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A quantitative pharmacological analysis of some excitatory amino acid receptors in the mouse neocortex *in vitro*

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1 The effects of 2-amino-5-phosphonovalerate and kynurenate, either alone or in combination, were tested on responses evoked by the excitatory amino acid agonists quinolinate, ibotenate, N-methyl-D-aspartate and N-methyl-DL-aspartate by use of an *in vitro* preparation of mouse neocortex and artificial cerebrospinal fluid nominally free of magnesium.

2 Schild plots for 2-amino-5-phosphonovalerate, using each of the excitatory amino acids, were linear and had a slope not significantly different from one. The apparent pA_2 values for 2-amino-5-phosphonovalerate using each of the excitatory amino acids were 4.98 (quinolinate), 5.00 (N-methyl-DL-aspartate), 4.92 (N-methyl-D-aspartate) and 5.05 (ibotenate). The apparent pA_2 obtained using ibotenate was distinct from that of N-methyl-D-aspartate but there were no significant differences between pA_2 estimates for quinolinate, N-methyl-D-aspartate or N-methyl-DL-aspartate.

3 Schild plots for kynurenate, using each of the excitatory amino acids, were linear and had a slope of 1.36 ± 0.03, significantly greater than one. The estimated apparent pA_2 values for kynurenate were 3.65 (quinolinate), 3.71 (N-methyl-DL-aspartate), 3.65 (N-methyl-D-aspartate) and 3.89 (ibotenate). The apparent pA_2 obtained using ibotenate was distinct from that of the other agonists.

4 Experiments using combinations of 2-amino-5-phosphonovalerate and kynurenate indicated that both antagonists apparently acted competitively at receptors activated by ibotenate or by quinolinate.

5 These results indicate that ibotenate acts at a site distinct from that of quinolinate, N-methyl-D-aspartate and N-methyl-DL-aspartate.

Introduction

Receptors for excitatory amino acids have on pharmacological evidence been divided into at least three separate classes, namely N-methyl-D-aspartate (NMDA), kainate and quisqualate; furthermore ibotenate and quinolinate are probably agonists at the NMDA receptor (for recent reviews see Watkins & Evans 1981; McLennan 1983; Stone & Connick, 1985; Mayer & Westbrook, 1987).

The existence of two NMDA receptor subtypes was proposed by Perkins & Stone (1983a,b) on the basis of regional variations in neuronal sensitivity to NMDA and quinolinate. This idea was developed further by Stone *et al.* (1987) to account for data from both neurotoxic and neuro-excitatory studies, phenomena linked by the excitotoxic hypothesis (Olney, 1983). In particular the model predicts that: (1) NMDA or quinolinate but not ibotenate activate one receptor subclass which is blocked by 2-amino-5-phosphonovalerate (APV) and kynurenate, and (2) NMDA or ibotenate but not quinolinate activate another receptor subclass which is blocked by APV but not by kynurenate.

Consistent with this proposal are electrophysiological data where kynurenate reduced neuronal responses to quinolinate more than those to NMDA in rat hippocampus *in vivo* (Perkins & Stone, 1985; but see also Ganong *et al.*, 1983), and autoradiographic data where NMDA and ibotenate showed different regional profiles for the displacement of [^{3}H]-glutamate (Greenamyre *et al.*, 1985).

A study showing that APV reduced neuronal responses to NMDA more than those to quinolinate in rat pyriform cortex (Ffrench-Mullen *et al.*, 1986)

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implies that APV has different affinities for the proposed quinolinate-activated and ibotenate-activated NMDA receptor subtypes.

This model therefore predicts that APV will reduce neuronal responses to ibotenate more than those to NMDA or quinolinate, and that kynurenate will reduce responses to quinolinate more than those to NMDA, but will not affect responses to ibotenate.

Two drugs acting at the same receptor should be blocked to the same extent by a competitive antagonist acting at the receptor. If two different pA_2 values are obtained then the drugs act at different sites, whereas if the same pA_2 is obtained the evidence is strongly suggestive that they act at the same site (Waud, 1968). Since APV (Mayer & Westbrook, 1987) and kynurenate (Ganong & Cotman, 1986) are competitive receptor antagonists, the most direct test of the predictions of the above model is to estimate the pA_2 values for each antagonist, such as APV at the proposed quinolinate or ibotenate receptors. Though cellular uptake of amino acids may disguise actions at NMDA receptors (Garthwaite, 1985), there is no evidence for effective uptake of ibotenate, NMDA, N-methyl-DL-aspartate (NMDLA) and quinolinate (Balcar & Johnston 1972; Collins *et al.*, 1985).

