The role of Ca^{2+} in the protoveratrine-induced release of γ -aminobutyrate from rat brain slices

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1. Protoveratrine A increased the release of γ -amino[^3H]butyrate from small slices of rat cerebral cortex. This effect increased with increasing protoveratrine concentration, reaching a maximum at $100\mu\text{M}$. 2. Removal of Ca^{2+} from the superfusing medium did not change the increase in release due to $10\mu\text{M}$ -protoveratrine; however, the Ca^{2+} antagonists, compound D-600, La^{3+} , Mn^{2+} , Mg^{2+} and also high Ca^{2+} concentration inhibited the effect of the alkaloid, as did procaine. 3. Protoveratrine A increased the uptake of $^{22}\text{Na}^{+}$ into the slices with a similar dose–response curve to that found for γ -aminobutyrate release. For the most part, the substances that inhibited protoveratrine-stimulated γ -aminobutyrate release also inhibited $^{22}\text{Na}^{+}$ uptake, although the correlation was not perfect. 4. Although extracellular Ca^{2+} is not required for protoveratrine-induced γ -aminobutyrate release, an increase in Na^{+} influx that is susceptible to inhibition by some Ca^{2+} antagonists does appear to be associated with this phenomenon. However, the possibility remains that changes in the free intracellular Ca^{2+} concentration may be important for transmitter release induced by depolarizing veratrum alkaloids.

Several of the veratrum alkaloids have been shown to depolarize excitable tissues, a phenomenon that is abolished by removal of Na^{+} from the external medium or by the addition of tetrodotoxin, suggesting an interaction with Na^{+} fluxes across the cell membrane (Ulbricht & Flacke, 1965; Wellhöner, 1970; Ohta *et al.*, 1973). This is supported by measurements of radioactive Na^{+} fluxes across a variety of tissue membranes; in particular, veratridine increases $^{22}\text{Na}^{+}$ uptake and the Na^{+} content of synaptosomes (Li & White, 1977) and protoveratrine A enhances Na^{+} uptake into brain slices (Okamoto & Quastel, 1970); both of these effects were decreased by tetrodotoxin. In addition, veratridine increased the depolarization-induced fluorescence of synaptosomes that had been treated with a voltage-sensitive fluorescent probe (Blaustein & Goldring, 1975) and this too was inhibited by tetrodotoxin. These experiments strongly suggest that certain veratrum alkaloids depolarize excitable tissues by increasing their membrane Na^{+} permeability. In synaptosomes, veratridine-induced depolarization is accompanied by an increase in Ca^{2+} influx (Blaustein, 1975) and an increase in the efflux of endogenous noradrenaline (Blaustein *et al.*, 1972). Other neurotransmitters are released from synaptosomes or brain slices by depolarizing vera-

trum alkaloids, by a process that is dependent on the presence of Ca^{2+} in the external medium, for example, acetylcholine release from brain slices (Grewaal & Quastel, 1973), dopamine from synaptosomes (Patrick & Barchas, 1976) and various amino acids from synaptosomes (Nadler *et al.*, 1977; Hammerstad *et al.*, 1979). However, dependence on external Ca^{2+} has not always been demonstrable for veratrum alkaloid-induced transmitter release. Thus, Benjamin & Quastel (1972) found that removal of external Ca^{2+} caused an increase in protoveratrine-stimulated efflux of glutamate, aspartate and γ -aminobutyrate from brain slices and Levi (1978), Szerb (1979) and Neal (1979) have recently shown that veratridine-induced release of exogenously accumulated γ -amino[^3H]butyrate from synaptosomes and brain slices was similarly enhanced in the absence of Ca^{2+} in the external medium. On the other hand, veratridine-induced release of endogenous amino acids from brain slices was found to be neither decreased nor enhanced when Ca^{2+} was removed from the superfusing medium (Cutler & Young, 1979). Furthermore, White (1978) has demonstrated that the veratridine-induced efflux of ATP from synaptosomes was enhanced by removal of Ca^{2+} from the superfusing fluid.

The present experiments were designed to attempt to resolve the ionic dependence of neurotransmitter release induced by protoveratrine A from rat brain slices; a preliminary account of this work has been published previously (Minchin, 1979).

