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STIMULATION OF RAT CEREBELLAR GUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE (CYCLIC GMP) LEVELS: EFFECTS OF AMINO ACID ANTAGONISTS

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1 The ability of glutamate, aspartate and related neuroexcitants to produce large calcium-dependent increases in the levels of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) in immature rat cerebellar slices has been demonstrated.

2 These effects were inhibited by selective antagonist compounds, indicating the presence of at least two types of excitatory amino acid receptor mediating the cyclic GMP response.

3 Protoveratrine also produced large increases in cyclic GMP, and this action was antagonized by L-glutamate diethylester suggesting that released endogenous glutamate, subsequently interacting with its postsynaptic receptors, is the predominant mechanism.

4 The kinetic characteristics of several of the inhibitor compounds were investigated.

Introduction

The excitatory parallel and climbing fibre input to the rat cerebellum may use glutamate and aspartate as their respective transmitters (Young, Oster-Granite, Herndon & Snyder, 1974; Hudson, Valcana, Bean & Timiras, 1976; Nadi, McBride & Aprison, 1977; Foster & Roberts, 1980a). Administration of glutamate *in vivo* (Biggio & Guidotti, 1976; Briley, Kouyoumdjian, Haidamous & Gonnard, 1979) or its addition to cerebellar slices (Schmidt, Ryan & Molloy, 1976; Schmidt, Thornberry & Molloy, 1977) produces a significant increase in levels of guanosine 3′,5′-cyclic monophosphate (cyclic GMP). Particularly large increases in cyclic GMP can be elicited by glutamate in slices of rat cerebellum during a short period of postnatal development, coincident with the morphological and biochemical maturation of the cerebellum (Garthwaite & Balázs, 1978).

Recently we reported that a range of excitatory amino acids and analogues, including aspartate, (±)-ibotenate, DL-homocysteate, N-methyl-D-aspartate, L-cysteine sulphinate, l-amino-cyclopentane-1,3,-dicarboxylic acid (cyclopentyl glutamate) and kainate were also able to produce an increase in cyclic GMP concentrations. In general, the rank order of potency was close to that for their ability to excite mammalian neurones (Foster & Roberts, 1980b) and preliminary qualitative data indicated that L-glutamate diethylester (but not D-α-aminosuberate) was an effective antagonist of the effects of L-glutamate and L-aspartate.

The object of the present study was to investigate in detail the effects of a range of compounds that have been suggested to act as specific excitatory amino acid receptor antagonists, on the stimulation of cerebellar slice cyclic GMP levels by L-glutamate, L-aspartate and related compounds. We have also used protoveratrine, a drug that releases endogenous transmitters from nerve terminals (Grewaal & Quastel, 1973; Hammerstad, Cawthorn & Lytle, 1979; Minchin, 1980), in an attempt to elucidate whether an endogenous transmitter is involved in the cyclic GMP response, and to investigate its pharmacological characteristics.

Methods

Preparation of tissues

Eight-day or 15-day old female Wistar rats were killed by decapitation and their cerebella removed. After slicing (0.5 × 0.5 mm) on a McIlwain chopper, the tissue was preincubated in Krebs-bicarbonate buffer (composition mM: NaCl 114, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.6, glucose 11.7, and $NaHCO_3$ 25; pH 7.4) (20 mg tissue/ml) at 37°C, under an atmosphere of 95% O_2 : 5% CO_2 in small conical flasks. After 90 min, glutamate or other agonist drug was added and the incubation continued for a further 5 min. The effects of the antagonists were investigated generally by their inclusion in the incubation medium 10 min before addition of the agonist.

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The incubation was terminated by aspiration of the Krebs-bicarbonate medium, and replacement with 0.05 M Tris-HCl (pH 7.5) containing 4 mM disodium edetate (EDTA) which was then heated at 100°C for 10 min. After homogenization and centrifugation in a Beckman microfuge, 50 μl aliquots of the supernatant, containing between 0.05 pmol to 10 pmol cyclic GMP, were taken for analysis of cyclic GMP.