Methods

Full details of the mouse neocortex preparation and a qualitative description of its properties appears elsewhere (Burton *et al.*, 1987).

Tissue preparation

Male TO mice (4–8 weeks old) were killed by cervical dislocation, decapitated and the whole brain removed quickly into ice-cold artificial cerebrospinal fluid (ACSF). Coronal sections about 500 μm thick were cut using a Vibroslice (Campden Instruments). Acceptable sections had as rostral and caudal limits the genu of the corpus callosum and the rostral region of the cerebral ventricles respectively. These were transferred to an incubation chamber containing a 95% O_2, 5% CO_2 atmosphere at room temperature (20–24°C). A suitable slice was subsequently divided in the midline and further cuts were made to produce several wedge shaped pieces of tissue about 1.5 mm wide at the pial surface and about 1 mm wide at the corpus callosum. One such wedge was transferred to a two chamber bath, so that most of the cortical tissue was contained in one chamber, and the corpus callosum and a little cortical tissue contained in the other. A high resistance seal between the two compartments was achieved using high vacuum silicone grease (BDH). Each chamber was continuously perfused at 2.5 ml min^{-1} via droppers with ACSF gassed with a mixture of 95% O_2 and 5% CO_2.

Experimental protocol

The d.c. potential between the two compartments was continuously monitored via Ag/AgCl electrodes and a high input impedance amplifier. It was displayed on a storage oscilloscope and for permanent records a chart recorder. Agonists applied to the cortical tissue produced a negative d.c. potential relative to the callosal tissue. Since tetrodotoxin did not measurably affect drug-induced responses (Harrison & Simmonds, 1985; Burton *et al.*, 1987) it was not included in the ACSF in these experiments. After an incubation of at least 1 h in ACSF the cortical chamber was perfused with nominally Mg-free ACSF (to relieve the Mg suppression of responses to NMDA receptor activation – Mayer & Westbrook, 1987) for 1–2 h. Excitatory amino acid agonists dissolved in Mg-free ACSF were subsequently applied at various concentrations by 2 min superfusion to the cortical chamber, usually at 10–15 min intervals, to construct suitable dose-response curves. APV or kynurenate, dissolved in Mg-free ACSF, was then superfused for 15–30 min, and further dose-response curves constructed in the presence of 1–4 concentrations of APV or kynurenate. Subsequently the cortical chamber was perfused with Mg-free ACSF for 1–2 h and another, recovery, dose-response curve constructed. The concentration range over which data points for Schild plots could be obtained was limited by the poor solubility of kynurenate and quinolinate (see Merck Index, 9th Edition, Merck, Sharpe & Dohme Ltd) and the expense of APV.

Composition of the artificial cerebrospinal fluid

ACSF had the following composition (in mM): NaCl 123.8, KCl 3.3, $MgSO_4 \cdot 7H_2O$ 1.0 or nominally 0, KH_2PO_4 1.22, $CaCl_2$ 2.5, D-glucose 10, and was continuously bubbled with 95% O_2 and 5% CO_2 to bring the pH to about 7.4. The ACSF was approximately 325 mOsm. The magnesium content of the nominally Mg-free ACSF, prepared by omission of $MgSO_4$, was not greater than 11.3 μM (estimated from manufacturer's data) and typically was 2-3 μM (estimated using Mg absorption spectrum).

Drugs and chemicals

Standard laboratory chemicals were obtained from BDH. Sigma supplied NMDA, N-methyl-DL-aspartate (NMDLA), quinolinate and kynurenate. Aldrich supplied quinolinate. Cambridge Research

Biochemicals supplied ibotenate and the racemic mixture of APV.

Data analysis

The absolute d.c. shift obtained in response to an agonist was strongly influenced by the effectiveness of the grease seal; therefore measured responses were normalised to the maximum obtained in the control or recovery periods. Data points for the linear region of each log dose-response curve were fitted by regression lines with the weighted common slope (where the slopes were obviously different the data were discarded).

