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NOVEL KAINIC ACID ANALOGUES

EFFECTS ON CYCLIC GMP CONTENT OF ADULT RAT CEREBELLAR SLICES

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Abstract—On the basis of previous electrophysiological studies, it has been proposed that there are three main classes of excitatory amino acid receptor in the mammalian central nervous system, which are activated preferentially by kainic acid, quisqualic acid and *N*-methyl-D-aspartate respectively. Although the pharmacology of the *N*-methyl-D-aspartate receptor has been investigated extensively, potent and selective ligands which act at the kainate or quisqualate sites are lacking. In this study, we report that a number of novel kainate analogues possess either agonist or antagonist activity in a system which permits investigation of receptor-mediated coupled responses, viz. the ability of excitatory amino acids to elevate cyclic GMP concentrations in incubated cerebellar slices prepared from the adult rat. The data reported here provide some clues as to the likely structural requirements for developing effective kainate antagonists.

It is now generally accepted that at least 2 amino acids (L-glutamate and L-aspartate) have major neurotransmitter roles within the mammalian central nervous system [1, 2]. Classification of the receptors through which these compounds mediate their responses has been based predominantly on electrophysiological data, which have suggested that there are three principal types, viz. those activated preferentially by the ligands *N*-methyl-D-aspartate (NMDA), kainic acid (KA) and quisqualic acid (QA) [3].

Despite considerable effort, with the exception of NMDA, there still exists a lack of potent and selective QA and KA antagonists. This has precluded further detailed characterization of these receptors.

In view of this, the present series of experiments were carried out in order to investigate a series of recently synthesized KA analogues (Fig. 1) in relation to their ability to alter adult rat cerebellar cyclic GMP concentrations. Excitatory amino acids are known to increase rat cerebellar cyclic GMP levels via receptors displaying a pharmacological profile similar to that found in electrophysiological studies. Measurement of this nucleotide has served as a useful biochemical approach in assessing coupled excitatory amino acid receptor function [4–6].

MATERIALS AND METHODS

(a) *Incubation procedures*. Male adult Wistar rats were killed by decapitation and their cerebella excised rapidly. The pial meninges were removed and 300 μm thick parasaggital slices were individually prepared on a Vibroslice (Camden Instruments U.K.) from tissues maintained at 37° 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; $NaHCO_3$: 25; pH 7.4). Slices were transferred to small conical flasks containing fresh Krebs bicarbonate buffer and preincubated at 37° under an atmosphere of 95% O_2:5% CO_2.

After 90 min, KA, NMDA, QA and other agonists were added and the incubation continued for a further minute. The effects of antagonists were investigated by their inclusion in the incubation medium one minute prior to the addition of agonists.

The incubation was terminated by aspiration of the Krebs medium and replacement with hot 0.05 M Tris–HCl buffer (pH 7.5) containing 4 mM EDTA, and then heated at 100° for 10 min. After homogenization and subsequent centrifugation in a Beckman microfuge, 50 μl aliquots of supernatant were taken for analysis of cyclic GMP.

(b) *Antibody binding of cyclic GMP*. Antibodies were raised to succinyl cyclic GMP – human serum albumin conjugate, using a modification of the method of Steiner *et al.* [7], as previously described [8]. Following reconstitution in the original volume of distilled water, the antiserum was diluted with 0.9% w/v NaCl solution to a final concentration of approximately 1:90, together with 0.4 pmol [8-^{3}H]-guanosine-3′,5′ cyclic monophosphate (15 Ci/mmol) (Radiochemical Centre, Amersham) and assay sample (in 50 mM Tris–HCl buffer, pH 7.5 containing 4 mM EDTA). After incubating for approximately 18 hr at 4°, 200 μl of a stirred charcoal solution (1 g activated charcoal, 25 mg dextran (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 sec, 300 μl of the supernatant was added to 3 ml scintillant (LKB Optiphase 'RIA') and radioactivity determined by liquid scintillation counting.

Chemicals. Kainic acid (batch No. 32F-0687) and quisqualic acid were obtained from Sigma. NMDA was obtained from Tocris Chemicals (Merseyside, U.K.) and the novel kainate analogues were prepared in our laboratories.