Antibody binding of cyclic GMP

The raising of antibodies to cyclic GMP was based on the method of Steiner, Parker & Kipnis (1972). Twenty milligrams of human serum albumin (Calbiochem) followed by 10 mg l-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (Sigma) were dissolved in 2.05 ml of a solution of 15 mM succinyl cyclic GMP (Boehringer) in distilled water. The mixture was adjusted to pH 5.5, incubated in the dark at 24°C for 20 h and then dialysed at 4°C for 48 h, with 5 changes of dialysing solution (0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl). Equal volumes of the conjugate solution and complete Freund's adjuvant (Difco) were emulsified, and 0.1 ml containing 0.25 mg of conjugate was injected subcutaneously at each of 4 different sites on the backs of New Zealand White rabbits. Six weeks later each rabbit received two booster injections of 0.1 ml emulsion subcutaneously on the back. After a further two weeks, the animals were bled from the marginal ear vein, and the serum separated and stored at 4°C.

Serum was diluted with 0.9% w/v NaCl solution (saline) to a final concentration of approx. 1:80 in the assay, together with 0.4 pmol [8-^{3}H]-guanosine 3',5'-cyclic monophosphate (58 Ci/mmol) (Radiochemical Centre, Amersham) and sample, in 50 mM Tris-HCl buffer, pH 6.5, containing 4 mM EDTA. After incubating for approx. 18 h at 4°C, 200 μl of a stirred charcoal solution (1 g activated charcoal; 25 mg dextran, mol. wt. 70,000 (Sigma) in 100 ml Tris-HCl/EDTA buffer) was added and left to stand for 5 min. After centrifugation at 12,000 *g* for 90 s, the supernatant was added to 8 ml scintillant (xylene 6.67 ml, Synperonic NXP 3.33 ml, PPO 40 mg and dimethyl POPOP 5 mg) and radioactivity determined by liquid scintillation counting.

Recovery of internal standards added at the 100°C heating stage was approximately 90% and a linear relationship between sample volume and cyclic GMP was demonstrated. Sensitivity of the assay was better than 100 fmol cyclic GMP. The amounts of possible interfering substances which displaced [^{3}H]-cyclic GMP binding with an equivalent potency to 4 pmol unlabelled cyclic GMP were as follows: 10 μmol ATP, 2 μmol AMP, 8 μmol IMP, 640 nmol GMP, 400 nmol GTP, 400 nmol GDP, 240 nmol cyclic AMP and 10 nmol theophylline.

Drugs and their abbreviations

L-Glutamate (L-Glu), L-aspartate (L-Asp), kainate, protoveratrine A/B (PTV), glutamate diethylester (GDEE) were all obtained from Sigma.

2-Amino-3-phosphonopropionate (APP) and 2-amino-4-phosphonobutyrate (APB) were both from Calbiochem.

2-Amino-5-phosphonovalerate (2APV), D-α-aminoadipate (DαAA), DL-α-aminosuberate (DLαAS), γ-D-glutamylglycine (γDGG), *cis*-piperidine-2,3,-dicarboxylic acid (*cis*-PD), 3-amino-l-hydroxypyrrolidone (HA966) and N-methyl-D-aspartate (NMDA) were all generous gifts from Dr J.C. Watkins (Bristol).

(±)-Ibotenate ((±)-Ibo) was a kind gift from Dr C.H. Eugster (Zurich).

Results

Calcium dependence of stimulation of cyclic GMP

As found previously (Foster & Roberts, 1980b), L-glutamate produced a dose-related increase in cyclic GMP concentrations, in cerebellar slices from 8-day old animals. The cyclic GMP levels rose from basal (0.5 ± 0.05 pmol/mg protein, $n = 42$) to a nearly half-maximal increase (41.5 ± 4.0 pmol/mg) protein, $n = 15$) at 1 mM glutamate, and to a maximal increase at 3 mM glutamate of 96 ± 8.0 pmol/mg protein ($n = 15$). EC_{50} values for a variety of agonists are shown in Table 1. The enhancement of cyclic GMP levels by all of the amino acids tested was maximal at 2–5 min. The effect of glutamate has now been found to be strongly calcium-dependent and the presence of 1 mM glutamate with zero-calcium, or 1 mM verapamil (calcium antagonist), or 2 mM Mg^{2+}, resulted in cyclic GMP levels of 0.3, 2.0 and 30.9 pmol/mg protein respectively. The response to kainate was also almost completely abolished by removing calcium, or by verapamil. The calcium ionophore, A23187 up to 0.1 mM was found to stimulate cyclic GMP in a dose-related fashion. However, the maximal effect observed, at 10 μM, was less than one tenth of the maximal stimulation caused by glutamate.