Data were accepted only if the dose-ratio of recovery/control (R/C) was less than two. Thereafter, the extent of the rightward shift of the log dose-response curve relative to the control log dose-response curve gave the log dose-ratio, and its antilog the estimated dose-ratio (DR_E) for a given concentration of antagonist. However, there was usually a small difference in the positions of the log dose-response curves for the control and recovery periods. When $R/C > 1$, this will tend to overestimate, and when $R/C < 1$ will tend to underestimate, the rightward shift due to the antagonist and hence DR_E. When comparing different data groups there will therefore be a systematic bias unless the mean R/C for each group is the same. This can be corrected by using the geometric mean of the dose-ratios relative to control and recovery periods for each wedge, a procedure easily shown to be equivalent to adjusting the DR_E by $(R/C)^{-1/2}$; where more than one concentration of antagonist is used this generalises to $DR_E\,(R/C)^{-i/n+1}$ where i is the i^{th} trial, and n is the number of trials, in the presence of antagonist. Such dose-ratios were used to construct Schild plots from which the slopes and apparent pA_2 values were calculated.

The most appropriate way to combine data in the form of ratios is to use the geometric, not the arithmetic mean; similarly standard errors or confidence intervals will be geometrically but not generally arithmetically symmetric about the mean. Such data are easily manipulated by using a logarithmic transformation. Data presented in Tables 1 and 3 are therefore antilogs of logarithmic means, with attached 95% confidence intervals.

If one competitive antagonist produces a dose-ratio of DR_1, and a second competitive antagonist produces a dose-ratio of DR_2, when they are both applied together they will produce a combined dose-ratio of $(DR_1 + DR_2 - 1)$ if they act competitively at the same receptor or $DR_1 \times DR_2$ if they do not (Barlow, 1980). The mode of action of APV and kynurenate at the proposed quinolinate and ibotenate type receptors could therefore be inferred from dose-ratios obtained when these antagonists were applied together.

Statistical analysis

Statistical procedures may be found in Armitage (1985) and also Finney (1978). Data were collected in the form of arrays to which linear regression lines were fitted using standard least-squares procedures. This assumption of linear regression was tested using analysis of variance with a test for linearity. The slopes of the Schild plots should be the same, and this null hypothesis was tested by an analysis of variance for differences between regression slopes. The null hypothesis of coincident regression lines was tested by analysis of covariance and subsequent inference using differences and standard errors of corrected means. All hypotheses were tested by use of the appropriate F-test or t-test, and by Fisher's rule for simultaneous inference where appropriate to adjust the significance level.

Results

Spontaneous depolarizing shifts (DS) appeared in most wedges during the first 1–2 h of perfusion of the cortical tissue with Mg-free ACSF; these DS were allowed to develop fully before the experiment was started. Once reproducible depolarizing responses to the excitatory amino acid agonists were obtained, a control dose-response curve was constructed; further dose-response curves were subsequently made in the presence of, and after washout of, one or more doses of antagonist.

The most medial wedges used contained area 8, and the next most lateral wedge areas 6 and 24 (Caviness, 1975), but no pharmacological differences were seen between these two types of neocortical wedge. The data from the two groups were therefore pooled. The ED_{50} estimates (mean ± s.e. mean), calculated from the control curve alone, were (in μM) quinolinate 624 ± 86 ($n = 32$), ibotenate 49 ± 13 ($n = 20$), NMDA 22 ± 5 ($n = 20$) and NMDLA 33 ± 4 ($n = 16$). These should be regarded as minimum estimates since it was difficult to establish a clear plateau in many wedges and high doses of the excitatory amino acids induced an irreversible loss of responsiveness.

