutyric acid (Baclofen, Loiresal) (see Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull 1980). Bowery has called this novel type of receptor the $GABA_B$ receptor. Electrophysiological studies in the hippocampus have demonstrated that baclofen activates a potassium conductance (Gahwiler & Brown 1985, Newberry & Nicoll 1985) which leads to a hyperpolarization of these neurones. $GABA_B$ receptors may also exist on the terminals of hippocampal neurones, since baclofen can inhibit the release of excitatory transmitter in this preparation (Ault & Nadler 1982, Inoue, Matsuo & Ogata 1985). Autoradiographic labelling using ^{3}H-baclofen and ^{3}H-GABA has confirmed the electrophysiological studies suggesting that $GABA_B$ receptors were preferentially located on the dendritic rather than on the somatic portions of pyramidal neurones. Newberry & Nicoll (1985) have suggested that $GABA_B$ receptors mediate the 'slow' IPSP observed in these cells.

It has been proposed that baclofen reduces excitatory transmitter release from primary afferent terminals in the spinal cord (e.g. Pireau & Zimmermann 1973) and this may account for the clinical effects of baclofen which is to cause a reduction in skeletal muscle tone and reflex activity. It has been suggested that baclofen exerts this action by directly causing a reduction in the calcium current in the terminals of primary afferent neurones (e.g. Dunlap & Fischbach 1978, Robertson & Taylor, see

Appendix D). It seems then that $GABA_B$ receptors are coupled to potassium channels in central neurones (hippocampal pyramidal cells) and calcium channels in peripheral (primary afferent) neurones. It is likely that this anatomical distinction is not rigid however. There may be a second messenger sytem, possibly a guanine nucleotide associated N-protein, coupling the $GABA_B$ receptor with the voltage gated calcium channel in dorsal root ganglion neurones (e.g. Holz, Rane, & Dunlap 1986, Dolphin & Scott 1986). The system linking $GABA_B$ receptors and potassium channels in hippocampal neurones has yet to be determined.

The $GABA_B$ receptor must have a structure and conformation quite different from the agonist acceptor site on $GABA_A$ chloride channels, since the $GABA_B$ receptor may only be activated by GABA and baclofen. The other $GABA_A$ type agonists are generally ineffective at this site. Baclofen is not able to activate Cl^- responses, so in some way the chlorophenyl group attached to the GABA backbone reduces affinity at $GABA_A$ receptors, but does not alter binding to $GABA_B$ receptors.

The $GABA_B$ type receptor must have originated long ago in our evolutionary history, since this receptor is found to modulate a potassium current in the neurones of the mollusc Onchidium (Shimizu, Akaike, Oomura, Maruhashi & Klee 1983), and baclofen has a presynaptic action at crayfish motor nerves, where it reduces transmitter release (Barry 1984).

It may be worth pointing out that GABA has the ability to act as an inhibitory substance in 'fast' and 'slow' manner, and is active in both pre- and postsynaptic neural structures, possibly by acting on different receptors and channels. It will be interesting to determine how these different mechanisms of neural inhibition evolved, and why GABA appears to be ubiquitously inhibitory in function.

Presynaptic inhibition: chloride, calcium or both?

It has been strongly proposed that GABA mediates presynaptic inhibition in the spinal cord, being released from axo-axonic synapses on the central terminals of primary afferent fibres and depolarizing the terminals by increasing membrane chloride conductance (see e.g. Curtis 1978, Curtis & Lodge 1982). Most of the evidence for this however is indirect, and may only be unequivocally obtained by recording from primary afferent terminals themselves. This would of course be a difficult if not impossible task *in vivo*. There is a wealth of evidence that GABA depolarizes peripheral nerve fibres (e.g. Barker & Nicoll 1973, Brown & Marsh 1978) and reduces transmitter release from autonomic terminals (e.g. Bowery & Hudson 1979, Kato & Kuba 1980), but absolute proof of the GABA mediated depolarization mechanism is sadly still unavailable (see Nicoll & Alger 1979 for a review). Although there is even less evidence for the $GABA_B$ type mechanism of presynaptic inhibition in the spinal cord *in*

vivo, we may consider some of the advantages and disadvantages of each system.

Depolarization of primary afferent terminals by GABA would be generated by a Cl^- efflux. Chloride concentration inside peripheral neurones is higher than in central neurones, and higher than would be predicted from passive distribution across the membrane. The high intracellular chloride level must be maintained by an inwardly directed chloride pump (Keynes 1963, Adams & Brown 1975, Gallagher et al. 1983a, Alvarez-Leefmans 1986). Even though only a very small fraction of chloride inside the postsynaptic region of the axo-axonic specialization would be lost in the GABA-induced depolarization, to reach equilibrium again this chloride must be actively reaccumulated. Even if the chloride transporter itself is an antiport mechanism, the energy to provide the gradient for the 'antiportee' must be generated by ATP hydrolysis.

The reduction in transmitter release caused by the membrane depolarization is thought to be brought about by a reduction in action potential height. (However, at the accessible squid giant synapse, depolarization of the presynaptic terminal actually increases transmitter release). Depolarization of the somatic membrane of DRG neurones results in a very marked prolongation of the calcium action potential (Heyer & MacDonald 1982b, and my own personal observations), which could presumably result in a larger influx of calcium ions (since calcium channels activate more at positive potentials). As yet, a

detailed study has to be performed in which transmitter release from primary afferent terminals is measured when these terminals are depolarized. However, McBurney & Neering (1985) have reported that the rate of change of intracellular calcium as measured by aequorin is reduced when the calcium action potential is broadened by membrane depolarization. This would suggest that calcium flux is actually reduced, possibly because the driving force is decreased or because the intracellular calcium buffering system is altered by membrane potential.

As an alternative to the 'GABA depolarization' mechanism, it is relatively simple to envisage a situation in which transmitter release is modulated by controlling the calcium influx into the terminal directly. GABA could activate $GABA_B$ type receptors, which would decrease the number of available calcium channels, possibly by a second messenger system. Such a mechanism would be economical in energetic terms, and would allow quite a fine control over transmitter release that may be harder to obtain with a gross depolarization. A similar mechanism is now well established for presynaptic inhibition in Aplysia neurones (Shapiro, Castellucci & Kandel 1980).

It must be borne in mind however that the arguments presented above are speculative, but it should be possible to test these hypotheses relatively easily in the sytems of co-cultures of DRG and motoneurones presently available.

The small amount of evidence available to date would

suggest that both $GABA_A$ and $GABA_B$ type mechanisms exist in cat spinal cord. It is possible to remove a great deal of presynaptic inhibition with bicuculline (e.g. Curtis 1978), but there is a residual component that is insensitive to this antagonist. There may be local differences in the proportion of A and B type recptors, since bicuculline is less effective at antagonizing presynaptic inhibition of the input to the dorso-spinocerebellar tract neurones than at motoneurones (Curtis, Gynther & Malik, in press).

Anaesthetic actions on the GABA receptor-channel complex.

Mode of action of anaesthetic and other agonists.

The anaesthetics (±)pentobarbitone, (+)etomidate, α chloralose and alphaxalone all acted as GABA receptor channel agonists on mammalian DRG neurones. The antehelmintic avermectin B_{1a} also activated these channels. These agonist-like actions were completely antagonized by bicuculline and picrotoxinin. It is unusual that these substances, which have widely differing chemical structures (see Appendix D) should activate the GABA channel in such a specific manner, especially as this receptor is normally so selective. Previous results obtained by other authors for pentobarbitone (Huang & Barker 1980) and etomidate (Evans & Hill 1978) suggest that the optical isomers of these anaesthetics are not

able to induce GABA-like conductance changes. The stereospecificity displayed for these agonists would imply that there is some 'receptor' for these anaesthetic agonists on the GABA receptor channel. It is widely held that bicuculline acts at the receptor portion of the GABA complex, while the picrotoxinin binding site is believed to be associated with the ionophore (see e.g. Olsen 1981) (But see also McBurney, Barker & Mathers 1983 for picrotoxinin). If this is the case, then one would expect that the agonist action of the anaesthetics would be blocked by a 'channel blocker', since the ionophore would longer be able to conduct although the agonist may still be bound. However, the rapid antagonism of the response by bicuculline would tend to suggest that the receptor is the site of action of the foreign agonist. Agonist dose-response experiments will have to be performed with and without bicuculline to determine the nature of the competition (if any) for the binding site between bicuculline and the agonist. If the GABA receptor portion is not involved in the agonist response, then bicuculline may allosterically inhibit the response to these agents. Bowery & Dray (1978) found that pentobarbitone is able to reverse the antagonism of GABA by convulsants including bicuculline, and since pentobarbitone did not always enhance GABA action on the superior cervical ganglion, these authors concluded that pentobarbitone interacts with the antagonist site rather than the receptor site. Brown & Constanti (1978) did not observe the agonist like action of pentobarbitone on

superior cervical ganglion cells, even at concentrations up to 1mM, and Feltz (cited in Brown & Constanti 1978) also found no action of pentobarbitone on rat DRG cells *in vivo*. Huang & Barker (1980) and Mathers & Barker (1980) have also reported that cells possessing GABA receptors did not always respond to pentobarbitone, and suggest that the agonist effects of this drug are not mediated through GABA receptors, but possibly some other receptor site. In contrast, in the present study, pentobarbitone responses were never elicited if GABA responses (and presumably receptors) were absent, and all of the cells responding to GABA also produced conductance changes on application of pentobarbitone. A similar situation was found for all the anaesthetic GABA-mimetics examined.

Binding studies with GABA and pentobarbitone have revealed that pentobarbitone does not compete with GABA for a binding site, but pentobarbitone actually *enhances* GABA binding (Willow & Johnston 1981). This again suggests that some site in the GABA receptor channel complex other than the receptor site may be where pentobarbitone (and possibly the other agonists) binds and consequently opens the ionophore.

Since pentobarbtone and other anaesthetics prolong the open time of GABA channels (cf. Segal & Barker 1984b, and the present study) it might be suggested that these agents stabilize the channel in an open conformation. If GABA channels open spontaneously in the absence of agonist then this could provide a mechanism whereby the

anaesthetics tested could appear to open the GABA channel. We can consider this hypothesis briefly.

Channels are likely to have a significant energy barrier between non-conducting and conducting states, and this barrier is overcome by the binding of the normal agonist. However, from thermodynamic principles spontaneous fluctuations to the conducting state are possible, due to random thermal agitation of the complex. We would imagine that these spontaneous openings would be infrequent and short lived, since effective agonists hold the channel in the open conformation for longer periods of time (vide infra). If GABA channels do open occasionally in the absence of agonist, the presence of anaesthetic molecules could stabilize the channel in the open conformation once it had spontaneously crossed the open/closed transition state. Spontaneous openings of the Ach receptor in embryonic muscle cells have been reported by Jackson (1984), who found that frequencies of opening in patch recordings were between 0.2-4.7sec^{-1} (unfortunately no information was given concerning the lifetime of these spontaneous openings). Similar spontaneous openings of agonist-activated channels have been observed by Sakmann, Noma & Trautwein (1983), Brehm, Moody, Corbett & Kullberg (1983) and Hille, Sanchez, Siemen & Dani (1983).

If this hypothesis were applied to GABA channels, we would predict that: A) If the driving force for chloride ions were maximised, it should be possible to detect a small decrease in cell conductance when the GABA blockers

bicuculline and picrotoxinin were applied. B) The stabilization of spontaneously opening channels would result in a slow build up of current, as sufficient numbers of channels were absorbed into the open state. C) Other substances which increase GABA channel open time would also act as agonists.

Point A) was tested experimentally. The holding potential was made either +40 or -100mV; at -100mV the driving force for chloride was over 100mV, which would maximize the current that would be blocked by the antagonists. No change was observed. At +40mV, the spontaneous channels would be open for longer, given the normal voltage sensitivity of channel lifetime. This would again increase the current to be blocked. Once again application of the antagonists failed to change membrane conductance.

The rise time of the whole cell currents induced by anaesthetics was generally slower than similar magnitude GABA currents, and was accelerated by increasing the concentration of anaesthetic. Rise times with high concentrations of pentobarbitone were of the order of a few seconds, which probably reflects bath solution changeover times. Slow rises in etomidate induced currents are explained by the marked blocking activity of this compound. However, very slow rise times were observed for avermectin B_{1a} - it is possible that this agent may be acting by stabilizing spontaneous channel openings. It will be necessary to perform patch clamp experiments in the abscence of possible GABA agonists to

determine if spontaneous openings do occur, but if their open time is very short, then recording these events may be difficult because of limited bandwidth with this technique. A further check could be performed by looking for changes in the 'resting' conductance of small, high input impedance cells in whole cell clamp or in isolated patches when GABA antagonists are applied. (Mathers (1985) has reported spontaneously opening channels similar to GABA channels in cultured
spinal cord neurones, but as yet there is no evidence to suggest that these are GABA channels).

Finally, as a means of testing conjecture C), halothane and ketamine were each applied at a concentration of 1mM. Both halothane and ketamine lengthen IPSCs in the hippocampus (present study) and enhance GABAergic conductance changes (vide infra), presumably by stabilizing open GABA channels. However, neither of these anaesthetic substances were effective in activating a GABA-like conductance.

In the absence of further experimental evidence, the simplest hypothesis would be that the anaesthetics directly activate the GABA receptor channel complex, and this action occurs through some site whose nature has yet to be determined.

The activation of GABA channels by some anaesthetic substances, whose structures vary widely and are not easily reconciled with the classical requirements of GABA agonists is unusual for the agonist gated channels studied

to date. There are a few precedents however. Israel & Meunier (1979) have found that the local anaesthetic procaine can act as an agonist on the Ach-mediated H and D responses of Helix ganglion neurones. This action of procaine may be explained if the drug binds directly to the Ach receptor - indeed both procaine and Ach have a charged terminal nitrogen and a carboxyl group about 0.6nm from this terminal nitrogen (Steinbach 1968). It has now become clear that curare, the 'classical' competitive antagonist at nicotinic Ach receptors can also act as an agonist. This agonist like activity was first reported by Ziskind & Dennis (1978), and more recently examined in detail by Takeda & Trautmann (1984). Takeda & Trautmann have hypothesized that one curare molecule binds to the receptor causing a brief opening of the ionophore, a second molecule may then bind stabalising this opening, and a third molecule may bind and block the channel. Interestingly these studies have also revealed developmental and species differences in the receptor structure, for curare is a more potent agonist on embryonic rather than adult rat muscle and is active on rat and human myotubes but not on chick myotubes.

It will be of interest to extend these studies of species and developmental differences of agonist sensitivity to activation of GABA channels by foreign agonists, and determine if and perhaps why such differences exist for the GABA chloride channel.

Blocking behaviour by the anaesthetic agonists.

Current responses induced by pentobarbitone, chloralose, alphaxalone and in particular etomidate, were consistent with blocking of the GABA receptor channel complex by the anaesthetic compounds. With etomidate this blocking activity was displayed at concentrations as low as 60μM. In contrast, there was no evidence of block by GABA of these receptor channels, even at concentrations as high as 1mM. Blockage of receptor channel complexes by agonists has been obseved previously by several other authors. Adams & Sakmann (1978) found that the partial agonist decamethonium could both open and block endplate channels, and the voltage dependence of channel lifetime and the blocking rates were simliar to those previously observed with full agonists and local anaesthetics. Other more recent studies have investigated the kinetics of block by the more potent agonists Ach, carbachol and suberyldicholine. Adams & Colquhoun (1983) found that high concentrations of these agonists produced inverse relaxations on voltage jumps, and concluded that Ach caused brief, voltage dependent blockages of the Ach channel, while suberyldicholine produced longer blockages. Sine & Steinbach (1984) have carried out similar studies on the voltage and concentration dependence of Ach block at its channels on BC3H1 cells, and found that block resulted in a marked depression of the single channel conductance at negative

potentials. Interestingly, this block could be relieved at extreme negative potentials (~ -200mV), and Sine & Steinbach concluded that the Ach was being 'sucked' through the channel at these potentials. The zwitterionic nature of GABA, and the extreme pK_a values for its carboxyl and amine groups means that it would be difficult to achieve any marked alteration of charge and hence to assess the magnitude of any voltage dependent blocking.

A recent study by Vyklicky, Vyklicky, Vysocil, Vlachova, Ujec & Michl (1986) has suggested that excitatory amino acid agonists can activate and block their receptor/channel complex at concentrations as low as 100μM. These authors have demonstrated that when the agonists are applied and removed quickly enough a 'bounce' response is seen with NMDA, aspartate, glutamate and quisqualate, which is similar to those responses seen with etomidate and pentobarbitone in the present report. It is possible that these concentrations of agonist are achieved transiently at excitatory synapses in the CNS, and it will be useful to determine the full concentration and voltage dependence of block by these acidic amino acids.

The results obtained for pentobarbitone suggest that at low concentrations the drug potentiates GABA responses, and at higher concentrations it acts as an agonist with weak blocking activity. A depressant or blocking action of pentobarbitone has been observed at other agonist (Ach)

gated chloride channels. Adams, Gage & Hamill (1982) observed that 100μM pentobarbitone produced biphasic decays in IPSCs in Aplysia neurones, and raising the anaesthetic concentration produced an acceleration of the fast phase of IPSCs and a slowing of the slower phase. This behaviour is similar to that seen by Adams (1976) for procaine block at the neuromuscular junction, and may be approximated by a sequential block of the chloride channel by pentobarbitone molecules. Judge & Norman (1982) and Wachtel & Wilson (1983) have also observed a depression of Ach evoked chloride currents by barbiturates in Helix and Aplysia neurones respectively. The concentrations of barbiturates employed in all three studies were similar to those required to potentiate IPSCs in the hippocampus, suggesting that if the action of pentobarbitone is at the ionophore, then there must be significant differences in the structure of the synaptic chloride channel between molluscs and mammals. It is possible that molluscan chloride channels are simply more sensitive to the block produced by barbiturates, since the blocking behaviour was only observed at fairly high concentrations in the present study.

The nature of the 'blocking site' for pentobarbitone has yet to be determined. It is possible that it is equivalent to the 'low affinity' site observed by Willow & Johnston (1981) in binding studies. A full examination of the stereoselectivity of the site, and a complete study of the potential dependence of the block (the latter providing information as to its equivalent electrical

distance across the membrane field) could provide clues as to the possible nature and location of the blocking site. The barbiturate modulatory site associated with the GABA receptor is also incorporated with the 'GABA complex' manufactured by oocytes using chick brain (Smart, Constanti, Bilbe, Brown & Bernard 1983) and rat brain (Houamed, Bilbe, Smart, Constanti, Brown, Barnard & Richards 1984) messenger RNA as a blueprint. It should be easy to detemine if the proposed agonist and blocking sites for pentobarbitone are also expressed on these 'transplanted' receptor channels. If so, then site directed mutagenesis could be used to identify and isolate these site(s).

The non-barbiturate hypnotic etomidate showed the most dramatic channel blocking behaviour observed with whole cell currents, with pronounced 'bounce' on washout of the agonist. Once again, the agonist actions of this agent were abolished by perfusion of picrotoxinin and bicuculline. These results are similar to those obtained by Evans & Hill (1978) who observed a GABA-mimetic action in rat superior cervical ganglion and reticular formation neurones, and in the frog hemisected spinal cord. These authors found that the agonist effects were apparent at concentrations similar to those measured in the rat brain during behavioural and hypnotic effects. There was no evidence for the blocking action of etomidate in the report of Evans & Hill (1978), but the responses evoked by this agonist were much longer than those elicited by pentobarbitone or GABA.

There are other reports which suggest that etomidate may block chloride current responses. Judge & Norman (1982) found that etomidate at 100-500μM depressed Ach responses in D1 and D2 cells of Helix, and Cullen & Martin (1982) have reported that metomidate (an analogue of etomidate) depressed GABA induced chloride responses in lamprey medullary neurones. In the latter study no potentiation but only inhibition of GABA responses was observed, even at concentrations as low as 18μM. Preliminary results obtained from guinea-pig hippocampal neurones suggest that addition of etomidate to the perfusate results in biphasic IPSCs, showing both fast and very prolonged slow phases (F. Edwards, personal communication).

The binding studies of Willow (1981) have indicated that etomidate (6-100μM) enhances GABA binding to receptors on rat brain membranes, and is more effective than pentobarbitone in this action. However, Willow suggests that etomidate increases GABA binding by increasing the number of sites available for GABA binding rather than their affinity, and this action is insensitive to bicuculline and picrotoxin. It is difficult to reconcile this result with the electrophysiological observations.

In 1941 the endocrinologist Selye observed that intraperitoneal administration of high doses of pregnane and androstane steroids produced hypnosis and anaesthesia in rats (see Gyermek & Soyka 1975). Subsequent

pharmacological research led to the development of potent steroid anaesthetics for clinical practice. Harrison & Simmonds (1984) observed that low doses of the steroid anaesthetic alphaxalone potentiated the depolarizing responses to GABA and muscimol in rat cuneate neurones. These authors also found that doses of alphaxalone at 10μM and above could directly activate GABA receptor/channels, and that this activation was only seen with the α and not the β isomer. The experiments reported here suggest that alphaxalone also activates GABA channels in DRG neurones, and this activation is antagonised by bicuculline and picrotoxinin. Similar observations have recently been made by Barker, Harrison, Lange, Majewska & Owen (1986) and by Cottrell,
Lambert & Peters (1986) on central neurones and chromaffin cells respectively. Barker et al. (1986) also reported that 1μM alphaxalone increased the mean open time of GABA activated channels from 30 to 74ms. In the present study, there was no significant 'bounce' on washout of alphaxalone, suggesting that alphaxalone is not a rapid blocker like etomidate.

The stereospecificity of the response (Harrison & Simmonds 1984) would suggest that some specific 'receptor' site is involved in the response, rather than some 'degenerate' action of this anaesthetic (see below and Richards et al. 1980). To date, we have no clues as to the nature of this site. Binding studies have indicated that alphaxalone, but not betaxolone increases the affinity of the GABA receptor in rat brain

membranes for ^{3}H-muscimol (Harrison & Simmonds 1984) while the affinity of other agonist receptors (muscarinic, α_2 adrenergic and glycinergic) is unaltered. Similarly the 'benzodiazepine receptor' was not involved in the response. The stabalization of the GABA receptor site may account for the action of this agonist in potentiating GABA responses (by increasing GABA channel open time) by analogy with the pentobarbitone effects. Barker and his colleagues (Majewska, Harrison, Schwartz & Paul 1986) have some evidence suggesting that the potentiating effect of alphaxalone is shared by metabolites of endogenous steroids, and have proposed that GABA receptors may be modulated physiologically by circulating steroids. If this is the case, then it would be important to determine if this action occurs in vivo since it could have implications for the regulation of inhibition and inhibitory disorders in physiological and pathological conditions.

The results presented in this study indicate that the general anaesthetic α chloralose was also effective in activating GABA channels directly. Simliar results have been made previously by Nicoll & Wojtowicz (1980) and Nicoll & Madison (1982). Chloralose was more potent than equivalent concentrations of pentobarbitone both in the present study and that of Nicoll & Wojtowicz (1982). The anaesthetic also exhibited blocking behaviour, and it would be of interest to examine concentrations higher than used in the present study to determine just how much 'blockage' of these channels can be produced. Chloralose is well known for its 'excitatory' actions when used during anaesthesia, and the basis for this action should be investigated at the electrophysiological level. It is possible that the GABA blocking action of chloralose becomes significant at higher doses, leading to a temporary reduction in inhibition which could lead to an increase in excitability (e.g. Nicoll, 1972). This is of course purely speculative, but it would be useful to examine the effects of chloralose on IPSCs in the hippocampus to determine if this anaesthetic can potentiate and or block central GABA receptor channels. The concentration of the anaesthetic used here would be similar to that obtained during induction of anaesthesia with chloralose, since very high concentrations (50-70 $mg.kg^{-1}$) have to be applied to cats for neurophysiological procedures.

Avermectin B_{1a} was able to slowly activate GABA channels in DRG neurones. It took many seconds for the agonist induced current to reach a peak, and was slowly reversible on washing out. The activation was rapidly and completely antagonized by bicuculline and picrotoxin. Avermectin B_{1a} is a potent antehelmintic and insecticide, and its mode of action on the latter is believed to be through blockade of signal transmission from central command interneurones to peripheral motoneurones, resulting in paralysis of the nematodes (Kass, Wang, Walrond & Stretton 1980). In lobster muscle membrane avermectin blocks EPSPs and IPSPS by reducing the membrane resistance (Fritz, Wang & Gorio 1979). The action on lobster muscle and Ascaris neurones has been explained by the GABA agonist like actions of avermectin on these membranes, since the conductance was Cl^- dependent and blocked by picrotoxin. Avermectin binds specifically to brain membranes, and enhances spicific GABA binding to these membranes (e.g. Wang & Pong 1982). Supavilai & Karobath (1981) have also suggested that avermectin enhances benzodiazepine binding to a novel modulatory site on the GABA-benzodiazepine-receptor-complex. Additionally the antehelmintic increases the release of GABA from brain synaptosomes and presumed GABA-ergic neurones in the myenteric plexus (Kerr & Ong 1986), and this may account for some of its agonist like activities if GABA containing terminals are present in the test preparation. It is unlikely that significant mounts of GABA would be released in isolated dorsal root ganglia

to cause a large conductance change. It has been reported that under certain conditions some agents can produce agonist like effects in sympathetic ganglia through stimulation of GABA efflux from the glial cells (e.g. Bowery, Jones & Neal 1976). However this release depends on external Na^+ (the work reported here was performed in Na^+ free media) and the glial cells have to be 'loaded' with GABA previously in order to obtain measurable release. In conclusion therefore, it is likely that avermectin has a direct agonist action by interacting with some part of the GABA complex to either open, or stabalize spontaneous channel openings. It will be of interest and possibly also of clinical relevance to further examine the actions of avermectin on mammalian GABA receptors, since derivatives may be effective anxiolytic or anticonvulsant agents.

On the mechanism of action of anaesthetic substances.

As discussed previously (see General Introduction) it is held that certain general anaesthetics can either depress excitatory neurotransmission (at pre or postsynaptic sites) or enhance inhibitory neurotransmission, or both. The results presented here for the effects of some anaesthetics on IPSCs suggest that pentobarbitone, halothane and ketamine can enhance inhibition to varying degrees principally by prolonging the open time of GABA activated channels. Possible presynaptic effects of these anaesthetics were not

examined. Experimental results from DRG neurones indicated that the anaesthetics pentobarbitone, chloralose, etomidate and alphaxalone could directly activate GABA channels, often at clinically relevant concentrations. This action was not shared by ketamime, halothane or diazepam. In addition to activating the channels, the anaesthetics also blocked the conducting GABA channel to varying degrees. Although we do not yet know the site of these actions, it is likely that they will be specific regions on the protein complex itself. We may briefly discuss the implications of these results for some of the hypotheses advanced to explain the 'mechanism' of anaesthesia.

There are a variety of 'lipid' hypotheses of anaesthesia, which have their basis in the strong correlation between lipid solubility of an anaesthetic and its potency as a depressant. Although there is no doubt as to the validity of the correlation itself, these theories overwhelmingly neglect the 'inactivity' of isomers of anaesthetics, which are as lipid soluble as their active partners but have no anaesthetic properties on their own.