Effects of antagonists

Table 1 shows the ability (expressed as IC_{50} values) of a number of proposed excitatory amino acid antagonists to influence the cyclic GMP responses to the agonists L-glutamate, L-aspartate, N-methyl-D-aspartate (NMDA) ibotenate and kainate. L-Glutamate and L-aspartate were antagonized to a similar degree by all of the compounds tested, with the exceptions of 2-amino-5-phosphonovalerate (2 APV) and γ-D-glutamylglycine (γDGG) which were

Table 1 Inhibition of the stimulation of cyclic GMP by excitant amino acids

	Agonist IC_{50} (mM)				
Antagonist	*L-Glu*	*L-Asp*	*NMDA*	(±)-Ibo	*Kainate*
GDEE	0.25	1.0	>1.0	»3.0	0.1
APB	2.90	>3.0	>3.0	»3.0	»3.0
APP	>3.0	2.5	3.0	3.0	1.9
DαAA	»3.0	»3.0	»3.0	2.0	N.T.
DLαAS	»3.0	»3.0	»1.0	»3.0	N.T.
2APV	3.2	0.25	0.054	0.08	0.01
γDGG	»3.0	0.25	0.55	1.0	0.074
Cis-PD	»3.0	»3.0	1.2	0.3	0.3
HA 966	>3.0	1.3	0.19	0.95	0.25

IC_{50} values were determined from log dose-percentage inhibition plots over a wide range of inhibitor concentrations (triplicate incubations at each of 4 inhibitor concentrations) at an agonist concentration of 1 mM (L-Asp, L-Glu, Ibo and kainate) or 0.3 mM (NMDA). EC_{50} values and maximally stimulated levels of cyclic GMP for each agonist were, respectively: L-glutamate, 1.22 mM, 96 ± 8.0 pmol/mg protein; L-aspartate, 1.4 mM, 122 ± 12.0 pmol/mg protein; NMDA, 0.06 mM, 81 ± 8.0 pmol/mg protein; (±)-ibotenate, 0.17 mM, 110 ± 11 pmol/mg protein; kainate, 1 mM, 40 ± 5 pmol/mg protein. The number of incubations was at least 6 in each case, and values varied by less than 15%. Abbreviations of the antagonists are listed in Methods.

more potent against aspartate, and L-glutamate diethylester which was the only agent to be particularly effective in antagonizing glutamate responses.

2APV was a very potent antagonist of both 0.3 mM NMDA and 1 mM ibotenate, which were also strongly antagonized by 3-amino-l-hydroxy-2-pyrrolidone and moderately antagonized by γDGG and *cis*-piperidine dicarboxylic acid (*cis*-PD). With the exception of the antagonism by *cis*-PD, the pharmacological profile of the two agonists was very similar.

A more detailed investigation of the antagonism of NMDA response by 2APV was carried out, in order to determine the nature of the inhibition. A plot of V/V_I against antagonist concentration I (Barlow, 1980) where V is the cyclic GMP accumulation with agonist alone, and V_I is the accumulation in the presence of inhibitor, reveals whether the antagonism is non-competitive (the lines overlap). Conversely, if the slopes of the lines decrease with increasing agonist concentration, then inhibition is likely to be competitive. In the case of NMDA and 2APV (Figure 1) increasing the concentration of NMDA resulted in a reduced inhibition of 2APV. Assuming therefore that the antagonism is wholly competitive, the K_i for 2APV may be calculated according to the equation

$$K_i = \frac{IC_{50}}{1 + \frac{NMDA}{K_m}}$$

(Cheng & Prusoff, 1973) where the apparent K_m for NMDA is approx. 0.06 mM. For NMDA concentrations of 0.1, 0.3 and 1.0 mM apparent K_i values of 19, 9 and 17 μM respectively were obtained.

Kainate was exceptional amongst the agonists in that apart from 2-amino-4-phosphonobutyrate (APB), each of the antagonists tested was effective in inhibiting the cyclic GMP response elicited by this compound. In contrast to the antagonism of NMDA

