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A comparison of excitatory amino acid antagonists acting at primary afferent C fibres and motoneurones of the isolated spinal cord of the rat

[1]R.H. Evans, S.J. Evans, P.C. Pook & D.C. Sunter

Department of Pharmacology, the Medical School, University Walk, Bristol, BS8 ITD

1 The potency of 7 excitatory amino acid antagonists has been measured at kainate receptors on primary afferent C fibres using isolated dorsal roots from immature rats. Two of the compounds were tested as antagonists of excitant amino acids at motoneurones using isolated hemisected spinal cord preparations.

2 Mean dose-ratios for antagonism of kainate at C fibres, produced by 1 mM antagonist in at least four preparations, ranged from 2.1 ± 0.5 (mean ± s.e.mean) with 2-amino-5-phosphonopentanoate (AP5) to 17.6 ± 2.4 with 1-(*p*-bromobenzoyl)-piperazine-2, 3-dicarboxylate (BBpzD). The rank order of potency of antagonists at C fibres was similar to that obtained previously for antagonism of kainate at motoneurones.

3 The potency of kynurenate as an antagonist of kainate at C fibres (apparent K_d 70 ± 4.3 μM (mean ± s.e.mean), $n = 12$) was significantly different ($P < 0.005$, Wilcoxon rank sum) from its potency at motoneurones (apparent K_d 164 ± 14 μM, $n = 13$). Kynurenate also was significantly ($P < 0.025$ Wilcoxon rank sum) more potent at antagonizing kainate- than quisqualate (apparent K_d 258 ± 28 μM, $n = 12$)-induced depolarization of motoneurones.

4 Kynurenate and BBpzD (0.25, 1.0 and 4.0 mM) were compared as antagonists of *N*-methyl-D-aspartate (NMDA) at motoneurones and the slope of the Gaddum-Schild plot for kynurenate was markedly greater than 1 (2.01 ± 0.22, 95% confidence limits). A greater than additive antagonism of NMDA-induced depolarizations was produced by combinations of kynurenate with, either AP5, or magnesium ions.

5 The results suggest that the kainate receptor on C fibres may be different from the kainate receptor on motoneurones and that antagonism of NMDA-induced depolarization by kynurenate may not be entirely competitive.

