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## Effect of Pressure on the Release of Radioactive Glycine and $\gamma$ -Aminobutyric Acid from Spinal Cord Synaptosomes

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**Abstract:** Exposure to high hydrostatic pressure produces neurological changes referred to as the high-pressure nervous syndrome (HPNS). Manifestations of HPNS include tremor, EEG changes, and convulsions. These symptoms suggest an alteration in synaptic transmission, particularly with inhibitory neural pathways. Because spinal cord transmission has been implicated in HPNS, this study investigated inhibitory neurotransmitter function in the cord at high pressure. Guinea pig spinal cord synaptosome preparations were used to study the effect of compression to 67.7 atmospheres absolute on [ $^3$ H]glycine and [ $^3$ H] $\gamma$ -aminobutyric acid ([ $^3$ H]GABA) release. Pressure was found to

exert a significant suppressive effect on the depolarization-induced calcium-dependent release of glycine and GABA by these spinal cord presynaptic nerve terminals. This study suggests that decreased tonic inhibitory regulation at the level of the spinal cord contributes to the hyperexcitability observed in animals with compression to high pressure. **Key Words:** Glycine efflux— $\gamma$ -Aminobutyric acid release—High-pressure nervous syndrome—Amino acid transport—Spinal cord. Gilman S. C. et al. Effect of pressure on the release of radioactive glycine and  $\gamma$ -aminobutyric acid from spinal cord synaptosomes. *J. Neurochem.* **49**, 1571–1578 (1987).

High hydrostatic pressure is recognized as the basic etiologic factor underlying nervous system changes referred to as the high-pressure nervous syndrome (HPNS), and as such is a major limiting factor in manned diving operations. HPNS is observed when human divers and experimental animals are exposed to pressures >27 atmospheres absolute (ATA).

The symptoms of HPNS are similar to other dysfunctional brain states such as the metabolic encephalopathies (e.g. hepatic encephalopathy, uremic encephalopathy, water intoxication, hypernatremia) (Hallenbeck, 1981). The disorder is progressive and appears in several stages. Initial manifestations are usually neuromuscular tremors succeeded by myoclonic episodes, EEG changes, and finally convulsions, as demonstrated in animals (Hunter and Bennett, 1974).

Several researchers have reported that exposure to high pressure depresses synaptic transmission at the neuromuscular junction, sympathetic ganglion, and other peripheral synapses (Kendig et al., 1975; Henderson et al., 1977; Athey and Akers, 1978; Wann et

al., 1980; Ashford et al., 1982). Unfortunately, little is known about the effects of pressure on synaptic transmission in the CNS. In a recent study (Gilman et al., 1986b), we reported that pressure reduced the calcium-dependent release of the inhibitory neurotransmitter,  $\gamma$ -aminobutyric acid (GABA), by central presynaptic nerve terminals isolated from the cerebral cortex. This suggests that disinhibition, or the loss of tonic regulation by central inhibitory neuronal systems, may be etiologically significant in HPNS.

Kaufmann et al. (1979) have demonstrated HPNS symptoms caudal to spinal transections, indicating that the syndrome is not localized exclusively in structures rostral to the spinal cord. Because glycine apparently functions as the major inhibitory transmitter of the spinal cord activity, and is primarily active in the ventral horn (Aprison et al., 1975; Davidoff and Adair, 1976), this study investigated the extent to which the release of [ $^3$ H]glycine by synaptic vesicles isolated from the guinea pig spinal cord is affected by exposure to high pressure.

Richard and Little (1985) recently reported that

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**Abbreviations used.** ATA, atmospheres absolute; GABA,  $\gamma$ -aminobutyric acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPNS, high-pressure nervous syndrome.

exposure to 50 atm of helium had no effect on the evoked release of [ $^3\text{H}$ ]GABA from hemisected frog spinal cord preparations, whereas at 100 atm this was increased. Therefore, we also studied the effect of increased pressure on GABA release at presynaptic nerve terminals from the spinal cord.

