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Possible NMDA antagonist properties of drugs that affect high pressure neurological syndrome

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1 Previous studies have suggested that a series of drugs modelled on part of the strychnine molecule interfere with the development of high pressure neurological syndrome (HPNS) and it was presumed that this effect was via an action on inhibitory glycinergic transmission. We have now used the rat hippocampal slice preparation to examine the possibility that some of these drugs might instead have an action at the strychnine-insensitive (SI) glycine binding site associated with the NMDA receptor.

2 D-2-Amino-5-phosphonovalerate (AP5) and 7-chlorokynurenate (7CK) had no significant effect on the height of the population spike recorded from the CA1 region in 1 mM Mg^{2+} medium, but both blocked the multiple population spikes recorded in Mg^{2+}-free medium. The effect of 7CK, but not AP5, was reversed by 200 μM D-serine which is consistent with the known antagonist action of 7CK at the SI-glycine site.

3 A derivative of benzimidazole, which shows the clearest structural similarities to known SI-glycine site antagonists and ameliorates HPNS, mirrored the effects of 7CK although it was considerably less potent.

4 Gramine, which exacerbates HPNS, significantly increased the number of population spikes evoked in Mg^{2+}-free medium.

5 Mephenesin, which is the most potent known drug in ameliorating HPNS, had no significant effect on the response recorded in 1 mM Mg^{2+} and significantly reduced the number of population spikes recorded in Mg^{2+}-free medium, but this effect was only partially reversed by the addition of D-serine.

6 The results are consistent with the benzimidazole derivative, but not gramine, being an antagonist at the SI-glycine receptor. The results with mephenesin are equivocal but leave open the possibility that some of the drugs which are effective against HPNS act via an effect on excitatory NMDA receptor-mediated transmission, rather than on inhibitory glycine-mediated transmission.

Keywords: High pressure neurological syndrome; N-methyl-D-aspartate; glycine; strychnine-insensitive glycine site; mephenesin; gramine

Introduction

High pressure neurological syndrome (HPNS) describes the collection of symptoms including tremor and convulsions that are observed when animals or man are exposed to high barometric pressures (Halsey, 1982). Previous studies have shown that strychnine acts strictly additively with pressure in producing convulsions in mice (Bowser-Riley *et al.*, 1988) and that a series of drugs with molecular structures related to strychnine affect the threshold pressure for producing convulsions (Bowser-Riley *et al.*, 1989b). These observations led to the theory that increased hydrostatic pressure depresses inhibitory glycinergic synaptic transmission and that the drugs tested exert their effects via an action at the strychnine-sensitive glycine receptor.

More recently we observed that several of these drugs also show structural similarities with drugs known to be active at the strychnine-insensitive (SI) glycine binding site which is associated with the NMDA receptor (Thomson, 1990; Kemp & Leeson, 1993). This presents the possibility that the drugs which affect HPNS do so not by an action on inhibitory glycinergic transmission but by modulating excitatory NMDA receptor-mediated transmission.

As a preliminary way of testing this hypothesis we have examined the effects of a number of drugs on extracellular population spikes recorded in rat hippocampal slices. Under normal experimental conditions stimulation of the Schaffer collateral-commissural fibres at a low frequency evokes a single population spike recorded in the cell body region of the CA1 neurones. NMDA antagonists do not reduce the amplitude of the population spike and therefore the underlying excitatory postsynaptic potential (e.p.s.p.) under these conditions is thought to be mediated almost entirely by non-NMDA receptors (Collingridge *et al.*, 1983). However, perfusion with Mg^{2+}-free medium unblocks the NMDA gated channels so that stimulation evokes multiple population spikes that are blocked by NMDA antagonists (Coan & Collingridge, 1985). If the antagonist acts competitively at the glycine site then its efficacy is reduced by adding increased concentrations of glycine or the more selective agonist, D-serine (Bashir *et al.*, 1990). A profile showing (a) no effect on the single population spike evoked in normal medium, (b) blockade of the multiple population spikes evoked in Mg^{2+}-free medium and (c) a reduced effect in the presence of added D-serine, would therefore be consistent with that of an NMDA antagonist acting at the SI-glycine site.