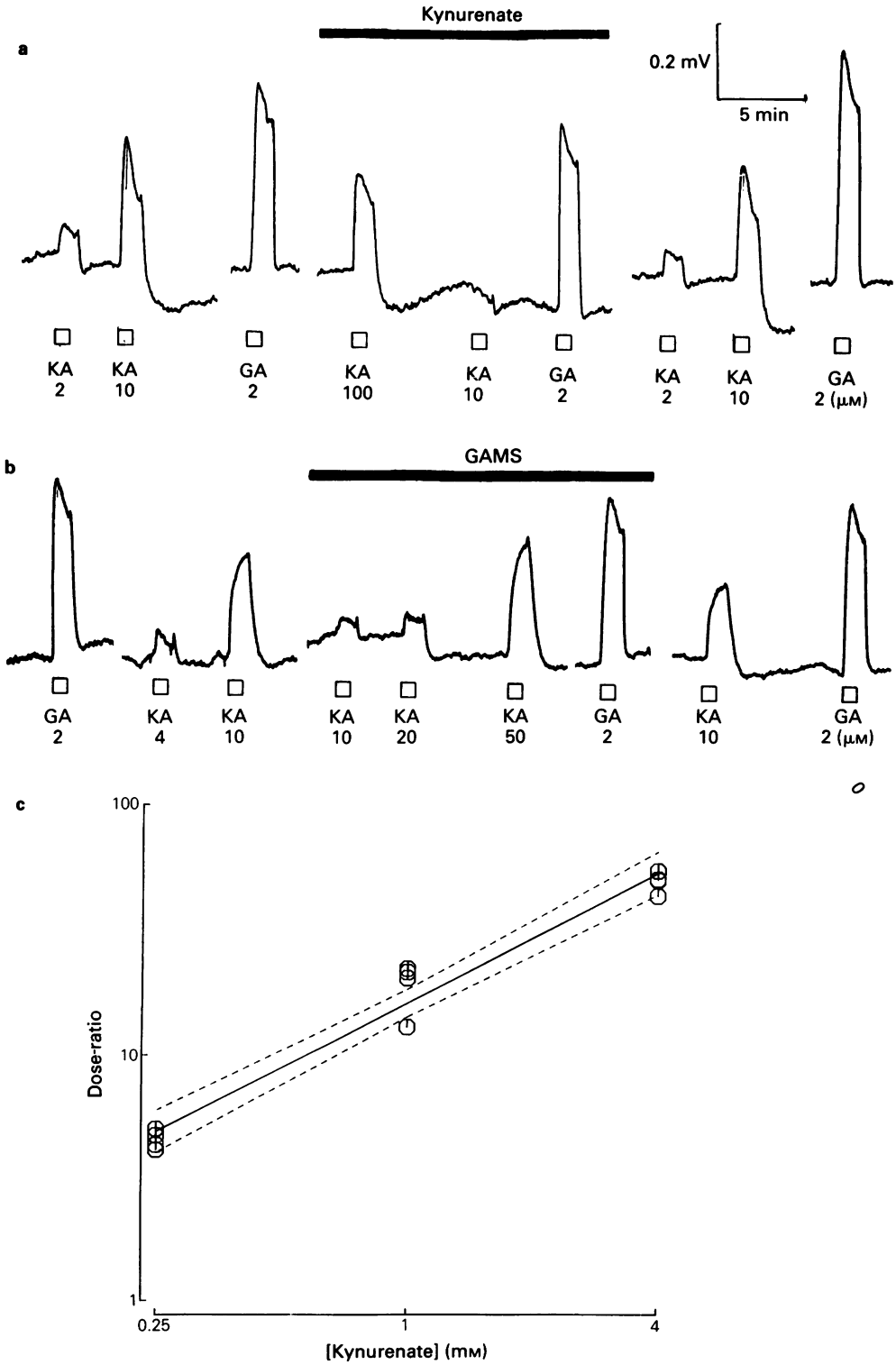
Introduction

A population of C fibres found in dorsal roots and peripheral nerves of the rat are depolarized by kainate and L-glutamate but not L-aspartate (Agrawal & Evans, 1986). Blockade of conduction associated with the selective depolarization of these nerve fibres could represent a potential mechanism for analgesic drug action. However, it would be necessary, for this purpose, to discriminate pharmacologically between the kainate receptors which mediate the depolarization of C fibres and those which mediate the depolarizing action of kainate at central neurones. The present paper describes a structure-activity study of reported antagonists of kainate acting at C fibre receptors.

The compounds which have been compared are as follows. The selective *N*-methyl-D-aspartate (NMDA) antagonist 2-amino-5-phosphonopentanoate (AP5) (Evans *et al.*, 1982); the dipeptides γ-D-glutamylglycine (DGG) and γ-D-glutamylaminomethanesulphonate (GAMS) (Jones *et al.*, 1984); the piperazines 1-(*p*-bromobenzoyl)-piperazine-2, 3-dicarboxylate (BBpzD) and 1-(*p*-chlorobenzoyl)-piperazine-2, 3-dicarboxylate (CBpzD) (Davies *et al.*, 1984); *cis*-2, 3-piperazine dicarboxylate (PDA; Davies *et al.*, 1981) and kynurenic acid (Perkins & Stone 1982; Ganong *et al.*, 1984). Apart from AP5 all these compounds have been shown to antagonize the neuroexcitant action of kainate to varying degrees.