Experimental

Adult male albino rats (Sheffield strain) were decapitated and their brains were removed on to a chilled surface where the cerebral cortices were dissected. The cortical tissue was trimmed of white matter, the pia were removed and it was chopped in two directions on a McIlwain tissue chopper to give small slices of dimension $0.1\text{ mm} \times 0.1\text{ mm} \times 1.5\text{ mm}$. For release experiments, these were then suspended in oxygenated Krebs-phosphate solution, and portions containing 50 mg of tissue were distributed volumetrically into bottles so that the final volume of Krebs-phosphate in each was 5 ml. The slices were preincubated at 37°C for 10 min in a shaking water bath after which a γ -aminobutyrate solution containing γ -amino[^3H]butyrate was added to give a final concentration of $0.1\text{ }\mu\text{M}$ and a specific radioactivity of 4 Ci/mmol. The incubation was continued for a further 15 min. The slices were then collected by filtration on to a glass-fibre filter (Whatman GF/A) and transferred to a Sartorius membrane-filter holder (Sartorius G.m.b.H., Göttingen, Germany) where they were superfused with oxygenated Krebs-phosphate or Krebs-Tris solution at 37°C (0.5 ml/min) by means of a peristaltic pump. All the solutions in these experiments contained $10\text{ }\mu\text{M}$ -amino-oxyacetic acid to inhibit γ -aminobutyrate metabolism by 4-aminobutyrate-2-oxoglutarate aminotransferase (EC 2.6.1.19). Fractions of the superfusion medium were collected every 3 min and the radioactivity was counted by liquid-scintillation spectrometry after the addition of 10 ml of 0.5% (w/v) 2,5-diphenyloxazole in a toluene/Triton X-100 (2:1) mixture. Counting efficiency was 22%. At the end of the superfusion, the radioactivity in the tissue was extracted into 1.5 ml of water and similarly counted. The radioactivity in each wash was then expressed as an approximate first-order fractional-release rate constant, which was calculated as $(\ln C_0/C_t)/t$, where C_0 = c.p.m. in tissue at start of superfusion period, C_t = c.p.m. in tissue at end of superfusion period and t = duration of superfusion period, in this case 3 min. Collection of fractions was routinely started 20 min after the start of superfusion to allow for the initial washout of radioactivity from the extracellular space and to enable efflux of γ -amino[^3H]butyrate from the tissue to reach a steady value.

In Na^+ -uptake experiments 20 mg of cerebral slices was preincubated for 10 min at 37°C in 5 ml of oxygenated Krebs-phosphate or Krebs-Tris solu-

tion after which $^{22}\text{Na}^+$ and [^3H]inulin were added to a specific radioactivity of $0.59\text{ }\mu\text{Ci/mmol}$ and 1.8 Ci/mmol respectively. Incubation was continued for various times, the slices were collected by filtration on to Whatman GF/A filters and the filters were washed with 2 ml of ice-cold saline (0.9% NaCl). The radioactivity in the tissue was extracted into 1.5 ml of water and counted after the addition of 10 ml scintillation 'cocktail'. ^3H was counted at 15.5% efficiency and ^{22}Na was counted at 70% efficiency; spillover of ^{22}Na into the ^3H channel was 1.2%, whereas that of ^3H into the ^{22}Na channel was negligible. The extracellular space of the slices was calculated as the tissue/medium ratio of [^3H]inulin, i.e. (d.p.m./g of tissue)/(d.p.m./ml of incubation medium), and the $^{22}\text{Na}^+$ present in the extracellular space was then subtracted from the whole tissue $^{22}\text{Na}^+$ content to give the amount of $^{22}\text{Na}^+$ taken up into the slices.

Materials

4-Amino[2,3- ^3H]butyric acid (sp. radioactivity 66 Ci/mmol), [^3H]inulin (sp. radioactivity 1.8 Ci/mmol) and $^{22}\text{NaCl}$ (sp. radioactivity 200 mCi/mg of Na) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Protoveratrine A was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and compound D-600 hydrochloride was a gift from Knoll AG, Ludwigshafen am Rhein, Germany. The composition of the Krebs-phosphate solution was 118 mM-NaCl, 4.8 mM-KCl, 1.2 mM- CaCl_2 , 1.2 mM- MgSO_4 , 5.6 mM-D-glucose and 15 mM-sodium phosphate buffer, pH 7.4. The sodium phosphate buffer was prepared by adjusting approx. 0.1 M- NaH_2PO_4 to pH 7.4 with NaOH solution and diluting to 15 mM. The Krebs-Tris solution used in these experiments had the following composition: 140 mM-NaCl, 4.8 mM-KCl, 1.2 mM- CaCl_2 , 1.2 mM- MgSO_4 , 5.6 mM-D-glucose and 15 mM-Tris/HCl, pH 7.4. The Tris/HCl buffer was made by adjusting approx. 0.1 M-Tris to pH 7.4 with HCl solution and diluting to 15 mM.