The presynaptic vesicle (synaptosome) preparation was selected because it retains many metabolic and transport properties of the presynaptic nerve ending in situ (Blaustein et al., 1972; Levy et al., 1973; Cotman et al., 1976), responding to depolarization with a release of neurotransmitter substances (deBelleruche and Bradford, 1973; Blaustein et al., 1977). Also, synaptosomes offer an advantage as an experimental model for studying amino acid neurotransmitter release, eliminating multineuronal or neuroglial interactions.

## MATERIALS AND METHODS

### Animals

Adult male Hartley guinea pigs (300–400 g) (Charles Rivers Breeding Lab, Willington, MA, U.S.A.) were housed at the Laboratory Animal Facility, Naval Medical Research Institute, under a 12-h dark cycle with food and water provided ad libitum.

### Materials

Radioactive [ $2\text{-}^3\text{H}$ ]glycine (sp act 30–60 Ci/mmol) and  $\gamma$ -[ $2,3\text{-}^3\text{H}$ ]aminobutyric acid (sp act 25–40 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

### Synaptosome isolation procedure

Each guinea pig was killed by rapid cervical dislocation. The medulla and cervical spinal cord to C4 were removed via dorsal laminectomy and placed in 10 volumes of ice-cold 0.32 M sucrose buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Synaptosomes were prepared by density gradient centrifugation using a previously described procedure (Gilman et al., 1986a). A motor-driven glass homogenizer was used to disperse the tissue. Centrifugations were carried out at 3°C in an ultracentrifuge (model OTD75B, Sorvall, Wilmington, DE, U.S.A.) using a  $w^2/dt$  digital integrator to obtain consistent centrifugation. The initial tissue homogenate was centrifuged at 1,100 g for 5 min to yield a crude nuclear pellet and a low-speed supernatant. The supernatant was transferred and centrifuged at 17,000 g for 10 min to yield a mitochondrial pellet containing synaptosomes. The pellet was resuspended in 0.32 M sucrose and layered over a discontinuous Ficoll gradient (7.5–13%) and centrifuged at 26,000 g for 30 min. After centrifugation, the middle band containing the purified synaptosomes was removed, diluted with sucrose, and pelleted at 11,500 g for 10 min. The resulting synaptosomal pellet was diluted in ice-cold incubation solution. The composition was (in mM): NaCl, 145; KCl, 5;  $\text{MgCl}_2$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2; Tris, 20; and no  $\text{Ca}^{2+}$ ; pH 7.4. The pellet was washed and centrifuged again at 11,500 g for 10 min.

### Radioisotope uptake

The method used for radioactive amino acid loading of the synaptosomes was a modification of the superfusion

method of Raiteri et al. (1974). The final synaptosome pellet was suspended in 10 volumes of a buffer medium consisting of a high-sodium, calcium-free solution. The composition was (in mM): NaCl, 145; KCl, 5;  $\text{MgCl}_2$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2; HEPES, 20; glucose, 10; pH 7.4. In the GABA release studies, 0.1 mM of aminooxyacetic acid and 0.01 mM GABA were included in the solution. The radio-labeled amino acid was added and the mixture incubated at 37°C for 20 min to allow uptake by the tissue.

### Compression studies

Following uptake, a 1.0-ml aliquot of the radioisotope-loaded synaptosome suspension was placed on a filter unit consisting of a 0.45  $\mu\text{m}$  nylon membrane filter positioned on a multiperforated support of a 10-ml perfusion chamber. The perfusion chamber was then connected to a polystaltic pump (model 39, Rainin Instrument, Woburn, MA, U.S.A.). The filters were immediately washed with 25 ml of buffer medium using the highest pump speed. Background radioactivity due to nonspecific binding of the radiolabeled amino acid to the filters was independently determined by perfusing blank filters with [ $^3\text{H}$ ]glycine or [ $^3\text{H}$ ]GABA, but no synaptosomes. This ensured that the wash technique used was sufficient to remove unbound radioactivity. The radioactivity on these filters was found to decline with each wash and the counts present in the final perfusate were the same as general background.