Kynurenate is used widely as an antagonist of non-NMDA receptors thus it was of interest, during the present investigation, to compare the relative antagonist potency of this compound against depolarization of spinal motoneurones produced by NMDA, kainate or

[1] Author for correspondence.


a
Kynurenate
0.2 mV
5 min
KA 2
KA 10
GA 2
KA 100
KA 10
GA 2
KA 2
KA 10
GA 2 (μM)
b
GAMS
GA 2
KA 4
KA 10
KA 10
KA 20
KA 50
GA 2
KA 10
GA 2 (μM)
c
100
10
1
Dose-ratio
0.25
1
4
[Kynurenate] (mM)

quisqualate. The results suggest that the behaviour of kynurenate was competitive as an antagonist of kainate at C fibres or motoneurones but was mainly non-competitive as an antagonist of NMDA at motoneurones.

Methods

Isolated dorsal roots (L5) or hemisected spinal cords, from 1 to 5 day old rats, were maintained at 25°C and superfused with gassed (95% O_2 plus 5% CO_2) medium of the following composition (mM): NaCl 138, KCl 3, $CaCl_2$ 2.5, $NaHCO_3$ 24, dextrose 12.

The methods of stimulation, recording, application of bathing medium and drugs and measurement of potency ratio have been described previously (Evans & Watkins, 1978; Agrawal & Evans, 1986). The spinal root under investigation either attached to a hemisected spinal cord, in the case of ventral roots, or isolated, in the case of the dorsal roots, was placed across a grease seal. The d.c. level was measured between the distal and the central end which was superfused with drugs and bathing medium. Agonists were applied for a contact time of 2 min.

Dose-ratios were interpolated from standard responses, obtained in antagonist-free medium, produced by agonist concentrations ranging from threshold up to 5 μM kainate, applied to dorsal roots, and up to 10 μM kainate, NMDA or quisqualate applied to spinal cords. Rightward shifts of the dose-response curves were parallel within these limits of interpolation and within the limits of variance. The *t*-distribution was used to test the significance of slopes of log dose-ratio versus log concentration plots. The size of groups was too small to allow tests for normality of distribution.

Mean apparent dissociation constant (K_d) values for receptor antagonist complex were calculated from pooled mean dose-ratios obtained at each concentration of antagonist tested on four or more preparations. Each preparation yielded a mean dose-ratio for each concentration of antagonist tested on it. Thus preparations which received more than one concentration of antagonist yielded a mean dose-ratio for each concentration of antagonist. These mean dose-ratios were ranked in order to show significance of differences between different preparations or treatments by use of Wilcoxon rank sum test.

Kynurenic acid, kainic acid, γ-aminobutyric acid (GABA) and picrotoxin were obtained from Sigma Chemical Company. The other amino acids were obtained from Tocris Chemicals.

Results

Application of kainate (1–20 μM) to isolated dorsal root preparations produced dose-dependent depolarizing responses, as previously shown (Agrawal & Evans, 1986). Examples of such responses and their antagonism by kynurenate and GAMS are presented in Figure 1. All antagonists tested produced, within the limits of experimental error and the range of agonist concentration used for interpolation, a parallel rightward shift of the dose-response curve. Antagonists were tested against depolarizing responses to GABA in order to show their selectivity towards kainate (Figure 1). In the series of compounds examined only picrotoxin (20 μM) produced a significant antagonism of GABA-induced responses, giving a dose-ratio, measured on four preparations, of 7.8 ± 0.6 (mean ± s.e. of mean). This concentration of picrotoxin produced no significant antagonism of kainate-induced responses. In contrast the seven amino acid analogues of the present series all produced significant antagonism of kainate at a concentration of 1 mM. Mean dose-ratios for this antagonism are presented in Figure 2. Kynurenate was tested over a sixteen fold range of concentrations. Figure 1c shows the plot of dose-ratio versus concentration of kynurenate. Apparent K_d values and slopes of log concentration versus log dose-ratio are presented in Table 1.

It was of interest to compare the antagonism produced at C fibres with that produced at motoneurones. The open circles in Figure 3 show mean dose-ratios for antagonism of kainate-induced depolarization of motoneurones. The apparent K_d, for 13 values obtained from 8 preparations at the 3 concentrations of kynurenate (Table 1) was significantly different from the value obtained with dorsal root fibres ($P < 0.005$, single tail Wilcoxon rank sum).