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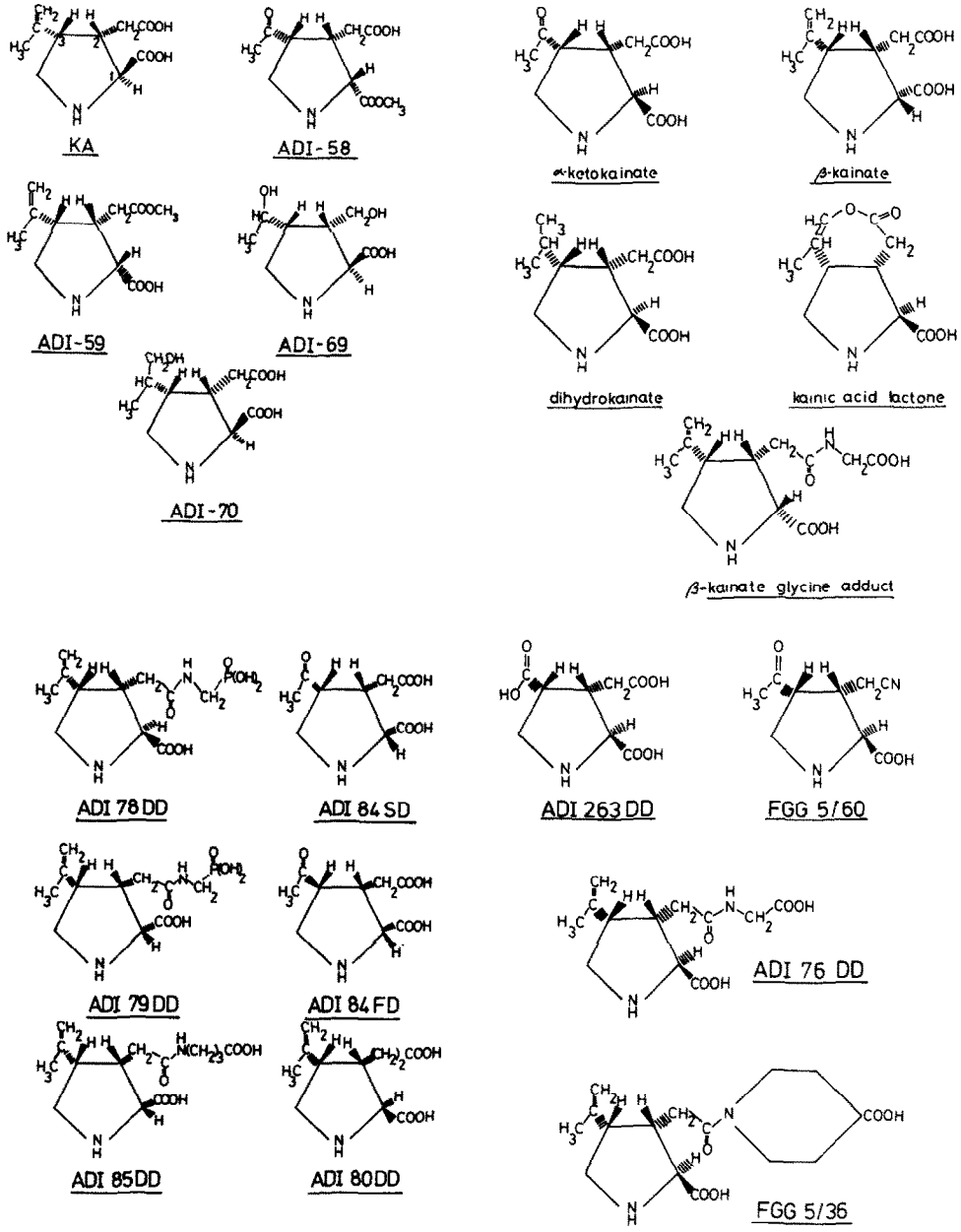

Fig. 1. Structures of synthetic kainic acid analogues investigated.

All other common laboratory reagents were obtained from BDH or Sigma (both of Poole, Dorset U.K.).

Protein was determined by the method of Lowry *et al.* [9], using bovine serum albumin (Sigma) as standard.

RESULTS

(a) *Histology*

Light microscopic examination of sections cut from adult rat cerebellar slices which had been incubated for 90 min in Krebs-bicarbonate medium, revealed a good morphological preservation (Fig. 2). Except in the very centre of the slices, there was minimal damage to any of the cell types. Similar cerebella slice preparations have been shown to be viable in electrophysiological studies [10]. Although wholly adequate slices can, with care, be prepared using buffer at 37°, more recently we have found that the process is facilitated at lower temperatures (we now routinely prepare slices at 15°).