After washing, the filter unit and superfusion apparatus were placed in a hyperbaric chamber (model 18361, Beth-lehem, Hellertown, PA, U.S.A.). A line from the filter unit was aligned directly over a scintillation vial inserted in a compartment of a turning carousel. This carousel was positioned on a Plexiglas stand, to which was mounted two stepping pump systems. Pump 1 was programmed to deliver 5 ml of buffer medium; pump 2 to deliver 10 ml of an efflux medium containing either 5 mM or 70 mM  $\text{K}^+$ . The composition was (in mM): NaCl, 145 or 80; KCl, 5 or 70;  $\text{CaCl}_2$ , 1.2;  $\text{MgCl}_2$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2; HEPES, 20; glucose, 10; pH 7.4. Lines from the pump systems were secured directly over the filter unit.

Ten milliliters of buffer medium was poured directly over the filter and the hyperbaric chamber was pressurized to 1.3 ATA using oxygen, then to a final depth of 67.7 ATA with helium, at a compression rate of 4.03 ATA/min. Oxygen partial pressure, as measured with a paramagnetic  $\text{O}_2$  analyzer (model F-3, Beckman, Silver Spring, MD, U.S.A.), was adjusted to 0.80% ( $0.49 \pm 0.1$  ATA) on reaching 67.7 ATA. A recirculation atmosphere control system was used to regulate  $\text{CO}_2$  at  $<0.0005$  ATA, as measured by an infrared analyzer (model 865, Beckman, Silver Spring, MD, U.S.A.).

During compression, the filter was washed continuously with buffer medium. On reaching 67.7 ATA, 5 ml of buffer medium from pump 1 was delivered over the filter unit and three "wash" perfusates collected directly into scintillation vials. Ten milliliters of a 5 mM  $\text{K}^+$ , nondepolarizing or 10 mM of a depolarizing, 70 mM  $\text{K}^+$  "efflux buffer" from pump 2 was then rapidly delivered over the filter unit. Nine fractions containing 500  $\mu\text{l}$  each of perfusate were collected every minute directly into scintillation vials.

During pressurization and the release studies, the temperature of the buffer media and perfusion chamber was constantly monitored by microthermistors and maintained at  $37^\circ\text{C} \pm 1^\circ\text{C}$  using YSI model 73A temperature control units. At the end of the release studies, the hyperbaric

chamber was decompressed to 1 ATA at the rate of 4.03 ATA/min. In all experiments, aliquots from the same synaptosome preparation were used for obtaining the 1 ATA and 67.7 ATA release values. The order of experimental exposures was randomized to eliminate any time effect. The control preparations were treated identically to the experimental group, except that pressure exposures were sham (1 ATA).

#### Assay of samples

Fifteen milliliters of Biofluor was added to each scintillation vial and radioactivity determined for each perfusate. The radioactivity remaining on the filters at the end of the superfusion was also counted. Each filter was placed in a scintillation vial containing 500  $\mu$ l of 1% sodium dodecyl sulfate. After agitation, 15 ml of Biofluor was added and the filter counted for radioactivity.

#### Expression of results

Fractional efflux was expressed as percentage of total radioactivity, i.e.,

$$\text{Efflux} = \frac{\text{cpm in 500 } \mu\text{l filtrate}}{\text{Total radioactivity}}$$

where total radioactivity was the sum of all fractional filtrate cpm values and cpm remaining on the filter. Statistical significances were determined by *t* tests. A *p* < 0.05 was considered significant.

## RESULTS

#### [<sup>3</sup>H]Glycine release at 1 ATA

The time course of the nondepolarized (i.e., spontaneous) and high  $K^+$  evoked (depolarized) efflux of [<sup>3</sup>H]glycine at 1 ATA from synaptosome fractions isolated from the guinea pig spinal cord is shown in Fig. 1, squares and triangles, respectively. Application of the high- $K^+$  medium containing 1.2 mM of  $Ca^{2+}$  (Fig. 1, triangles) initially induced a significant increase in the release of [<sup>3</sup>H]glycine by the synaptosomal preparation. After the first 3 min, however, the

release declined, approaching the nondepolarized efflux level within 8–9 min. This pattern of release is absent in the nondepolarized preparation (Fig. 1, squares).