Since lipid theories rely on fairly non-specific type mechanisms e.g. changes in fluidity, bilayer thickness, occupation of a critical volume, alteration of dielectric etc. then we can make predictions as to what effect these changes in lipid environment will have on different kinds of channels and under changed thermodynamic conditions. For instance the fact that halothane prolongs IPSCs would

suggest that this anaesthetic is not simply 'fluidizing' the postsynaptic membrane thereby facilitating conformational changes, and since ethanol was without effect on IPSC decay the simple model of increased dielectric constant by the alcohol does not work for these inhibitory synapses.

If the anaesthetics were dissolving in the membrane lipid to exert their actions on membrane channels, it is predicted that their potency should decrease with cooling or high pressure, since the partition of the anaesthetic into the membrane will be reduced. However, cooling appears to have little effect on potency (e.g. Richards, Martin, Gregory, Keightley, Hesketh, Smith, Warren & Metcalfe 1978) and similarly the effects of increased pressure on 'anaesthetic potency' are inconsistent with a purely lipoid site of action (e.g. Ashford, Macdonald & Wann 1984, Kendig, Trudell & Cohen 1975, Little & Thomas 1986).

It has been proposed (e.g. Richards et al. 1980) that anaesthetics exert their actions through specific modifications of membrane proteins. Franks & Lieb (1978) found from X-ray diffraction studies on membrane/protein sytems that general anaesthetics had no effect on lipid bilayer structures, and suggested that the primary site of anaesthetic action had characteristics similar to proteins. Anaesthetics and narcotic substances have

been shown to have specific sites on haemoglobin (e.g. Schoenburn & Nobbs 1966) and firefly luciferase enzyme (see Miller 1985 for a review). It has been shown that halothane can spicifically disrupt the proteins associated with microtubules and microfilaments, and these actions are manifested at clinical concentrations of this anaesthetic (Allison, Hulands, Nunn, Kitching & MacDonald 1970, Hinkley & Telser 1974).

Studies employing stereoisomers of anaesthetics (e.g. Butler 1950, Richards & Hesketh 1975) suggest that 'receptors' are involved in which specific structureal and electronic requirments must be met before the substance can be effective. Huang & Barker (1980) found that the (+) and (-) isomers of pentobarbitone produce quite different effects on isolated preparations. (The (-) isomer produced a transient excitatory response at subanaesthetic doses, followed by an increased chloride current. The (+) isomer produced a stronger excitatory response).

The absence of a charge on the pentobarbitone molecule appears also to be important. Adams (1976) and Narahashi, Frazier, Deguchi, Cleaves & Ernau (1971) suggest that the neutral form of pentobarbitone is responsible for blocking Ach and sodium channels. In the present study, it was the neutral form of pentobarbitone which had agonist activity on GABA channels on DRG neurones. It is possible that pentobarbitone is active on at least three separate sites on the GABA channel complex: the agonist site, another site to

stabalize the open channel, and a 'blocking' site. Two sites for pentobarbitone have been found in binding studies (Willow 1981, Willow & Johnston 1983). The action of a few of the anaesthetics to block the GABA channel may be taking place at a common site on the channel. Further data relevant to this could be obtained if these studies were extended.

It must be remembered that anaesthesia is a complex biological state, and different types of anaesthesia may be produced by different drugs. These substances are probably affecting a wide variety of membrane channels, on both pre- and postsynaptic structures and altering the function of these channels in subtly different ways. It is very unlikely that enhancement of synaptic inhibition is the basis for anaesthesia. For example, transmitter release is also affected (e.g. Weakly 1969), possibly by a reduction in calcium influx into the terminals (Nishi & Oyama 1983). Pentobarbitone decreases the response to excitatory amino acid transmitters at very low concentrations (Harrison 1985). The anaesthetic ketamine specifically antagonizes NMDA type amino acid receptors, yet high concentrations are necessary to cause a fairly small enhancement of inhibition in the hippocampus. No doubt further investigations will reveal even more specific effects of anaesthetics on membrane channels. At the neurophysiological level, the cause(s) of anaesthesia are likely to be due to a complex interplay of actions on many of the functions of neurones, and it is probably ill advised to use extreme reductionist arguments to explain the phenomenon.

Is there an endogenous 'anaesthetic' that interacts with GABA receptor channels? There are a number of studies suggesting that some anaesthetic substances interact specifically with parts of the GABA protein complex, and a few of these have already been discussed. We may speculate as to why these complex molecules interact so specifically with this channel: do these drugs bind to sites that are used physiologically? Recently there has been a great deal of effort directed towards finding an endogenous 'benzodiazepine' (see for instance Bowery 1985) to account for the very specific effects of these agents on GABA channels, and it is believed that the benzodiazepine complex is stucturally and functionally intimately associated with the GABA receptor channel (see e.g. Olsen 1981). On similar lines, the results obtained recently by Barker and his colleagues on the actions of the steroid anaesthetic alphaxalone and steroid hormone metabolites have led them to suggest that GABAergic transmission can be modulated by similar compounds in vivo. Many of the clinically useful anaesthetics have been found serendipitously by pharmaceutical chemists, and often we have no idea why some work while others do not. Is it purely fortuitous that some of these compounds interact so specifically with, for instance, GABA channels, or are they acting at sites that Nature has designed? Since it is unlikely that these hypothetical sites have evolved to be ready for the clinicians of the twentieth century then it may be possible that anaesthetics are mimics of some endogenous regulator.

In Conclusion .. . .

It was the aim of this work to describe some of the characteristics of GABA channels in freshly isolated mammalian neurones. To this end, the action of anaesthetics, anions, membrane voltage and agonists other than GABA were used to explore these channels. However, I am very conscious that there are now more questions posed than have been answered by this work. For instance, how do the anions act to alter GABA channel kinetics and voltage sensitivity, and how do the anaesthetics, which are very dissimilar to the natural agonist, gate (and 'block') these channels? Perhaps it is in the nature of science that a host of new questions arise as attempts are made to lay a few to rest. In this sense, the work to date on the biophysics and pharmacology of the nervous system probably represents no more than our initial, tentative steps in exploring the roughly-charted and wondrous continent that is the nervous system.

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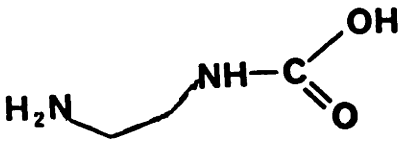
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APPENDIX A :

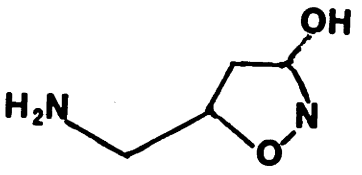
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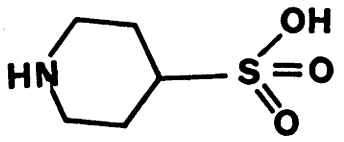
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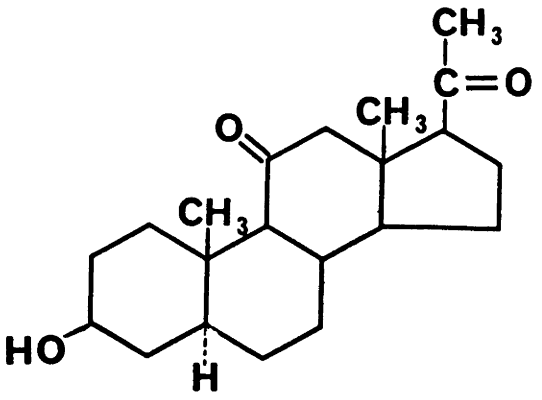
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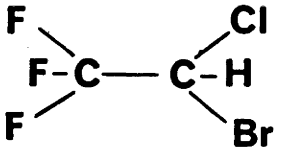
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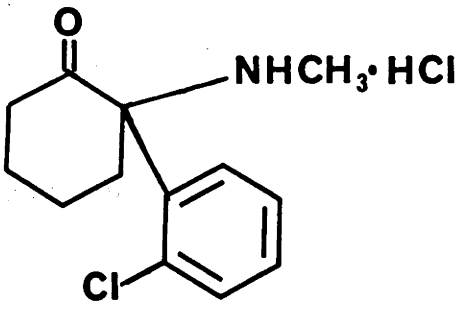
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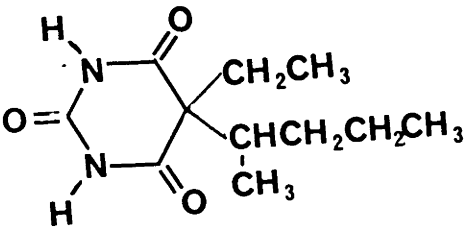
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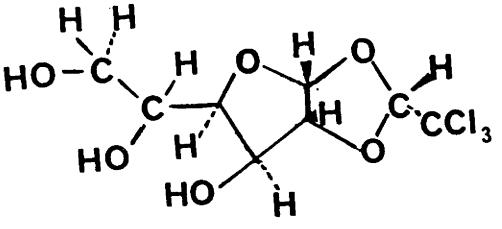
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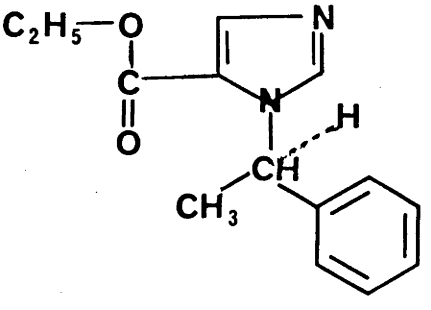
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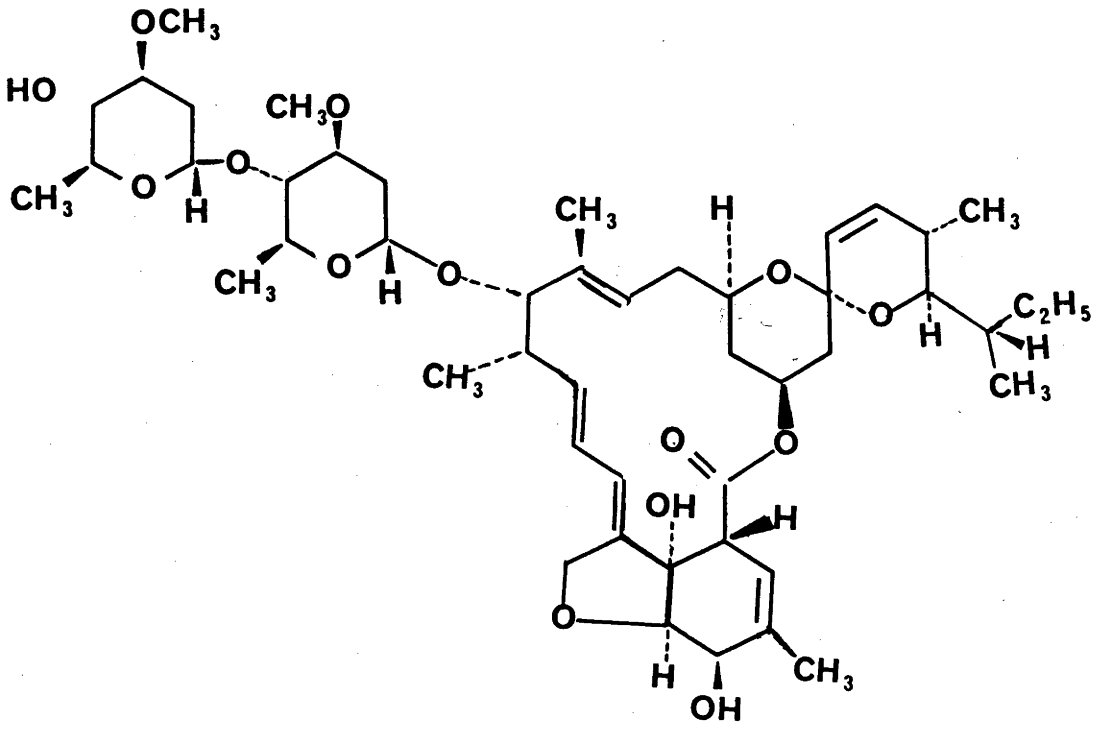
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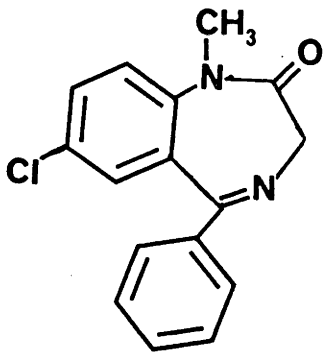
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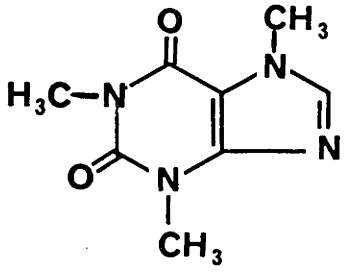
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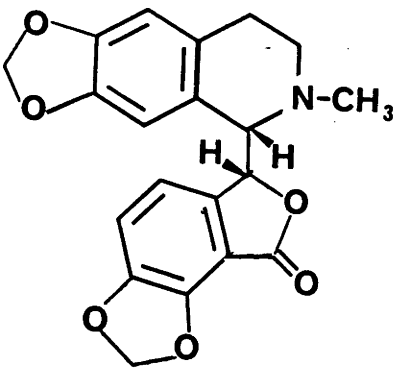
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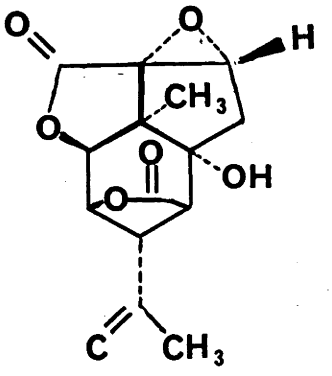
DIAZEPAM



CAFFEINE



BICUCULLINE



PICROTOXININ

APPENDIX B :

MODELS OF ANAESTHETIC - INDUCED CURRENTS

APPENDIX B.

This section presents the results of computer simulations of models representing the responses induced by anaesthetics. These simple models are extentions of the sequential blocking model first proposed for local anaesthetic block of cationic channels. It must be stressed that these are not unique solutions, but simply an example of how simple models and equations can begin to reproduce current responses qualitively similar to those observed experimentally.

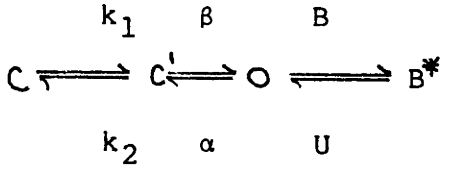
1) "THE ETOMIDATE BOUNCE."

A simple activation and blocking scheme was chosen,

$$C \rightleftharpoons C' \rightleftharpoons O \rightleftharpoons B^*$$

where C is the closed channel and binding of the anaesthetics results in an activated state (C') followed by an open channel (O). (Simulations with two equal closed states, i.e. an activation scheme requiring the binding of two agonist molecules made very little overall difference to the models response). Once the channel was open, it then entered a non-conducting or 'blocked' state (B^*). When the agonist concentration was reduced, the channels absorbed into the blocked state re-entered the open state and finally closed.

Fig. B1 represents a simulation of the membrane current in response to a pulse of agonist. Experimentally, etomidate responses were characterised by a slow rise to a fairly steady plateau, and a marked increase in current or bounce as the agonist concentration was suddenly reduced. The response in Fig.B1 is the result of a simulation of a sequential activation and block by etomidate with the following rate constants:

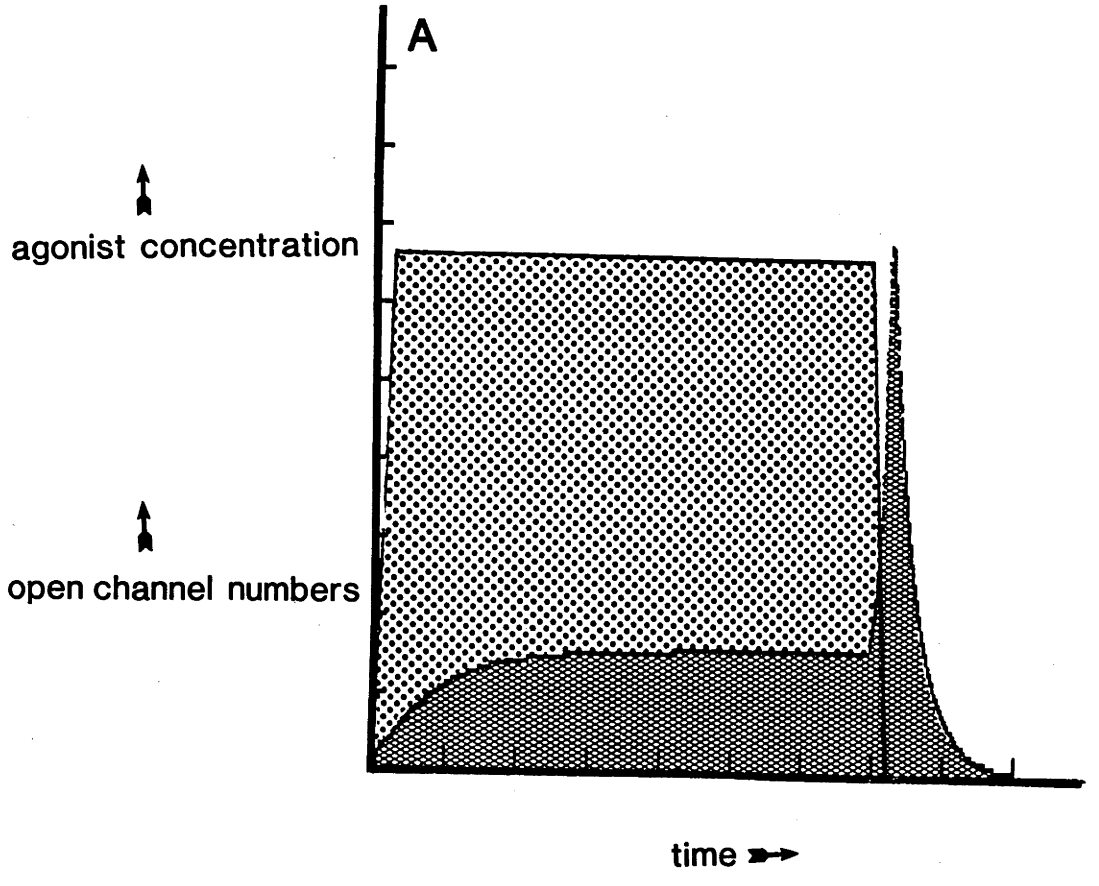


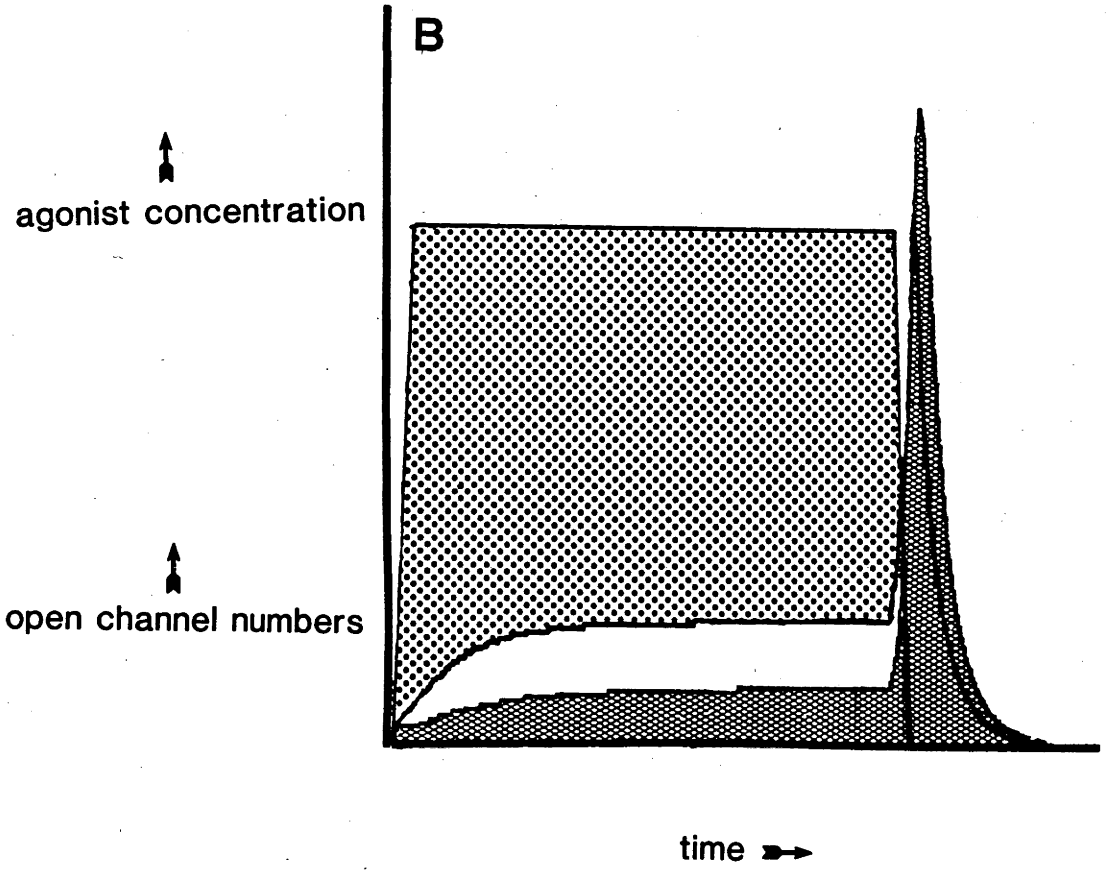
(k_1: 20, β: 0.006, B: 20, k_2: 0.4, α: 0.4, U: 4). (Note rate constants and the timing of the simulation are relative - they are not accurate values in real time units. It is the ratio between the rate constants that simulates the response). β could be varied between 0.006 and 1 to give a similar response. The most important consideration is that the blocking rate B be very fast (at least equivalent to the agonist binding rate constant k_1), and large relative to α, the channel closing rate. In this example, the magnitude of the bounce is approximately four times the plateau agonist response - this was within the range experimentally observed.

Fig. B2 illustrates the effect of changing the ratio of B to U. Decreasing backward rate constant U essentially increases the proportion of channels in the blocked state. This has the result of decreasing the plateau current and increasing the amplitude of the bounce.

FIGURE B1: Model of the current response to 60uM etomidate. Dotted area denotes agonist concentration with time, shaded area is the increase in current (open channel numbers produced by etomidate).

FIGURE B2: As B1, but with increased numbers of blocked channels (shaded area). The open area is the same response as B1.


A
agonist concentration
open channel numbers
time

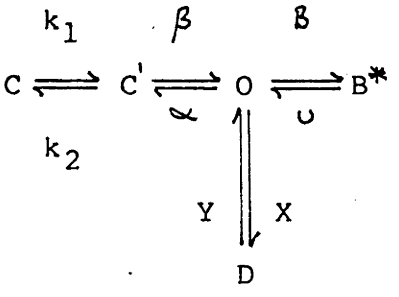

B
agonist concentration
open channel numbers
time

2) PENTOBARBITONE RESPONSES.

High concentration of pentobarbitone produced a sharp increase in current, which rapidly declined to a plateau. On wash-out of the agonist, a bounce was observed, though this was of much smaller amplitude than the initial peak current.

The pentobarbitone responses were modelled by including a fast desensitized state, absorbing from the open state, and a nonconducting state connected to the open state.

i.e.



(k_1:20, k_2:0.4, β:0.3, α:0.4, B:0.3, U:0.03, X: 1, Y:0.4)

Effectively, a 'desensitized' state has been included which reduces the peak current very quickly. Making Y, the exit rate from state D large, reduces the level of the plateau, increases the peak current and decreases the bounce. It is not known if state D is a desensitized state, or simply another blocked state. B^* could be the desensitized state. Further experiments will be necessary to describe current responses in pentobarbitone more reliably.

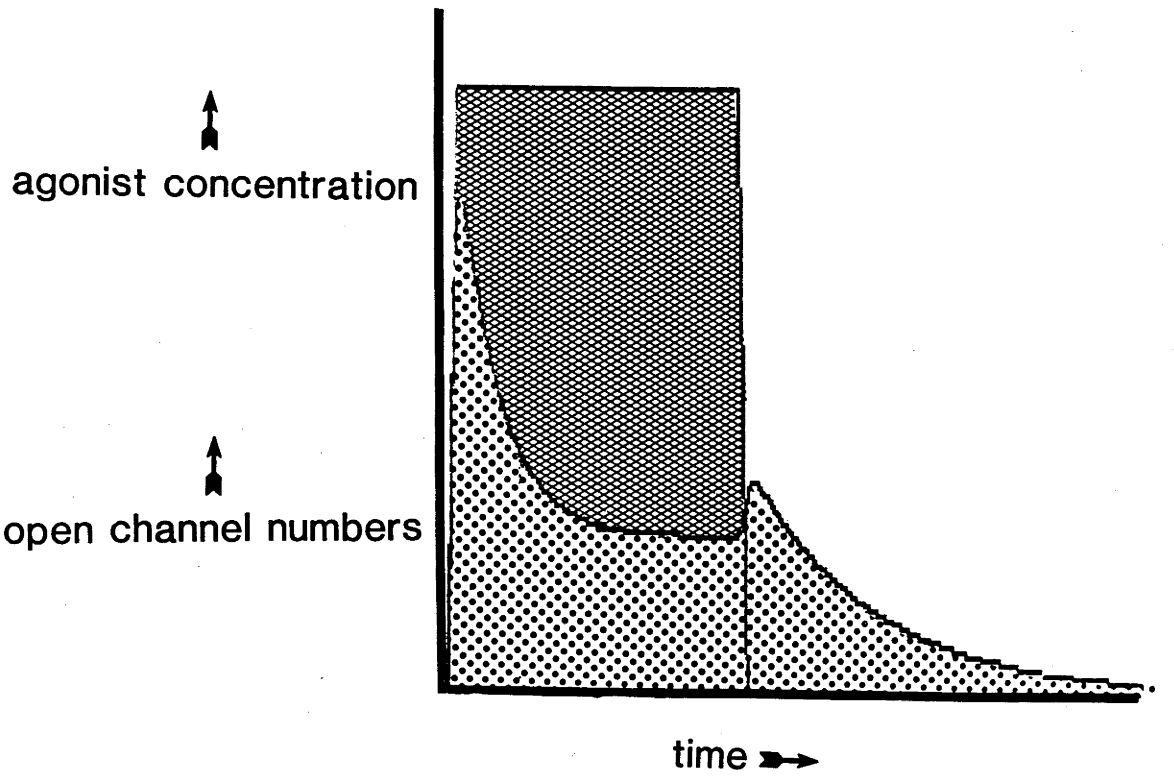


FIGURE B3: Model of the current induced by 1mM pentobarbitone. Shaded area is the agonist concentration profile, dotted area is the number of channels opened by pentobarbitone.

3) VOLTAGE JUMP RELAXATIONS WITH A VOLTAGE SENSITIVE BLOCKER.