Results

γ -Amino[^3H]butyrate efflux

Exposure of the brain slices to protoveratrine A for 9 min caused a rapid increase in the rate of γ -amino[^3H]butyrate efflux and a slow return to the baseline value (Fig. 1a); the dose-response curve was sigmoid with a maximum at approx. $100\text{ }\mu\text{M}$ -protoveratrine (Fig. 1b).

In an attempt to establish whether Ca^{2+} was necessary for this phenomenon, various alterations were made to the perfusing medium; removing all Ca^{2+} from the medium did not significantly decrease the increase in γ -amino[^3H]butyrate efflux caused by

Fig. 1. The effect of protoveratrine A on γ -aminobutyrate release from brain slices

Slices of cerebral cortex were incubated with γ -amino[^3H]butyrate and superfused with Krebs-phosphate solution as described in the text. (a) Protoveratrine A ($10\mu\text{M}$) was added to the superfusing medium for the time indicated by the horizontal line. The points represent the results of a single experiment. (b) At 32 min after the start of superfusion the slices were exposed to various concentrations of protoveratrine A for 9 min. The maximum value of the efflux rate constant achieved during exposure to protoveratrine was divided by the mean of the two values immediately preceding addition of the alkaloid to give the relative efflux coefficient. Each point represents the mean for the number of experiments shown in parentheses, with the s.e.m. indicated by vertical bars, except where these are smaller than the symbol.

$10\mu\text{M}$ -protoveratrine, even if the tissue was bathed in the Ca^{2+} -free solution for a prolonged time (Table 1). If, in addition to removing Ca^{2+} from the medium, 1 mM -EGTA was added in an attempt to ensure removal of any Ca^{2+} ions that might diffuse from intracellular stores into the superfusing fluid, and the slices were superfused with this solution for a long time before exposure to protoveratrine, the effect of the depolarizing drug was not significantly changed, although short exposure to this medium did result in a diminution of the increase in γ -amino-

Fig. 2. $^{22}\text{Na}^+$ uptake into cerebral cortical slices. Slices were incubated with $^{22}\text{Na}^+$ for various times in Krebs-phosphate solution and the amount of Na^+ taken up into the tissue was calculated as described in the text. Each point represents the mean for the number of experiments shown in parentheses with the s.e.m. indicated by vertical bars.

[^3H]butyrate release (Table 1). However, high concentrations of the Ca^{2+} antagonist, Mg^{2+} , added to the superfusing medium with or without Ca^{2+} , significantly decreased the effect of protoveratrine. Furthermore, the local anaesthetic procaine and the organic Ca^{2+} antagonist compound D-600 both inhibited protoveratrine-induced γ -amino[^3H]butyrate efflux, and similarly, the inorganic Ca^{2+} antagonists La^{3+} and Mn^{2+} , tested in Krebs-Tris buffer to avoid precipitation of inorganic phosphates, also significantly inhibited this effect, as did Mg^{2+} in this buffer (Table 1). Moreover, Ca^{2+} itself, if present at high concentration, inhibited the increase in γ -amino[^3H]butyrate efflux caused by protoveratrine. Nevertheless, in Krebs-Tris medium, Ca^{2+} -free medium again failed to alter the γ -amino-[^3H]butyrate-efflux rate constant, whereas EGTA was effective at inhibiting protoveratrine-induced γ -amino[^3H]butyrate release irrespective of the time

Table 1. *Effects of drugs and ions on protoveratrine-stimulated γ -amino[^3H]butyrate efflux*