To determine the extent to which the increase in release observed with depolarization was calcium dependent, synaptosomes were perfused with a depolarizing, 70 mM  $K^+$  calcium-free medium, with  $MgCl_2$  replacing the omitted  $CaCl_2$  (Fig. 1, circles). The removal of calcium from the depolarizing medium reduced initial (i.e., first 2 min of depolarization) [<sup>3</sup>H]glycine release by synaptosomes at 1 ATA by approximately 50%.

#### Compression effects on [<sup>3</sup>H]glycine release

Compression to 67.7 ATA did not cause any significant changes in the spontaneous (i.e., nondepolarized) efflux of [<sup>3</sup>H]glycine from synaptosomes isolated from the spinal cord. However, this level of compression did suppress the high- $K^+$ -evoked release of glycine in the presence of 1.2 mM  $Ca^{2+}$  (Fig. 2A, circles). Data analysis showed that the depression was significant at the 4- and 5-min superfusion times. On the other hand, [<sup>3</sup>H]glycine release was not significantly affected by compression to 67.7 ATA in the absence of  $Ca^{2+}$  (Fig. 2B).

To determine if pressure might be selectively depressing the glycine efflux that is dependent on calcium, fractional calcium-dependent [<sup>3</sup>H]glycine efflux was calculated as the difference between the percent of total radioactivity released from synaptosome preparations that were depolarized in the presence of 1.2 mM  $Ca^{2+}$  (Fig. 2A) and the percent of the total released from preparations that were depolarized in a calcium-free medium (Fig. 2B). That is, data obtained at 1 ATA from  $Ca^{2+}$ -containing media (Fig. 2A, triangles) minus data obtained at 1 ATA from  $Ca^{2+}$ -free media (Fig. 2B, triangles) were used to generate data representing the calcium-dependent release

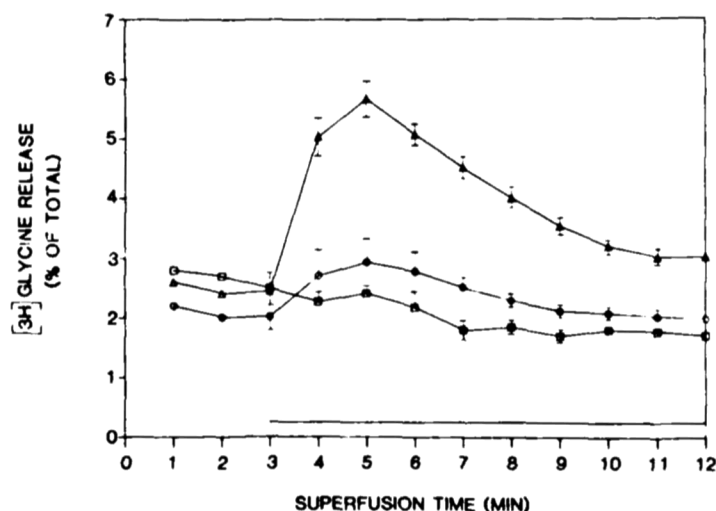


FIG. 1. Release of [<sup>3</sup>H]glycine from guinea pig spinal cord synaptosome fractions. Depolarized,  $Ca^{2+}$  present ( $\Delta$ ); depolarized,  $Ca^{2+}$  absent ( $\circ$ ); nondepolarized,  $Ca^{2+}$  present ( $\square$ ). After preloading with [<sup>3</sup>H]glycine, synaptosome aliquots were superfused with a high-sodium, calcium-free buffer medium for 3 min, then 70 mM  $K^+$ -containing medium with or without  $Ca^{2+}$  or 5 mM  $K^+$ -containing medium with  $Ca^{2+}$  was similarly superfused for the 9 min marked by horizontal bar (i.e., 4–12 min). Each point represents mean  $\pm$  SEM of six experiments for each condition.