In the present study we have chosen to investigate three test drugs; (i) a benzimidazole derivative which shows the clearest structural similarities to known SI-glycine site antagonists and is also effective against HPNS (Helliwell, 1990); (ii) gramine which has the opposite effect and facilitates HPNS (Bowser-Riley *et al.*, 1989b) and (iii) mephenesin which is the most effective drug against HPNS (Bowser-Riley *et al.*, 1989b). We have also compared the effects of these drugs with those of the competitive NMDA antagonist D-2-amino-5-phosphonovalerate (AP5), and a known antagonist at the SI-glycine site, 7-chlorokynurenic acid (7CK). Some of these results have previously been published in abstract form (Shuker *et al.*, 1992).

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Methods

Transverse rat hippocampal slices (400 μm thick) were cut and maintained in a recording chamber at the interface between a humidified oxygen enriched atmosphere and a modified Krebs solution. The CA3 region of the slice was routinely removed to prevent the propagation of spreading depression which often originates in this area. The composition of the Krebs was (mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_2$ 1, D-glucose 10, which was continuously bubbled with 95% O_2/5% CO_2, maintained at 30°C, and perfused through the chamber at approximately 2 ml min^{-1}. Mg^{2+}-free Krebs solution consisted of the same solution with no added $MgSO_4$. The Schaffer collateral-commissural pathway was stimulated with bipolar silver electrodes and extracellular population spikes were recorded from the cell bodies of the CA1 region by use of 3 M NaCl filled glass electrodes (resistance approx. 5 MΩ). AP5, D-serine and 7CK (all from Tocris Neuramin, U.K.) were made up as 1000 × concentrated stock solutions and diluted into the perfusing medium, whereas gramine (Sigma), mephenesin (Sigma) and the benzimidazole derivative (HCl salt from Dr J. Lewis, University of Aberdeen Chemistry Department) were all dissolved directly in the Krebs solution. The structures of these three compounds are shown in Figure 1. Slices were always left for at least 60 min after changing to Mg^{2+}-free solution, or to D-serine containing solution, to allow full equilibration. Where possible, to exclude the effects of inter slice variation, the effects of the test drug were examined under the three conditions (i.e. in 1 mM Mg^{2+} medium, in Mg^{2+}-free medium, and in Mg^{2+}-free medium in the presence of added D-serine) on the same slice. In the case of mephenesin, where two doses of the test drug were used, this usually made the experiments impractically long. Therefore, in most instances, on any one slice the effect of mephenesin in 1 mM Mg^{2+} ws compared with that in Mg^{2+}-free medium, *or* the effect in Mg^{2+}-free medium was compared with that in Mg^{2+}-free medium with added D-serine. Data in the text are expressed as mean ± s.e.mean and tests of statistical significance were made by a paired Wilcoxon matched pairs test on the original data.

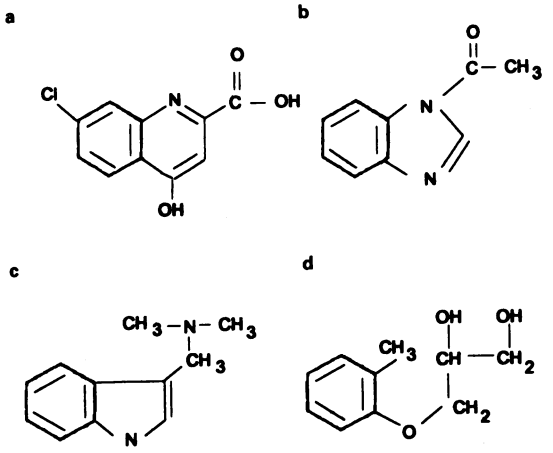

Figure 1 Structures of the compounds used in the present study: (a) 7-chlorokynurenic acid (7CK); (b) benzimidazole derivative; (c) gramine; (d) mephenesin.