Many of the voltage jump relaxations with the anaesthetic agonists showed an outward relaxation, i.e. an exponential increase in current on hyperpolarizing steps. Often depolarizing steps showed an initial decrease in current after the ohmic jump. This decrease was then followed by an increase to a new steady state value. A simple interpretation of these responses is that the 'blocking' effect of the anaesthetic is voltage sensitive, such that blocking is enhanced by depolarization and reduced at hyperpolarized potentials. This hypothesis was modelled and the resulting simulation shown in Fig. B4. No adjustment was made to the reaction α and β, similarly the voltage sensitivity of the closing rate α was set at 100mV (i.e. an e fold decrease in α for a 100mV depolarization). By introducing comparatively weak voltage sensitivity into the unblocking reaction, relaxations of the appropriate sign can be obtained. In Fig. B4 the unblocking reaction U, e.g.

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O \underset{U}{\overset{B}{\rightleftharpoons}} B^{*}$$

has a voltage sensitivity of -200mV. H is still 100mV. Note that ohmic jumps are not included in this figure. A voltage pulse of varying amplitude is applied to the channels at steady state. On hyperpolarizing jumps, there is an initial increase in the channel numbers following the voltage jump, and a slow relaxation back to a new

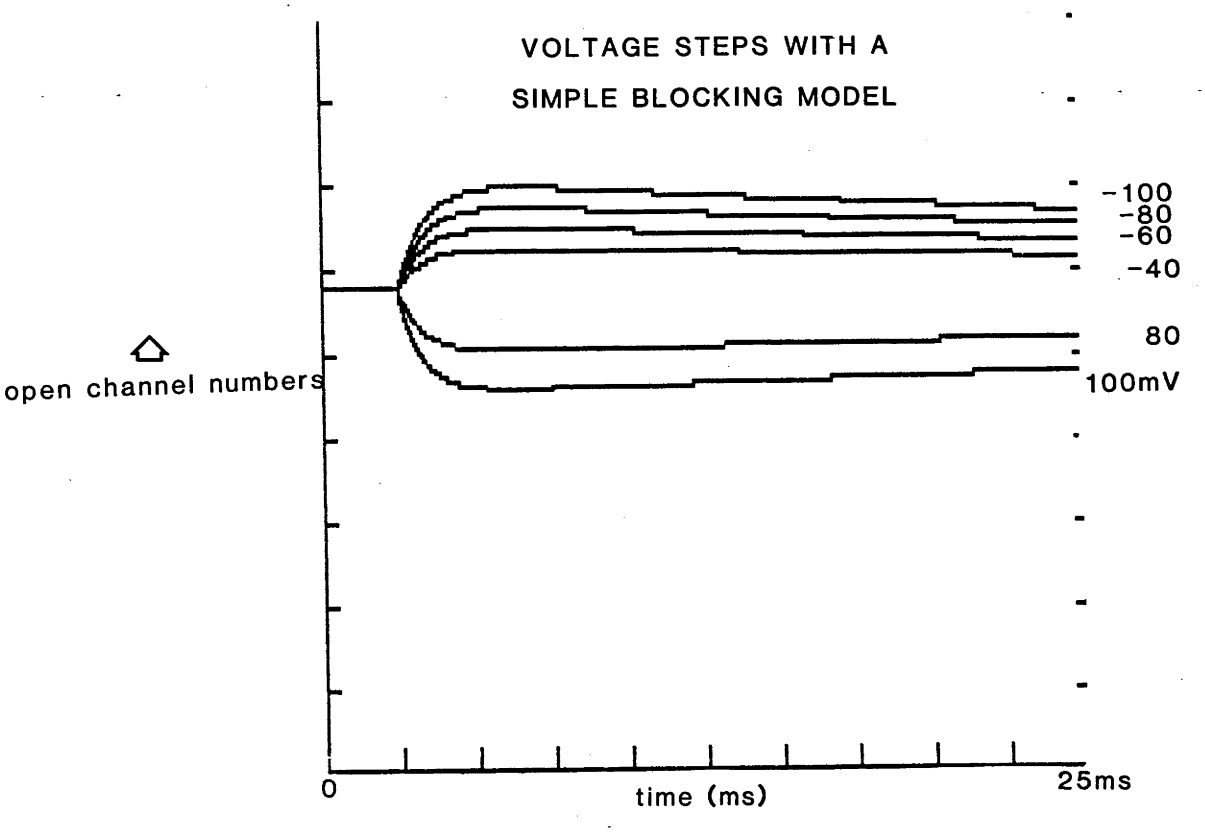


FIGURE B4: Simulation of current responses to voltage jumps with anaesthetic agonists. The voltage step is applied at steady state, and the relaxations corresponding to changes in open channel numbers are shown. The magnitude of the voltage step is illustrated at the right hand side of the figure. Note that instantaneous current jumps are not included in this figure.

equilibrium. In depolarizing steps, after an initial instantaneous current, there is a decrease in channel numbers, followed by a slow increase in current, dependent on the magnitude of α and H. These responses are qualitively similar to those relaxations observed with the anaesthetic agonists. It is likely that the anaesthetics also change α, the open channel lifetime. The normal voltage dependence of α may be unaffected. If the anaesthetics do keep channels open for longer, then the relaxations could appear to be almost flat after the initial re-equilibration of the blocking reaction.

APPENDIX C : STOP PRESS.

Since the completion of the literature survey for this thesis, some other relevent papers have appeared.

Spontaneous channel openings by the acetylcholine receptor have been studied in more detail by M.B. Jackson (1986) [Biophys. J. 49 663-672]. Jackson suggests that an individual Ach channel will spontaneously open at a rate of 2×10^{-3} . sec^{-1} , and that the voltage dependence of spontaneous channel openings was greater than agonist activated channels. Ligand binding markedly increases the rate of channel opening, and increases open channel lifetime. Thus the effect of ligands is to destabilize the closed channel rather than stabilize the open channel. Not enough data is available at present to apply these results to GABA channels, and in particular activation of these channels by anaesthetics, but it is conceivable that pentobarbitone, chloralose etc. bind at some sites in the channel (other than the receptor) to destabilize the closed ionophore. Further experiments with anaesthetics on GABA channels should be exciting.

The theoretical treatment of J.A. Dani (1986 Biophys. J. 49 607-618) is of relevence to the discussion on the effects external anions to increase Cl^- efflux at hyperpolarized potentials. Dani has shown that

alteration of ion charge in the vestibule of an ionic channel, even with a very small change in composition of the external solution, can markedly alter the conductance through that channel.

It was noted in the text that no subconductance levels for GABA have yet been observed in freshly isolated neurones. S.G. Cull-Candy & A. Mathie (1986, Neurosci. Lett. 66 275-280) have recorded GABA activated single channels in freshly dissociated rat sympathetic ganglion neurones. No evidence for subconductance levels was obtained, but conductance levels varied between ~24 and 33pS in different patches.

Suzdak, Schwartz, Skolnick & Paul (1986, PNAS 83 4071-4075) have reported that low concentrations of ethanol (20-50mM) increase chloride uptake in rat brain synaptosomes induced by GABA. Uptake activated by muscimol and pentobarbitone was similarly increased by ethanol. These authors have suggested that ethanol does affect GABA receptor channels, and may potentiate the effectiveness of GABA. In contrast, the results presented in Section B suggested that ethanol had no action on GABA-mediated currents at concentrations from 10-200mM. In view of these opposite results, it may be worth reinvestigating the actions of ethanol on synaptic inhibition.

APPENDIX D:

PAPERS AND ABSTRACTS PUBLISHED DURING THESIS WORK,
MODEL OF GABA RECEPTOR-CHANNELS
PRESENTED IN A SEMINAR AT THE JOHN CURTIN SCHOOL OF MEDICAL RESEARCH, NOVEMBER 1985

[*From the Proceedings of the Physiological Society*, 16–17 *December* 1983
Journal of Physiology, **349**, 31*P*]

Inhibitory synaptic currents recorded in rat hippocampal slices

By G. L. Collingridge*, P. W. Gage and B. Robertson (introduced by R. M. McAllen). *Nerve-Muscle Research Centre, University of New South Wales, P.O. Box 1, Kensington, NSW 2033, Australia*

Inhibitory synaptic currents in the mammalian central nervous system have not previously been characterized. We report here the properties of spontaneous synaptic currents recorded at 21–25 °C from rat CA1 hippocampal neurones *in vitro* using a single electrode voltage clamp (Axon Instruments, 10 kHz switching rate, 30 % duty cycle).

When electrodes contained chloride ions (3 M-KCl or 3 M-CsCl) spontaneous inward currents were recorded (Fig. 1). These currents were blocked by 10^{-5} M-bicuculline and considerably reduced in frequency by 5×10^{-7} M-tetrodotoxin. These results are consistent with the observations of Alger & Nicoll (1980) that γ-aminobutyric acid-mediated inhibitory synaptic responses can be recorded in chloride loaded CA1 neurones.

Over 100 cells were studied and in every case currents varied widely in amplitude, from just being distinguishable above the baseline noise ($\sim$ 0·1 nA) to 0·5–2·5 nA in size. At a holding potential of −80 mV most currents had growth times (20–80 %) of less than 2·0 ms and had decay phases which were exponential with a single time constant (τ_D) of 7–15 ms. Both the growth and decay phase decreased with hyperpolarization, while the mean current amplitude increased. H values (change in membrane potential for an e-fold increase in τ_D) ranged from 100 to 200 mV.

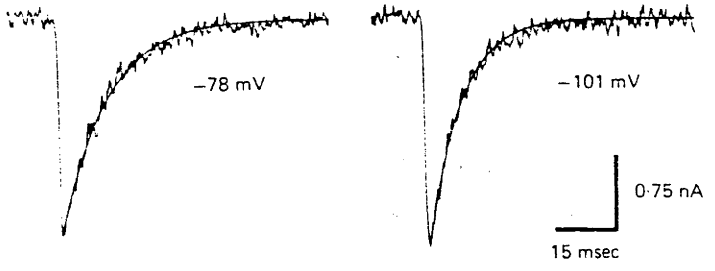


Fig. 1. Spontaneous synaptic inward currents recorded from the same cell at different holding potentials. The lines through the decay phases are single exponentials of best fit (least squares). τ_D 10·3 ms at −78 mV, 7·3 ms at −101 mV.

REFERENCE

Alger, B. E. & Nicoll, R. A. (1980). *Brain Res.* **200**, 195–200.

* Permanent address: Dept. of Pharmacology, University of Bristol, Bristol BS8 1TD, U.K. (In receipt of a travel grant from the Wellcome Trust.)

THE ACTION OF SOME DRUGS AND ANIONS ON INHIBITORY SYNAPTIC CURRENTS IN RAT HIPPOCAMPAL CA1 NEURONES *IN VITRO*

E. Robertson and P.W. Gage, Nerve-Muscle Research Centre, School of Physiology and Pharmacology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia.

The existence of spontaneous, γ-aminobutyric acid (GABA)-mediated synaptic potentials in rat hippocampal CA1 neurones has previously been noted (Alger & Nicoll, 1980). Using a single microelectrode voltage clamp, we have characterized the synaptic conductance change underlying these potentials (Collingridge, Gage & Robertson, 1984). GABA mediated inhibitory postsynaptic currents (IPSCs) have an exponential decay, which becomes shorter with membrane hyperpolarization and at higher temperatures. We believe that this system will prove useful in the study of molecular mechanisms of drug action in the mammalian central nervous system and have tested the effects of some drugs and general anaesthetics on the time course of the IPSCs.

Pentobarbitone(50-200 μmol l^{-1}) lengthened the decay of the IPSCs, and the effect was graded with concentration. The dissociative anaesthetic ketamine, at a concentration of 0.5 mmol l^{-1}, increased τ_D ~ 1.7-fold. This result is in contrast to the findings of Lodge & Anis (1982) who reported no enhancement of GABA-mediated inhibition by ketamine on spinal neurones. The hippocampal slices were exposed to an atmosphere containing clinically relevant concentrations of the inhalational anaesthetics halothane (1-5%) and nitrous oxide (75-80%). Halothane prolonged the IPSCs in a dose-dependent manner: in an atmosphere containing 3% halothane τ_D increased approximately two-fold. In contrast, nitrous oxide appeared to have no significant effect on IPSCs. A molecular model involving an increase in membrane dielectric constant has been proposed to explain the marked effects of ethanol on synaptic current decay in peripheral, excitatory synapses (see eg. Gage & Hamill, 1981). However, we have found no effect of ethanol on IPSCs at concentrations from 10 to 200 mmol l^{-1}. Nistri & Berti (1983) have reported that the central stimulant caffeine could potentiate GABA responses postsynaptically at low concentrations but blocked them at higher concentrations. We found neither of these effects with caffeine concentrations between 10 μmol l^{-1} and 5 mmol l^{-1}.

The effects of injecting different anions into CA1 neurones suggest that the chloride channels mediating the IPSCs are impermeable to sulphate, methylsulphate, acetate and citrate. However, bromide, azide, iodide, thiocyanate, chlorate and nitrate are permeable. The decay of IPSCs appeared to be influenced by the nature of the permeant anion: for example, τ_D in cells injected with iodide were up to five times longer than normal.

Alger, B.E. & Nicoll, R.A. (1980) *Brain Research* 200, 195-200.
Collingridge, G.L., Gage, P.W. & Robertson, B. (1984) *Journal of Physiology* (Proceedings) (In press).
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Lodge, D. & Anis, N.A. (1982) *British Journal of Anaesthesia* 54, 228P.
Nistri, A. & Berti, C. (1983) *Brain Research* 258, 263-270.

ELECTROPHARMACOLOGY OF THE IN VITRO SYNAPSE
ST. ANDREWS, U.K. AUG. 1984.

EFFECTS OF PREJUNCTIONALLY ACTIVE SNAKE TOXINS ON INHIBITORY SYNAPTIC CURRENTS IN RAT HIPPOCAMPAL SLICES

By Alan L. Harvey and Brian Robertson, Department of Physiology and Pharmacology, University of Strathclyde, U.K. and Department of Physiology, Australian National University.

Presynaptic toxins from snake venoms are potent inhibitors of acetylcholine release at the neuromuscular junction. β-Bungarotoxin has been reported to reduce the release of several neurotransmitters in the C.N.S. (Halliwell and Dolly, 1982; Halliwell, Tse, Spokes, Othman and Dolly, 1982). In order to examine more precisely the central effects of such toxins, we have studied the actions of β-bungarotoxin and taipoxin on spontaneous inhibitory synaptic currents recorded from CA1 neurones in rat hippocampal slice preparations. These currents are mediated by GABA (Collingridge, Gage and Robertson, 1984).

Currents were recorded at room temperature using a single electrode voltage clamp with electrodes containing 3M KCl. After control currents had been recorded, solution containing toxin was continuously superfused. Recordings were made from single cells for 90-120 min.

β-Bungarotoxin (230-460nM) caused a slow decrease in the amplitude and frequency of the synaptic currents. The maximum decrease was about 50-60%. After about 1h in toxin, there were often bursts of very large currents, perhaps due to nerve terminal damage. However, there was no evidence that the postsynaptic CA1 neurones were damaged as membrane potentials and resistances remained constant in the presence of toxin, and action potentials could still be elicited. There was no change in the time course of the decay of the inhibitory synaptic currents, suggesting a lack of a direct effect on GABA receptors.

Taipoxin acted similarly although it appeared to be more potent: 400nM reduced most currents to levels barely above baseline noise in less than 20 min. There were fewer high frequency bursts in the presence of taipoxin.

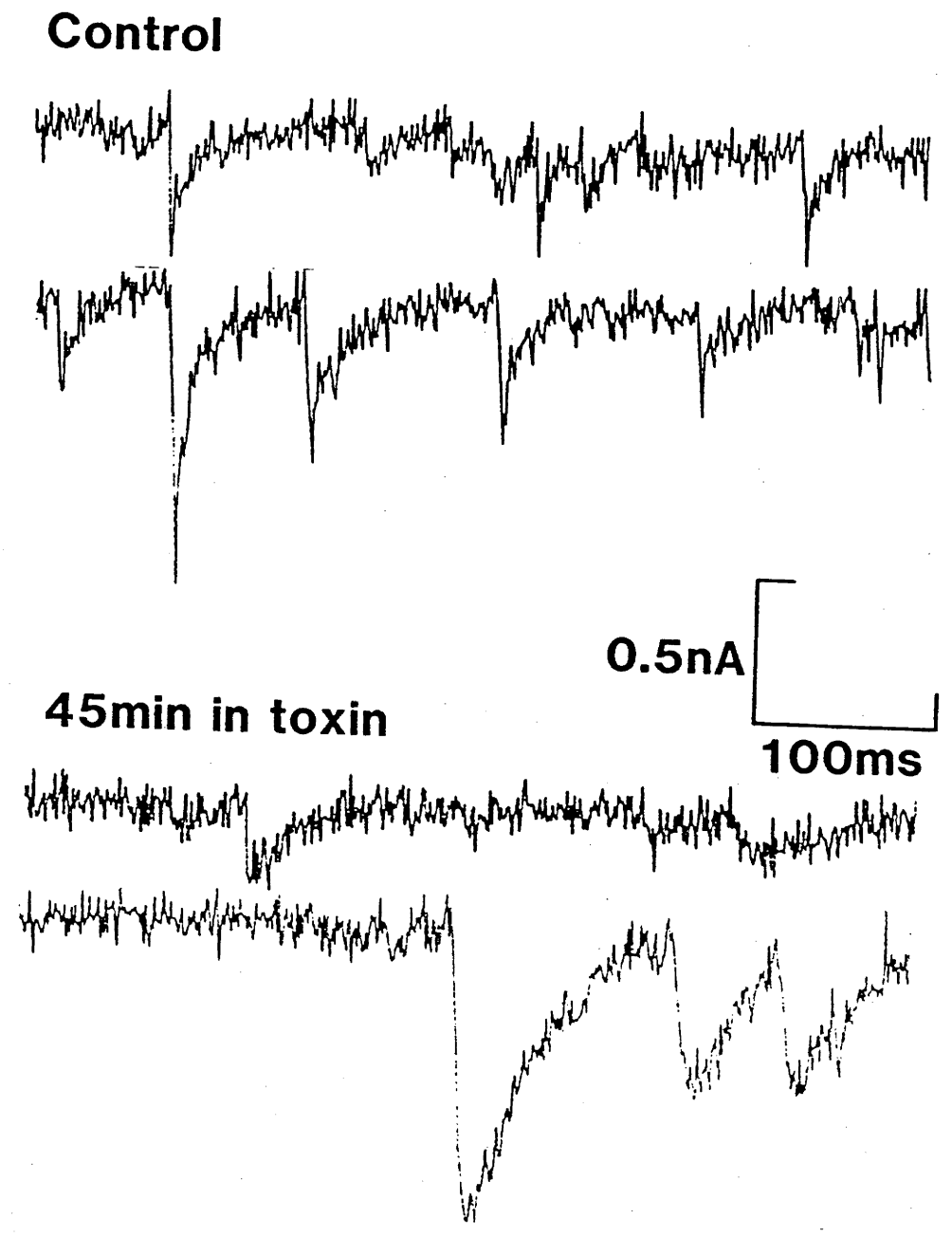
We thank Prof. P.W. Gage for providing the facilities for these experiments, and the Wellcome Trust and the Ramaciotti Foundation for support.

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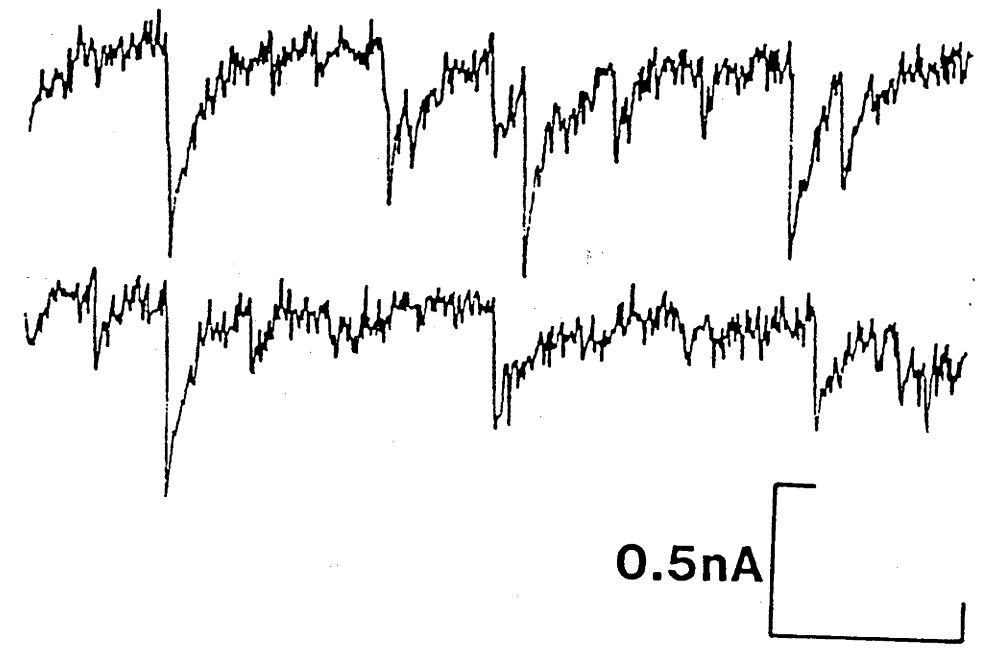
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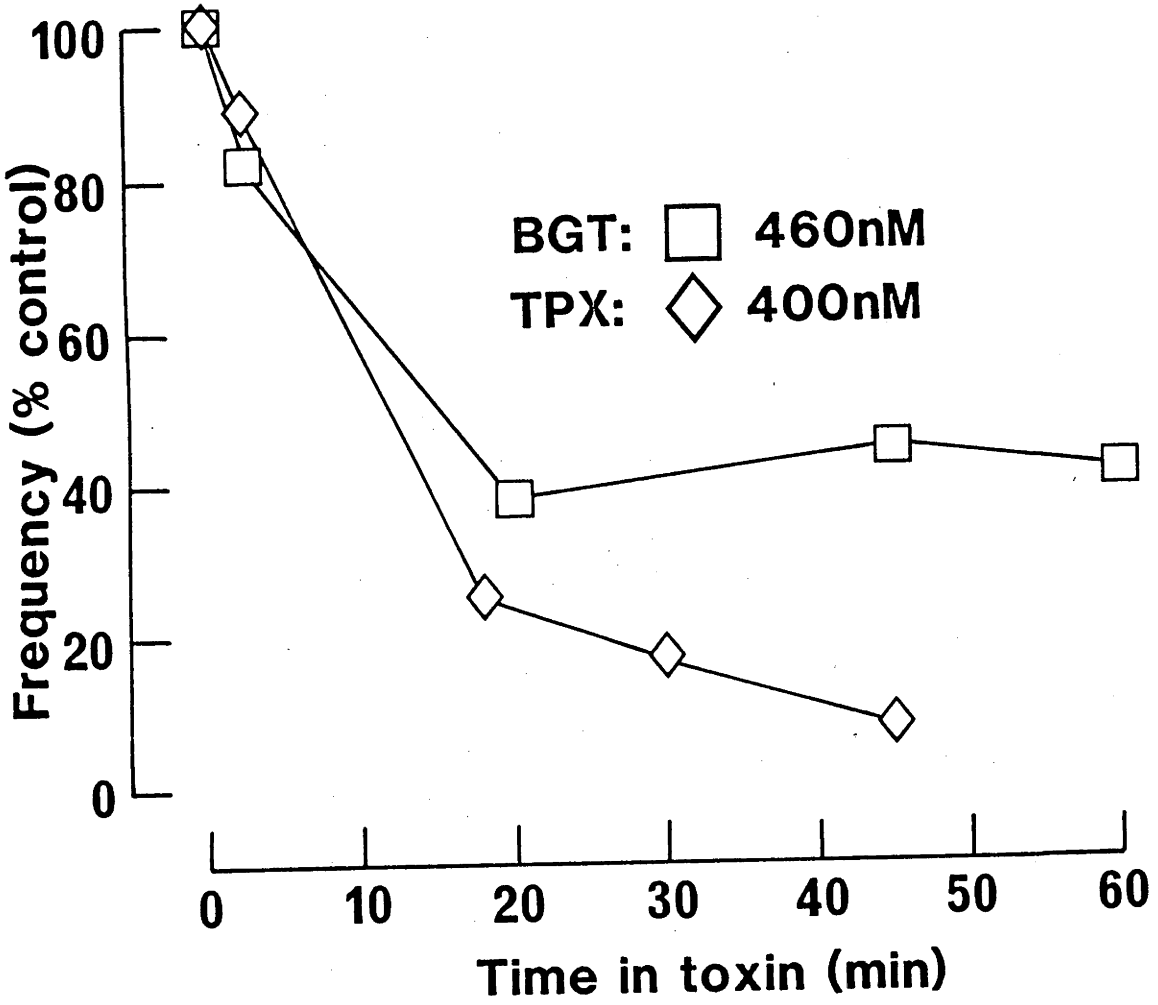
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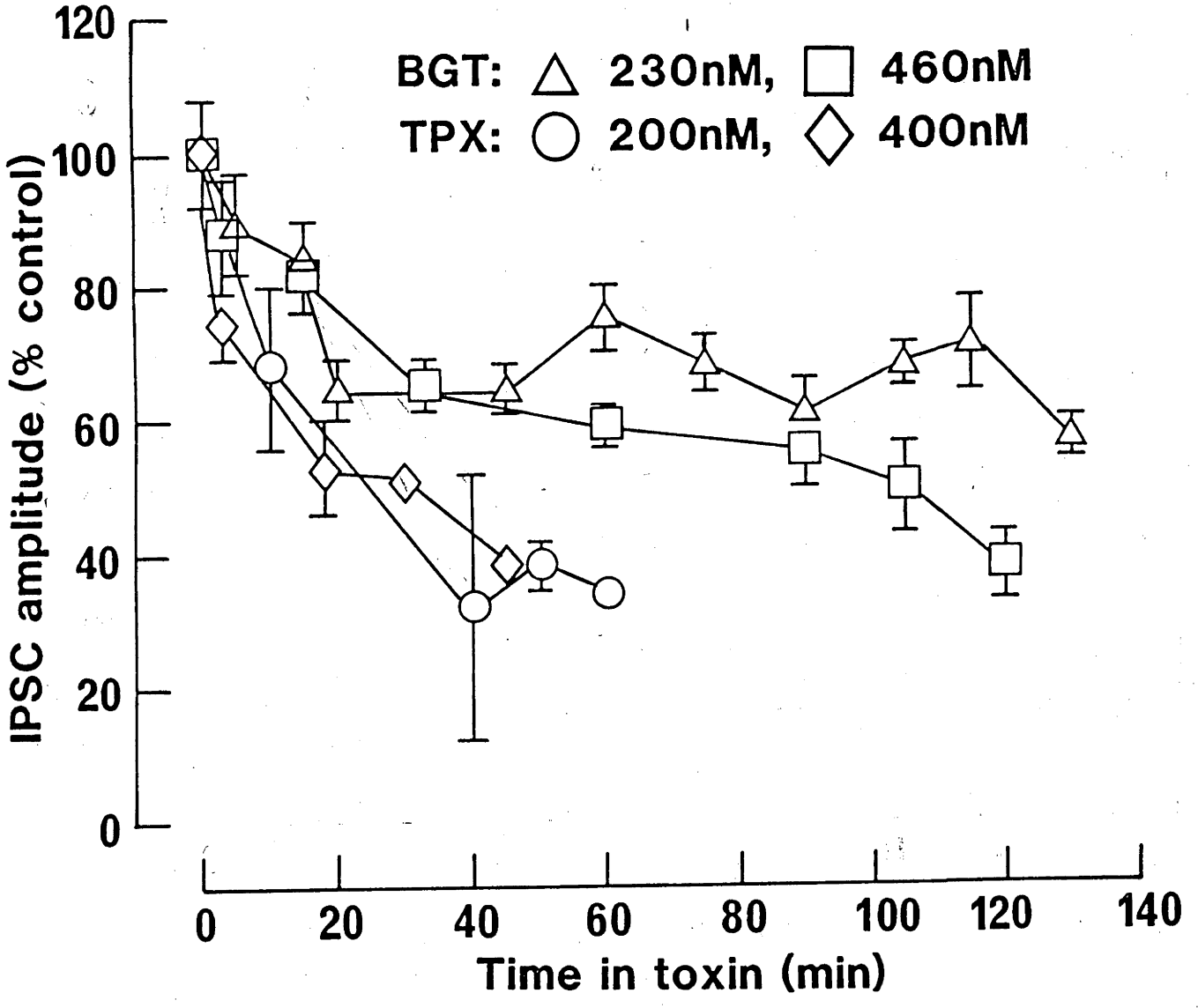
HALLIWELL, J.V., TSE, C.K., SPOKES, J.W., OTHMAN, I. and DOLLY, J.O. (1982) J. Neurochem., 39, 543-550.