(b) *cGMP response to excitatory amino acids*

KA, NMDA and QA each produced a dose-dependent increase in cyclic GMP when added for 1 min to incubated adult rat cerebellar slices (Fig. 3). KA evoked a maximum stimulation at a concentration of 100 μm, elevating cyclic GMP from basal levels of about 5 pmol/mg protein to about 160 pmol/mg protein, with an EC_{50} of 14 μm, rep-

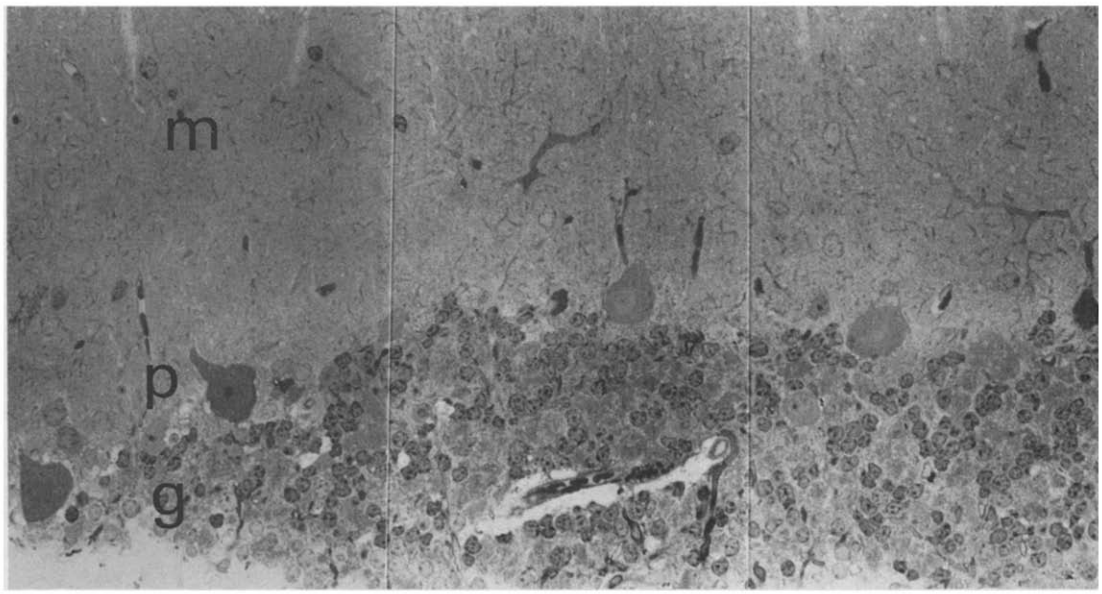

Fig. 2. Light microscopic appearance of slices of adult rat cerebellum incubated in Krebs-bicarbonate medium under 95% O_2:5% CO_2 for 90 min at 37°. Following fixation and embedding of slices in resin using conventional procedures, 1.0 μm sections were cut and stained with toluidine blue for light microscopy. Note healthy, uniform appearance of molecular (M), Purkinje cell (P) and granule cell (G) layers. Pyknotic cells (mainly granule cells) were observed only at the cut edges of slices (not shown). Magnification × 400.

resenting over a 30-fold stimulation. QA and NMDA were less potent than KA, evoking maximum stimulations at 300 μM (80 pmol cyclic GMP/mg protein) and 1000 μm (55 pmol cyclic GMP/mg protein) respectively.

The EC_{50} values were approximately 100 μm for both. The quisqualate receptor agonist AMPA, and the NMDA-type agonist, quinolinic acid were ineffective.

(c) *Time course to excitatory amino acids*

The effect of KA in elevating cerebellar cyclic GMP was rapid, peaking within 1 min and then gradually declining over a 10 min drug exposure (Fig.