Brain slices were incubated with γ -amino[^3H]butyrate and superfused as described in the Experimental section. They were exposed to the test substances for 6 min before and during exposure for 9 min to $10\text{ }\mu\text{M}$ -protoveratrine except in the case of long exposure to low Ca^{2+} media when the superfusing medium was changed 32 min before addition of protoveratrine. The maximum value of the efflux rate constant achieved during protoveratrine stimulation was divided by the baseline rate constant to give the fractional increase due to protoveratrine; in each experiment the control values were averaged and the increase in efflux caused by protoveratrine in the presence of the test substance was expressed as a percentage of the control. The average relative efflux coefficient due to protoveratrine of all the controls in Krebs-phosphate medium was 4.02 ± 0.31 (mean $\pm$ S.E.M., $n = 42$), whereas in Krebs-Tris it was 2.07 ± 0.08 (mean $\pm$ S.E.M., $n = 21$). The test substances did not alter the spontaneous release of γ -amino[^3H]butyrate, except 10 mM-Mg^{2+} , which decreased spontaneous efflux to $79 \pm 2\%$ (mean $\pm$ S.E.M., $n = 3$) of control and 1% procaine, which decreased spontaneous γ -amino[^3H]butyrate release to $64 \pm 4\%$ (mean $\pm$ S.E.M., $n = 3$) of control. The control values for the spontaneous γ -amino[^3H]butyrate efflux rate constant were $0.104 \pm 0.004\% \cdot \text{min}^{-1}$ (mean $\pm$ S.E.M., $n = 79$) in Krebs-phosphate and $0.064 \pm 0.003\% \cdot \text{min}^{-1}$ (mean $\pm$ S.E.M., $n = 42$) in Krebs-Tris medium. The statistical significance of the results, when compared with controls, was estimated by Student's t test. The number of experiments is indicated in parentheses. Abbreviation: N.S., not significant.

Krebs-phosphate	Efflux (% of control)	P
Zero Ca^{2+}	115 ± 9 (5)	N.S.
Zero Ca^{2+} (long exposure)	94 ± 12 (3)	N.S.
EGTA (1 mM, no Ca^{2+})	57 ± 7 (6)	<0.05
EGTA (1 mM, no Ca^{2+} , long exposure)	89 ± 6 (12)	N.S.
Compound D-600 (0.1 mM)	27 ± 1 (4)	<0.01
Mg^{2+} (10 mM, no Ca^{2+})	61 ± 6 (3) [†]	<0.05
Mg^{2+} (10 mM, normal Ca^{2+})	60 ± 4 (3) [†]	<0.01
Procaine (1%, w/v)	0 (3)*	
Krebs-Tris		
Zero Ca^{2+}	104 ± 9 (3)	N.S.
EGTA (1 mM, no Ca^{2+})	62 ± 0 (3)	<0.05
EGTA (1 mM, no Ca^{2+} , long exposure)	62 ± 3 (3)	<0.01
La^{3+} (1 mM)	56 ± 2 (6)	<0.001
Mn^{2+} (5 mM)	65 ± 3 (6)	<0.001
Ca^{2+} (10 mM)	67 ± 2 (3)	<0.01
Mg^{2+} (10 mM, normal Ca^{2+})	74 ± 2 (3) [†]	<0.05
Procaine (1%, w/v)	0 (3)*	

* Procaine delayed the peak increase in rate constant so that at the peak time in control samples there was no increase in rate constant in procaine-treated samples. The late increase seen in experiments with procaine was probably due to washout of procaine exposing the decaying phase of the rise in rate constant caused by protoveratrine, as shown in Fig. 1(a).

[†] Mg^{2+} caused a delay in the peak increase in rate constant; the results reported here were taken at the time at which the maximum rate constant occurred in control samples.

the slices were exposed to it (Table 1). The nature of the superfusing buffer made no difference to the effect of procaine, however.

Compared with protoveratrine-induced efflux, the increase of γ -amino[^3H]butyrate release caused by 40 mM-K^+ (as KCl replacing NaCl in Krebs-phosphate medium) was more sensitive to Ca^{2+} -deficient media. In control experiments 40 mM-K^+ caused a 7.0 ± 0.8 -fold (mean $\pm$ S.E.M., $n = 12$) increase in the efflux rate constant. Removing Ca^{2+} from the superfusing fluid for 6 min before exposing the slices to 40 mM-K^+ decreased the K^+ -evoked release of γ -amino[^3H]butyrate to $41 \pm 7\%$ of the control value (mean $\pm$ S.E.M., $n = 3$). Similarly, 1 mM-EGTA inhibited the γ -amino[^3H]butyrate efflux caused by 40 mM-K^+ , decreasing it to $59 \pm 10\%$ (mean $\pm$ S.E.M., $n = 3$) and $68 \pm 9\%$ (mean $\pm$ S.E.M., $n = 6$) of the control value after 6