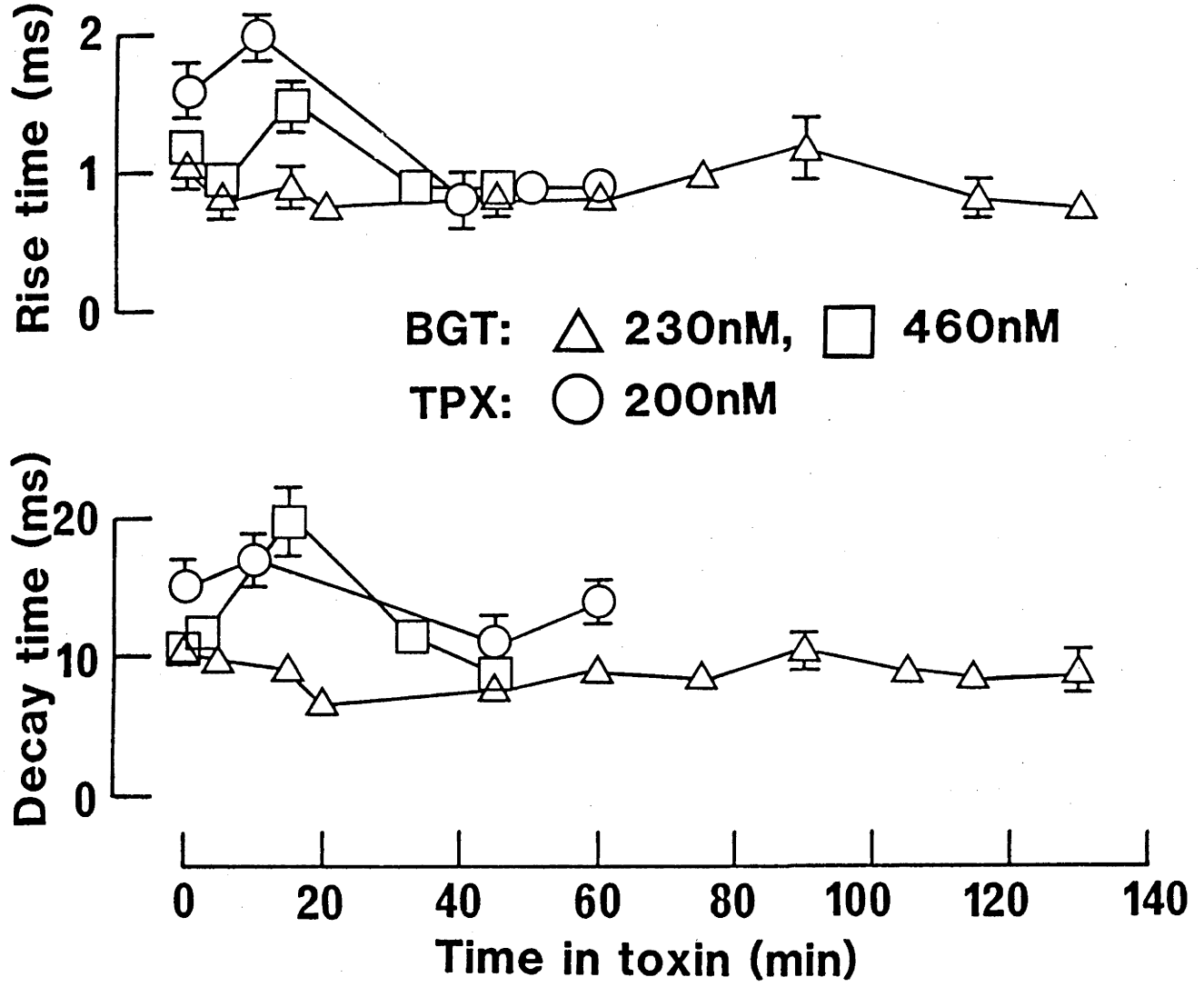

BGT
Control
0.5nA
100ms
45min in toxin
60min in toxin

TPX




100
80
60
40
20
0
Frequency (% control)
BGT: 460nM
TPX: 400nM
0
10
20
30
40
50
60
Time in toxin (min)


BGT: △ 230nM, □ 460nM
TPX: ○ 200nM, ◇ 400nM
IPSC amplitude (% control)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time in toxin (min)


Rise time (ms)
2
1
0
BGT: △ 230nM, □ 460nM
TPX: ○ 200nM
Decay time (ms)
20
10
0
0
20
40
60
80
100
120
140
Time in toxin (min)

ELECTROPHARMACOLOGY OF THE IN VITRO SYNAPSE
ST. ANDREWS, U.K. AUG. 1984.

THE EFFECTS OF SOME DRUGS AND ANAESTHETICS ON INHIBITORY SYNAPTIC CURRENTS IN RAT HIPPOCAMPAL CA1 NEURONES *IN VITRO*

By Brian Robertson and Peter W. Gage, Department of Physiology John Curtin School of Medical Research, Australian National University, Canberra, Australia.

The existence of spontaneous, GABA-mediated synaptic potentials in rat hippocampal CA1 neurones has previously been noted (Alger and Nicoll, 1980). We have previously characterized the synaptic conductance change underlying these potentials (Collingridge, Gage and Robertson, 1984). We believe that this system will prove useful in the study of the molecular mechanisms of drug action in the mammalian CNS and consequently have tested the effects of some drugs and anaesthetics on the time course of the IPSCs.

Pentobarbitone lengthened the decay of IPSCs and the effect was graded with concentration (50-200μM). The dissociative anaesthetic ketamine (0.5mM) increased τ_D about 1.7 fold. The hippocampal slices were exposed to an atmosphere containing clinically relevant concentrations of the inhalational anaesthetic halothane (1-5%) and nitrous oxide (75-80%). Halothane prolonged the IPSCs in a dose-dependent manner. In contrast, nitrous oxide appeared to have no significant effect on IPSCs. A molecular model involving an increase in membrane dielectric constant has been proposed to explain the marked effects of ethanol on synaptic current decay in peripheral excitatory synapses (e.g. Gage and Hamill, 1981). However, we find no effect of ethanol on IPSCs at concentrations from 10 to 200mM. Nistri and Berti (1983) have reported that the central stimulant caffeine could potentiate GABA responses postsynaptically at low concentrations but blocked them at higher concentrations. We found neither of these effects with caffeine concentrations between 10μM and 5mM.

B.R. would like to thank Beecham Research Laboratories (Australia) and Merck, Sharp and Dohme (Australia) Pty. Ltd. for travel grants.

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J. Physiol. (1984), **356**, *pp.* 551–564
With 7 text-figures
Printed in Great Britain

INHIBITORY POST-SYNAPTIC CURRENTS IN RAT HIPPOCAMPAL CA1 NEURONES

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(*Received 7 February 1984*)

SUMMARY

1. Spontaneous synaptic currents were recorded in voltage-clamped CA1 neurones in rat hippocampal slices at room temperature (21–25 °C). The currents, which could be seen at the resting membrane potential only when cells were loaded with chloride ions, were blocked by bicuculline. It was concluded that they were inhibitory post-synaptic currents (i.p.s.c.s) generated by the opening of chloride-selective channels activated by γ-aminobutyric acid (GABA).

2. In twenty-nine cells, ten to forty-five i.p.s.c.s were recorded at a potential between −75 and −85 mV. In every cell, i.p.s.c.s varied widely in amplitude with an average coefficient of variation of 28·7 %. The mean amplitude varied from 0·22 to 1·16 nA with an average of 0·52 nA.

3. Reversed (outward) currents could be recorded from cells voltage clamped at positive potentials. Null potentials varied between −20 and +20 mV, the variability being attributed to differences in chloride loading.

4. Most currents had a rapid growth phase with a mean growth time (20–80 % of peak) of 1·1 ms followed by a slower decay phase. The decay phase was exponential with a single time constant. The average decay time constant (τ_D) ranged from 8·3 to 16·2 ms with a mean value of 11·0 ms.

5. The rate of decay of currents was affected by membrane potential. τ_D decreased exponentially with hyperpolarization in the range from +40 to −120 mV with an average volt constant (H value) of 146 ± 9·6 mV (mean ± 1 S.E. of mean, $n = 17$). The mean value of τ_D at 0 mV was 19 ms. In some cells, growth times also decreased with hyperpolarization.

6. The decay of currents was faster at higher temperatures but remained exponential. At 32 °C, the average τ_D at 0 mV was 8·3 ms ($n = 5$) giving a Q_{10} value of 3·3 for the decay time constant at 0 mV.

7. The frequency and mean amplitude of i.p.s.c.s were reduced by tetrodotoxin (TTX) or cadmium, indicating that many of the currents were generated by action potentials in presynaptic terminals. The spontaneous 'miniatures' remaining had the same time course and voltage sensitivity as currents recorded in normal solutions.

8. Pentobarbitone (50–100 μM) greatly prolonged the decay of i.p.s.c.s but had no discernible effect on their amplitude or growth phase.

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INTRODUCTION

It has previously been reported that spontaneous inhibitory potentials can be recorded from pyramidal neurones in rat hippocampal slices (Alger & Nicoll, 1980). It is likely that inhibitory synapses on pyramidal cells in the hippocampus are predominantly on or near the soma (Anderson, Eccles & Loyning, 1964*a*). The occurrence of spontaneous potentials generated at synapses on the soma of these cells provides a very favourable situation for employing voltage-clamp techniques to determine the time course and amplitude of the underlying inhibitory currents. An additional advantage is that spontaneous synaptic potentials are not accompanied by contamination from excitatory inputs or from large extracellular field potentials that are normally produced by electrical stimulation.

We have therefore recorded spontaneous inhibitory currents in CA1 cells in rat hippocampal slices in order to determine characteristics of the post-synaptic conductance change at an inhibitory synapse in a mammalian central nervous system where the neurotransmitter is probably γ-aminobutyric acid (GABA) (Curtis, Felix & McLennan, 1970). Some of these results have appeared in a preliminary form (Collingridge, Gage & Robertson, 1984).

METHODS

Experiments were carried out on rat hippocampal slices. The hippocampus from adult, male rats (150 g, Wistar strain) was quickly dissected out and sliced transversely into 400 μm thick sections with a hand-operated chopper. The slices were then transferred to an experimental chamber where they were maintained at an interface with a humidified O_2/CO_2 mixture (Spencer, Gribkoff, Cotman & Lynch, 1976). The standard bathing medium contained (mM): NaCl, 124; KCl, 5; $NaHCO_3$, 26; Na_2HPO_4, 1·25; $MgSO_4$, 2; $CaCl_2$, 2; D-glucose, 10, and was maintained at pH 7·4–7·5 with O_2/CO_2 (95 %/5 %). Generally, the slices were continuously perfused at a constant rate of approximately 1 ml min^{-1} and maintained at room temperature (21–25 °C) unless otherwise stated. Recordings were obtained from approximately 1 h after the death of the animal. Successful experiments were completed in 139 cells in which the mean resting potential was −45 mV (S.D. = 10 mV) and action potentials had amplitudes greater than 60 mV. The lower resting potentials were associated with the use of low resistance electrodes (less than 50 MΩ) which allowed better voltage control under voltage clamp. Currents recorded in initially depolarized cells were indistinguishable from those with better resting membrane potentials. Slices remained viable for up to 24 h, as determined by intracellular recording of membrane potentials, action potentials and inhibitory post-synaptic currents (i.p.s.c.s). The drugs used in this study were added to the standard solution to give the following concentrations: bicuculline, 10^{-5}–10^{-6} M (Sigma); tetrodotoxin (TTX), 0·5–1·0 × 10^{-6} M (Sankyo) and Na pentobarbitone, 0·5–1·0 × 10^{-4} M (Prosana).

Conventional intracellular recording techniques were employed. Electrodes (25–90 MΩ) were pulled from 1 mm 'Omegadot' glass tubing (Clark Electromedical) on a Brown–Flaming micro-electrode puller (Model P-77, Sutter Instrument Co.) and were filled with 3 M-KCl or 3 M-CsCl, unless otherwise stated. Neurones were impaled in the stratum pyramidale region using a hydraulic stepping microdrive (Narishige) assisted by rapid 'zapping' with the electrode capacity neutralization control.

A single micro-electrode voltage clamp (Axoclamp 1, Axon Instruments, 1437 Rollins Road, Burlingame, CA 94010, U.S.A.) was used for the experiments reported here. This instrument permits voltage clamping with one electrode which is switched at high frequency between current passing and voltage recording modes. By selecting lower resistance micro-electrodes, and keeping the slices at the solution interface (to minimize the fluid level and hence the input capacitance), it was possible to use routinely a switching rate of 10 kHz. Procedures used to optimize the performance of the voltage clamp were similar to those employed elsewhere (Finkel & Redman, 1983). In particular,

the voltage recorded at the headstage was continually monitored to ensure that the voltage had settled to a steady value before it was sampled.

Current and voltage traces were continuously displayed on an oscilloscope and events of interest were captured and stored on magnetic disks using a microcomputer (Biosystems Processor 2950, P.O. Box 221, Kensington 2033, Australia) which was also used to measure the amplitude and time course of i.p.s.c.s.

A horizontal base line generated by the computer was displayed on the oscilloscope. Its level was adjusted using the computer until it passed through the true base line for an i.p.s.c. as determined visually. The decay of i.p.s.c.s could be well fitted by an exponential function with single time constant. The time constant of decay of currents was determined by finding a τ_D that gave the best (least-squares) fit of measured currents to:

$$I(t) = I(0)\exp(-t/\tau_D), \quad (1)$$

where $I(0)$ is the current measured from the base line at $t = 0$, a time selected after the peak and marked by a cursor. The least-squares fit was made between the first cursor and a second cursor placed at about 20% of the peak height. The selected τ_D was used with $I(0)$ to generate an exponential curve through the current on the the oscilloscope screen so that the goodness of fit could be validated by eye (see, e.g. Fig. 4).

RESULTS

Inhibitory post-synaptic potentials

As has been found previously (Alger & Nicoll, 1980), spontaneous transient depolarizations of variable amplitude were seen in CA1 neurones penetrated with micro-electrodes filled with 3 M-KCl or 3 M-CsCl (Fig. 1*A*). Some were as large as 20 mV in amplitude.

The lower the resistance of micro-electrodes, the earlier these were seen following impalement. Spontaneous potentials typically occurred within several seconds to several minutes after a cell was penetrated with a low resistance electrode (25–50 MΩ) but only after 20 min or more when a higher resistance electrode was used (greater than 70 MΩ). Their frequency varied from 0·1 to 50 s^{-1}. As these spontaneous depolarizations were not seen when intracellular electrodes contained the sulphate, citrate, acetate or methylsulphate salt instead of the chloride salt of potassium, even when the membrane potential was varied over a wide range, it was concluded that they were due to outward movement of chloride ions from cells that had been loaded with chloride from the micro-electrode (Fromm & Schultz, 1981).

The spontaneous i.p.s.p.s disappeared in solutions containing the GABA antagonist bicuculline (10^{-5}–10^{-6} M) and became larger and longer in the presence of pentobarbitone (10^{-4} M). These results confirm previous observations by Alger & Nicoll (1980), who suggested that the spontaneous depolarizations are 'inhibitory' post-synaptic potentials (i.p.s.p.s) generated by GABA secreted from the terminals of interneurones in response to action potentials. We have seen persistent, but generally smaller and invariably less frequent i.p.s.p.s, in cells exposed to solutions containing TTX ($0{\cdot}5$–$1{\cdot}0 \times 10^{-6}$ M) or $CdCl_2$ (0·2 mM) suggesting that some of the i.p.s.p.s are produced by GABA secreted spontaneously rather than in response to action potentials.

Inhibitory post-synaptic currents

When cells in which these i.p.s.p.s were recorded were voltage clamped, spontaneous, transient inward currents were seen. As these currents were abolished by bicuculline

(10^{-5}–10^{-6} M, twelve cells) and also depended on chloride loading the cells, it was assumed that they were i.p.s.c.s. Spontaneous currents could also be recorded when electrodes contained Cs_2SO_4, but only at positive membrane potentials: the time course of these currents was similar to those recorded with CsCl electrodes at the same

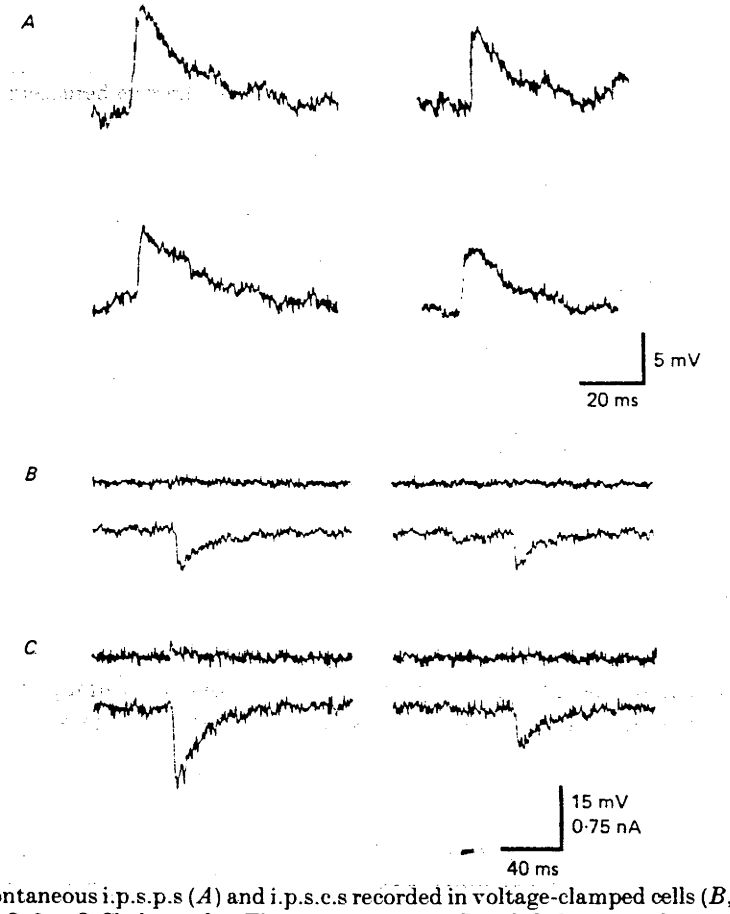


Fig. 1. Spontaneous i.p.s.p.s (*A*) and i.p.s.c.s recorded in voltage-clamped cells (*B*, 3 M-KCl electrode; *C*, 3 M-CsCl electrode). The upper traces in *B* and *C* show membrane potential (clamped at −90 mV in *B*, −67 mV in *C*) and the lower traces show i.p.s.c.s. Switching rate 10 kHz. Temperature 22 °C.

potentials, suggesting that chloride loading does not radically alter the properties of the GABA-activated currents, at least at positive potentials. Examples of typical currents recorded with KCl and CsCl electrodes are shown in Fig. 1*B* and *C*. In general, the membrane potential was well controlled, even during the rapid growth phase of the currents. Occasionally there was a small, rapid deflexion in the voltage trace during the growth phase of larger currents, as can be seen in Fig. 1*C*.

Following impalement of a cell, the amplitude of currents increased at first to a relatively stable level and then showed little or no further increase. The amplitudes of i.p.s.c.s during a recording period of 15 min are shown in Fig. 2*A* for a cell

voltage clamped at −97 mV. It can be seen that there was little change in current amplitude as i.p.s.c.s were sampled over this period of time.

The size of the i.p.s.c.s in any one cell was very variable. At potentials of −70 to −100 mV some were as small as 0·1 nA while others as large as 2·5 nA were occasionally seen in some cells. The distribution of amplitudes seen in one of these

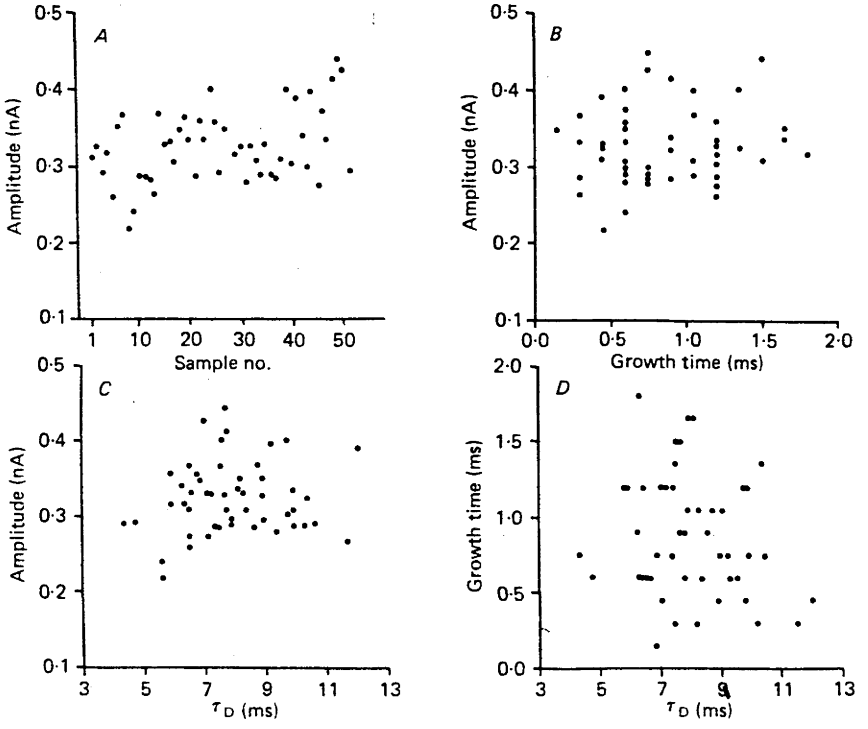


Fig. 2. *A*, a graph of peak amplitude of successive i.p.s.c.s. recorded over a period of 15 min. *B*, a graph of amplitude *versus* growth time. *C*, a graph of amplitude *versus* time constant of decay (τ_D). *D*, a graph of growth time *versus* τ_D. I.p.s.c.s recorded from the same cell as for Fig. 3. Clamp potential −97 mV, temperature 25 °C.

cells voltage clamped at −97 mV is shown in Fig. 3*A*. During the period of recording in this cell, the amplitude of i.p.s.c.s ranged generally from 0·2 to 0·45 nA: occasional currents were as large as 0·7 nA.

I.p.s.c.s were recorded in twenty-nine cells voltage clamped at −75 to −85 mV. The amplitude, rise time and time constant of decay were measured for each i.p.s.c.: mean values and standard deviations were obtained from ten to forty-five i.p.s.c.s in each cell. The average i.p.s.c. amplitude varied from 0·22 to 1·16 nA and the average coefficient of variation was 28·7 %. The mean of the twenty-nine average amplitudes was 0·52 nA (S.D. 0·21 nA).

Most of the currents had growth times (20–80 % of peak height) of less than 2 ms. Mean growth times in the twenty-nine cells voltage clamped at −75 to −85 mV (22–25 °C) ranged from 0·76 to 1·65 ms. with an average value of 1·1 ms and S.D. of 0·26 ms. The distribution of growth times in one cell (the same as in Fig. 3*A*) can

be seen in Fig. 3*B*. The average coefficient of variation was 41·5%. Sometimes currents (not included in the histogram) had growth times longer than 2 ms. These currents were not analysed because it was thought that they were probably generated in areas not adequately voltage clamped, for example on dendrites. No correlation between growth time and amplitude, or growth time and rate of decay, was detected in the currents that were analysed, as can be seen in Fig. 2*B* and *C*. This suggests that these currents were generated in adequately voltage-clamped regions of the cell.

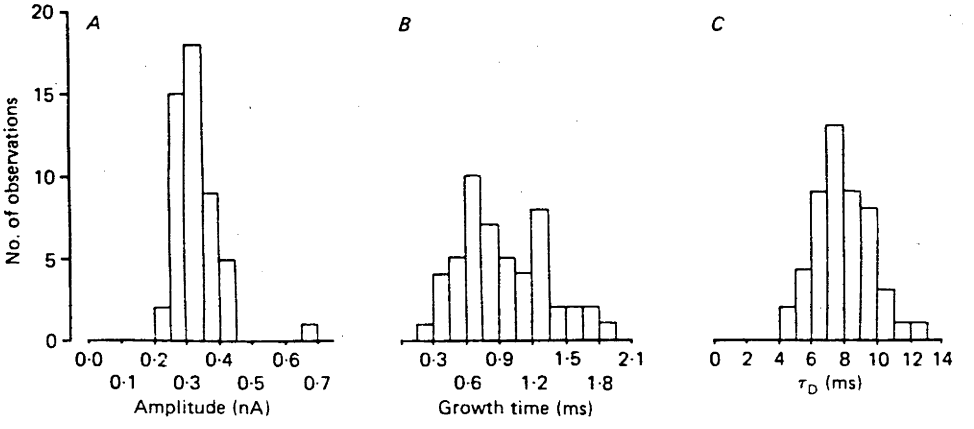


Fig. 3. Frequency histograms of amplitude (*A*), growth time (*B*) and time constant of decay (*C*) of spontaneous i.p.s.c.s recorded from a CA1 neurone voltage clamped at −97 mV. Temperature 25 °C.

The decay of i.p.s.c.s could be well fitted by an exponential with single time constant, τ_D (see Methods, eqn. (1)). Examples of exponential fits to the decay of i.p.s.c.s are shown in Fig. 4. The currents in Fig. 4*A* were recorded at 25 °C, those in Fig. 4*B* at 32 °C. As can be seen in Fig. 4, the conformity to a single exponential decay was very good at both temperatures and for both inward and outward currents. Under constant conditions (temperature and clamp potential) the range of τ_Ds in any one cell was quite small, as shown in the histogram in Fig. 3*C*. The mean coefficient of variation in the twenty-nine cells at −75 to −85 mV was 19·3%. The average τ_D ranged from 8·3 to 16·2 ms with a mean value of 11·0 ms and S.D. of 1·9 ms.