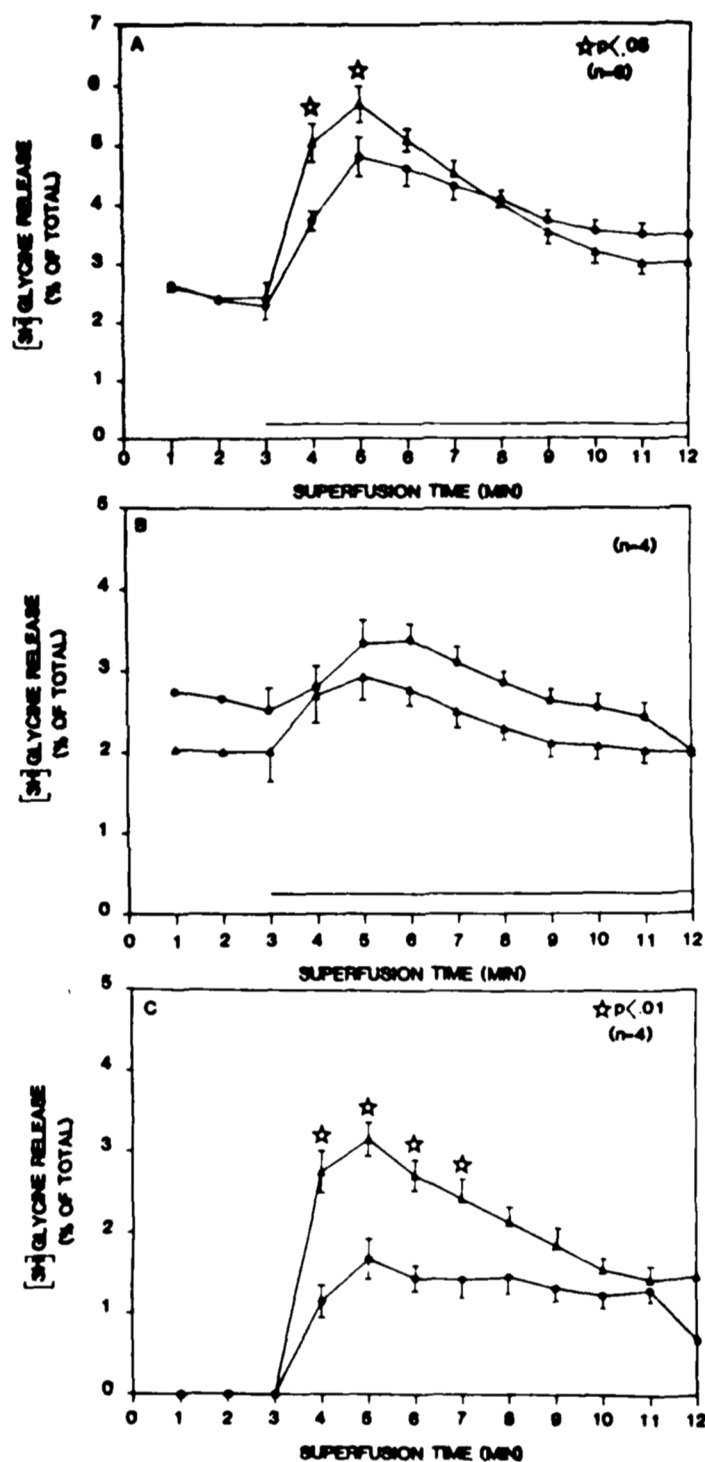


FIG. 2. Effect of compression to 67.7 ATA on the high- $\text{K}^+$ -evoked release of [ $^3\text{H}$ ]glycine from spinal cord synaptosomes. A: In the presence of  $\text{Ca}^{2+}$ . B: In the absence of  $\text{Ca}^{2+}$ . C:  $\text{Ca}^{2+}$ -dependent release, i.e., the difference between (A) and (B). 1 ATA ( $\Delta$ ); 67.7 ATA ( $\bullet$ ). Each point represents six experiments for each condition.

at 1 ATA (Fig. 2C, triangles). A similar manipulation was performed for data obtained at 67 ATA to yield Fig. 2C, circles. Data points were handled as discrete populations and prior to arithmetical manipulation each datum point was normalized by subtraction of the release value obtained in the final pretreatment

"wash" perfusate to account for nonspecific [ $^3\text{H}$ ]glycine activity in the perfusate. Statistical analysis revealed that compression to 67.7 ATA significantly reduced the calcium-dependent component of stimulated [ $^3\text{H}$ ]glycine release at the 4- and 5-min superfusion times (Fig. 2C).