and 32 min exposure respectively. However, $100\text{ }\mu\text{M}$ -ouabain, which also enhanced γ -amino[^3H]butyrate efflux [by 7.6 ± 0.8 times (mean $\pm$ S.E.M., $n = 3$) in Krebs-Tris buffer], did not demonstrate a Ca^{2+} -sensitive component in its effects, 1 mM-EGTA causing the efflux to increase slightly to $148 \pm 20\%$ of the control value (mean $\pm$ S.E.M., $n = 3$) after prolonged exposure.

The possibility was considered that a Ca^{2+} -sensitive component of protoveratrine-induced γ -amino[^3H]butyrate release may exist at short times after depolarization and that later events, such as movement of Ca^{2+} from internal stores, may mask this dependence when efflux is measured over comparatively long (3 min) periods. Therefore, a fast perfusion experiment was performed in which the perfusion rate was increased to 2.5 ml/min and fractions were collected every 30 s. In Krebs-Tris

Table 2. *Effects of drugs and ions on protoveratrine-stimulated $^{22}\text{Na}^+$ uptake*

Brain slices were incubated in Krebs-Tris medium for 10 min with $^{22}\text{Na}^+$ and [^3H]inulin as described in the Experimental section. Protoveratrine A (100 μM) was added at the same time as the radioactive substances to estimate the increase in Na^+ uptake caused by depolarization; this was achieved by subtracting the Na^+ uptake found in the absence of protoveratrine. Test substances were present throughout the 10 min preincubation period as well as during exposure of the slices to protoveratrine and radioactivity, and the protoveratrine-induced Na^+ uptake found in the presence of these substances was then expressed as a percentage of that found in their absence. The average control Na^+ uptake, i.e. that due to the effects of protoveratrine alone was $2.14 \pm 0.18 \mu\text{mol}$ of Na^+ ·g of tissue $^{-1}$ ·min $^{-1}$ (mean $\pm$ s.e.m., $n = 10$), which was twice the blank value for uptake. The statistical significance of the results, when compared with controls, was estimated by Student's t test, with the number of experiments in parentheses. Abbreviation: N.S., not significant.

Test substance	Uptake (% of control)	P
Compound D-600 (0.1 mM)	11.5 ± 2.9 (4)	<0.001
La^{3+} (1.0 mM)	26.3 ± 8.7 (4)	<0.005
Ca^{2+} (10.0 mM)	44.0 ± 7.4 (4)	<0.005
EGTA (no Ca^{2+}) (1.0 mM)	79.0 ± 1.7 (3)	<0.01
Mn^{2+} (5.0 mM)	81.5 ± 1.0 (4)	N.S.
Mg^{2+} (10.0 mM)	117.8 ± 17.8 (4)	N.S.
Procaine (1%, w/v)	0 (4)	*

* Procaine abolished all uptake due to protoveratrine and in addition decreased spontaneous Na^+ uptake by 46%.

Fig. 3. *The effect of protoveratrine A on $^{22}\text{Na}^+$ uptake into brain slices*

Cerebral-cortex slices were incubated for 10 min with $^{22}\text{Na}^+$ and various concentrations of protoveratrine A in Krebs-phosphate solution. The amount of Na^+ taken up into the tissue was calculated as described in the text. This value was divided by the value for the amount of Na^+ taken up in a 10 min control period when protoveratrine was absent to give the fractional increase in sodium uptake caused by protoveratrine. The extracellular space of control slices constituted $77 \pm 2\%$ (mean $\pm$ s.e.m., $n = 31$) of the total tissue volume and 100 μM -protoveratrine did not significantly alter this value [$75 \pm 3\%$ (mean $\pm$ s.e.m., $n = 16$)]. Each point represents the mean for the number of experiments shown in parentheses with vertical bars indicating the s.e.m.

$159 \pm 14\%$ (mean $\pm$ s.e.m., $n = 3$) of the control response to protoveratrine.