Changes in membrane potential affected the amplitude and time course of i.p.s.c.s. When the intracellular electrode contained CsCl, it was possible to shift the membrane potential sufficiently positive to reverse i.p.s.c.s. I.p.s.c.s recorded over a range of membrane potentials, including a 'reversed' current recorded at +29 mV, are shown in Fig. 5*A*.

There was a non-linear relation between mean peak current amplitude and membrane potential, the conductance becoming greater at more positive potentials. The curved line in Fig. 5*B* was fitted by eye and gave a null potential of about 0 mV. The null potential varied from cell to cell, but was normally within the range of −20 to +20 mV, the variability presumably being due to differences in the degree of loading of cells with chloride ions.

The decay of i.p.s.c.s was slower at more positive (depolarized) potentials. For

example the i.p.s.c. on the left in Fig. 4*B*, recorded at a membrane potential of −58 mV, has a decay time constant of 5·0 ms whereas the i.p.s.c. recorded at −12 mV has a decay time constant of 9·8 ms. Similarly, it can be seen in Fig. 5*A* that the decay of the currents became slower as the membrane potential became more positive.

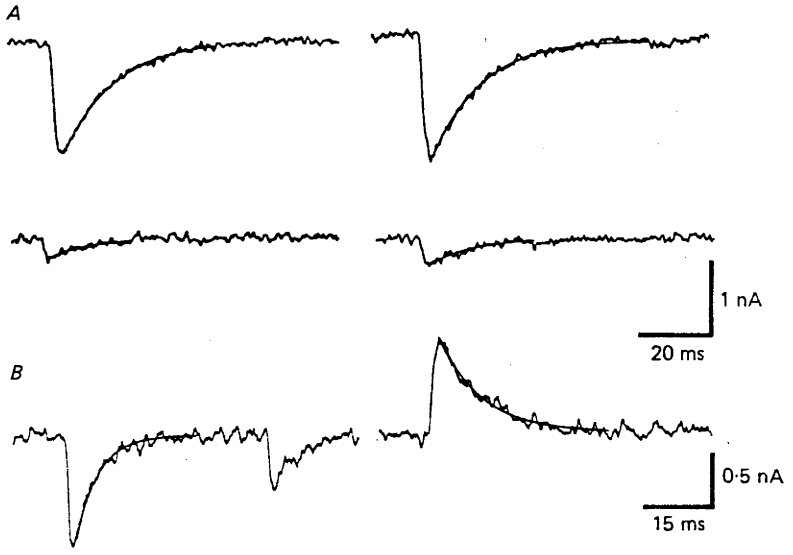


Fig. 4. Records of i.p.s.c.s. at 25 and 32 °C illustrating their exponential decay and the faster decay at the higher temperature. The records in *A* were obtained from a cell at 25 °C, voltage clamped at −99 mV. The lines through their decays show exponentials of best fit (see eqn. (1), Methods). The records in *B* were obtained in another cell at 32 °C. The record on the left in *B*, obtained at a membrane potential of −58 mV gave a τ_D of 5·0 ms whereas the inverted i.p.s.c. on the right, recorded at −12 mV, gave a τ_D of 9·8 ms. Vertical calibrations: 1 nA for *A*, 0·5 nA for *B*. Horizontal calibrations: 20 ms for *A*, 15 ms for *B*.

Mean τ_Ds were measured at several different membrane potentials in seventeen cells at 25 °C. These are plotted semilogarithmically against membrane potential in Fig. 6. The straight line through the points is the regression line

$$\ln \tau_D = \ln \tau_D(0) + V/H \qquad (2)$$

in which $\tau_D(0) = 20{\cdot}5$ ms and $H = 128$ mV ($r = 0{\cdot}898$). The results indicate an exponential relation between τ_D and membrane potential. An exponential relation between τ_D and V was also seen in each of the seventeen cells examined individually. The average $\tau_D(0)$ for these cells was $19{\cdot}0 \pm 1{\cdot}5$ ms and the average H value was $146 \pm 9{\cdot}6$ mV (mean ± S.E. of mean).

In four of seven cells in which the effect of membrane potential on the growth time was studied in detail, the growth time of i.p.s.c.s became greater at more positive potentials. In the other three cells, there was no detectable change in τ_D with membrane potential.

Most of the experiments described above were done at room temperature (21–25 °C) rather than at higher temperatures, so that currents would be slower allowing better

voltage control. In several experiments, the temperature was raised to 32–35 °C in order to determine whether the characteristics of i.p.s.c.s were different at higher temperatures. The decay of currents was clearly much faster at the higher temperatures, as can be seen by comparison of the i.p.s.c.s recorded at 25 and 32 °C

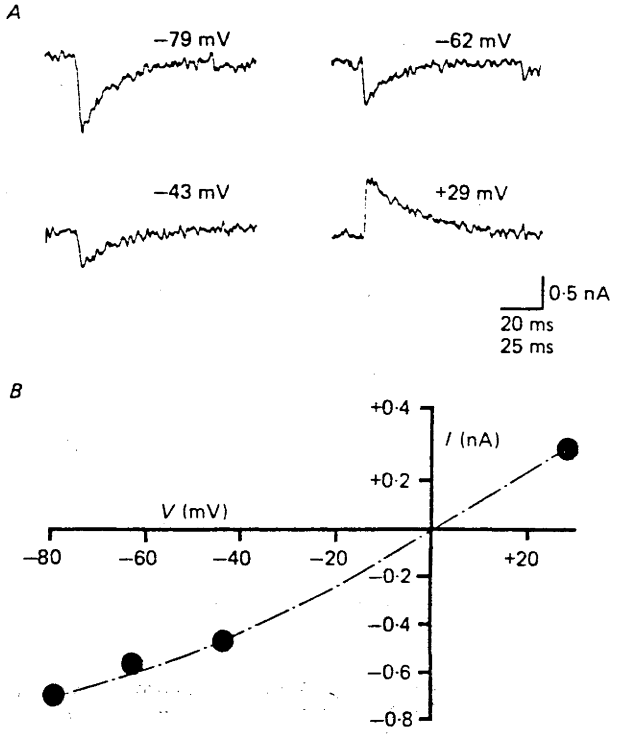


Fig. 5. *A*, i.p.s.c.s recorded at membrane potentials of a cell voltage clamped at −79, −62, −43 and +29 mV in one cell (CsCl electrode). Vertical calibration, 0·5 nA. Horizontal calibration, 20 ms for all i.p.s.c.s except that recorded at +29 mV where the calibration denotes 25 ms. *B*, the relation between average peak amplitude of i.p.s.c.s and membrane potential. The symbols show the average amplitude of eight to twenty-four i.p.s.c.s at each potential and are larger than the standard error bars. The interrupted line, drawn by eye, gave a null potential of 0 mV.

in Fig. 4*B*. The decay of currents was exponential at the higher temperatures as at lower temperatures, again illustrated in Fig. 4*B*. In five cells in which i.p.s.c.s were recorded over a range of membrane potentials at 32 °C, fitting eqn. (2) to average τ_D values gave an H value of 208 ± 103 mV and a $\tau_D(0)$ value of 8·3 ± 0·8 ms (mean ± 1 S.E. of mean). Comparison of the mean $\tau_D(0)$ values of 19·0 and 8·3 ms at 25 and 32 °C gives a Q_{10} of 3·3 for τ_D (at 0 mV).

In order to test whether the 'spontaneous' i.p.s.c.s were generated by action potentials in presynaptic nerves, some preparations were exposed to solutions containing TTX ($0·5–1·0 \times 10^{-6}$ M). We have found that even the lower concentration is sufficient to abolish sodium currents and action potentials in the soma of these cells.

The frequency and amplitude of i.p.s.c.s was clearly reduced in solutions containing TTX. Although there appeared to be a decrease in the modal amplitude of i.p.s.c.s (to approximately one-half, $n = 3$) in the presence of TTX, their time course was not detectably changed. The decay remained exponential and their growth times and decay time constants were within the range observed without TTX. In spite of the presence of TTX, there was still a large variability in the amplitude of i.p.s.c.s. The relation between τ_D and V remained exponential.

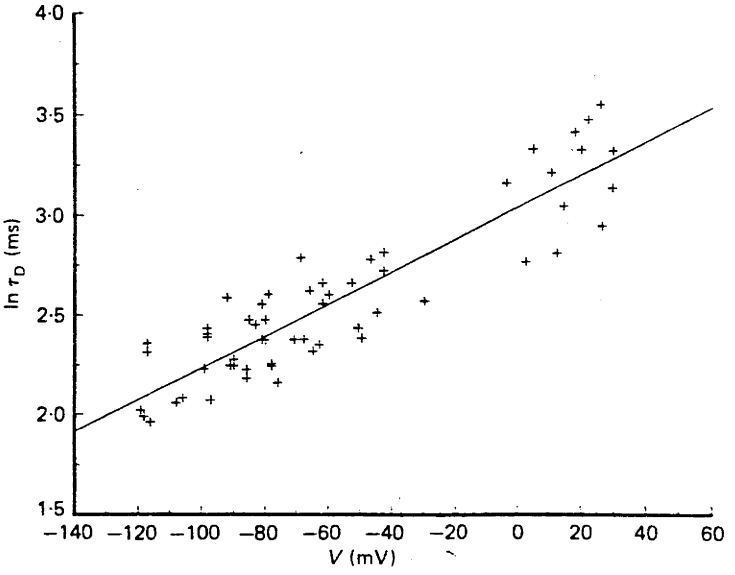


Fig. 6. A semilogarithmic plot of decay time constants (τ_D) obtained at three to five potentials in each of seventeen cells, against clamp potential (V). The symbols show the mean τ_D for eighteen to sixty-nine i.p.s.c.s recorded at each potential. The line through the points is a linear regression line giving an H value of 128 mV, $\tau_D(0)$ of 20·5 ms and a correlation coefficient of 0·898.

In some other experiments, 0·2 mM-$CdCl_2$ was added to the TTX solution to ensure that 'spontaneous' i.p.s.c.s were not being elicited by calcium spikes, which can be very large in some hippocampal neurones. We have found that this concentration of cadmium completely blocks calcium currents in the soma of these cells. Cadmium should also inhibit evoked transmitter secretion by suppressing calcium movement into nerve terminals. Small i.p.s.c.s persisted despite the presence of cadmium, suggesting that they were being generated by spontaneous secretion of packets of GABA.

The increase in amplitude and prolongation of the time course of spontaneous i.p.s.p.s produced by pentobarbitone (Nicoll, Eccles, Oshima & Rubia, 1975) suggested that the underlying currents were prolonged by the drug. This was indeed found to be the case. In the presence of anaesthetic concentrations of pentobarbitone (50–100 μM), the decay of i.p.s.c.s was markedly prolonged but remained exponential. The prolongation of i.p.s.c.s produced by pentobarbitone is illustrated in Fig. 7*A*.

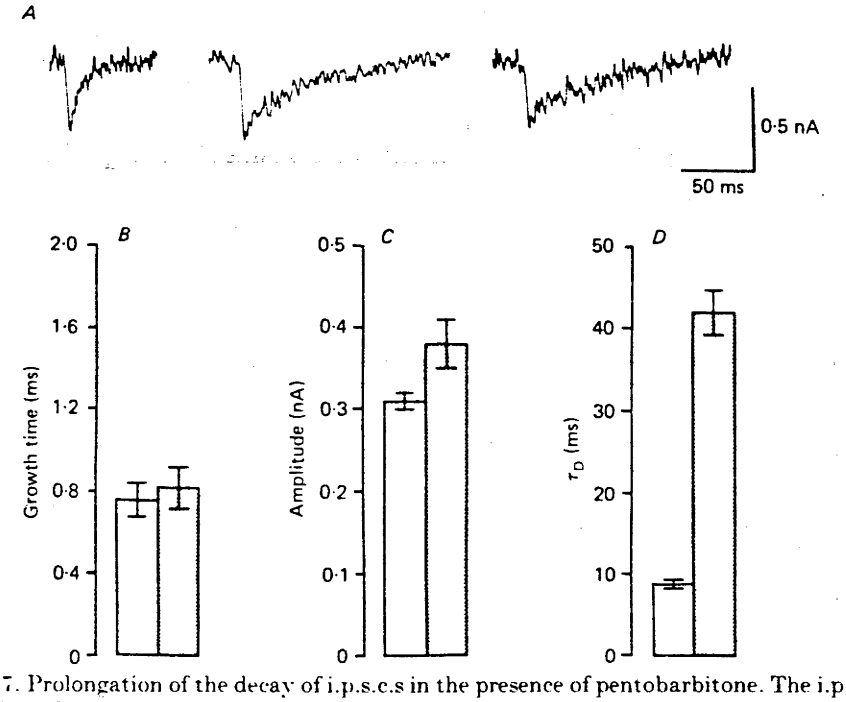


Fig. 7. Prolongation of the decay of i.p.s.c.s in the presence of pentobarbitone. The i.p.s.c. on the left in *A* was recorded before exposure to pentobarbitone, the other two after exposure to 50 μM-pentobarbitone. The average growth time (*B*), amplitude (*C*) and τ_D (*D*) recorded in another cell before (open bars) and after (hatched bars) exposure to the 100 μM-pentobarbitone show that the drug causes a large increase in τ_D but no significant change in average growth time or amplitude. The vertical lines at the top of the bars show ± 1 S.E. of mean.

There was no significant change in growth time (Fig. 7*B*) or amplitude (Fig. 7*C*) of i.p.s.c.s after exposure to pentobarbitone. However, τ_D increased more than fourfold in the presence of 100 μM-pentobarbitone, as illustrated in Fig. 7*D*.

DISCUSSION

Spontaneous excitatory and inhibitory potentials have previously been observed in mammalian hippocampal neurones (Nicoll *et al.* 1975; Brown, Wong & Prince, 1979; Alger & Nicoll, 1980; Lebeda, Hablitz & Johnston, 1982). Our aim was to record the ionic currents underlying the spontaneous i.p.s.c.s mediated by GABA in CA1 neurones. It proved very difficult to see i.p.s.c.s when electrodes contained anions other than chloride, even when the holding potential was varied over a wide range. We have no explanation to offer for this. In order to record i.p.s.p.s over a range of membrane potentials, it was necessary to use electrodes containing 3 M-KCl (Alger & Nicoll, 1980) or, preferably, 3 M-CsCl in spite of their drawbacks. When cells are penetrated with chloride-containing electrodes, chloride ions leak from the electrode tip (Fromm & Schultz, 1981) so that the chloride concentration in the cell rises (chloride loading), shifting the chloride equilibrium potential to more positive values

than normal. As a result, i.p.s.c.s become depolarizing at the normal membrane potential (Coombs, Eccles & Fatt, 1955). In spite of this abnormal intracellular chloride concentration, the time course of i.p.s.c.s did not appear to be changed: i.p.s.c.s recorded in several experiments with Cs_2SO_4 electrodes appeared to have the same time course as those recorded with chloride-containing electrodes.

In previous studies of synaptic channels activated by GABA in a variety of cells, single channel conductance (γ) has been reported to range from 16 to 26 pS (Cull-Candy & Miledi, 1981; Barker, McBurney & Macdonald, 1982; Bormann, Sakmann & Seifert, 1983; Martin, 1983; Segal & Barker, 1983; Gray, Kellaway & Johnston, 1983). The small amplitude of some of the inhibitory currents seen in the present study would suggest that they were generated by the opening of relatively few channels. An i.p.s.c. of 0·3 nA (e.g. Fig. 2) at -100 mV represents a peak conductance increase of 3×10^{-9} S (assuming a null potential of 0 mV). If each open channel had a conductance between 15 and 25 pS, only 120–200 channels would have been open at the peak of the i.p.s.c. This is much less than the 1000–2000 channels activated by a single quantum of acetylcholine at the vertebrate end-plate and is also in striking contrast to values obtained for quantal inhibitory events in other vertebrate central neurones. Faber & Korn (1982) calculated a value of 1000–2000 channels in the goldfish Mauthner cells, which is similar to the value reported for quantal i.p.s.c.s in the Muller neurones of the lamprey (Gold & Martin, 1983). Our estimate of the number of channels activated by a 'quantum' of GABA is of the same order as the estimate of 250 channels for a quantal current at the locust neuromuscular junction (Cull–Candy & Miledi, 1982).

Kandel, Spencer & Brinley (1961) found that very large i.p.s.p.s could be recorded in hippocampal pyramidal cells and later investigators (Andersen *et al.* 1964*a*, *b*) suggested that these i.p.s.p.s were due to the activation of a recurrent inhibitory system which terminated on or near the soma of the pyramidal cells. It was proposed that the basket cell, which makes a plexus round the pyramidal cell soma, formed these inhibitory synapses. However, more recent work has suggested that interneurones other than the basket cell may be involved in inhibition of pyramidal cells (see, e.g. Schwartzkroin & Mathers, 1978; Knowles & Schwartzkroin, 1981). For instance, the initial segment of pyramidal cells is heavily invested with inhibitory terminals (Andersen, 1976; Somogyi, Smith, Nunzi, Gorio, Takagi & Wu, 1983) and evidence is accumulating to suggest that both the apical and basal dendrites of pyramidal neurones receive GABAergic inhibitory innervation (see e.g. Alger & Nicoll, 1982*a*, *b*). If it is assumed that the interneurones responsible for dendritic inhibition have some degree of spontaneous activity, it is probable that i.p.s.c.s. are being generated on the pyramidal cell dendrites and initial segment as well as on the cell soma. It is unlikely that areas very far from the somatic recording site would be adequately voltage clamped during i.p.s.c.s; consequently, i.p.s.c.s recorded with an electrode in the soma would be distorted (Johnston & Brown, 1983). In some cells, currents *were* observed with slower than normal growth and decay phases. These i.p.s.c.s were rejected as it was assumed that they originated at areas remote from the soma. The lack of correlation between growth time, amplitude and decay time constant in the remaining currents (the vast majority) would strongly suggest that these were generated in well-clamped regions of membrane.

The rate of decay of the i.p.s.c.s was clearly voltage sensitive, in accord with previous observations of GABA-activated currents in crayfish muscle (Dudel, 1977; Onodera & Takeuchi, 1979) and stretch receptor neurones (Adams, Constanti & Banks, 1981). Although the lifetime of channels activated by GABA in cultured rat spinal neurones appears not to be voltage sensitive (Barker *et al.* 1982), voltage sensitivity qualitatively similar to that reported here has been described for i.p.s.c.s in cultured hippocampal neurones (Segal & Barker, 1983). In some cells, the growth time of i.p.s.c.s appeared longer at more positive voltages as has been observed at other inhibitory synapses (Dudel, 1977; Onodera & Takeuchi, 1979; Cull-Candy, 1983). This observation needs to be treated with some caution, however, since it is difficult to control the membrane potential effectively during fast events with the single-electrode voltage clamp.

The decay of inhibitory currents in CA1 neurones is much slower than the decay of excitatory currents at peripheral (Takeuchi & Takeuchi, 1959) or central synapses (Finkel & Redman, 1983) recorded under similar conditions. Since the decay phase of these i.p.s.c.s. is sensitive to membrane potential, has a high Q_{10} and is invariably exponential in form with a single time constant, it is likely that the decay reflects the rate of closing of a population of open channels, as at the motor end-plate (Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973; Gage & McBurney, 1975). We have no direct evidence for this assumption, but it is pertinent that there is reasonably close agreement between the time constant of decay of i.p.s.c.s and the average open time of GABA-activated channels obtained by noise analysis in other central neurones (cultured hippocampus, Segal & Barker, 1983; cultured spinal cord, Barker & McBurney, 1979; goldfish Mauthner cells, Faber & Korn, 1980; lamprey Muller cells, Gold & Martin, 1983).

Anaesthetic concentrations of pentobarbitone have previously been reported to increase dramatically the duration of recurrent inhibition in the hippocampus (Nicoll *et al.* 1975; Wolf & Haas, 1977) and olfactory cortex (Scholfield, 1978). In particular, Nicoll *et al.* (1975) reported that spontaneous inhibitory potentials were prolonged by pentobarbitone. Inhibitory currents in cultured neurones are also prolonged by barbiturates (e.g. Barker & McBurney, 1979). We have found that clinically relevant concentrations of pentobarbitone specifically prolong the decay of i.p.s.c.s with little or no effect on growth time or peak amplitude, and that the effect is concentration dependent. It is likely that pentobarbitone is having a direct action on the post-synaptic channels in the CA1 neurones, as inhibition of GABA uptake (e.g. Aickin & Deisz, 1981) appears not to be involved (Jessel & Richards, 1977).

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Prolongation of inhibitory postsynaptic currents by pentobarbitone, halothane and ketamine in CA1 pyramidal cells in rat hippocampus

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1 Spontaneous inhibitory postsynaptic currents (i.p.s.cs) were recorded in voltage-clamped CA1 neurones in rat hippocampal slices.

2 The exponential decay of i.p.s.cs was prolonged by concentrations of sodium pentobarbitone as low as 50 μM. With concentrations up to 100 μM, there was no change in the amplitude or rise time of the currents but current amplitude was depressed at 200 μM. The prolongation of currents increased with drug concentration within the range tested (50 to 200 μM).

3 Halothane, at concentrations from 1 to 5%, also increased the time constant of decay of i.p.s.cs. The effect increased with concentration and was fully reversible.

4 Ketamine, at a concentration of 0.5 mM, increased the time constant of decay of i.p.s.cs by 50 to 80% and the effect was reversible.

5 Ethanol (10–200 mM), nitrous oxide (75–80%), and caffeine (10 μM–5 mM) had no detectable effect on the i.p.s.cs.

6 It is suggested that pentobarbitone, halothane and ketamine increase the time constant of decay of the i.p.s.cs by stabilizing the open state of channels activated by γ-aminobutyric acid.

Introduction

Although anaesthetic drugs (e.g. ethanol) have been used for centuries to allow otherwise painful surgical procedures, the mechanisms involved in producing anaethesia are still uncertain. It has been known for some time that anaesthetics decrease the effectiveness of excitatory synaptic transmission (Sherrington 1906; Winterstein 1919; Larabee & Posternak 1952) at doses lower than those required to block axonal conduction. More recently, use of voltage clamp and patch clamp techniques at the neuromuscular junction has revealed that a variety of general anaesthetics depress transmission at this synapse by reducing channel open time (for a review, see Gage & Hamill, 1981). At least part of the depressant action of anaesthetics at central excitatory synapses is likely to be due to a similar mechanism. There is also compelling evidence that some general anaesthetics alter transmission at inhibitory synapses. For instance, early studies on the actions of pentobarbitone (Eccles & Malcolm 1946; Eccles *et al.*, 1963) suggested that this anaesthetic enhances γ-aminobutyric acid (GABA)-mediated inhibitory neurotransmission.

It has been difficult to use biophysical techniques similar to those used at the neuromuscular junction to study the effect of anaesthetics on synaptic transmission in the mammalian central nervous system. However, voltage clamp studies of cultured neurones have given useful information about possible synaptic actions of anaesthetics (for a review, see Mathers & Barker, 1982) and now, with the advent of single electrode voltage clamp techniques and isolated brain slice preparations, it has become possible to examine the effects of anaesthetics on postsynaptic conductance changes at central synapses. We have employed these techniques to study the effects of several anaesthetics on GABA-mediated synaptic currents in rat hippocampal slices. Some of these results have been presented briefly elsewhere (Robertson & Gage, 1984).

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Methods

Spontaneous inhibitory postsynaptic currents (i.p.s.cs) were recorded in the stratum pyramidale region of rat hippocampal slices using methods described previously (Collingridge *et al.*, 1984). Briefly, cells were voltage-clamped with a single microelectrode voltage clamp (ASFI, Axon Instruments Ltd) using switching rates between 10 and 16 kHz. Adequate settling of the current injection before voltage sampling was ensured at all times by continuously monitoring the headstage voltage at high gain. Currents were recorded digitally with a sample rate of 6.25 kHz and the decay of each current was fitted with a single exponential, as described elsewhere (Collingridge *et al.*, 1984). The rise time was measured from 20 to 80% of the peak amplitude by fitting a line (least squares fit) to the data points on the rising phase from 10 to 90% of peak amplitude. The temperature in all experiments was 20–25°C.

Drugs and anaesthetics used in this study were made up fresh during each experiment and dissolved in standard physiological salt solution (Collingridge *et al.*, 1984) immediately before use. The inhalational anaesthetic halothane was administered with a vaporizer (Fluotec 3, Cyprane) placed in the O_2/CO_2 supply circuit, so that the gas was administered both in the atmosphere above the hippocampal slices and in the inflow reservoir allowing the gas to dissolve in the perfusion solution. Nitrous oxide was supplied in a similar manner.

There are significant problems with the diffusion of drugs to cells in the slice preparation (see Nicholson & Hounsgaard, 1983), particularly with slices maintained at an interface between the bathing solution and a humidified atmosphere. The slices used in this study were 400 μm thick, and solution flow rates were 1 to 2 ml min^{-1}. The effects of any drug were always detected within 10 min of changing the solution and the maximum, stable effect was usually seen after approximately 20 min. Except for halothane and N_2O, all of the results presented were obtained from cells that had been exposed to a drug for at least 30 min. Even if diffusional barriers were still a significant problem after this time, drug concentrations at the cell membrane could only have been lower than the bath concentration.

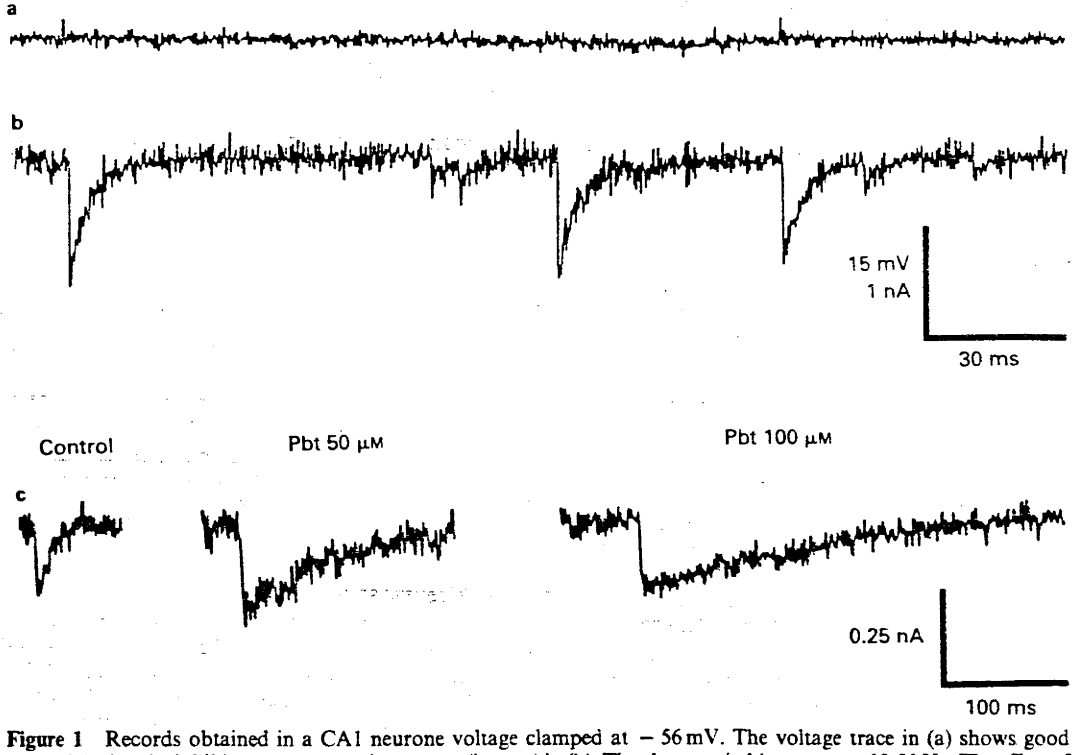


Figure 1 Records obtained in a CA1 neurone voltage clamped at − 56 mV. The voltage trace in (a) shows good control during the inhibitory postsynaptic currents (i.p.s.cs) in (b). The clamp switching rate was 13.5 kHz. The effect of pentobarbitone (Pbt) on the decay of i.p.s.cs recorded in another cell is shown in (c). Typical i.p.s.cs, recorded in control solution and solutions containing 50 μM and 100 μM pentobarbitone, illustrate the striking lengthening of the decay phase produced by this drug. Clamp potential, − 41 mV; clamp switch rate, 11.5 kHz.