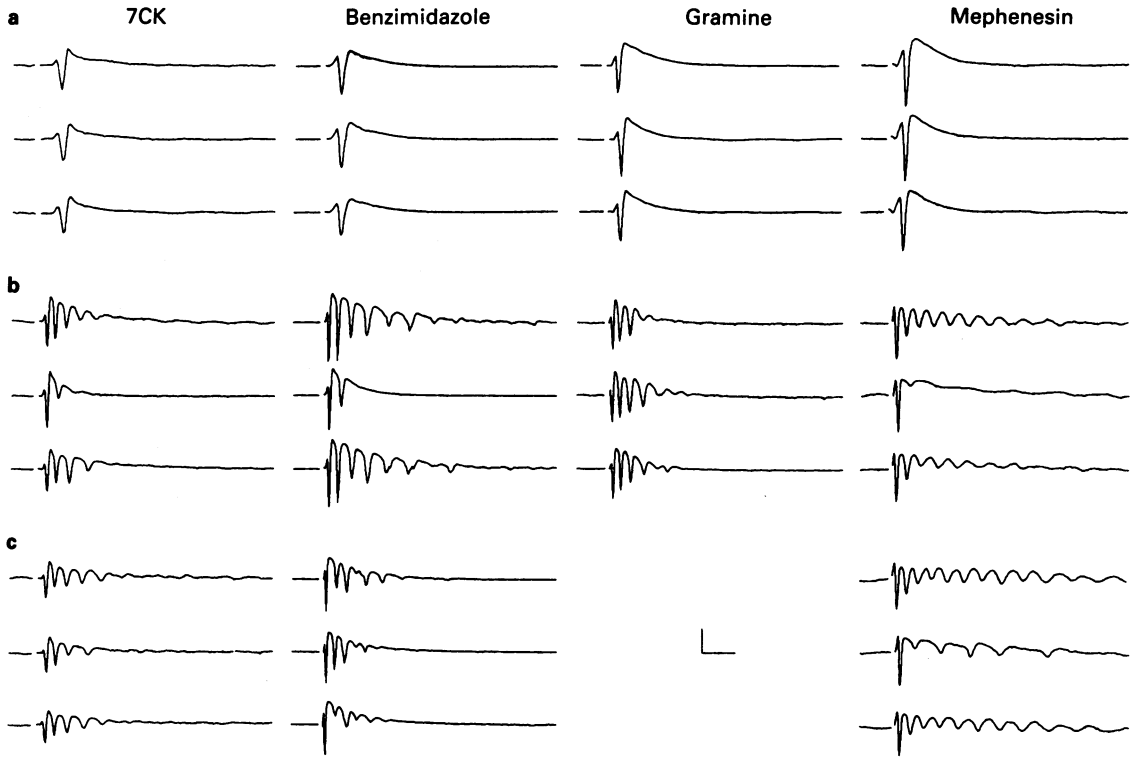

Figure 2 Representative traces showing the effects of a single dose of, 7-chlorokynurenic acid (7CK) (40 μM), the benzimidazole derivative (2 mM), gramine (500 μM), and mephenesin (1 mM) on synaptic responses recorded in 1 mM Mg^{2+} (a); Mg^{2+}-free medium (b); and in Mg^{2+}-free medium containing 200 μM D-serine (c). Each set of 3 traces shows control, drug and wash responses. Each drug was tested on 1 slice under all three perfusing conditions, except for mephenesin where the responses in 1 mM Mg^{2+} medium come from a separate slice. Each trace is the average of 3–5 consecutive recordings and stimulation artifacts have been blanked for clarity. Scale bar represents: (a) 10 ms; (b) and (c) 20 ms; 7CK, 2 mV; benzimidazole derivative, 10 mV; gramine, 5 mV; mephenesin, 2 mV.