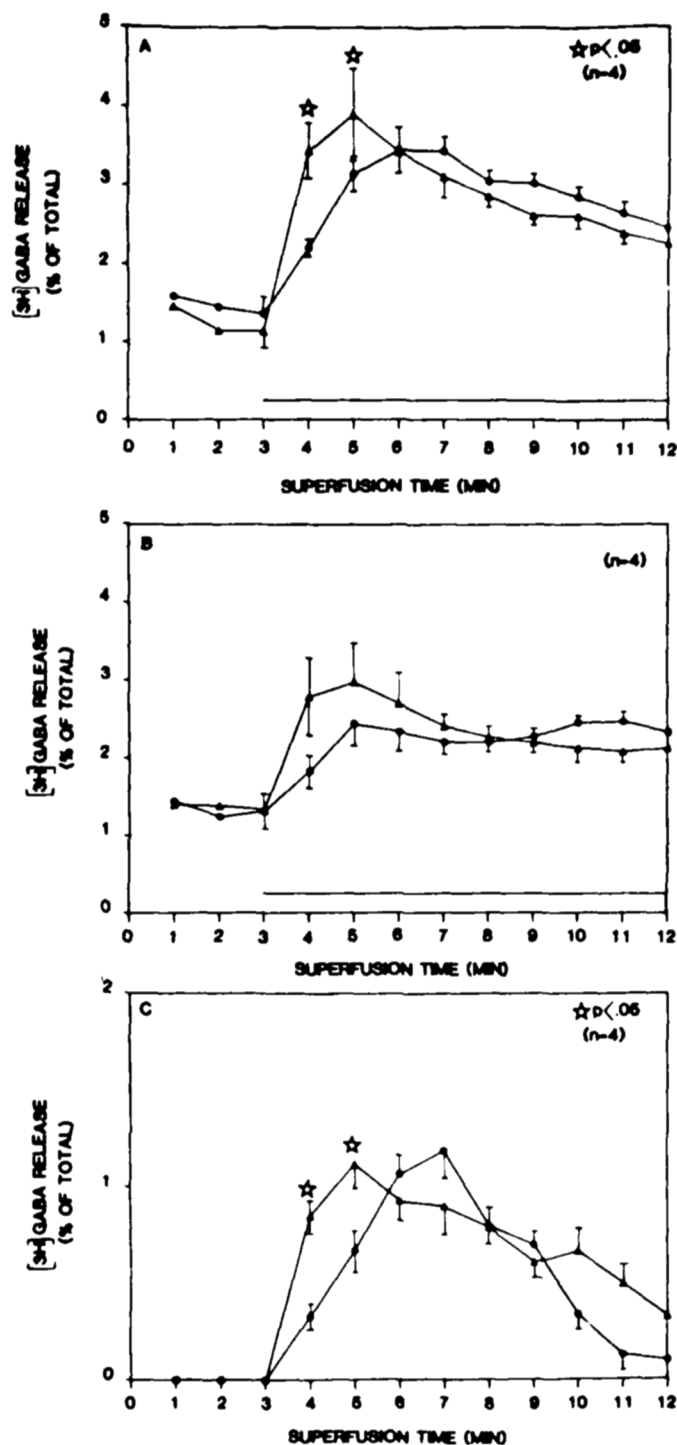


FIG. 3. Effect of compression to 67.7 ATA on the stimulated release of  $[^3\text{H}]\text{GABA}$ . A:  $\text{Ca}^{2+}$  present. B:  $\text{Ca}^{2+}$  absent. C:  $\text{Ca}^{2+}$ -dependent. 1 ATA ( $\Delta$ ); 67.7 ATA ( $\bullet$ ). Each point represents six experiments for each condition.