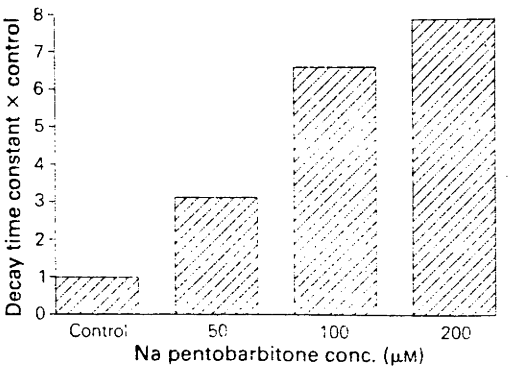


Figure 2 The increase in the average time constant of decay of inhibitory postsynaptic currents produced by pentobarbitone. The results were obtained in 5 voltage clamped cells exposed to 2 or more concentrations of pentobarbitone. The s.e. means for 50 μM and 100 μM pentobarbitone were 0.4 and 1.1 respectively. The two values at 200 μM pentobarbitone were the same (7.9).

Results

Pentobarbitone

As has been described previously (Collingridge *et al.*, 1984), the decay of spontaneous i.p.s.cs in hippocampal CA1 neurones is exponential with a single time constant that is presumed to be determined by the open time of ion channels, as at the endplate (Anderson & Stevens, 1973). It was also noted that sodium pentobarbitone increased the time constant of decay of the i.p.s.cs, but the effect was not examined over a range of concentrations. This observation has been extended in further experiments.

Examples of i.p.s.cs recorded from a cell voltage clamped at − 56 mV in control solution are shown in Figure 1b. The upper trace (Figure 1a) shows the voltage control during the currents. Typical examples of i.p.s.cs recorded from a cell before and after exposure to pentobarbitone can be seen in Figure 1c. The decay of the currents could be well fitted with a single exponential (Collingridge *et al.*, 1984) but the decay time constant was increased by 50 μM pentobarbitone and even more so by 100 μM pentobarbitone. Similar prolongation of i.p.s.cs in solutions containing pentobarbitone (50 to 200 μM) was seen in 15 cells. The effect could be fully reversed by perfusing the slice with control solution for more than 30 min.

The relationship between the average decay time constant and pentobarbitone concentration, recorded in 5 cells in which it was possible to change solutions without dislodging the electrode, is illustrated in Figure 2. It can be seen that pentobarbitone had a very large effect on the decay time constant which was increased on average more than three fold by a concentration as low as 50 μM and almost eight fold by

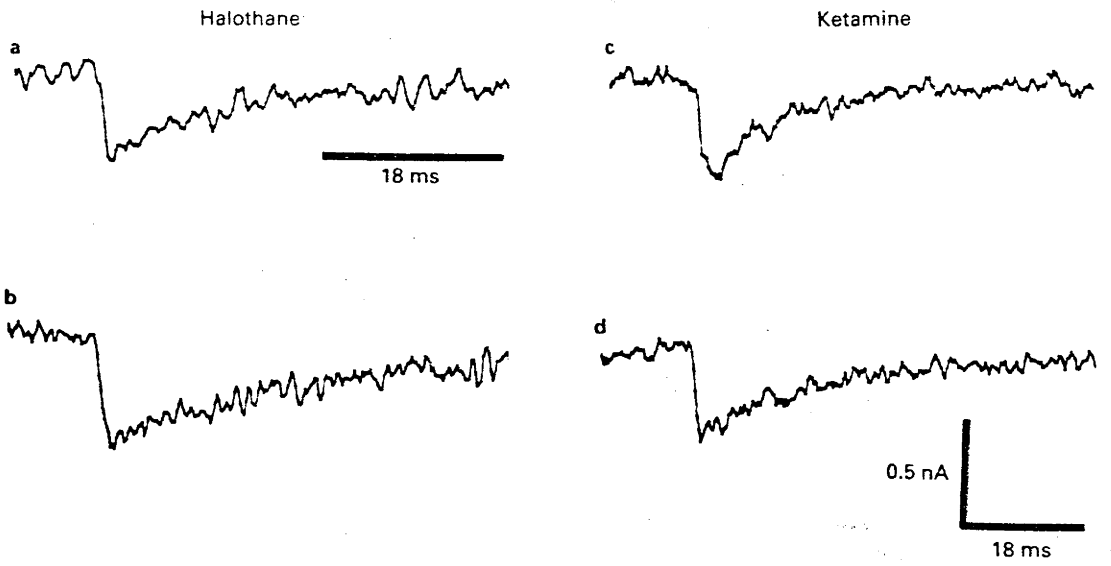


Figure 3 Slowing of the decay of inhibitory postsynaptic currents (i.p.s.cs) produced by halothane and ketamine. The decay of a typical i.p.s.c. in control solution (a) was clearly prolonged in the presence of 3% halothane (b). Note the difference in time scale for (a) and (b). In this cell, the clamp potential was − 54 mV and the switching rate 11.5 kHz. Similarly, the control i.p.s.c. (c) recorded in another cell was prolonged by ketamine 0.5 mM (d). Clamp potential, − 53 mV; switching rate, 11.3 kHz. The horizontal calibration bars denote 18 ms.

a concentration of 200 μM. At concentrations of 50 and 100 μM, pentobarbitone had little effect on the rise time or amplitude of i.p.s.cs: for example, in one cell, the average amplitude and rise time were 0.31 ± 0.1 nA and 0.76 ± 0.08 ms (n = 23) before, and 0.38 ± 0.03 nA and 0.83 ± 0.07 ms (n = 11) after, exposure to 100 μM pentobarbitone (means ± s.e.mean). With 200 μM pentobarbitone, the amplitude was invariably decreased.

Halothane

Halothane, like pentobarbitone, slowed the decay of i.p.s.cs. The effect, which was observed in all 15 cells studied, is illustrated in Figure 3. In this experiment, the average decay time constant before exposure to halothane was 9.9 ± 0.6 ms (mean ± 1s.e.mean, n = 15): one of the currents is shown in Figure 3a. Halothane (3%) was then applied directly in the atmosphere around the hippocampal slice and prolongation of currents was observed within a few seconds of exposure to the anaesthetic. Currents were captured for analysis when the effect had stabilized after 5 min in halothane. One of these currents is shown in Figure 3b. This i.p.s.c. was clearly prolonged. The average decay time constant had increased more than two fold to 21.5 ± 0.8 ms (mean ± 1s.e.mean, n = 30).

The sequential effects of a range of halothane concentrations on the decay time constant of i.p.s.cs are illustrated in two separate cells in Figure 4. Each point shows the mean from 9 to 35 i.p.s.cs: s.e.means varied from 0.3 to 1.2 ms. The effects of halothane obviously increased with concentration and were reversible. There was no significant change in the amplitude or rise time of i.p.s.cs. For example, in the experiment illustrated in Figure 4a, the average amplitude and rise time (mean ± 1 s.e.mean) were 0.45 ± 0.02 nA and 0.78 ± 0.07 ms (n = 15) in control solution, 0.51 ± 0.02 nA and 0.91 ± 0.04 ms (n = 30) with 3% halothane, 0.42 ± 0.02 nA and 0.45 ± 0.02 ms (n = 9) back in control solution, and 0.45 ± 0.02 nA and 0.92 ± 0.11 ms (n = 20) with 2% halothane. In most of the cells exposed to the anaesthetic, it was noticed that there was an increase in holding current indicating an increase in membrane conductance. This effect was not pursued, although the result was consistent with the increase in potassium conductance observed by Nicoll & Madison (1982). The effects of halothane were reversed within 5 min when a slice was flushed with control solution.

Ketamine

Previous studies have shown that ketamine, at low concentrations, reduces the time constant of decay of miniature endplate currents at the neuromuscular junction (Torda & Gage, 1977). In contrast, ketamine

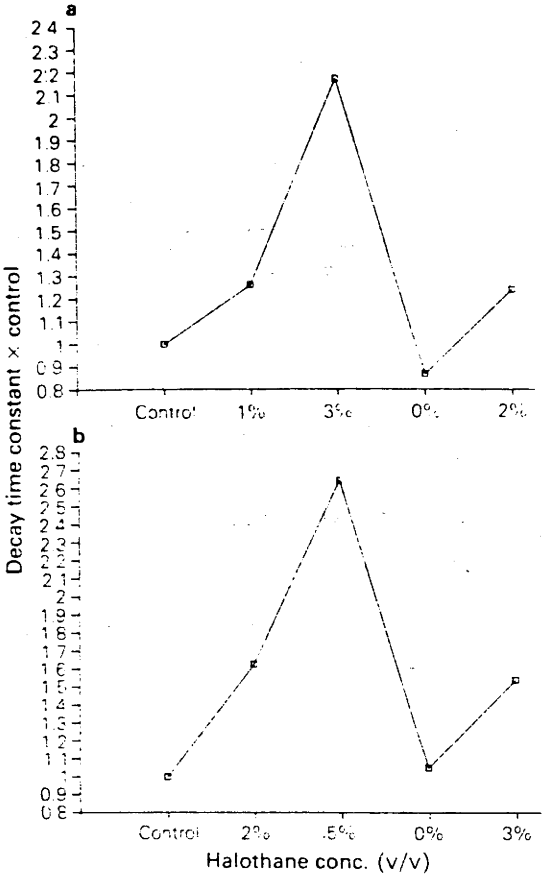


Figure 4 The effects of a range of halothane concentrations on the average time constant of decay of inhibitory postsynaptic currents (i.p.s.cs) in two cells. Each point shows the average decay time constant of 9 to 35 i.p.s.cs. In (a), the clamp potential was − 54 mV and switching rate 11.5 kHz. The control time constant was 9.9 ± 0.6 ms (mean ± 1 s.e.mean, n = 15). In (b), the clamp potential was − 72 mV and the switching rate 12.8 kHz. The control time constant was 11.5 ± 0.4 ms (mean ± 1 s.e.mean, n = 27).

potentiates inhibitory potentials in the olfactory cortex (Scholfield, 1980). Ketamine caused prolongation of i.p.s.cs, though to a lesser extent than pentobarbitone or halothane. The effect of ketamine (0.5 mM) on the decay of i.p.s.cs is illustrated in Figure 3. A current recorded after exposure to ketamine (Figure 3d) clearly decays more slowly than a current recorded before introduction of the drug (Figure 3c). In three experiments, 0.5 mM ketamine increased the average decay time constant of i.p.s.cs by 57%, 74% and 81%, while i.p.s.c. amplitude was not significantly affected. The increase in decay time constant was completely

reversible when slices were perfused with control solution for more than 30 min. No change in holding current (membrane conductance) was noted during exposure to ketamine.

Ethanol and nitrous oxide

Ethanol, at concentrations as high as 200 mM, caused no perceptible change in the decay time constant, rise time or amplitude of i.p.s.cs. Similarly, there were no detectable changes in i.p.s.cs in the presence of nitrous oxide (75–80% in carbon dioxide/oxygen).

Caffeine

Caffeine is a methylxanthine with central stimulatory activity (Snyder, 1981). Recent reports have suggested that this drug can selectively enhance or inhibit GABA responses, depending on the concentration applied (Nistri & Berti, 1983). Caffeine, at concentrations of 10 μM (10 cells), 50 μM (2 cells) and 500 μM (1 cell), had no effect on the time course or amplitude of i.p.s.cs. Furthermore, normal i.p.s.cs were observed in 7 cells exposed to 5 mM caffeine. The amplitude and time course of i.p.s.cs were within the normal range even after 90 min with this high concentration of caffeine. These results are in marked contrast to the effects of GABA, described by Nistri & Berti (1983), on the frog spinal cord. It is possible that the result may reflect a difference between the GABA receptors and channels of different species (as discussed by Nistri & Constanti, 1979).

Discussion

In this study, several general anaesthetics (pentobarbitone, halothane and ketamine), when applied at low, 'clinical' concentrations, were found to prolong spontaneous i.p.s.cs in hippocampal CA1 neurones by increasing their decay time constant. Such an effect would potentiate inhibition caused by GABA in these neurones. Pentobarbitone, in particular, must cause a profound potentiation of GABA-mediated inhibition in these cells when used clinically. It seems likely that similar effects may occur at GABA synapses elsewhere in the central nervous system. Indeed, it has been found previously that barbiturates and halothane potentiate GABA-mediated inhibition (Galindo, 1969; Nicoll, 1972; Nicoll *et al.*, 1975; Scholfield, 1980). On the other hand, ketamine has been found not to potentiate inhibition caused by GABA (Lodge & Anis, 1982; Anis *et al.*, 1983; but see Scholfield, 1980). However, it should be noted that the concentration used in our experiments may be somewhat higher than the concentration in rats anaesthetized with ketamine (Cohen *et al.*, 1973).

It is interesting that the three anaesthetics that potentiate inhibition by increasing the decay time constant of i.p.s.cs, depress excitatory transmission at the neuromuscular junction by reducing the decay time constant of excitatory currents. A drug that had both these effects (depression of excitation and potentiation of inhibition) at central synapses would have a powerful depressant action on transmission of signals in neuronal networks.

Ethanol has a depressant effect on the central nervous system but its mechanism of action is at present unknown. Previous results on the effects of ethanol on inhibitory transmission are conflicting. Nicoll (1972) found that ethanol, unlike several other anaesthetic substances tested, did not prolong synaptic inhibition in the rabbit olfactory bulb. Carlen *et al.* (1982) and Gruol (1982) have also shown that ethanol has no effect on GABA-mediated i.p.s.cs. However, Newlin *et al.* (1981) found that ethanol increased inhibition in the CA3 region of the hippocampus, and Nesteros (1980) has suggested that ethanol enhances GABA-mediated neurotransmission. Our results clearly show that ethanol has no effect on the GABA-activated inhibitory currents.

This lack of effect of ethanol was surprising, although Finger & Stettmeier (1984) have also found recently that ethanol has no effect on GABA-mediated i.p.s.cs in crayfish muscle. Ethanol increases channel open time at nicotinic neuromuscular junctions, where membrane hyperpolarization also increases channel open time, and has the opposite effect at glutaminergic junctions in crustacea where hyperpolarization decreases channel open time (Adams *et al.*, 1979). It has been suggested that all of these effects can be explained by the hypothesis that ethanol, by increasing the polarizability (dielectric constant) of lipids adjacent to receptor-channel complexes, stabilizes the state of the complex which is also stabilized by membrane hyperpolarization (Gage *et al.*, 1975). This hypothesis would predict that ethanol should reduce channel open time at GABA synapses where hyperpolarization reduces channel open time (Collingridge *et al.*, 1984). It could be argued that the decay of the i.p.s.cs is not determined by channel open time but this seems unlikely, as discussed below. The lack of any effect of ethanol on the decay of i.p.s.cs in which the decay time constant is voltage-sensitive weakens an attractive hypothesis.

It has been shown that many anaesthetics reduce the decay time constant of excitatory currents at the neuromuscular junction by reducing channel open time (for a review, see Gage & Hamill, 1981). Although there is no direct evidence, it seems likely that the increase in decay time constant of the i.p.s.cs described here is due to an increase in channel open time. However, it could be argued that the prolongation of the decay of the currents is due to inhibition of the reuptake of GABA: such an effect of pentobarbitone has

been demonstrated at inhibitory synapses on the crayfish stretch receptor (Aickin & Deisz, 1981). Nevertheless, there is much evidence against this possibility. Jessel & Richards (1977) found no effect of pentobarbitone, in the concentration range studied here, on GABA uptake in the hippocampus. Furthermore, in cultured hippocampal neurones, the increase in decay time constant of i.p.s.cs caused by barbiturates has been shown to be explained by an increase in channel open time (Segal & Barker, 1984). Finally, the GABA uptake inhibitor, nipecotic acid, at concentrations of 10 μM and 1 mM (Krogsgaard-Larsen & Johnston, 1975), causes no prolongation of spontaneous i.p.s.cs in rat hippocampal CA1 neurones (B. Robertson & I. Spence, unpublished observations). Pentobarbitone, halothane and ketamine could increase channel open time by stabilizing the open states of GABA-activated channels in many different ways e.g. by potentiating agonist binding (Willow & Johnston, 1980), by an allosteric effect on the receptor-channel complex or by changing the properties of 'boundary lipids'. There is, for instance, strong evidence that clinical concentrations of halothane can specifically disrupt the state of other protein complexes (Allison *et al.*, 1970; Hinkley & Telser, 1974).

It has been suggested that anaesthetics may decrease channel open time at the neuromuscular junction by blocking open channels (Adams, 1976) or by making the membrane more fluid (Gage & Hamill, 1975). Neither of these explanations can suitably account for an increase in channel open time at these inhibitory synapses. It seems more likely that anaesthetics can bind to hydrophobic sites on (or close to) receptor-channel complexes and, by so doing, stabilize channels in a closed (as at the motor endplate) or open (as at the GABA synapse) conformation.

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EFFECTS OF $GABA_B$ RECEPTOR ACTIVATION ON CALCIUM AND POTASSIUM CURRENTS IN CAT DORSAL ROOT GANGLION (DRG) NEURONES IN VITRO

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GABA is now believed to activate two different receptor types: $GABA_A$ receptors open chloride channels, while bicuculline insensitive $GABA_B$ receptors have been implicated in the modulation of potassium and calcium conductances. Systemic or oral administration of baclofen (Loiresal), a specific $GABA_B$ agonist, results in a reduction in skeletal muscle tone and reflex activity. This action is believed to be caused by a reduction in excitatory transmitter release. A decrease in transmitter release could be brought about by a reduced calcium influx into the neurone terminals by decreasing the calcium current directly or indirectly by increasing a potassium current. We have used single electrode voltage clamp and suction electrode techniques to examine these two possibilities. By blocking other ionic conductances internally and externally, we have been able to examine calcium and potassium currents in isolation. 100μM (-) baclofen and 100μM GABA were found to reduce the peak amplitude of the sustained calcium current - an effect which became more pronounced with increasing concentrations of external magnesium (1-5mM). Baclofen and GABA had no effect on the voltage activated potassium current, and neither agonist changed the resting potassium conductance. We suggest that, in the spinal cord, synaptic excitation may be diminished through $GABA_B$ receptor activation acting to directly reduce calcium influx into primary afferent terminals.

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IONIC CONDUCTANCES IN MAMMALIAN DORSAL ROOT GANGLION NEURONES

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Suction electrode techniques were applied to enzymatically dispersed cat and rat dorsal root ganglion neurones. When the cell soma was isolated from the axon the membrane could be voltage clamped rapidly and uniformly, using a single electrode.

We have characterized these neurones in terms of the ionic conductances observed in the cell bodies. There appear to be two sodium currents, a predominant TTX sensitive current and residual TTX insensitive current abolished when sodium is replaced by TRIS-Cl. In Na free solutions, approximately 80% of the cells had calcium spikes. The underlying current was the slowly inactivating "sustained" calcium current. A small number of neurones also had a low threshold, rapidly inactivating "transient" calcium current. The delayed rectifier appeared to be the most prominent potassium current and was blocked by external TEA or internal dialysis with Cs solutions. Occasionally a small transient potassium current (I_A) was activated by depolarising steps from hyperolarised potentials (-80mV). No evidence for a large, Ca-activated potassium current was found although a Ca-activated chloride conductance was often observed. Additionally, external Cs^+ activates a chloride current (1), which is not blocked by the GABA channel blocker picrotoxin, but is reduced in amplitude by 1mM $CdCl_2$. GABA activates a chloride channel in these cells but they do not respond to glycine or taurine.

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EFFECTS OF γ-AMINOBUTYRIC ACID AND (-)-BACLOFEN ON CALCIUM AND POTASSIUM CURRENTS IN CAT DORSAL ROOT GANGLION NEURONES IN VITRO

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SUMMARY

1. Calcium and potassium currents were examined in dialysed, isolated cat dorsal root ganglion neurones using a single electrode voltage clamp.

2. Two calcium currents were identified, a low threshold inactivating (transient) current and a higher threshold slowly inactivating (sustained) current. Both currents were blocked by 1 mM cadmium and replacement of calcium with manganese, which revealed an underlying nonspecific outward current. The sustained current disappeared with internal dialysis over a period of 20 to 30 minutes.

3. (-)Baclofen (100μM) and GABA (100μM) were found to reduce the peak amplitude of the sustained calcium current, an effect which became more pronounced with increasing concentrations of external magnesium (1-5 mM). In 5 mM external magnesium, 100μM baclofen reduced the calcium current by 28%.

4. The voltage activated delayed rectifier appeared to be the most prominent potassium current in these cells. We were unable to find any evidence for a significant contribution from calcium activated potassium conductance or a transient potassium conductance under our recording conditions.

5. Baclofen and GABA at 100μM had no consistent effect on the voltage activated potassium current. Baclofen did not change the resting potassium conductance.

INTRODUCTION

The amino acid γ-aminobutyric acid (GABA) is now believed to activate two different receptor types (Bowery, Hill & Hudson 1983). $GABA_A$ receptors are bicuculline sensitive and are coupled to chloride channels, whilst activation of $GABA_B$ receptors (bicuculline insensitive) has been implicated in the modulation of potassium and calcium conductances (eg. Gahwiler & Brown 1985, Fischbach & Dunlap 1981). Baclofen (β-p-chlorophenyl-γ-aminobutyric acid), a synthetic analogue of GABA, specifically activates $GABA_B$ receptors (Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980). The physiological significance of $GABA_B$ receptors has yet to be determined. Systemic or oral administration of baclofen results in a reduction in skeletal muscle tone and reflex activity (Birkmayer 1972) and electrophysiological investigations have revealed that baclofen reduces monosynaptic and polysynaptic excitation in the spinal cord (Pierau & Zimmermann 1973, Fox, Krnjevic, Morris, Puil & Werman, 1978) and higher centres (Ault & Nadler, 1982, Inoue, Matsuo & Ogata 1985). In particular, it has been proposed that baclofen has a presynaptic action on the terminals of primary afferent fibres, causing a reduction in excitatory transmitter release (eg Curtis, Lodge, Bornstein & Peet 1981, Davies 1981). Of relevance to this hypothesis is the finding that GABA (Dunlap & Fischbach 1978) and baclofen (Dunlap 1981; Desarmenien, Feltz,

Occhipinti, Santangelo & Schlichter 1984) shorten the duration of calcium dependent action potentials in dorsal root ganglion (DRG) cell bodies. These results are consistent with the notion that baclofen and GABA could reduce the release of excitatory transmitter from primary afferent terminals by reducing the calcium influx into the nerve terminal, although a depolarising action of GABA (which is not seen with baclofen) on primary afferent terminals has also been suggested as a mechanism for presynaptic inhibition (Curtis 1978, Curtis et al. 1981). Dunlap & Fischbach (1981) found that GABA reduces the calcium current, without any action on the potassium currents in cultured chick DRG neurones. Deisz and Lux (1985) also reported that GABA and baclofen reduced the calcium current, although these authors did not examine the potassium currents. In contrast, Schlichter, Demeneix, Desarmenien, Desaulles, Loeffler & Feltz (1984) suggest that the shortening of calcium spikes in rat DRG neurones by baclofen and GABA is due to a potentiation of the potassium conductance. Recent electrophysiological studies on hippocampal neurones also indicate that activation of $GABA_B$ receptors leads to an increase in potassium conductance (Newberry & Nicoll 1984; Gahwiler & Brown 1985).

Here we have examined the effects of GABA and baclofen on calcium and potassium conductances in freshly isolated cat DRG neurones in vitro, using voltage clamp techniques and specific channel blockers to dissect out the various voltage activated

conductances. Cat neurones were used since these cells are large and exhibit strong calcium currents. The use of freshly isolated neurones avoids problems associated with changes in the receptor profile which can occur over time in cultured preparations (see eg. Dunlap, 1981). In contrast to the results for central neurones and rat DRG neurones, we found a reduction in the Ca conductance with no change in the resting or voltage activated K conductances.

METHODS

Dorsal root ganglia (L_6, L_7 and S_1) with portions of central and peripheral nerve trunks were removed after dorsal laminectomy in adult cats (1.6-2.2 kg) anaesthetized with sodium pentobarbitone. Most of the connective tissue forming the capsule of the ganglion was removed with fine scissors and forceps. The cell membranes were then cleaned by enzyme treatment at 30° C. In the initial experiments each ganglion was exposed to 1% trypsin (Type IX, Sigma) in standard solution for one hour; in later experiments, a combination of 0.5% collagenase (Type 1A, Sigma) and 0.5 % trypsin was used. The latter treatment was found to be sufficient to permit good electrode sealing resistances while improving cell viability. The enzyme treatment was essential for dispersal of the neurones and for obtaining adequate sealing resistances (>20 MΩ) between the suction electrode and the cell membrane.

Enzymes were washed out of the preparation by perfusing with approximately 20 ml of standard solution before recording was attempted.

Ganglia were pinned to a Sylgard (Dow Corning) base in a recording bath fitted with a peltier temperature controller (20-22°C). Single neurones were then gently dispersed with a fine needle, under a binocular microscope (80x magnification). Bath volume was 0.4 ml and the total dead space between a solution multiplexer and the bath was 0.5 ml. Solutions were continually perfused at a rate of 2 to 5 ml per minute. The isolated neurone was separated from the ganglia thus ensuring that the exchange of solution around the cell was rapid and complete.

Solutions had the following compositions (in mM):

External solutions:

Standard solution: NaCl 140, $CaCl_2$ 5, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 5, glucose 10, KCl 2, pH 7.4 adjusted with NaOH.

Calcium current solution: Tris(hydroxymethyl)aminomethane chloride (TRIS-Cl) 110, $CaCl_2$ 10, HEPES 5, glucose 10, CsCl 2, tetraethylammonium bromide (TEA-Br) 25, pH 7.4 adjusted with HCl.

Potassium current solution: As for the standard solution with 1 to 3 μM tetrodotoxin (TTX, Sankyo) (or Na replaced with TRIS) and 1 mM $CdCl_2$ (or $CaCl_2$ replaced with $MnCl_2$).