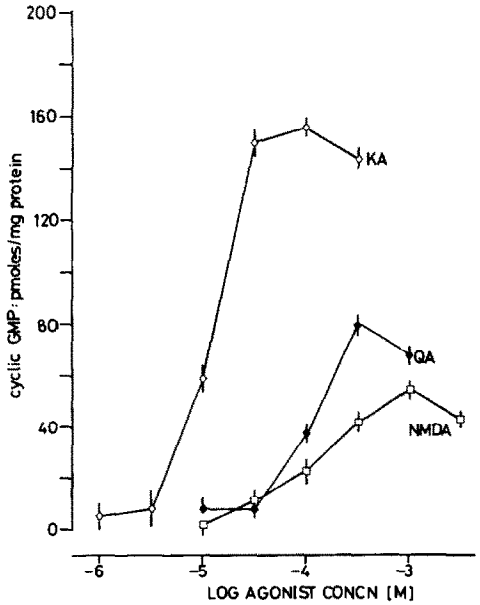

Fig. 3. Concentration dependence of the rise in cyclic GMP in adult rat cerebellar slices evoked by KA, NMDA and QA. Drugs were added to the slices in 10 μl neutralized solution for 1 min at 37° as described in the text. Each point represents the mean ± S.E.M. of 3 separate experiments assayed in triplicate.

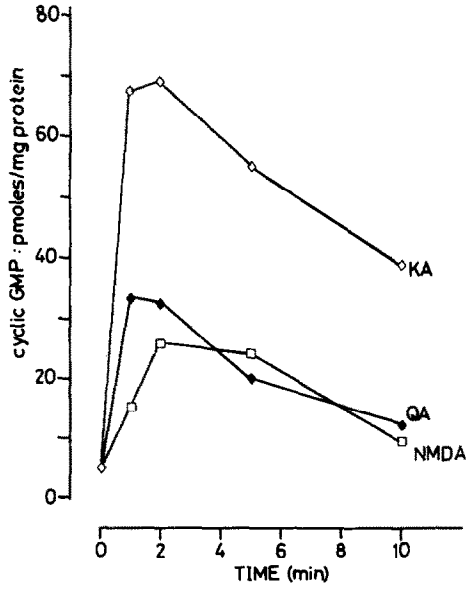

Fig. 4. Time course of the rise in cyclic GMP in response to KA, NMDA and QA, tested at their respective EC_{50}s of 14, 100 and 1000 μm, in adult rat cerebellar slices incubated at 37°. Each point represents the mean of 2 separate experiments assayed in triplicate, with S.E.M. of the mean about 15%.

Table 1. Calcium dependence of stimulation of adult rat cerebellar cyclic GMP concentrations by excitatory amino acids

Treatment	Cyclic GMP (pmol/mg protein)
Basal	5.3 ± 1.2
Kainic acid (14 μm)	74.1 ± 13.2
Kainic acid (14 μm) + 1 mM EGTA (Ca^{2+} free medium)	21.5 ± 4.8
Quisqualic acid (100 μm)	39.0 ± 3.4
Quisqualic acid (100 μm) + 1 mM EGTA (Ca^{2+} free medium)	12.1 ± 1.9
N-methyl-D-aspartate (100 μm)	24.4 ± 4.3
N-methyl-D-aspartate (100 μm) + 1 mM EGTA (Ca^{2+} free medium)	8.2 ± 5.4

Cerebellar slices were prepared and incubated at 37° as described in the text. The calcium-free medium used was Krebs solution containing a final concentration of 1 mM EGTA. Each result is the mean ± S.E.M. of two separate experiments assayed in triplicate.

4). Whilst QA and NMDA displayed a similar rapid onset and decay, as expected, the responses to these agonists (each tested at their EC_{50} concentration) were markedly less than with kainate.

(d) *Calcium dependency of the response to excitatory amino acids*

As reported previously for immature cerebellar slices [8], the ability of KA, NMDA and QA to increase cerebellar cyclic GMP was strongly dependent on the presence of calcium ions (Table 1). Responses to KA, NMDA and QA (tested at their respective EC_{50} values) in a calcium free medium containing 1 mM EGTA were reduced by 72%, 68% and 67% respectively.

(e) *Agonist activity of synthetic KA analogues*

The synthetic KA analogues displayed a differential ability to increase adult rat cerebellar cyclic GMP concentrations (Figs 5a–d and Table 2). ADI-59 was the most potent of the compounds tested with an EC_{50} of 10 μm, evoking a maximal stimulation of 100 pmol cyclic GMP/mg protein at 30 μm, whilst ADI-79DD was the least effective with an EC_{50} of 1778 μM and a maximal stimulation of 55 pmol cyclic GMP/mg protein at a 10 mM concentration.

ADI-70 exhibited a rather different profile to the other compounds in that it was active up to a concentration of 10 μm, with its response subsequently declining at higher concentrations.