$^{22}\text{Na}^+$ uptake

Another possibility considered was that since protoveratrine depolarized excitable tissues by increasing membrane Na^+ conductance, the Ca^{2+} antagonists may interfere with this process directly. Accordingly the various Ca^{2+} antagonists were tested against $^{22}\text{Na}^+$ uptake induced by protoveratrine. $^{22}\text{Na}^+$ was taken up by the brain slices almost linearly over 30 min (Fig. 2) and protoveratrine increased this uptake. The dose-response curve for the effect of protoveratrine was sigmoid and reached a maximum at a concentration of 100 μM (Fig. 3). Several substances inhibited the protoveratrine-induced increase in $^{22}\text{Na}^+$ influx (Table 2). Procaine was the most effective, decreasing spontaneous Na^+ influx in addition to abolishing that due to protoveratrine. Compound D-600 was also very effective, whereas La^{3+} and high Ca^{2+} concentration were less so. EGTA slightly inhibited protoveratrine-induced $^{22}\text{Na}^+$ influx, whereas Mn^{2+} and Mg^{2+} did not alter it significantly.

Discussion

Veratrum alkaloids are finding increasing use as depolarizing agents in models of neurotransmitter release *in vitro* since they distinguish between neuronal and glial release by having no discernible effect on the latter (Minchin, 1975, 1977; Neal & Bowery, 1979). However, to be plausible, models of neurotransmitter release should demonstrate that depolarization-induced efflux is dependent on the presence of extracellular Ca^{2+} since most known

medium exposure of the slices to 100 μM -protoveratrine for 30 s caused a 4.3 ± 0.2 -fold (mean $\pm$ s.e.m., $n = 3$) increase in the efflux rate constant, and 1 mM-EGTA (17 min exposure before depolarization) caused an increase in this to

neurohumoral release processes are thought to require Ca^{2+} (Rubin, 1970). Results from several studies indicate that the Ca^{2+} dependency of veratrum alkaloid-induced transmitter release varies considerably; in general that of acetylcholine (Grewaal & Quastel, 1973) and noradrenaline and dopamine (Blaustein *et al.*, 1972; Patrick & Barchas, 1976) is always dependent on extracellular Ca^{2+} , whereas that of amino acids is not, and is even sometimes enhanced in the absence of Ca^{2+} (Benjamin & Quastel, 1972; Neal, 1979). It has recently been suggested (Minchin, 1980) that some of the contradictions concerning the Ca^{2+} dependency of veratrum alkaloid-induced γ -aminobutyrate release may be due to loss of tissue γ -aminobutyrate stores during certain preparative and handling conditions causing the unmasking of a releasable pool that is inhibited by Ca^{2+} ions. In any event, the nature of the veratrum alkaloid-sensitive releasable pools of acetylcholine and the catecholamines on the one hand, and amino acids on the other, is clearly different and may represent different subcellular sites. In the case of amino acid transmitters, De Belleruche & Bradford (1977) have provided evidence that veratrine promotes release from a cytoplasmic pool in synaptosomes. The present results confirm previous experiments that showed that γ -aminobutyrate release from brain slices induced by veratrum alkaloids was not decreased in the absence of Ca^{2+} in the external medium (Benjamin & Quastel, 1972; Szerb, 1979; Cutler & Young, 1979; Neal, 1979), although the addition of EGTA to the superfusing medium gave conflicting results. For reasons that are not immediately apparent, the effectiveness of EGTA in decreasing protoveratrine-induced γ -amino[^3H]butyrate efflux depended on the duration of exposure and the nature of the buffer superfusing the slices. Interestingly, inhibition of K^+ -stimulated γ -amino[^3H]butyrate efflux also decreased with increasing exposure to EGTA in phosphate buffer. Others have shown that EGTA can inhibit veratrum alkaloid-induced transmitter release (Blaustein *et al.*, 1972; Nadler *et al.*, 1977; Hammerstad *et al.*, 1979), but none has described a dependency on time of exposure to the chelating agent. In any event, the effectiveness of EGTA in inhibiting release does not appear to be related simply to its ability to chelate extracellular Ca^{2+} .