#### $[^3\text{H}]\text{GABA}$ release at 1 ATA

The time course of high- $\text{K}^+$ -evoked efflux of  $[^3\text{H}]\text{GABA}$  at 1 ATA from synaptosome fractions isolated from the spinal cord is shown in Fig. 3A, triangles. Application of the high  $\text{K}^+$  medium containing  $\text{Ca}^{2+}$  initially induced an increase in the release of  $[^3\text{H}]\text{GABA}$  by these synaptosomes. After the first 2 min,

however, the evoked release rapidly declined, returning within 6–8 min to the resting efflux levels.

Figure 3B, triangles, also shows the effect of calcium removal on depolarization-induced  $[^3\text{H}]\text{GABA}$  release at 1 ATA. The removal of calcium from the

depolarizing medium reduced initial [ $^3\text{H}$ ]GABA release by only 25%. Thus, a large  $\text{Ca}^{2+}$ -independent component appears to be present in this preparation.

#### Compression effects on [ $^3\text{H}$ ]GABA release

Pressure did not have a significant effect on the spontaneous, nondepolarized efflux of [ $^3\text{H}$ ]GABA from synaptosomes isolated from the spinal cord. Figure 3A, circles shows the effect of compression to 67.7 ATA on the release of [ $^3\text{H}$ ]GABA stimulated by a 70 mM  $\text{K}^+$  depolarizing medium. As with the [ $^3\text{H}$ ]glycine release, compression significantly reduced the initial (first 2 min) calcium-dependent component of high- $\text{K}^+$ -evoked [ $^3\text{H}$ ]GABA release from spinal cord synaptosomes (Fig. 3C).

### DISCUSSION

Electron microscopy and the measurement of cytoplasmic markers have shown that the methods used in this study produce a synaptosomal preparation that retains an intact plasma membrane, with no apparent nonselective changes in permeability (Gray and Whittaker, 1962; Gurd et al., 1974; Dodd et al., 1981; Gilman et al., 1986a,b). Furthermore, we have shown that the pressures used in this study have not been found to have an apparent disruptive effect on the morphology of the synaptosomal plasma membrane or membrane marker enzymatic activity (Gilman et al., 1986a,b). This is consistent with Bichard and Little (1985), who report the absence of nonselective permeability changes in isolated frog spinal cord exposed to as much as 100 ATA.

The purified synaptosomes were superfused in an apparatus similar to that described by Raiteri et al. (1974). In our study, a remote-controlled turntable and three separate perfusion pumps were used to collect serial samples in a closed and pressurized hyperbaric chamber. This arrangement of the equipment allowed the synaptosomal preparation to come into equilibrium with the gaseous environment, as well as permitting the collection of the transmitter substance at the desired pressure and therefore eliminating the effects of decompression.

Once the synaptosomal preparation was perfused with a depolarizing medium (70 mM  $\text{K}^+$ ) containing  $\text{Ca}^{2+}$ , and the effluent serially collected, it was possible to construct a standard release curve for both [ $^3\text{H}$ ]glycine and [ $^3\text{H}$ ]GABA. The pattern of release (i.e., a relatively rapid increase in transmitter efflux within the first 1–2 min followed by a slow decline in transmitter efflux over the subsequent 10–15 min), was similar to that described by other investigators using this perfusion technique (Raiteri et al., 1974; Redburn et al., 1975; Klaff et al., 1982).

A substantial calcium-independent component for [ $^3\text{H}$ ]glycine or [ $^3\text{H}$ ]GABA efflux was observed when the preparation was perfused with 70 mM  $\text{K}^+$  in a calcium-free medium. Calcium-independent release accounted for 50% of [ $^3\text{H}$ ]glycine efflux and up to