Results

Effects of AP5 and 7CK

Stimulation in 1 mM Mg^{2+} medium evoked a single population spike 4–13 mV in amplitude and this was not significantly affected by perfusion of 20 μM AP5 (control: 8.4 ± 1.5, AP5: 7.8 ± 1.5 mV, $n = 5$) or by 20 or 40 μM 7CK (7.1 ± 0.4 to 6.2 ± 0.2 mV, $n = 4$ (2 each at 20 μM and 40 μM) Figures 2 and 3). Stimulation in Mg^{2+}-free medium evoked between 2 and 16 population spikes and these were significantly blocked by both 20 μM AP5 (5.1 ± 1.1 and 1.4 ± 0.3 spikes, $n = 7$) and by 20 or 40 μM 7CK (6.0 ± 0.6 to 2.2 ± 0.6 spikes, $n = 4$, Figures 2 and 3) with good recovery after washout. Over the course of the experiments we noticed that D-serine tended to increase the number of population spikes recorded in Mg^{2+}-free medium, but with a dose of 200 μM D-serine this was not statistically significant in any of the groups tested. The multiple population spikes evoked in Mg^{2+}-free medium and 200 μM D-serine were still effectively blocked by 20 μM AP5 (10.0 ± 2.0 to 2.5 ± 1.5 spikes, $n = 2$), but not by 20 or 40 μM 7CK (7.0 ± 0.6 to 5.7 ± 1.1 spikes, $n = 4$, Figures 2 and 3).

Effects of the benzimidazole derivative

Perfusion of the benzimidazole derivative (2 mM) had no significant effect on the height of the single population spike recorded in 1 mM Mg^{2+}-medium (11.9 ± 1.6 to 11.5 ± 1.5 mV, $n = 6$). In the same group of slices the derivative significantly reduced the number of population spikes evoked in Mg^{2+}-free medium (9.3 ± 1.2 to 4.0 ± 0.8 spikes, $n = 6$) in a reversible manner. However, also in the same group of slices, in the presence of D-serine the effect of the derivative was attenuated such that it was no longer statistically significant (8.2 ± 1.3 to 6.8 ± 1.0 spikes, $n = 6$, Figures 2 and 3).

Effects of gramine

Perfusion of gramine (250 and 500 μM, cumulative doses) had no significant effect on the height of the single population spike evoked in 1 mM Mg^{2+}-medium (6.3 ± 0.9 to 6.8 ± 1.0 to 6.9 ± 1.1 mV, $n = 9$). However, gramine (250 and 500 μM) consistently increased the number of population spikes evoked in Mg^{2+}-free medium and this was statistically significant at the 250 μM dose (3.6 ± 0.8 to 5.7 ± 1.2 to 5.3 ± 0.6, $n = 8$, Figures 2 and 3). Little or no recovery from this effect of gramine was observed over the course of 1 h. The effect of gramine in Mg^{2+}-free medium with added D-serine was not examined.

Effects of mephenesin

Perfusion of mephenesin (0.5 and 1 mM, cumulative doses) in 1 mM Mg^{2+} medium had no significant effect on the height of the single population spike (9.4 ± 1.0 to 8.1 ± 0.7 to 7.9 ± 0.8 mV, $n = 7$, Figures 2 and 3), however in the same 7 slices perfused with Mg^{2+}-free medium, mephenesin significantly and reversibly reduced the number of population spikes evoked (6.3 ± 1.4 to 3.3 ± 0.9 to 1.2 ± 0.4 spikes, Figures 2 and 3). In a separate group of 8 slices the same 2 doses of mephenesin significantly reduced the number of population spikes evoked in Mg^{2+}-free medium (8.0 ± 0.7 to 5.1 ± 1.2 to 3.0 ± 0.7 spikes), and had a smaller, but still statistically significant effect, in the presence of 200 μM D-serine (10.0 ± 1.6 to 8.0 ± 1.4 to 6.3 ± 1.0 spiked). In this group of 8 slices, as in all other groups, the increase in number of population spikes induced by perfusion of D-serine alone was not statistically significant (10.0 ± 1.6 compared to 8.0 ± 0.7 spikes); however, the number of spikes recorded in 1 mM mephenesin was significantly higher in the presence, as compared to the absence, of D-serine (6.3 ± 1.0 compared to 3.0 ± 0.7 spikes). There is therefore some evidence for a partial reversal of the effect of mephenesin by D-serine.