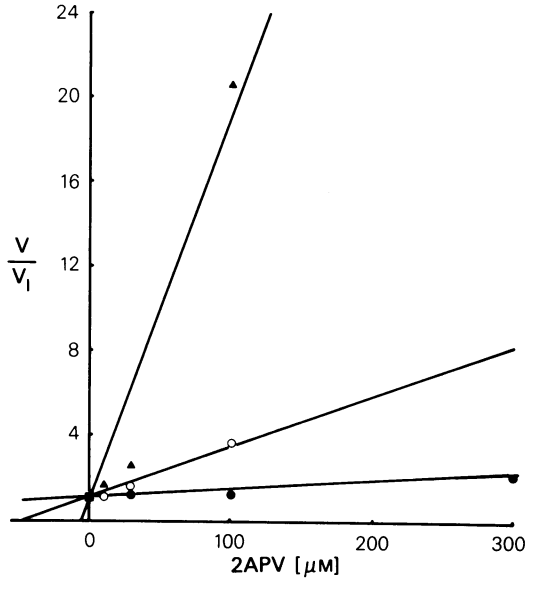

Figure 1 Modified Dixon plot (Barlow, 1980) of V/V_I against concentration of 2-amino-5-phosphonovalerate (2APV) for 3 different N-methyl-D-aspartate (NMDA) concentrations. V is cyclic GMP level in cerebellar slices in presence of NMDA alone, and V_I that seen after the addition of 2APV. Incubation and assay procedures were as described in methods. Slopes and the 95% confidence limits of the slopes were found by regression analysis and were as follows:- 0.1 mM NMDA (▲), 0.181 ± 0.07; 0.3 mM NMDA (○), 0.0246 ± 0.01; 1.0 mM NMDA (●), 0.005 ± 0.001.

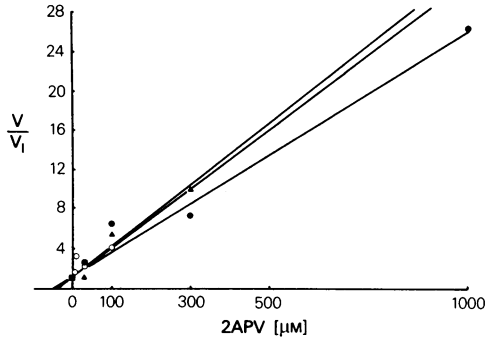

Figure 2 Plot of V/V_I against concentration of 2-amino-5-phosphonovalerate (2APV) for 3 different concentrations of kainic acid. Methods and statistical analysis were the same as for Figure 1. Slopes and 95% confidence limits were:- 0.3 mM kainate (○), 0.031 ± 0.0022; 1.0 mM kainate (●), 0.025 ± 0.0048; 3.0 mM kainate (▲), 0.030 ± 0.0075.

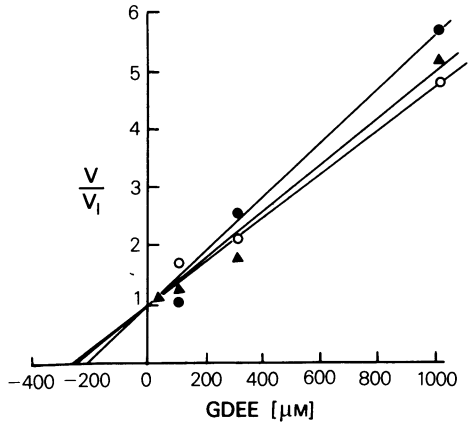

Figure 3 Plot of V/V_I against concentration of glutamate diethylester (GDEE) for 3 different concentrations of kainic acid. Methods and statistical analysis were the same as for Figure 1. Slopes and 95% confidence limits were:- 0.5 mM kainate (▲), 0.0040 ± 0.0008; 1.0 mM kainate (●), 0.0047 ± 0.001; 3.0 mM kainate (○), 0.0038 ± 0.0007.

by APV, however, the antagonism of kainate was independent of kainate concentrations in the range of 0.3–3 mM (Figure 2) as there was no significant difference in the slopes of the three lines. When all the points were plotted as a single line, the intersection with the ordinate axis occurred at −39.2 μM. The apparent K_i for 2APV against kainate was therefore 39.2 μM somewhat greater than the apparent K_i against NMDA.

Similarly, the inhibition by glutamate diethylester (GDEE) was also independent of the kainate concentration between 0.5–3.0 mM (Figure 3) and the point of intersection of the line through the combined points corresponded to an apparent $K_i = 241$ μM.

If the kainate and 2APV concentrations were fixed at 1 mM and 30 μM respectively, a reduction in the effect of 2APV was observed with increasing external calcium concentrations (Table 2). For example, at a calcium concentration of 0.1 mM, inhibition was almost complete (basal levels only detected) while at a concentration of 10 mM Ca^{2+}, the inhibition was only 4%.