As can be seen in Fig. 5, the maximal responses to these compounds varied considerably and thus presentation of EC_{50} values alone as calculated from these plots, is unlikely to provide an accurate indication of the compounds' potencies. Care is therefore required in comparing these data.

Whilst QA and NMDA were apparently not as potent as some of the KA analogues, they were more potent than dihydrokainate and β-kainate in

Table 2. Effects of excitatory amino acids and analogues on adult rat cerebellar slice cyclic GMP concentrations

Drug	EC_{50} (μm)	Maximal response (pmol cyclic GMP·mg protein)	occurring at concn (μm)
ADI-59	11	160	30
Kainic acid	14	160	100
ADI-58	20	95	100
ADI-69	30	60	100
FGG-5/60	56	110	300
ADI-70	60	100	300
ADI-84SD	67	55	3000
Quisqualic acid	100	80	300
NMDA	100	55	1000
ADI-80DD	100	200	3000
ADI-263DD	100	80	300
α-Ketokainate	141	210	1000
Dihydrokainate	150	100	300
β-Kainate	158	50	3000
ADI-85DD	178	80	300
ADI-76DD	178	50	1000
ADI-78DD	200	120	3000
Kainic acid lactone	251	25	3000
ADI-79DD	1778	55	10000

EC_{50} values (concentrations of agonists producing 50% of their maximal responses) were determined from the appropriate log dose–effect curves. Because of the large variation in the maximal responses achieved, concentrations required to achieve maximal effect are also shown. Data were derived from 3 independent experiments performed in triplicate. Compounds FGG-5/36, quinolinic acid and AMPA were inactive.