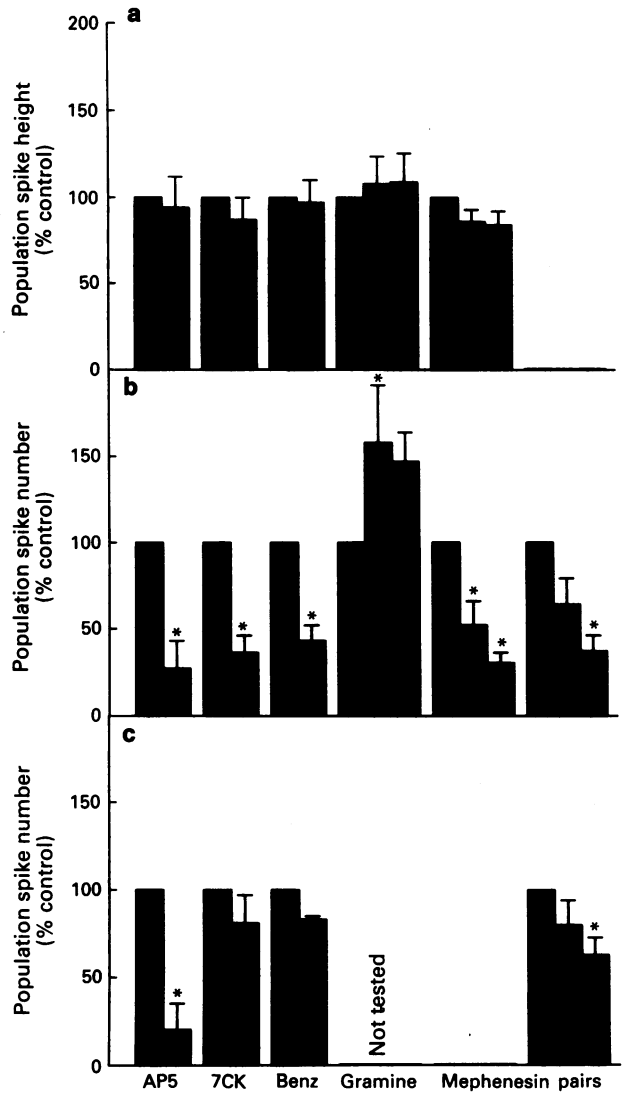

Figure 3 Effects of D-2-amino-5-phosphonovalerate (AP5, 20 μM), 7-chlorokynurenate (7CK, 20 or 40 μM), the benzimidazole derivative (2 mM), gramine (250 and 500 μM) and mephenesin (0.5 and 1 mM) on the population spike amplitude recorded in 1 mM Mg^{2+} (a), the number of population spikes evoked in Mg^{2+}-free medium (b), and the number of population spikes evoked in Mg^{2+}-free medium containing 200 μM D-serine (c). In order to aid comparison, the data are expressed as percentage of control, calculated from the figures given in the text. Each pair (or triplet) of histograms represents the normalized control response, together with the response in the presence of 1 (or 2) concentrations of drug. Bars denote s.e.mean. *Indicates $P < 0.05$, as determined by the Wilcoxon matched pairs test on the original data.

Discussion

The results with AP5 and 7CK agree with those of previous authors (Bashir *et al.*, 1990) and confirm that our preparation reveals the expected profile for known competitive NMDA antagonists and SI-glycine site antagonists respectively.

The effects of the benzimidazole derivative closely mirrored those of 7CK (see Figure 3) and are therfore consistent with the benzimidazole derivative having an antagonist action at

the SI-glycine site, although it was considerably less potent than 7CK in our preparation.

The results with mephenesin were somewhat equivocal. Like 7CK and the benzimidazole derivative, mephenesin selectively reduced the number of population spikes recorded in Mg^{2+}-free medium, but the 1 mM dose still had a significant effect in the presence of 200 μM D-serine. However, a comparison of the number of spikes evoked in the presence of 1 mM mephenesin with or without D-serine, shows that significantly more spikes were evoked in the presence of D-serine and that this could not be explained by an action of D-serine on the control responses. Of course, not all drugs that are effective against HPNS need work by the same mechanism; mephenesin is used as a centrally acting muscle relaxant, which may indicate an action at $GABA_B$ receptors, and this could explain some of its effects against HPNS. We are also aware of one report that the weak NMDA antagonist properties of mephenesin are not reversed by D-serine in a grease-gap preparation of the rat spinal cord (Pralong *et al.*, 1992).