75% for [ $^3\text{H}$ ]GABA efflux. Similar findings for calcium-independent release have been reported for glycine, GABA, and dopamine in brain slices, spinal cord slices, retinal tissue, and brain synaptosomes (Moscowitz and Cutler, 1980; Cunningham and Neal, 1981; Sandoval et al., 1985; Arias and Tapia, 1986; Okuma and Osumi, 1986; Jonsson et al., 1986). The calcium-independent release appears to be associated with the particular transmitter type, since acetylcholine and norepinephrine are reported to be highly calcium dependent and the release of these compounds is essentially inhibited when calcium is absent (Blaustein et al., 1972; Cunningham and Neal, 1981; Arias and Tapia, 1986; Okuma and Osumi, 1986). The mode of stimulation also affects release. For example, Okuma and Osumi (1986) demonstrated that calcium-independent release of endogenous dopamine from rat hypothalamic slices was induced by high  $\text{K}^+$  but not by electrical stimulation. Hammerstad et al. (1971) made a similar observation when they showed that glycine and GABA were highly calcium dependent when electrically evoked from rat spinal cord slices. However, glycine, GABA, and dopamine all show calcium independence when stimulated with high  $\text{K}^+$  (i.e., >30 mM  $\text{K}^+$ ), veratridine, ouabain, quinidine, or monensin (Sandoval, 1980; Cunningham and Neal, 1981; Sandoval et al., 1985). The mechanism of the calcium-independent release is currently unclear. The reversal of a bidirectional sodium-dependent membrane-bound carrier for glycine or GABA, or the mobilization of cytosolic calcium from intraterminal stores cannot adequately explain this phenomenon (Moscowitz and Cutler, 1980; Sihra et al., 1984; Arias and Tapia, 1986). There is evidence, though, that intraterminal sodium content is an important factor contributing to the calcium-independent release (Minchin, 1980; Cunningham and Neal, 1981; Sihra et al., 1984; Sandoval et al., 1985; Arias and Tapia, 1986).

The most significant finding in this study was that a helium/oxygen gas mixture at high pressure (67.7 ATA) produced an initial depression in calcium-dependent [ $^3\text{H}$ ]glycine or [ $^3\text{H}$ ]GABA release from spinal cord synaptosomes. For glycine, there was both a significant decrease in the quantity of glycine released over the initial 4 min of efflux and a marked depression in the rate of release during the first minute. For GABA, there was a significant decrease in transmitter efflux over the first 2 min as well as a concomitant decrease in the rate of release during the same time period. A similar pattern of release has been observed for GABA efflux from guinea pig cerebrocortical synaptosomes when exposed to 67.7 ATA helium (Gilman et al., 1986b). One possible explanation of the effect of helium pressure on transmitter release is that inward-directed calcium movement may be impaired due to an alteration of voltage-sensitive calcium channels in the presynaptic terminals. However, data extrapolated from observation of synapto-

some preparations from cerebral cortex may alter this hypothesis. In a previous study (Gilman et al., 1986a), we have shown that the first minute of  $^{45}\text{Ca}^{2+}$  uptake into synaptosomes at 67.7 ATA was not significantly different from  $^{45}\text{Ca}^{2+}$  uptake at 1 ATA. This does not coincide with the depressed rate of release in the first minute of efflux for both glycine and GABA in both cortex and spinal cord preparations. In addition, preliminary unpublished studies using the calcium ionophore A23187 indicate that at 67.7 ATA the initial first minute of release remains depressed even though the ionophore enhances the overall response at 1 ATA. Therefore, we conclude that the fall in  $[^3\text{H}]$ glycine and  $[^3\text{H}]$ GABA release is not entirely a  $\text{Ca}^{2+}$  channel gating problem, but also due to some other aspect of the intraterminal cascade for the calcium-dependent release of these two transmitters.

Glycine and GABA are considered to be major inhibitory neurotransmitters in the spinal cord (Curtis et al., 1967, 1968; Graham et al., 1967; Werman et al., 1968; Aprison et al., 1975) and thus play an important modulatory role in motor function. Of particular interest in this study is the depression in the initial rate of release of the calcium-dependent component for both transmitters. The timing of inhibitory transmitter release is also critical to its function as a modulator of excitatory neurotransmission. Alteration in release of either glycine or GABA could have profound effects on motor function in the spinal cord. It is unknown whether there is an analogous pressure-dependent depression of depolarization-induced, calcium-dependent release of glycine or GABA by presynaptic nerve terminals in the whole, intact brain. It is also unknown if this phenomenon is a contributory factor in the development of the symptoms of HPNS. On the other hand, such change could contribute to the hyperexcitability associated with high pressure exposure.

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