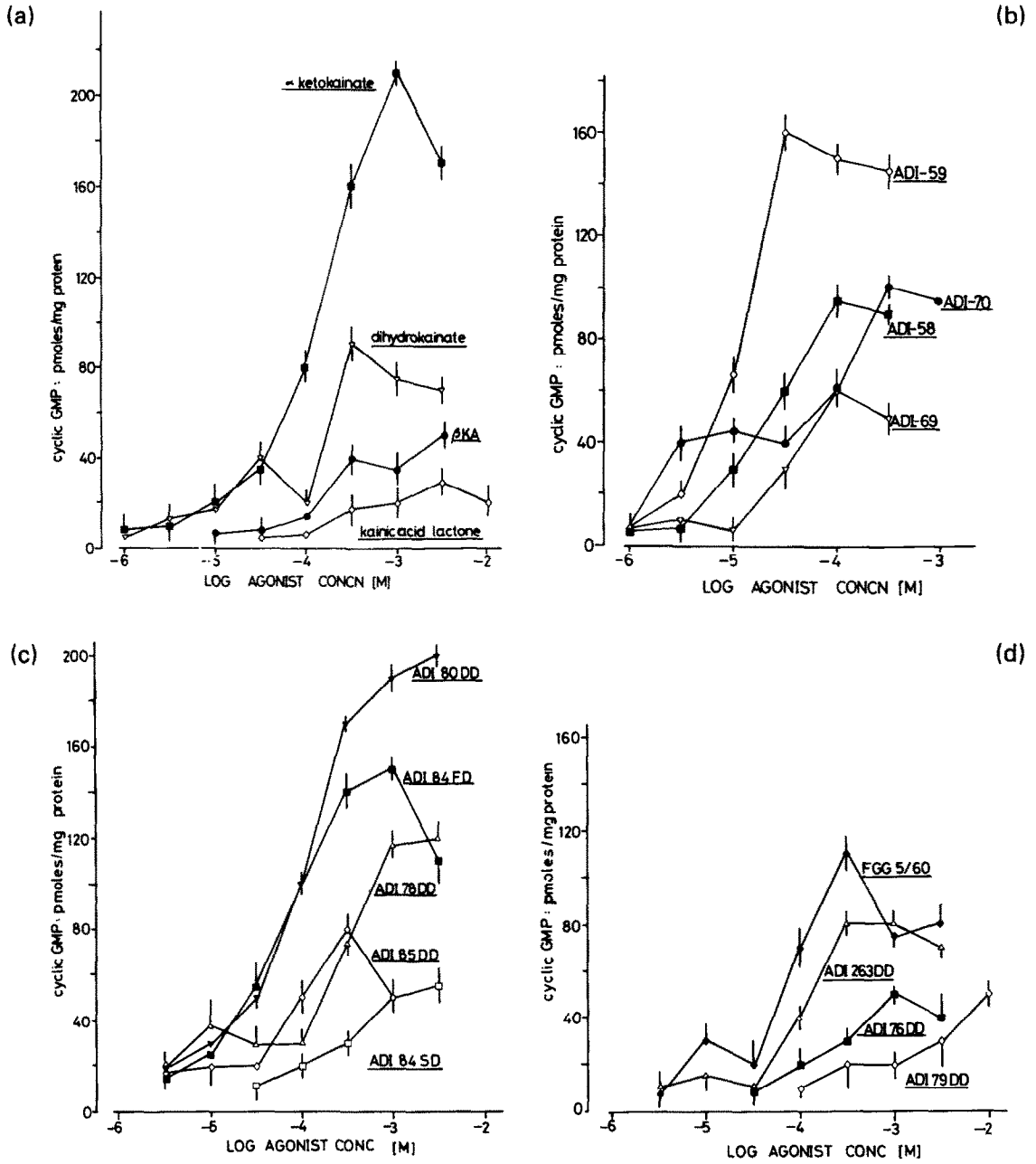

Fig. 5. (a–d) Concentration dependence of the rise in cyclic GMP in adult rat cerebellar slices evoked by KA analogues. Drugs were added to the slices for 1 min at 37° as described in the text. Each point represents the mean ± S.E.M. of 3 separate experiments assayed in triplicate.

increasing cyclic GMP levels. α-Ketokainate evoked the largest maximal response of 210 pmol cyclic GMP/mg protein, but with an EC_{50} of 110 μm, was approximately 9 times less potent than KA.

(f) *The effects of antagonists*

A number of compounds, including the KA analogues, were tested over a wide concentration range (100–1000 μm) for their ability to inhibit excitatory amino acid-evoked increases in adult rat cerebellar cyclic GMP concentrations (Table 3). γ-DGG was able to antagonize responses to KA, NMDA and QA to a similar degree, whilst (−)APV was a potent and selective inhibitor of NMDA-induced responses. GAMS was potent in antagonizing KA responses, but was less so against QA. GDEE appeared to antagonize KA and QA to a similar extent. Neither of these latter antagonists was effective against NMDA. In this study the KA analogues were tested specifically against KA responses. Of these compounds, ADI-76DD, ADI-71DD and FGG-5/36 displayed moderate antagonist activities with IC_{50}s of about 270 μm. Other compounds such as ADI-70, α-kainyl glycine, and ADI-69 were only weakly effec-

Table 3. Inhibition of the stimulation of adult rat cerebellar cyclic GMP concentrations

Antagonist	IC_{50} (μm) KA (14 μm)	NMDA (100 μm)	QA (100 μm)
ADI-76DD	265		
ADI-79DD	285		
FGG-5/36	285		
ADI-78DD	300		
GAMS	320	inactive	850
β-Kainate	320		
ADI-85DD	410		
ADI-70	600		
β-Kainyl glycine	600		
Cis PDA	740	820	inactive
ADI-69	780		
γDGG	850	780	>1000
Dihydrokainate	870		
ADI-84FD	920		
α-Ketokainate	920		
ADI-80DD	>1000		
GDEE	>1000	inactive	680
ADI-59	inactive	<1000	
(−)APV	inactive	210	

GAMS, γ-D-glutamylaminomethyl sulphonate; cisPDA, *cis*-2,3-piperidine dicarboxylic acid; γDGG, γ-D-glutamylglycine; GDEE, L-glutamate diethyl-ester; (−)APV, (−)-2-amino-5-phosphonovaleric acid.

Cerebellar slices were incubated at 37°. Antagonists (concentration range 100–1000 μm) were added 1 min prior to agonist (tested at their respective EC_{50}s (calculated from Fig. 4)) as described in the text. IC_{50}s (the concentration required to produce 50% inhibition for that compound) were calculated from log concentration percentage inhibition plots obtained from 3 independent experiments, with SEMs less than 15%.

ADI-84SD, ADI-263DD, FGG-5/60, Kainic acid lactone and ADI-58 were ineffective against KA.

tive, with IC_{50} values ranging from 600 to 800 μm. ADI-80, ADI-59, ADI-84SD, ADI-263DD, FGG-5/60, kainic acid lactone and ADI-58 were all ineffective as antagonists of kainate-induced responses.