In our experiments gramine significantly increased the number of population spikes evoked in Mg^{2+}-free medium. Therefore, this drug, which lowers the threshold pressure for convulsions, clearly did not show the profile expected of an NMDA antagonist at any site.

Structure-activity relationships

Structure activity relationships triggered this study; the test drugs used being modelled on the strychnine molecule and in particular a negatively charged group spaced 4.5 angstroms from an aromatic group (Bowser-Riley *et al.*, 1989b), but it transpired that this shows marked similarities to the more recently proposed antagonist pharmacophore at the SI-glycine site associated with the NMDA receptor (Moselley *et al.*, 1992; Kemp & Leeson, 1993). The benzimidazole derivative closely fits the model whereas gramine clearly does not. Compared to the other drugs used, mephenesin is conformationally unrestricted, and one of the possible conformations (Figure 1) approximates to the model, but in this case the negatively charged group lies too far away from the aromatic group. This may explain the equivocal results obtained in our experiments. The obvious similarities between the strychnine-sensitive and SI-glycine sites make it likely that some drugs (e.g. glycine) will have activity at both. In the context of ameliorating HPNS, a combined action of agonist at the inhibitory strychnine-sensitive site, and antagonist at the excitatory SI-site, would theoretically be most effective.

Mechanism of effect of pressure

The initial observation that the effect of strychnine was strictly additive with pressure led to the theory that increased hydrostatic pressure might have some effect on inhibitory glycinergic transmission (Bowser-Riley *et al.*, 1988). However, the present experiments suggest that some of these drugs might instead act at the excitatory SI-glycine site associated with the NMDA receptor. How can this be reconciled with the observation that strychnine and pressure act strictly additively? There are two possibilities that might account for a common mechanism between the two treatments: (i) There is evidence that increased hydrostatic pressure directly potentiates NMDA receptor-mediated responses (Fagni *et al.*, 1987; Zinebi *et al.*, 1988). In addition, NMDA receptor gated channels are indirectly regulated by the state of GABAergic (and presumably glycinergic) inhibition which affects the voltage-dependent Mg^{2+} block of the channels (Nowak *et al.*, 1984; Collingridge *et al.*, 1988). Thus the common mechanism might be the opening of NMDA receptor gated channels by pressure acting directly on the NMDA receptor gated channels, and by strychnine acting on the state of glycinergic inhibition. (ii) Alternatively, high hydrostatic pressure might, as initially suggested, affect glycinergic inhibition itself. Here the common mechanism would still be the opening of NMDA receptor-gated channels, but in this case by pressure and strychnine both blocking the glycinergic inhibition.

Both these hypotheses predict that NMDA antagonists would be effective against HPNS, and indeed they are (Meldrum *et al.*, 1983; Milan *et al.*, 1990). However, the facts that bicuculline (Bowser-Riley *et al.*, 1989a) and picrotoxin (Bowser-Riley *et al.*, 1988) do *not* act additively with pressure, and that pressure selectively inhibits currents evoked by glycine (as opposed to GABA or kainate (Daniels *et al.*, 1993)), both favour the second hypothesis; that pressure reduces glycinergic inhibition and hence indirectly regulates the NMDA gated channel.

The results presented here suggest that an antagonist action at the SI-glycine site might have a role to play in the HPNS protecting effects of some of these drugs. However, none of the results using this preparation can be considered conclusive, but only consistent or inconsistent with an action at the SI-glycine site. For instance, due to the time course of the excitatory and inhibitory components of the synaptic response, it is possible that a drug which potentiates $GABA_B$ receptor-mediated responses would have little effect on the single population spike evoked in Mg^{2+} containing medium, but would affect the multiple population spikes evoked in Mg^{2+}-free medium. These pilot studies have, however, encouraged us to set up a grease-gap recording preparation in which to study more quantitatively the pharmacological actions of these and other drugs that interfere with HPNS.

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