High concentrations of Mg^{2+} and various other Ca^{2+} antagonists, both organic and inorganic, were effective in decreasing the evoked γ -aminobutyrate efflux. In addition, the local anaesthetic procaine completely abolished the evoked efflux and this suggested the possibility that these substances were acting by interfering with the increased Na^+ influx caused by protoveratrine rather than the increased Ca^{2+} influx. If this were the case, a parallel might

then exist between ouabain-induced transmitter release and protoveratrine-stimulated release, since in the former, an increased intracellular Na^+ content appears to be responsible for induction of release and extracellular Ca^{2+} is not required (see also Baker & Crawford, 1975). There is much evidence demonstrating an antagonism between Na^+ and Ca^{2+} for Na^+ channels at the neuromuscular junction (Kelly, 1965; Gage & Quastel, 1966), and if Ca^{2+} were to compete with Na^+ for Na^+ channels in brain tissue then one might expect that increased Ca^{2+} concentration would also inhibit protoveratrine-induced γ -aminobutyrate efflux. This was found to be the case and so the hypothesis was tested directly by investigating the effects of the Ca^{2+} blocking agents on $^{22}\text{Na}^+$ influx into the brain slices, although the possibility remains that alterations of Na^+ influx may be due to competition with protoveratrine for its site of action.

In agreement with the findings of Shankaran & Quastel (1972) protoveratrine caused an increase in Na^+ uptake into the slices; moreover, the dose-response curves for the effect of protoveratrine on Na^+ uptake and γ -amino[^3H]butyrate release were remarkably similar. For the most part the substances that inhibited protoveratrine-induced γ -aminobutyrate release also inhibited protoveratrine-stimulated Na^+ uptake, and their rank order of potency was similar in the two circumstances, implying a causal relationship between Na^+ uptake and transmitter release. However, the correlation was not perfect; both Mg^{2+} and Mn^{2+} were quite effective as inhibitors of γ -aminobutyrate efflux, whereas they did not significantly inhibit Na^+ influx. It remains unclear why Mn^{2+} and Mg^{2+} , which were found by Catterall (1975) to be potent and relatively less potent competitive inhibitors respectively of activation of the Na^+ ionophore in cultured neuroblastoma cells by veratridine, failed to significantly inhibit Na^+ influx caused by protoveratrine in the present study. However, the inhibition by compound D-600 of Na^+ uptake described in the present paper confirms the recent experiments of Nachshen & Blaustein (1979), in which the organic Ca^{2+} antagonists verapamil and compound D-600 diminished the veratridine-induced increases in fluorescence seen in synaptosomes that had been treated with a voltage-sensitive fluorescent dye. Since enhanced fluorescence in this preparation is proportional to depolarization of the tissue it was suggested that the Ca^{2+} antagonists were blocking Na^+ channels. The effect of verapamil, which is closely related structurally to compound D-600, was independent of veratridine concentration, implying that the Ca^{2+} antagonist did not interfere with the binding of veratridine.

It appears therefore that most of the Ca^{2+} antagonists that inhibited protoveratrine-induced

γ -aminobutyrate efflux did so not by blocking Ca^{2+} uptake but by inhibiting the Na^{+} influx associated with the action of the alkaloid. Previous experiments have demonstrated that a decrease in Na^{+} influx, caused either by removal of extracellular Na^{+} (Redburn *et al.*, 1976) or by tetrodotoxin (Grewaal & Quastel, 1973; Redburn *et al.*, 1976), similarly inhibits veratrum alkaloid-induced transmitter release. However, the inhibition of release seen with Mn^{2+} and Mg^{2+} cannot be explained in this way.

Despite the failure of removal of extracellular Ca^{2+} to inhibit protoveratrine-stimulated γ -aminobutyrate release it is possible that intracellular Ca^{2+} plays an important role in this process, particularly since the experiments of Meves (1966) suggest that the depolarizing veratrum alkaloids have an intracellular site of action. In these experiments veratridine was supplied to the squid giant axon by internal perfusion and the resultant after-depolarization was found to be more rapidly reversed on washing than that caused by external application. Mn^{2+} and Mg^{2+} may therefore have inhibited γ -aminobutyrate release by an action at intracellular sites, for example, by preventing mobilization of Ca^{2+} from intracellular stores. Moreover, $\text{Na}^{+}/\text{Ca}^{2+}$ exchanges have been described in a number of tissues (Blaustein, 1974) and therefore the increased intracellular Na^{+} concentration caused by protoveratrine may contribute to Ca^{2+} mobilization by exchanging with Ca^{2+} present in intracellular stores such as mitochondria.

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