Figure 1 Antagonism of kainate-induced depolarization of dorsal root fibres. (a) and (b) Records from separate isolated dorsal root preparations from 4 day old rats. Kainate (KA) and GABA (GA) were applied for the periods indicated below the records at the concentrations shown (μM). (a) Kynurenate (1 mM) and γ-D-glutamylaminomethanesulphonate (GAMS, 1 mM) were applied as indicated by the solid bar. Recordings are shown 15 min after introduction of antagonist and 15 min after washout of antagonist. Data from these recordings are included in Figure 2. (c) Log-log plot of dose-ratios for antagonism of kainate-induced responses by kynurenate in six dorsal root preparations. The points at each concentration of kynurenate are from at least four preparations. The least squares linear regression line with 95% confidence limits (dashed lines) has been fitted to the points.

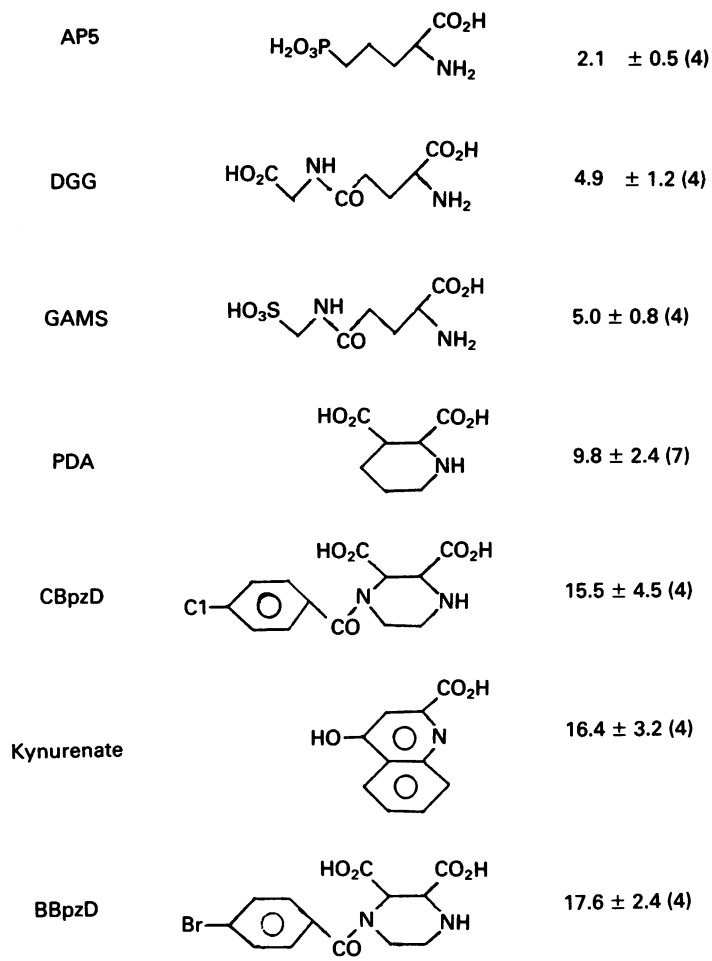


Figure 2 Chemical structure-activity for antagonism of kainate-induced depolarization of dorsal root fibres. Each antagonist was applied at 1 mM and the mean dose-ratios ± s.e.mean (number of preparations in parentheses) are presented on the right of each structure. AP5 = 2-amino-5-phosphonopentanoate; DGG = γ-D-glutamylglycine; CBpzD = 1-(*p*-chlorobenzoyl)-piperazine-2,3-dicarboxylic acid; GAMS = γ-D-glutamylaminomethanesulphonate; PDA = *cis*-2,3-piperazine dicarboxylate; BBpzD = 1-(*p*-bromobenzoyl)-piperazine-2,3-dicarboxylate.