Effects of protoveratrine

When added together with fresh medium to cerebellar slices from 15-day old rats, protoveratrine produced a large increase in cyclic GMP which was maximal after 8–10 min. Incubations of 8 min were therefore routinely used. The concentrations at which threshold, half-maximal and maximal stimulation occurred (3, 18 and 100 μM respectively), corresponded very closely with the concentration-dependency of transmitter release from perfused brain slices treated with protoveratrine (Minchin, 1980) (Figure 4). Of great importance is the observation that no enhancement of cyclic GMP levels occurred when protoveratrine was added to cerebellar slices from 8-day old rats (Figure 4) indicating that in these animals at least, it has no direct action on the guanylate cyclase. It is of interest that neither is there a K^+-evoked, Ca^{2+}-dependent glutamate release de-

Table 2 Effect of external Ca^{2+} on the stimulation of cyclic GMP by kainic acid in the presence and absence of (±)-2-amino-5-phosphonovalerate (2APV)

	Cyclic GMP (pmol/mg protein)		
Ca^{2+} (mM)	*Kainate* (1 mM)	*Kainate + 2APV* (30 μM)	*% Inhibition*
0.1	2.6	0.5	80
0.3	7.7	3.8	51
1.0	32.8	14.3	56
3.0	75.3	43.0	43
10.0	85.1	81.6	4

The stimulation of cerebellar cyclic GMP levels was determined as described in the text. Results are means of triplicate determinations which varied by less than 15%.

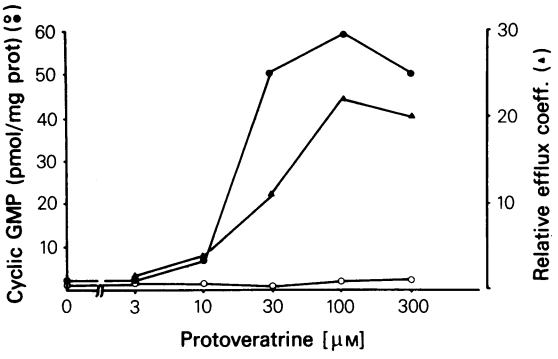

Figure 4 Plot of effects of protoveratrine (PTV) on cyclic GMP accumulation in 15-day (●) or 8-day (○) old rat cerebellar slices and on release of transmitter from rat brain slices (▲) (from Minchin, 1980), against PTV concentration. Incubation and assay for cyclic GMP were as described in Methods. Results for cyclic GMP are means of 4 determinations, whose standard errors were less than 15%.

monstrable in this age group (Roberts, Foster, McBean & Sharif, unpublished observations).

A limited number of antagonists have been studied for their effects on the protoveratrine-elicited increase in cyclic GMP. 2APV, DαAA and DLαAS, which are all proposed selective antagonists at NMDA ('aspartate type') receptors (Watkins, Davis, Evans, Francis & Jones, 1981) had no significant action on the stimulation by protoveratrine (Figure 5). On the other hand, GDEE, a 'glutamate type' receptor antagonist, was moderately potent in inhibiting the stimulation by protoveratrine with an

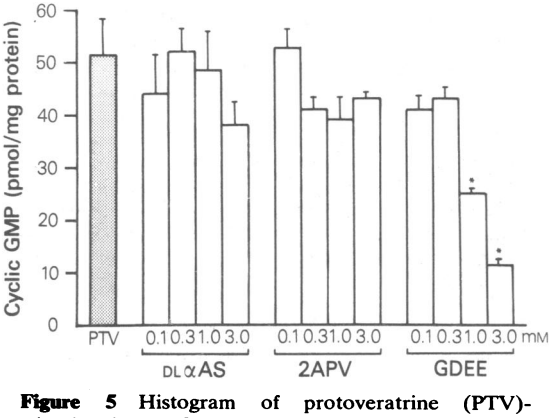

Figure 5 Histogram of protoveratrine (PTV)-stimulated cyclic GMP accumulation in cerebellar slices, in presence and absence of either glutamate diethylester (GDEE), 2-amino-5-phosphonovalerate (2APV) or DL-α-aminosuberate (DLαAS). Incubation and assay procedures were as described in methods. Standard error bars are shown, and level of significance calculated using Student's *t* test: $*P < 0.05$.

IC_{50} against 100 μM protoveratrine of approximately 1 mM. As yet, GDEE is the only drug tested that possessed any specificity against the glutamate-stimulated cyclic GMP accumulation.