H_2O_2 (0.001%) was sometimes included in solutions to increase O_2 tension and hopefully improve the viability of the neurones. $MgCl_2$ was included at concentrations of 1, 2.5, and 5 mM - $CaCl_2$ was reduced by a corresponding amount to maintain a constant divalent ion concentration.

Suction electrode internal solutions:

Calcium currents : CsCl 140, ethyleneglycol-bis-(β-aminoethylether)-N,N-tetra-acetic acid (EGTA) 11, HEPES 10, pH 7.3 adjusted with CsOH..

Potassium currents : KCl 140, EGTA 11, HEPES 10, pH 7.3 or K-methylsulphate 140, EGTA 2, HEPES 10, pH 7.3 adjusted with KOH.

(-)Baclofen hydrochloride was a gift from CIBA-GIEGY Switzerland. Bicuculline methiodide, picrotoxinin and GABA were purchased from Sigma.

Recording techniques

The experiments employed suction electrode techniques similar to those developed by Lee, Akaike & Brown (1980). Suction electrodes were made from Vitrex (Modulohm I/S Denmark) micro-haematocrit borosilicate glass using a David Kopf (model 700C) puller. Electrodes were scored against a glass coverslip and broken back to an outside diameter of 70 to 90 μm. The tips were then fire polished on a microforge to give an internal diameter of 10 to 20 μm. Suction electrode resistance

was usually 0.5 to 1.0 MΩ when filled with the internal solution (see above). Micro-electrodes were held in a suction electrode holder (WPI) and mounted directly onto the headstage amplifier. Neither the electrode nor the holder were shielded. The initial experiments were carried out using an Axon Instruments ASF 1 (maximum cycle rate 45 kHz) single electrode voltage clamp. Most of the experiments reported here were done using the ASF 2 model, which allowed switching rates of up to 65 kHz with complete settling of the electrode voltage between current injection. The feedback gain was 100 nA/mV and phase lag was used to prevent ringing during the steps while maintaining optimal clamp speed.

When the tip of the suction electrode was pressed onto the cell membrane, steady suction would generally result in a 20 to 40 MΩ seal between the electrode and the cell membrane. The input resistance of the cells was 20 to 60 MΩ. Sometimes the membrane under the tip would break down spontaneously within a few seconds of obtaining the seal but usually it was necessary to apply a brief depolarizing
current pulse (100 nA), to rupture the membrane. The cell soma was then isolated by breaking off the axon. An increase in cell input resistance was normally observed after the cell was isolated and the axon sealed over. Generally a length of axon remained attached to the cell body but this was never more than 100 µm and was usually less than 20 µm. In any case the remaining axon would be unlikely to have compromised the space

clamp of the somatic membrane (see Brown, Perkel, Norris & Peacock, 1981). Resting membrane potentials were approximately -40 to -60mV with this technique, which compares favourably with potentials of -50 mV we obtained with conventional intracellular recording.

Current and voltage signals were digitized on line and the records were stored on magnetic disc. A 6809 (Motorola) based microprocessor controlled the data acquisition through a 10 bit analogue to digital converter, and also provided the command voltages to the voltage clamp via a digital to analogue converter. Equal and opposite pulses were applied and the resulting current traces were subsequently summed to remove contributions from linear leakage and capacitive components. No voltage activated conductances were evident during the hyperpolarizing steps. Current and voltage signals were sampled at 160 μs per point for Ca^{2+} currents and 300 μs per point for K^+ currents. The current signal was low pass filtered at 2 kHz (4 pole Bessel), voltage was filtered at the output of the voltage clamp (30 kHz, single pole). Data was subsequently analysed on a PDP 11/44 computer. This involved subtracting the leakage and capacitive currents, and measurement of the peak amplitudes.

RESULTS

General observations.

In standard solution, cells had resting potentials of -40 to -60 mV and would usually fire a spike of up to 100 mV in amplitude in response to a depolarising current pulse. We do not believe that the enzyme treatment changed the electrophysiological properties of the neurones, because we found that the input resistance and active properties were similar to those of cells not treated with enzymes and examined with intracellular microelectrodes. A similar conclusion has been reached by other authors using enzyme pretreatment of mammalian DRG (Harper & Lawson 1985) and sympathetic neurones (Galvan & Sedlmeir 1984).

A major component of the action potential in normal solution is carried by Na^+ ions through TTX sensitive channels, since 1 to 3 μM TTX blocked the action potential and fast inward current. In many cells, Ca spikes could be elicited even with only partial block of the potassium channels. Additionally, a small spike of up to 20 mV was observed in the presence of TTX and Ca channel blockers (1 mM $CdCl_2$, or substitution of $CaCl_2$ with 10 mM $MnCl_2$). This spike could only be blocked by substituting TRIS for Na^+ ions. Therefore we suggest that some of these neurones possess a TTX insensitive Na current. We have made no detailed study of either sodium current. Figure 1 illustrates voltage activated currents elicited by a 70 mV depolarising step from -50 mV in control solution, during solution change and after complete change over (<3 minutes) to a "calcium current" solution.

(FIG. 1 NEAR HERE)

Calcium Currents

Calcium currents were recorded by blocking sodium and potassium current using internal and external Ca current solutions described in the methods section. Ca^{2+} currents were not seen in all cells, but were present in approximately 80% of the neurones studied. Figure 2A shows a plot of peak inward Ca current (recorded from a holding potential of -50 mV)

(FIG. 2 NEAR HERE)

versus membrane potential. The I-V relationship in this figure shows two distinct components, a shoulder at negative potentials characteristic of the recently discovered "transient" Ca current (Carbone & Lux 1984) and a maximum at 10 to 20 mV typical of the well established "sustained" Ca current (eg. Fenwick, Marty & Neher 1982). The transient current was never greater than 15 nA at -5 mV, whereas the sustained current reached a peak of 20 to 50 nA at 10 to 20 mV. We did not observe the transient current in all neurones where the sustained current was present. The transient current showed significant inactivation during a 50 ms pulse from -90 to -20 mV, unlike the sustained current which did not inactivate appreciably a during similar pulse from -50 to +20 mV (eg fig. 2B and 5A,C). Since the transient current was present in very few cells (about 20%) the results focus on the sustained calcium current which we will refer to as I_{Ca}.

I_{Ca} was difficult to work with because it "washes out" as the neurone is internally dialysed. Wash out is shown in figure 3; the points represent the peak inward current recorded on stepping to +20 mV (from -50 mV) at one or two

minute intervals. The current amplitude was stable for 10 minutes after which a steady decline was observed for the next 20 minutes. In order to allow for the wash out, applications of baclofen were bracketed by control measurements and the control level was taken as the average of the peak inward current before and after baclofen application.

(FIG. 3 HERE)

Since we were interested in measuring the effects of $GABA_B$ receptor activation on I_{Ca} we had to ensure that we had a pure calcium current. There were two possible sources of contamination of the Ca currents. Firstly, when Ca influx is blocked by Cd^{2+} (0.5-1 mM, fig. 4C) or by replacing Ca with Mn^{2+} (fig. 4A), a residual outward current (I_{ns}) can be seen. I_{ns} is similar to the nonspecific current previously observed by Byerly & Hagiwara (1982). The current becomes significant at positive potentials, showing voltage dependent activation (fig. 4B). This current had no effect on the measured peak I_{Ca} at potentials up to +30 mV as I_{Ca} activates much more rapidly than I_{ns} at these potentials. However, it undoubtedly contributes to the apparent inactivation of I_{Ca} during large depolarizing steps. A second possible source of contamination seen in some neurones (about 20% of the total) comes from a slowly decaying inward "tail" current observed upon repolarization after I_{Ca} (see fig. 4D). This inward tail current is blocked by Ca channel blockers, and declined in

parallel with I_{Ca}. The voltage sensitivity of the peak inward tail current followed that of the peak Ca current. It is probably not a slow calcium tail current as it was not present in every neurone where a Ca current was recorded. We believe this current is carried by chloride ions as it is recorded in solutions where Na and K currents were blocked, and the direction of the current is consistent with the null potential for chloride ions of ~0 mV in our recording situation. Since this Cl^- current would be an outward current at potentials positive to OmV, and therefore contaminate inward I_{Ca}, we discarded cells which showed a large slow inward component. This current appears similar to the Ca activated chloride conductance recently reported by Owen, Segal & Barker (1984) in cultured spinal neurones and by Mayer (1985) in cultured rat DRG. Cells showing large currents of this nature were discarded.

(FIG. 4 HERE)

Effects of GABA and (-)Baclofen on Ca currents.

(-)Baclofen and GABA were applied at a concentration of 100 μM throughout. This concentration was chosen as it gave a reduction in the calcium current which could be measured reliably. When GABA was applied, we included 10μM of bicuculline methiodide and picrotoxinin to block $GABA_A$ mediated chloride currents which can reach several nanoamps in these cells. Even with these precautions, a transient increase

in holding current was often observed, presumably due to residual activation of $GABA_A$ receptors. In contrast, no change in holding current was observed on application of baclofen, and we favoured the use of this agonist for this reason.

The effects of the agonists were assessed by measuring the change in the peak current during a step to +10 or 20 mV from a holding potential of -50 mV. Measurements were taken 1-2 minutes after changing solutions. The initial experiments were carried out without Mg ions in the external solution, and it was found that GABA and baclofen did not have a marked effect on either of the Ca currents (see table 1).

The results from one such experiment are shown in figures 5A and 5B and indicate that 100 μM baclofen has no obvious effect on either calcium current under these conditions. However, we found that both GABA and baclofen were able to reduce I_{Ca} in the presence of external Mg ions. Binding studies indicate that Mg^{2+} may potentiate binding of tritiated baclofen to crude synaptic membranes from rat brain (Bowery et al. 1983). Our results indicate that 100μM baclofen becomes more effective at reducing I_{Ca} as the external Mg concentration is raised. The results are shown in figures 5C and 5D and table 1. The effect shown in 5D did not reverse completely. This was probably due to rundown of the calcium current as it took several minutes to record and store three full I-Vs. The effects of GABA and baclofen were rapidly and

fully reversible (in absence of rundown), and the reduction appeared to be maintained as long as the agonist was present. There was no significant effect of baclofen on the rising phase of the calcium current.

(FIG 5 AND TABLE 1 HERE)

In some neurones no effect of baclofen was observed in spite of a high concentration of Mg, (eg figure 3 and table 1) and we attributed this to an absence of $GABA_B$ receptors on these cells. The variability in the responses to GABA and baclofen shown in table 1 may also reflect differences in receptor density in the population of neurones studied.

Baclofen also reduced the amplitude of the slow inward chloride tail current by a proportion similar to that of the sustained Ca current. For example, in the cell shown in figure 4D, baclofen reduced the slow current by 23% and the peak calcium current by 28%; the decrease was reversible for both currents. The suppression of the inward "tail" current by baclofen is similar to the depression of this current by adenosine in rat DRG neurones (Dolphin, Forda & Scott 1986).

Effects of baclofen and GABA on potassium currents

Potassium currents were studied in isolation by blocking Na and Ca currents. A variety of potassium currents have been reported in mammalian neurones; these include the delayed rectifier $I_{K(V)}$, a Ca activated K conductance $I_{K(Ca)}$ and a transient K current known as I_A. $I_{K(V)}$ was the most prominent

K current in the cat DRG neurones examined in this study and was often as large as 200 nA at +40 mV. Figure 6 shows typical traces and current-voltage plots for $I_{K(V)}$ in two cells. We found no evidence for a significant I_A in these neurones; steps from -90 mV to 0 mV did not activate an outward curren and no outward current was observed after a hyperpolarizing step to -120 mV from -50 mV. Both procedures should have revealed I_A if it were present (Adams, Brown & Constanti 1982, Belluzzi, Sacchi & Wanke 1985, Galvan & Sedlmeir 1984). We could find no evidence in our experiments for the anomalous rectifier reported by Mayer & Westbrook (1983) in cultured mouse dorsal root ganglion neurones. It is very difficult to separate any $I_{K(Ca)}$ from $I_{K(V)}$ as both are activated over the same voltage range. We consider that contribution from a Ca activated K conductance must be small, for the following reasons. Replacement of Ca with Mn or addition of 1 mM Cd^{2+} produced an increase in the total outward current, consistent with blocking a significant inward current and inconsistent with there being a large $I_{K(Ca)}$. The presence of a high concentration of internal EGTA (2 or 11 mM) might be expected to block a Ca activated potassium current. It seems however that the free calcium near the cell membrane was not well buffered, since we were able to record a Ca activated inward current (chloride current ?) under the same conditions. Figure 6 and table 2 show the effect of 100 µM baclofen and GABA on

activated K current. As can be seen there is no consistent effect of $GABA_B$ receptor activation on the K current with or without Mg ions. Similarly baclofen did not change the holding current, suggesting no effect on the resting K conductance.

(FIG. 6 AND TABLE 2 NEAR HERE)

DISCUSSION AND CONCLUSIONS

DRG neurones *in vitro* provide a convenient preparation for the application of voltage clamp techniques to adult mammalian nerve cell bodies. They are readily accessible and when the soma and adjacent axon are isolated, allow for excellent space clamp. We used this preparation to examine the effect of $GABA_B$ receptor activation on voltage activated conductances. Under our recording conditions, we observed six different conductances in these cells. The effects of TTX and Na replacement were consistent with there being two Na conductances, one blocked by TTX and the other insensitive to this toxin. Other workers have reported similar findings in sensory neurones (Gallego 1983, Yoshida, Matsuda & Samejima 1978). However, the TTX insensitive current in cat neurones is not blocked by calcium channel blockers, unlike that reported by Bossu & Feltz (1984). Two inward Ca currents were also apparent, the low threshold rapidly inactivating Ca current (Nowycky,Fox & Tsien 1984, Carbone & Lux 1984, Armstrong & Matteson 1985, Fedulova,Kostyuk & Veselovsky 1985), and the higher threshold slowly inactivating current. Outward K

currents were measured on depolarization to potentials greater than -20 mV, but no rigorous attempt was made to separate out the various components as has been done previously in mammalian neurones (eg Barrett & Barrett 1976, Freschi 1983). However our results suggested that transient I_A and $I_{K(Ca)}$, if normally present, are very small. Finally a Ca activated chloride current was observed as has been seen in egg cells (Barish 1983) cultured spinal neurones (Owen et al.1984), and cultured neonatal rat DRG (Mayer 1985).

Electrophysiological studies have shown that systemic and ionophoretic application of baclofen decrease excitatory neurotransmission in the spinal cord, both in vivo (Pierau & Zimmerman 1973, Fox et al. 1979, Curtis et al. 1981, Davies 1981) and in vitro (Ault & Evans 1981). Intracellular recording from motoneurones showed changes in postsynaptic membrane properties only with high concentrations of baclofen (Fox et al. 1979). However, Pierau & Zimmerman (1973) saw no changes in motoneurone properties with systemically applied baclofen, and suggested that the decrease in e.p.s.p. amplitude was due to an inhibitory action of baclofen on presynaptic primary afferent terminals. Curtis et al. (1981) and Curtis & Malik (1985) have shown that baclofen has no effect on transmitter release from excitatory spinal interneurones, cholinergic motor axon collaterals or from descending fibres in the dorsolateral funiculus (including the

rubrospinal, long propriospinal and corticospinal tracts). The results of Glavinovic (1979) also suggest that there is no significant effect of baclofen on transmitter release from motoneurone terminals. These results may imply that there is a differential distribution of $GABA_B$ receptors between central and peripheral neurones, which might reflect different mechanisms for reducing release of transmitter from terminals.

Previous authors have reported that GABA and baclofen decrease the duration of Ca action potentials in cultured chick (Dunlap & Fischbach 1978, Dunlap 1981) and rat DRG neurones (McBurney 1984; Schlichter et al. 1984). Spike measurements are potentially ambiguous due to the presence of potassium currents and the extremely labile nature of the underlying Ca conductance. Their duration depends critically on the frequency of stimulation (eg Neering & McBurney 1984) and on the membrane potential. In our experiments (unpublished observations) and those of others (eg Heyer & MacDonald 1982), changes in membrane potential as small as two millivolts were sufficient to cause profound alterations in Ca spike duration. We therefore used voltage clamp techniques to examine Ca and K conductances more reliably. Our results indicate that baclofen and GABA significantly reduced the amplitude of the sustained Ca current only when Mg ions were present in the external solution. Our findings are similar to those of Dunlap & Fischbach (1981) and Deisz & Lux (1985) who also observed that GABA reduced I_{Ca} in cultured chick DRG neurones. Cottrell &

Green (Physiological Society Proceedings, in press 1986) have found similar results in cultured mouse DRG neurones. It is interesting that the effects of $GABA_B$ agonists are potentiated by Mg ions, a finding which may complement the binding studies of Bowery et al. (1983) who have shown that Mg^{2+} potentiates the binding of baclofen to the $GABA_B$ receptor. Sine & Steinbach (1986) have reported that replacing Ca^{2+} with Mg^{2+} alters the binding of acetylcholine to its receptors. Mg ions also appear to be important in modulating responses to excitatory amino acids (Ault, Evans, Francis, Oakes & Watkins 1980), but the mechanism of action here has been shown to be at the level of the channel rather than at the receptor (Nowak, Bregestovski, Ascher, Herbet & Prochiantz 1984).

The results of Holz, Rane & Dunlap (1986), Dolphin, Forda & Scott (1986) and Dolphin & Scott (Physiological Society Proceedings in press, 1986) suggest that a second messenger is involved in $GABA_B$ receptor-mediated effects. The magnitude and variability of our results might be explained if important intracellular components were being removed from the cell with our recording procedure. Our preliminary experiments with intracellular electrodes also revealed a slight depression of calcium currents by GABA, but it is our experience that calcium currents are as labile with conventional intracellular techniques.

Activation of $GABA_B$ receptors in cat DRG neurones does

not lead to a change in either the resting or the voltage activated potassium conductances as has been suggested by Schlichter et al. (1984). Our results are therefore in contrast to those obtained in central neurones, where baclofen has been shown to hyperpolarize neurones (hippocampus: Newberry & Nicoll 1984;1985, dorsolateral septal nucleus: Gallagher, Stevens & Shinnick-Gallagher 1984, substantia nigra: Pinnock 1985), probably due to an increase in potassium conductance (Gahwiler & Brown 1985). Gahwiler and Brown observed no effect of baclofen on inward currents in cultured hippocampal neurones.

Although we observed only a modest effect of baclofen and GABA on the peak inward Ca current in cat DRG neurones, it is possible that baclofen could still produce its pharmacological effects by reduction of the presynaptic Ca influx if one accepts the following arguments. Firstly, this study measured the effects of activation of non-synaptic $GABA_B$ receptors, and if $GABA_B$ synapses exist on the primary afferent terminals within the spinal cord then it is probable that the density of receptors is much higher at the synapses than on the cell bodies. A higher density of receptors could cause a larger reduction of the Ca current in the terminals than we noted in the somata, thus reducing the evoked release of excitatory transmitter (Katz & Miledi 1965). Secondly, it appears that transmitter release is probably not directly proportional to Ca influx. Studies in accessible synapses such as the squid

giant synapse have revealed that postsynaptic responses (proportional to the amount of transmitter released) vary with the second (Augustine & Eckert 1984), or even the third power (Smith, Augustine & Charlton 1985) of presynaptic Ca current. A power function for transmitter release has also been suggested to account for the steep dependence of release on extracellular Ca concentration at the motor endplate (Jenkinson 1957; Dodge & Rahamimoff 1967; Katz & Miledi 1970). If a third power relation holds at primary afferent terminals in the spinal cord, then a reduction of I_{Ca} of 20% would reduce the postsynaptic response to about half of normal.

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Figure 1. Separation of conductances in an isolated DRG neurone. Currents were activated by a +70 mV step from -50 mV. The top trace shows the currents recorded in standard solution. There is an initial rapid inward current followed by a large outward current. The external solution was then changed to a "calcium current" solution. The middle trace was recorded during the exchange and the bottom trace upon completion. Potassium currents were completely suppressed by internal Cs^+ and external TEA-Br. Cell 82G, clamp switching rate 64 kHz, diameter 65 µm, holding potential -50 mV.

Figure 2. A. Current-voltage plot of peak inward Ca current, note the initial shoulder at potentials less than OmV. B. Sample current traces recorded at the potentials indicated. C. Voltage traces corresponding to the current traces illustrated. Cell 65K, clamp switching rate 64 kHz, diameter 65 µm, holding potential -50 mV throughout.

Figure 3. Illustration of the time course of the wash out of the calcium current in one cell. Each point is a single measurement obtained by stepping to +20 mV from -50 mV. Closed symbols control measurements, open symbols baclofen. Note that baclofen (100µM) had little or no effect on the calcium current in this cell. Cell 77T, clamp switching rate 60 kHz, diameter 80 µm.

Figure 4. Possible sources of Ca current contamination. A. Current-voltage plot before and after Ca^{2+} was replaced with Mn^{2+}. Block of the calcium current reveals the underlying nonspecific outward current, illustrated in B. I_{ns} shows voltage dependent activation but is slower to activate than the calcium current at similar potentials (see fig. 2B). Clamp switching rate 60 kHz, cell 65E, diameter 60 μm. C. Perfusion of 1 mM Cd^{2+} reversibly blocks I_{Ca}, and reveals I_{ns} in another cell. D. The trace illustrates the size and duration of the slow inward tail that was recorded in one cell, upon repolarization after a step to +20 mV from -50 mV. Clamp switching rate 64 kHz, cell 79D, diameter 35 μm, holding potential -50 mV throughout.

Figure 5. Baclofen (100μM) had no effect on the sustained (A) or the transient (B) calcium current in this cell with no external Mg^{2+}. Traces show single responses before, and during baclofen application superimposed. Calcium currents were activated by a 60 mV step from -50 mV (A), and a 70 mV step from -90 mV (B). Cell 65U, clamp switching rate 61 kHz, diameter 45 μm. C, shows I_{Ca} in response to a 70 mV step from -50 mV, before, during and after baclofen application, with 2.5 mM external Mg^{2+}. There is a clear reversible reduction in the peak inward calcium current, which was consistent over three applications (see table 1). Cell 74F, clamp switching rate 55 kHz, diameter 80 μm. D. Current-voltage plot from another cell, 82B. The solid circles show the control (1) and wash out (2). The current shows some rundown. The peak currents recorded during exposure to 100 μM baclofen are illustrated by the diamonds. 5mM Mg^{2+} present. Holding

Figure 6. A. Current-voltage plot of total potassium current, before (a) and during (b) exposure to 100 μM baclofen, with no external Mg^{2+}. Cell 62W, clamp switching rate 51 kHz. B. Sample traces recorded at -10, 10 and 30 mV from the cell illustrated in A , upper traces were recorded in control. C, shows a plot of peak potassium currents recorded before (a), during (b) and after baclofen application (c), with 5 mM external Mg^{2+}. D. Sample traces recorded at -20, 0 and 25 mV for cell 87R, illustrated in C. The inward tail currents on the repolarization are probably due to K^+ accumulation in the perineuronal space (see eg. Beluzzi et al. 1985). Clamp switching rate 65 kHz, diameter 65 μm, holding potential -50mV throughout.

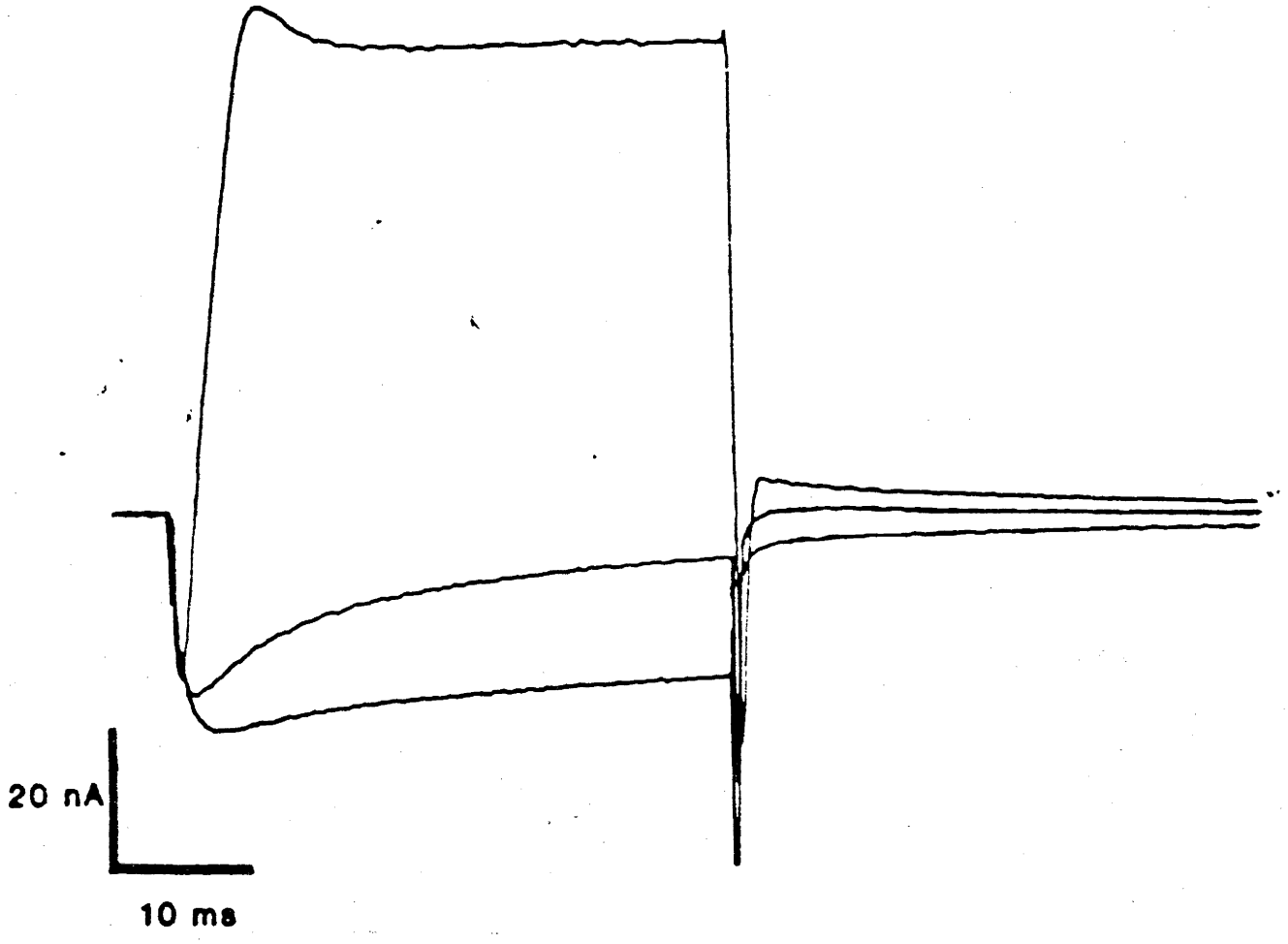
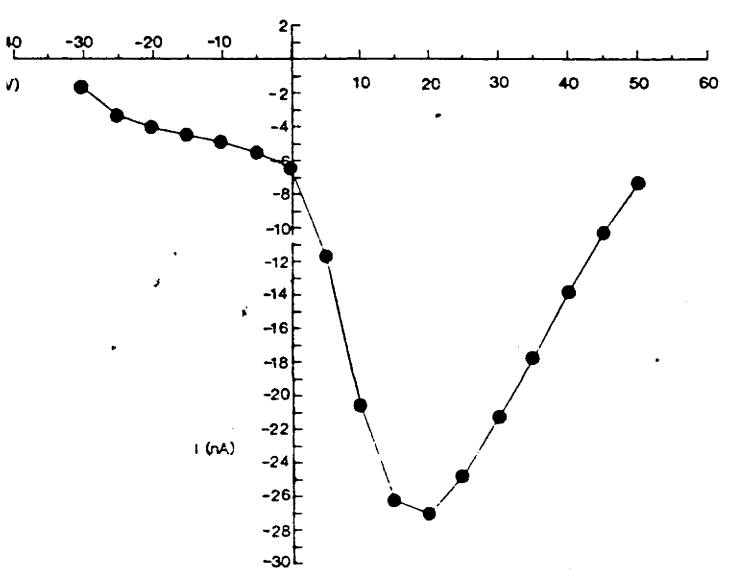
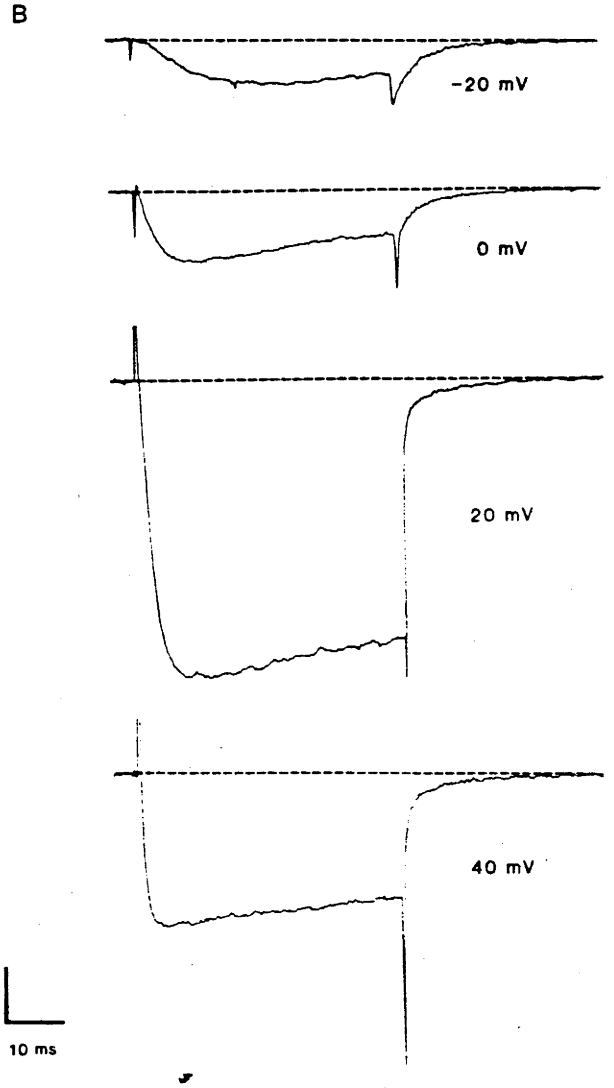