Table 1 Apparent K_d values and slopes of Gaddum-Schild plots for kainate (Kain), *N*-methyl-D-aspartate (NMDA) or quisqualate (Quis) antagonized by kynurenate (Kyn) or (1-(*p*-bromobenzoyl)-piperazine-2,3-dicarboxylate (BBpzD) at either C fibres or motoneurones

Site	*C fibres*		*Motoneurone*		
Antagonist	Kyn	Kyn	Kyn	Kyn	BBpzD
Agonist	Kain	NMDA	Kain	Quis	NMDA
n	12	24	13	15	12
K_d (μM)[a]	70 ± 4	35 ± 6	164 ± 14	258 ± 28	139 ± 18
Slope[b]	0.87 ± 0.11	2.01 ± 0.22	0.77 ± 0.18	0.6 ± 0.12	1.09 ± 0.18
P	<0.05	<0.0005	<0.025	<0.0005	<0.25

[a]Values are means ± s.e.mean. [b]Values are means ± 95% confidence internal.
Probability (*P*) is that for slope population mean = 1. Values calculated from data of Figures 1 and 3.

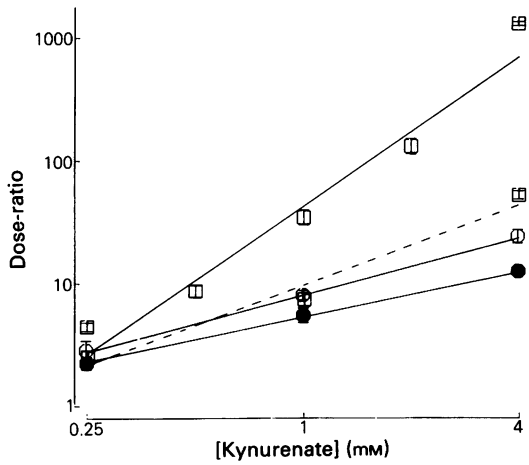


Figure 3 Dose-ratios for antagonism, by kynurenate, of kainate (○) –, quisqualate (●) – and *N*-methyl-D-aspartate (NMDA) (□)-induced depolarization of motoneurones. The broken line joins points showing the antagonism of NMDA-induced depolarizations by (1-(*p*-bromobenzoyl)-piperazine-2, 3-dicarboxylate (BBpzD). Each point is the mean ± s.e.mean obtained from at least four preparations. In most cases error bars are within the symbols. Regression lines have been fitted to each set of points as in Figure 1.

Kynurenate has been reported, on the basis of relative depression of depolarizing responses, to show selectivity towards kainate relative to quisqualate receptors (Ganong *et al.*, 1984). It was of interest, during the present study, to quantify this apparent selectivity. Thus dose-ratios for antagonism of quisqualate-induced depolarization of motoneurones were measured also at 0.25, 1.0 and 4 mM kynurenate (Figure 3). An apparent K_d value (Table 1) significantly different from that for kainate was obtained for the antagonism of quisqualate by kynurenate ($P < 0.025$, single tail Wilcoxon rank sum).

The Gaddum-Schild plots for antagonism of kainate and quisqualate by kynurenate have slopes equal to or less than one (Figures 1 and 3; Table 1). However, the apparent K_d for antagonism of NMDA-induced depolarization, of motoneurones by kynurenate, increased markedly with increasing concentrations of kynurenate (Figure 3 and Table 1). To determine if this difference in the nature of antagonism, towards NMDA as compared with non-NMDA receptors, was likely to be common to all the compounds of the present series a Gaddum-Schild plot was constructed for one other compound. The compound selected for this further test (BBpzD) was chosen because it had a similar antagonist potency to kynurenate at C fibre kainate receptors (Figure 2). It can be seen in Figure 3 that this piperazine analogue showed behaviour close to that expected for a competitive agent (Table 1).