Discussion

As we have previously shown (Foster & Roberts, 1980b), several amino acids with potent neuroexcitatory actions were able to produce substantial increases in cyclic GMP levels in tissue slices from cerebella of young (8-day old) rats.

The recent development of selective excitatory amino acid antagonists (Watkins, 1981; Watkins *et al.*, 1981) has led to the proposal of the existence of 3 main classes of receptor, with kainate, NMDA and quisqualate respectively, representing selective agonists for each receptor type. The new antagonists: (1) phosphonic acid analogues of monoamino dicarboxylic acids, (2) conformationally-restricted cyclic analogues of aspartate with the two carboxyl groups *cis*-related to each other and (3) ω-linked dipeptides of glutamate and aspartate, all block NMDA (aspartate-preferring) receptors. 2APV (the phosphonic acid analogue of α-aminoadipate), which is currently the most potent NMDA antagonist was highly effective in inhibiting the stimulation of cerebellar cyclic GMP by L-aspartate. NMDA and ibotenate. Neuronal excitation by ibotenate is antagonized well by DαAA, but only weakly by GDEE (McLennan & Lodge, 1979; Krogsgaard-Larsen, Honore, Hansen, Curtis & Lodge, 1980) and thus, like NMDA, may interact with aspartate-type receptors (Evans, Francis & Watkins, 1978) was also an effective antagonist of cyclic GMP responses to these amino acids. Surprisingly, both DαAA and DLαAS were devoid of significant antagonistic activity, since these have been reported to be effective, aspartate-preferring antagonists (Evans *et al.*, 1978; Biscoe, Davis, Dray, Evans, Martin & Watkins, 1978). The least selective of the newer antagonists, *cis*-PD, exhibited greatest activity against ibotenate, and kainate in this study. The finding that L-glutamate was not effectively antagonized by 2APV, γDGG and HA-966 indicates that the aspartate-preferring receptors involved in the stimulation of cyclic GMP levels are poorly able to accommodate L-glutamate. Indeed, the only antagonist to exhibit potent effects against L-glutamate was GDEE (selective for 'quisqualate type' receptors) (Watkins *et al.*, 1981). It would therefore appear that there are distinct populations of aspartate and glutamate receptors mediating cyclic GMP formation in the cerebellum possibly associated with the climbing fibre and the parallel fibre inputs respectively. Indeed, separate binding sites for these amino acids have been detected on cerebellar

synaptic membranes (Foster & Roberts, 1978; Sharif & Roberts, 1981).

The data for kainate in the young animals are anomalous on several counts. Firstly, compared to other amino acid agonists, kainate exhibited a flattened dose-response curve, shifted to the right of the glutamate curve (Foster & Roberts, 1980b) suggestive of partial agonist effects. Secondly, in this study, the effects of kainate were inhibited potently by both 'glutamate-preferring' and 'aspartate-preferring' receptor antagonists. During development, there appears to be an increase in sensitivity to kainate (Garthwaite & Balazs, 1981) which may be accompanied by qualitative changes in the kainate binding site. It is of significance that the antagonism by both GDEE and 2APV was of a non-competitive nature, and the marked reduction in the effect of 2APV observed with increasing external calcium concentrations, suggests that 2APV at least, may act at a calcium channel required for stimulation of cyclic GMP production.

Protoveratrine proved to be a useful tool in this study for investigating the possibility of increasing cyclic GMP levels indirectly, via released endogenous transmitter, and the finding that its effects were antagonised only by GDEE and not by the NMDA-type of inhibitor, implicates glutamate as the likely major transmitter involved in this response. Although veratridine-induced depolarization is accompanied by an increased Ca^{2+} influx (Blaustein, 1975) presumably at presynaptic sites, it is unlikely that this action is of major significance in the cyclic GMP response, because of the almost total abolition by the selective glutamate antagonist GDEE. In addition, the lack of effect observed in the immature animals, where depolarization-induced calcium-dependent glutamate release processes are absent, may be of significance.

Thus, in the present study, we have found that the antagonist-susceptibility of a number of excitatory amino acid agonists with regard to stimulation of cyclic GMP levels is in harmony with their electrophysiological effects. The findings with kainate suggest that this substance may either interact with other sub-populations of excitatory amino acid receptors in the immature cerebellum, or that during maturation, the kainate receptor characteristics are altered.

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