Figure 1


-30
-20
-10
10
20
30
40
50
60
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
-30
I (nA)


B
-20 mV
0 mV
20 mV
40 mV

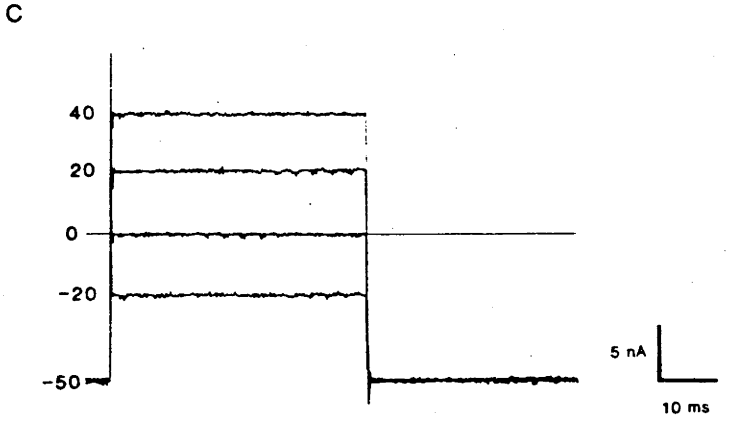

C
40
20
0
-20
-50
5 nA
10 ms

FIGURE 2-

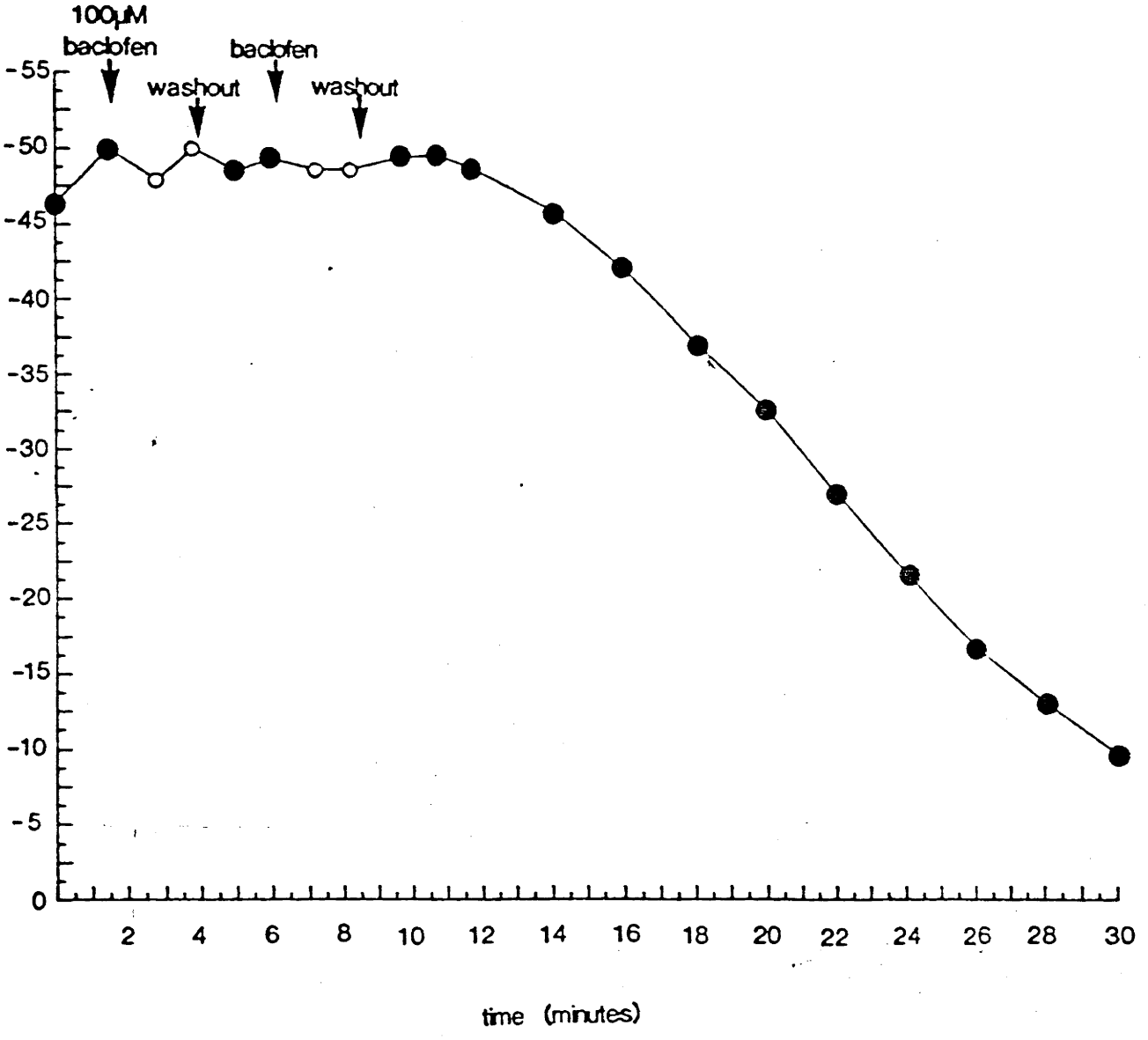
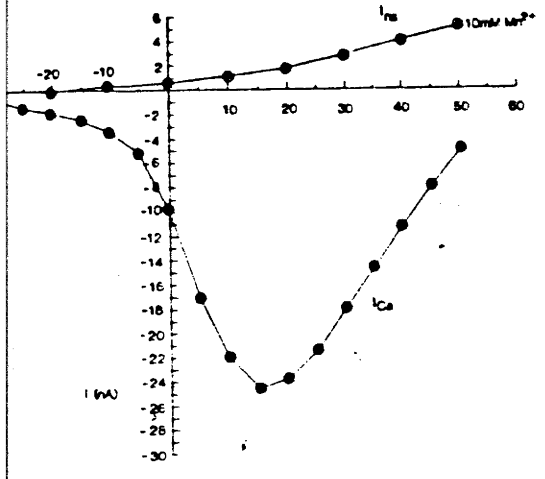
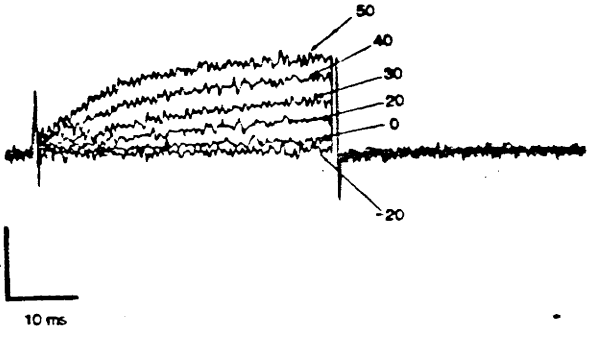
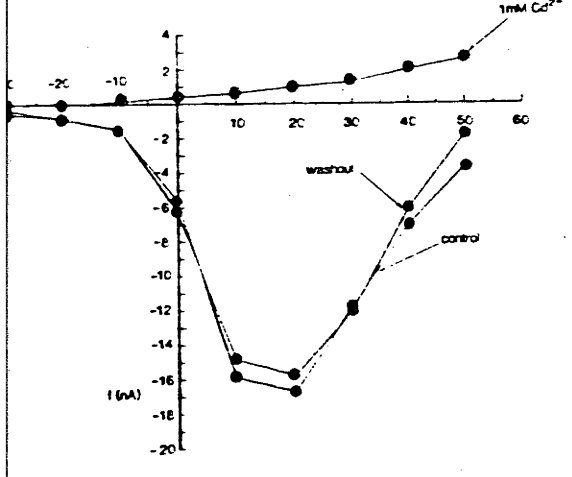

100μM
baclofen
baclofen
washout
washout
-55
-50
-45
-40
-35
-30
-25
-20
-15
-10
-5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
time (minutes)

FIG. 3.


I_{ns}
10mM Mn^{2+}
I_{Ca}
I (nA)

B


50
40
30
20
0
-20
4nA
10 ms


1mM Cd^{2+}
washout
control
I (nA)

D

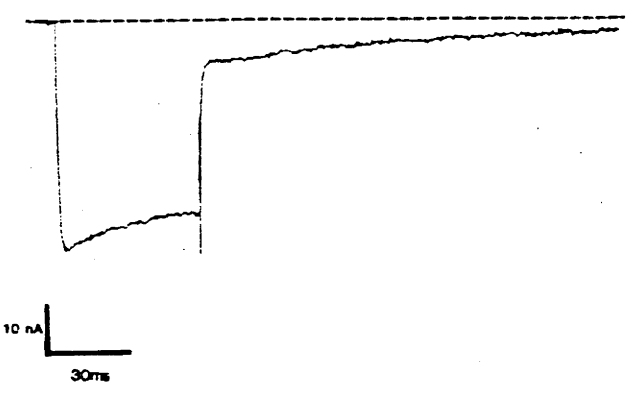
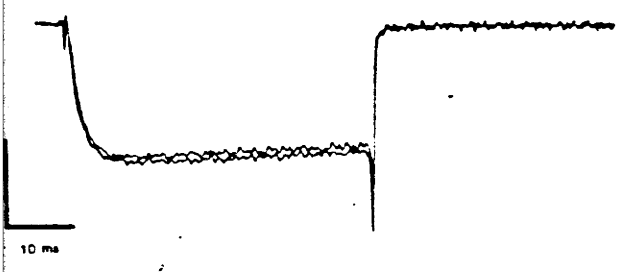
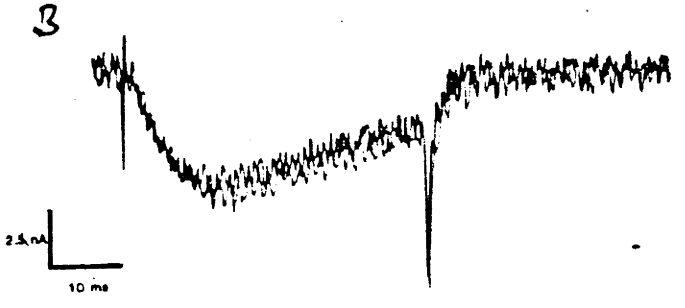
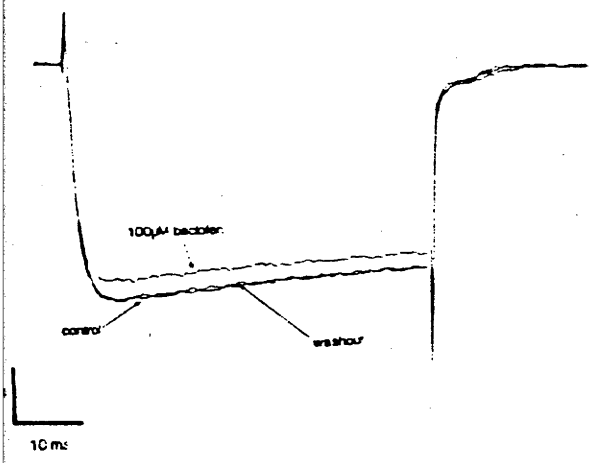

10 nA
30ms

FIG. 4.


10 ms


B
2.5 nA
10 ms


100μM baclofen
control
washout
10 ms

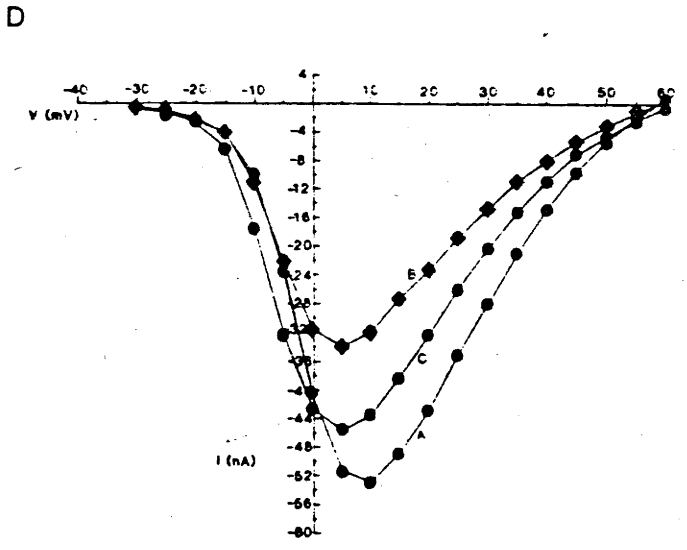
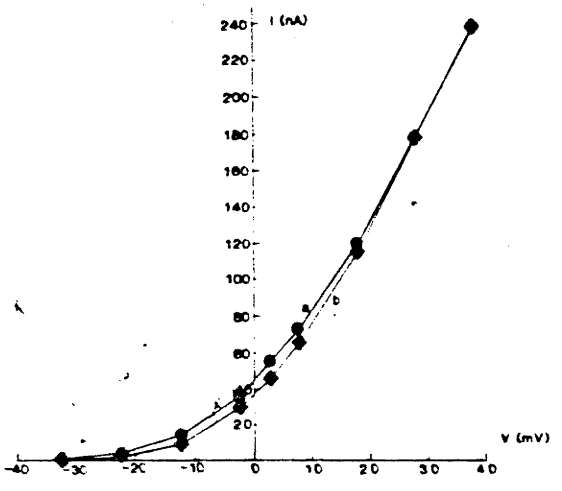
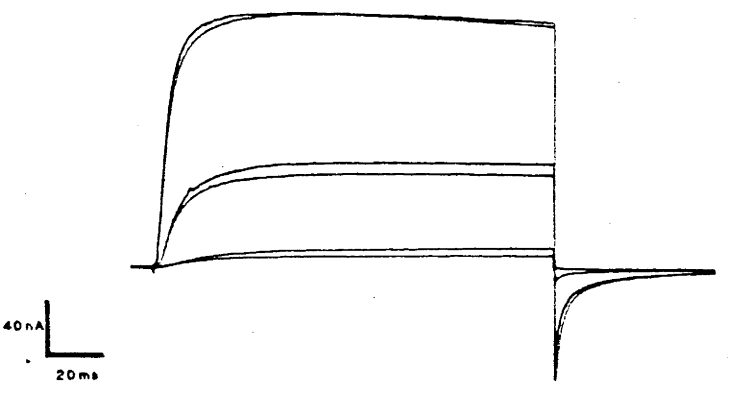
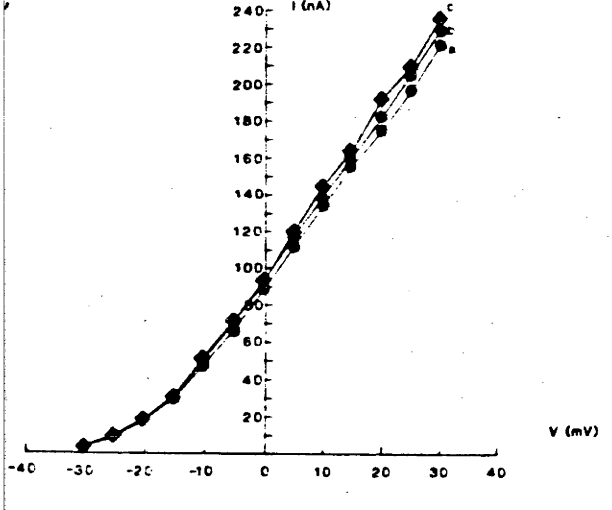

D
V (mV)
I (nA)
A
B
C

FIG. 5.


I (nA)
240
220
200
180
160
140
120
100
80
60
40
20
a
b
c
V (mV)
-40
-30
-20
-10
0
10
20
30
40

B


40 nA
20 ms


I (nA)
240
220
200
180
160
140
120
100
80
60
40
20
c
b
a
V (mV)
-40
-30
-20
-10
0
10
20
30
40

D

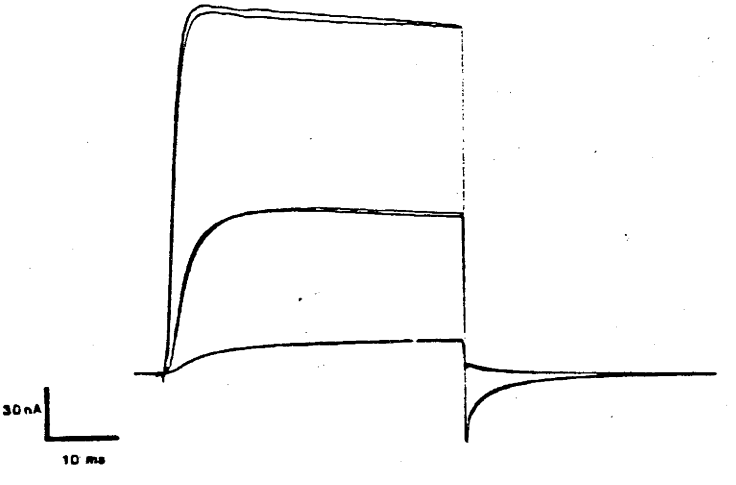

30 nA
10 ms

FIG. 6.

TABLE 1

Depression of peak inward calcium current by 100 μM baclofen or GABA. In order to allow for the effects of rundown, control values represent the average of peak I_{Ca} before and after the application of agonist. Values in agonist are usually the mean of two measures one minute apart.

Peak Current (nA).

Cell	Control	Baclofen or GABA (G)	% change
10 mM Ca, 0 mM Mg.			
68D	9.9	9.6	-3.0
68F	29.7	28.3	-4.7
69C	19.1	17.4 (G)	-8.9
69G	9.1	9.1 (G)	0.0
70C	5.4	4.9 (G)	-9.2
		Mean +- Std err.	-5.2 +- 1.8
9 mM Ca, 1 mM Mg.			
77A	6.6	6.0	-9.0
"	6.0	5.5	-8.3
77G	32.8	23.1	-29.6
77K	12.0	11.6	-3.3
"	11.0	10.8	-1.8
77N	24.2	21.3	-11.9
77T	49.2	49.0	-0.4
"	49.4	48.6	-1.6
		Mean +- Std err.	-8.2 +- 3.4
7.5 mM Ca, 2.5 mM Mg.			
73D	13.5	5.5	-59.2
"	12.4	9.1	-26.6
"	13.1	10.4	-20.6
73F	14.2	10.9	-23.2
74A	30.0	27.1	-9.7
74F	42.2	39.4	-6.6
"	44.1	40.6	-7.9
"	42.0	38.0	-9.5
74H	29.9	25.6	-14.4
"	31.0	27.8	-10.3
"	25.6	19.6 (G)	-23.4
74O	13.6	10.9	-19.8
"	11.4	9.1 (G)	-20.2
		Mean +- Std err.	-19.3 +- 3.8
5 mM Ca, 5 mM Mg.			
79A	5.1	3.5	-31.4
"	4.7	2.9	-38.3
79B	30.8	12.4	-59.7
"	23.6	16.6	-29.7
79C	44.1	31.6	-28.3
79M	29.8	26.2	-12.1
82B	48.2	31.9	-33.8
87E	43.2	36.2	-16.2
"	42.2	37.0	-12.3
87M	9.8	7.8	-20.4
		Mean +- Std err.	-28.2 +- 4.5

TABLE 2

Effect of 100μM baclofen or GABA on the peak potassium currents. The control values are mean measurements before and after the agonist.

Peak Current (nA).

Cell	Control	Baclofen or GABA (G)	% change
5 mM Ca, 0 mM Mg.			
63C	83.6	86.8	3.8
71D	62.8	65.2 (G)	3.8
71F	76.8	76.2 (G)	-0.8
"	60.5	58.3	-3.7
71J	130.6	127.7 (G)	-2.2
		Mean +- Std err.	0.2 +- 1.5
2.5 mM Ca, 2.5 mM Mg.			
75E	164.0	151.0	-7.9
"	184.0	151.0	-17.9
"	174.5	158.0 (G)	-9.5
"	151.3	144.1 (G)	-4.8
75N	41.9	39.2	-6.4
"	40.9	43.0	5.1
76C	215.5	206.1	-4.3
"	208.1	191.9	-8.0
76G	208.5	167.6	-19.6
"	202.9	174.2	-14.1
76I	215.3	214.7	-0.3
		Mean +- Std err.	-8.0 +- 2.2
5 mM Ca, 5 mM Mg.			
87Q	178.3	192.2	7.8
87W	205.5	209.4	1.9
87Z	55.3	49.8	-9.9
		Mean +- Std err.	-0.1 +- 5.2

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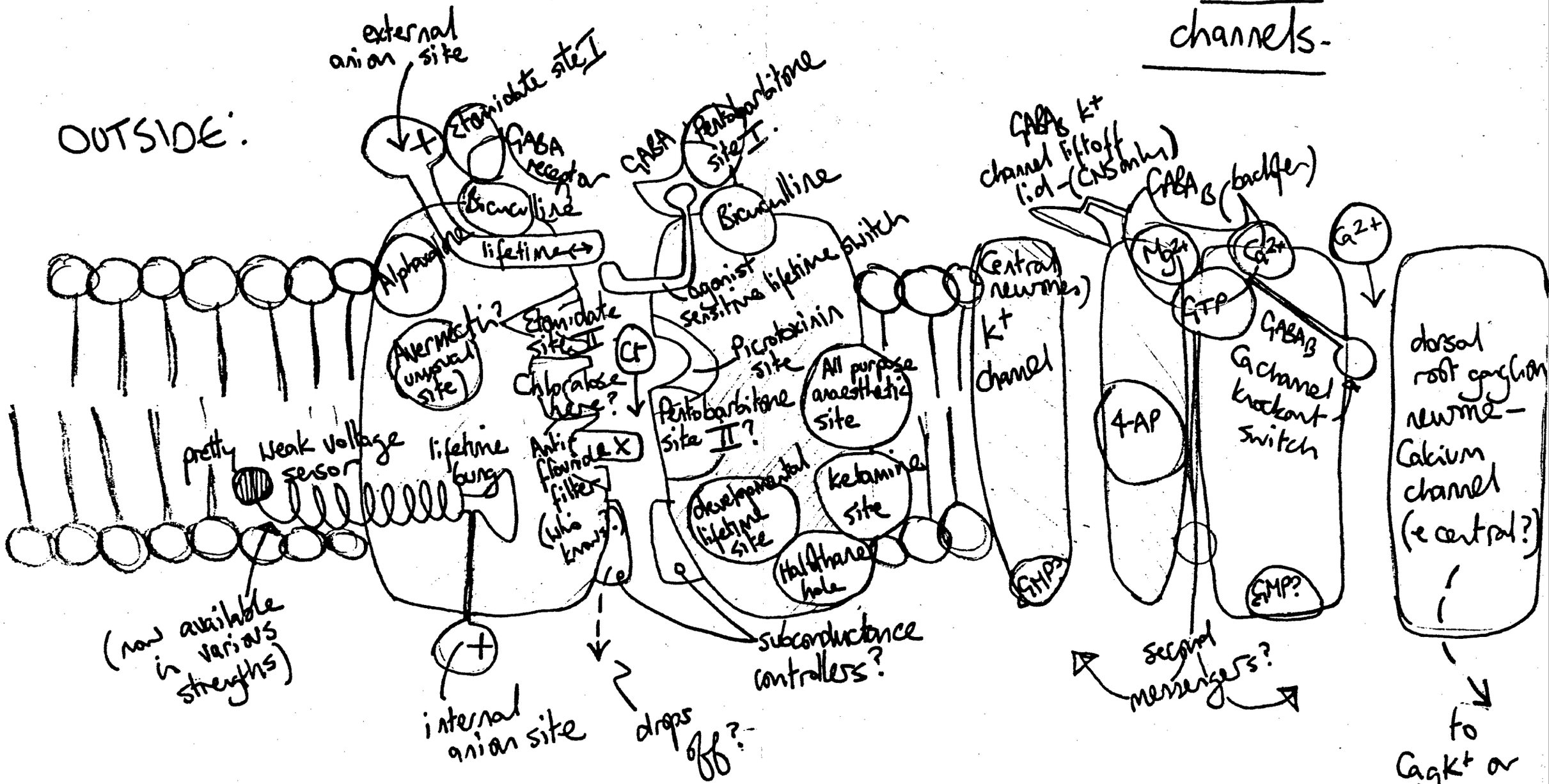
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$GABA_A$-chloride channel



$GABA_B$ channels-

(simplified scheme, Nov. 1985)