Competitive antagonists which act at the same site, should, when combined in mixtures, yield values of dose-ratio equivalent to the sum of the ratios which each component of the mixture would produce alone. Under similar circumstances a mixture of two antagonists which acted at independent sites should yield a dose-ratio approximating to the product. Three sites have been described at which antagonism of NMDA-induced responses can occur (Martin & Lodge, 1985). Magnesium ions produce a non-competitive action at one of these sites (Davies *et al.*, 1978) but AP5 competes with NMDA (Evans *et al.*, 1982; Harrison & Simmonds, 1984; Olverman *et al.*, 1984). Thus, in order to provide further information about the site at which kynurenate produces antagonism of NMDA-induced depolarization, dose-ratios produced by a mixture of kynurenate with AP5 and by a mixture of kynurenate with magnesium ions were measured.

In 4 spinal cord preparations 50 μM AP5 produced a mean dose-ratio of 7.6 ± 0.8 (s.e.mean) for antagonism of NMDA-induced responses. When a mixture of 50 μM AP5 and 1 mM kynurenate was applied to these preparations the mean dose-ratio increased to 157 ± 2.3. The mean dose-ratio produced by 1 mM kynurenate alone, from Figure 3, was 36.3 ± 5.2 ($n = 5$). Thus the ratio produced by the mixture (expected value of sum = 43, expected value of product = 234) is consistent with the non-competitive nature of the plot for kynurenate versus NMDA in Figure 3. Mg^{2+} 1 mM produced a dose-ratio for antagonism against NMDA of 4.8 ± 0.17 ($n = 4$) and a mixture of 1 mM Mg^{2+} and 1 mM kynurenate gave corresponding ratios higher than 1000 on all 4 preparations tested, indicating that kynurenate and Mg^{2+} do not have additive actions.

The piperazine derivative BBpzD, represented by the broken line in Figure 3, gave a ratio at 1 mM (from the data in Figure 3) of 7.6 ± 1.0. This concentration of piperazine in the presence of 50 μM AP5 gave ratios of 20.9 and 22.4 in two preparations. This contrasts with the behaviour of kynurenate.

Effect of kynurenate on conduction in C fibres

The depolarization of dorsal root fibres produced by kainate (Figure 1) has been located exclusively to C fibres because only the C fibre volley has been shown to be affected by kainate (Agrawal & Evans, 1986). It was of interest in the present series of experiments to see what effect an antagonist of kainate would have on the action potential of C fibres. In three isolated dorsal root preparations kynurenate (1 mM) alone had no apparent effect on the compound C fibre action potential. However, as expected, this level of kynuren-

ate reversed the kainate-induced depression of the C fibre compound action potential (Figure 4).

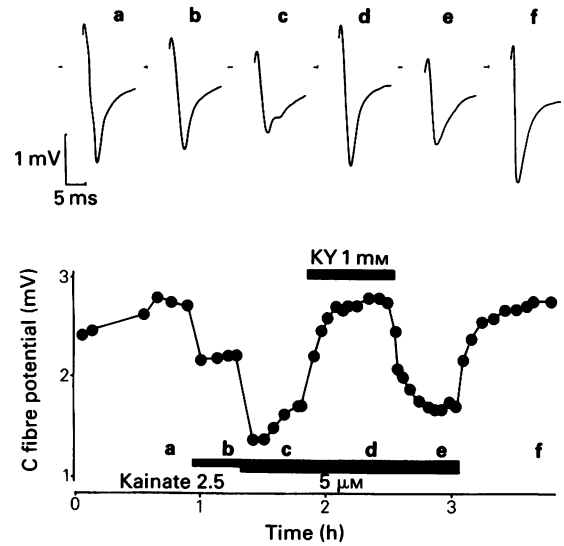


Figure 4 Reversal, by kynurenate (KY), of the depressant effect of kainate on the C fibre compound action potential of an isolated dorsal root (L5) from a 5 day old rat. Each point represents a single compound action potential, stimulation rate 0.05 Hz. Representative compound action potentials, recorded at the points indicated by the letters below the plot, are shown above. Introduction of kainate depressed the amplitude of the volley and introduction of kynurenate reversed this depression.