DISCUSSION

KA, NMDA and QA will produce large, rapid, Ca^{2+}-dependent increases in cyclic GMP concentrations in morphologically well-preserved adult rat cerebellar slices prepared on a Vibroslice. The localization of the KA receptors mediating this effect is believed to be on most cell types of the adult rat cerebellum, although Purkinje cells and inhibitory interneurones appear to be more sensitive than granule cells to KA [6] suggesting a predominant involvement of these cell types.

The rank order of potency for ability to increase cerebellar cyclic GMP concentrations was KA > QA ≥ NMDA. The variation in the maximum responses to NMDA and QA (even though the EC_{50} values were identical) might indicate differences in the number of receptors linked to the cerebellar cyclic GMP generating system. In addition, the receptor types are likely to have different anatomical distributions: for example a recent report [11] showed a differential sensitivity of adult rat cerebellar slices to the neurotoxic effects of KA, NMDA and QA. NMDA was toxic to only the inhibitory interneurones, while QA affected both Purkinje cells and inhibitory interneurones but spared granule cells. NMDA has previously been found to be less potent than glutamate in increasing cyclic GMP levels in both dissociated cerebellar cells [12] and in hand-cut adult rat cerebellar slices [5]. This substance is also relatively poor as an excitant of cerebellar Purkinje cells following ionophoretic application, in contrast to the more potent depolarizing effect of QA [10].

Pharmacological characterization of the receptors mediating excitatory amino acid-induced changes in cerebellar cyclic GMP concentrations suggests a close similarity to those described electrophysiologically. (−)APV selectively and potently inhibited the response to NMDA, as has been observed in the rat spinal cord [13]. GDEE attenuated QA responses to a greater degree than those of kainate, a finding in agreement with other workers [14]. The dipeptide γ-DGG, which electrophysiologically has been found to be more effective as an antagonist against NMDA than against QA or KA [15, 16], was in the present study found to inhibit the responses to all three agonists to a relatively similar degree.

GAMS on the other hand, was found to be an effective antagonist of KA responses on cerebellar cyclic GMP, but was considerably less potent against QA and had no effect on NMDA. This antagonist profile is similar to that observed in the cat spinal cord [17].

Marked increases in cerebellar cyclic GMP con-

centrations were evoked by those KA analogues which retained an electron withdrawing group at the C-3 position, e.g. ADI-59, ADI-69, ADI-84 FD and FGG 560. α-Ketokainate and ADI-80DD could also substantially increase cyclic GMP levels, but only at concentrations 10–30 times greater than that required of KA to observe a similar effect.

Reduction of the isopropylene side chain at C-3 as in dihydrokainate, a *cis* orientation of all three asymmetric centres as in β-KA or addition of long chain groups on the C-2 carboxyl terminal as in ADI-76DD and ADI-79DD, all appeared to result in a decreased ability of these compounds to evoke large changes in cerebellar cyclic GMP. Similarly, these modifications have been found to attenuate both the neuroexcitatory and neurotoxic potencies of these compounds [18].

When tested as potential antagonists against KA evoked increases in cerebellar cyclic GMP, those compounds which possessed extended side chains at C-2, such as ADI-76DD, ADI-78DD or ADI-79DD or groupings such as in FGG-5/36, which on their own were poor in ability to increase cyclic GMP, proved to be rather better in attenuating KA responses. Although these compounds did possess an electron withdrawing group at the C-3 position, the presence of the large chain groups at C-2 seemed to confer antagonistic activities. Due to the limited availability of compounds at this stage, it was not possible systematically to examine their selectivity of action at kainate receptors.

Those compounds which were more potent in evoking large increases in cyclic GMP such as ADI-59, ADI-84 or FGG-5/60 were, in turn, found to possess little intrinsic antagonistic activity. These studies with a number of novel kainate analogues may imply that in the design of potent KA antagonists, the compound should possess, together with a pyrolidine ring structure similar to that of KA, a reduced or non electron withdrawing side chain at position C-3, but perhaps additionally, long chain substitutions on the carboxyl terminals, with possibly a β-configuration.

Kainic acid lactone, where the C-2 and C-3 groups are joined together, whilst being an ineffective antagonist against KA was also poor in increasing cyclic GMP levels. Therefore, this type of chemical modification on the KA ring structure may not be appropriate.

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