Discussion

The excitatory amino acid receptors on C fibres have been identified as kainate selective sites because of their selectivity towards agonist (Agrawal & Evans, 1986). The rank order of potency of the antagonists shown in Figure 2, with AP5 having the lowest potency, is consistent with this classification and it is broadly similar to the rank order of potency for these compounds in antagonizing the kainate-induced depolarization of motoneurones (Jones *et al.*, 1984; Watkins *et al.*, 1986).

The range of potency reflected by the dose-ratios presented in Figure 2 is not very great but it may be significant that the three straight chain compounds are less potent at C fibres than the heterocyclic ones. Possibly the more compact conformation, with the three functional groups fixed in space by the ring structure, favours binding to the kainate site.

All seven compounds of the present study are structural analogues of excitatory amino acids and might have been expected to compete with agonists at receptors. The low potency of even the strongest agent of the present series did not allow measurements of competition to be made over a wide range of concentrations. Kynurenate was selected for closer study on account of its plentiful supply and relatively high potency at C fibre receptors.

The slopes of Gaddum-Schild plots for antagonism of kainate or quisqualate produced by kynurenate at C fibres or motoneurones were close to, but significantly less than, one (Table 1). It is likely, in view of the low potency of this antagonism over the narrow range of kynurenate concentrations tested, that these values of slope may not be inconsistent with competition between kynurenate and quisqualate or kainate at receptors on C fibres or motoneurones. The standard deviation of slopes, from which significance was calculated, is that for the slope at the mid-point of the range of antagonist concentration.

There was a very significant difference, between C fibres and motoneurones, in the apparent K_d for the kynurenate-kainate receptor (Table 1). Possibly this difference is caused by a difference between the structure of kainate receptors at these two sites. Alternatively, since kynurenate is an endogenous compound, it is conceivable that kynurenate could be actively removed from the vicinity of motoneurones thereby lowering its potency. Potency at dorsal root fibres, due to the absence of neuropil, would be affected less by such a consideration.

The value of the slope (Table 1) for kynurenate versus NMDA was considerably greater than one, indicating a clear difference between the nature of antagonisms produced by kynurenate acting at non-NMDA and NMDA sites. Such a difference is supported by the failure of the combination of AP5 and kynurenate to produce an additive antagonism. Therefore, it appears that the antagonism of NMDA-induced depolarization by kynurenate may be non-competitive. It is unlikely that this action of kynurenate is produced at the Mg^{2+}-sensitive site of the NMDA receptor/ionophore since the action of kynurenate was not additive with that of Mg^{2+}. Hence, kynurenate may be placed, in terms of its NMDA antagonist properties, in an ever increasing list of chemically disparate amines which act at a site or sites distinct from either the recognition site or the Mg^{2+} site of the NMDA receptor/ionophore. This site may be that characterized as the phencyclidine σ site by other workers (Anis *et al.*, 1983; Berry *et al.*, 1984). This non-competitive property was not shared by the only other member of the series which was tested as an *N*-methylaspartate antagonist, BBpzD, even though this compound was at least as potent as kynurenate when tested at kainate receptors (Figure 2). It is interesting that kynurenate and BBpzD were found to have similar, but weak, potencies in displacing a

labelled NMDA antagonist (AP5) from its binding sites (Watkins *et al.*, 1986). Thus it may be concluded that there is weak competition between kynurenate and *N*-methylaspartate but this is masked by the non-competitive action which prevails, particularly at higher concentrations of kynurenate.

Kynurenate has been found previously to show selectivity towards kainate compared with quisqualate (Ganong *et al.*, 1984). This selectivity would appear to be of a low order since in the present study a less than two fold difference between apparent K_d values for quisqualate and kainate was estimated (Table 1). The selectivity of the antagonism produced by kynurenate towards NMDA relative to the non-NMDA agonists, indicated by the K_d values in Table 1, was marked. However, reference to Figure 3 shows that this distinction was less evident at concentrations below 1 mM.

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