Effect of APV on responses to agonists

Figure 1 illustrates a representative experiment. A control dose-response curve for quinolinate was obtained, and four further two-point curves constructed in the presence of incremental concentrations of APV, followed after a suitable interval by a

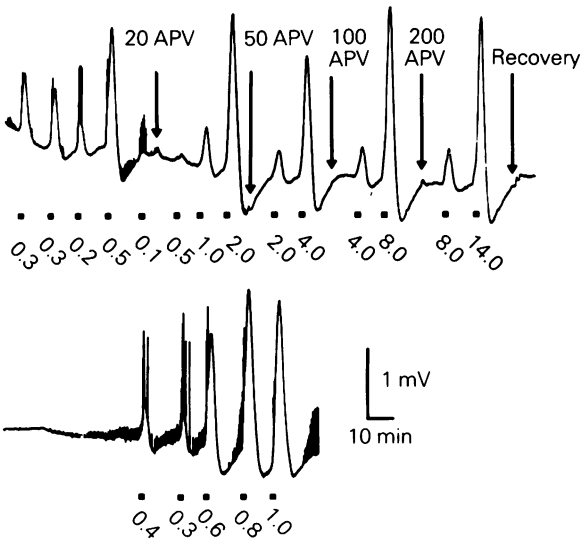

Figure 1 Depolarizing responses evoked by quinolinate from a tissue wedge prepared from coronal sections of mouse neocortex and their antagonism by 2-amino-5-phosphonovalerate (APV). Quinolinate (concentration in mM) was applied to the cortical tissue by 2 min superfusion as indicated beneath the record. Application of 2-amino-5-phosphonovalerate at concentrations (μM) shown began as indicated by the vertical arrow.

recovery dose-response curve where the depolarizing response to quinolinate could be fully explored. In other experiments only one or two concentrations of APV were used. Similar results were obtained using ibotenate, NMDA or NMDLA as the agonist. The dose-ratios obtained are presented in Table 1 as geometric means and 95% confidence intervals.

Figure 2 illustrates processed data from an experiment similar to that shown in Figure 1; in this case the log dose-response curves are for NMDLA in control and recovery periods, and in the presence of 50 μM APV. The ratio R/C in this case was 1.20. The parallel shift to the right from the geometric mean of control and recovery curves to that of the APV gave an estimate of the dose-ratio for 50 μM APV.

Schild plots for APV

The data presented in Table 1 were re-analysed and Schild plots of log (dose-ratio -1) versus log (APV concentration, M) were made for each of quinolinate, ibotenate, NMDA and NMDLA. These are illustrated in Figure 3. Regression lines were fitted to each data set. Each line was submitted to an analysis of variance with a test for linearity, and this indicated that there was no reason to doubt the basic assumption of linearity for any of the regression lines. An analysis of variance for difference between regression slopes did not indicate any significant difference between the individual slopes ($F_{3,76} = 0.4003$) and in no case was a slope significantly different from 1, the predicted slope of the Schild plot. The individual and pooled regression slopes are given in Table 2.

An analysis of covariance indicated that there was reason to reject the null hypothesis of coincident regression lines ($F_{3,79} = 5.2199$, $P < 0.01$). By use of Fisher's rule for simultaneous inference, the only significant contrast was between the regression lines for ibotenate and of NMDA ($P < 0.05$) though the ibotenate-quinolinate contrast was very nearly significant. Using a forced slope of 1, the intercept of the regression lines gave an apparent pA_2 value for APV; these are given for each agonist in Table 2.

Table 1 Dose-ratios for 2-amino-5-phosphonovalerate (APV) using amino acid agonists

	Quinolinate	*Ibotenate*	*NMDA*	*NMDLA*
APV 20	2.98 2.56–3.46 $n = 7$	3.15 2.52–3.92 $n = 5$	2.67 1.89–3.77 $n = 5$	3.05 1.94–4.81 $n = 4$
APV 50	6.30 5.39–7.36 $n = 7$	7.49 5.38–10.42 $n = 5$	5.17 3.73–7.17 $n = 5$	5.68 2.60–12.40 $n = 4$
APV 100	10.48 9.59–11.45 $n = 7$	12.23 10.83–13.82 $n = 5$	9.37 8.06–10.88 $n = 5$	11.36 7.49–17.24 $n = 4$
APV 200	18.06 14.93–21.86 $n = 7$	22.10 19.76–24.72 $n = 5$	17.92 15.57–20.64 $n = 5$	20.95 13.30–33.00 $n = 4$

Each entry contains from top downwards the geometric mean of the dose-ratios, the lower and upper 95% confidence limits and the number of data points. The concentrations of APV are in μM. NMDA = N-methyl-D-aspartate and NMDLA = N-methyl-DL-aspartate.

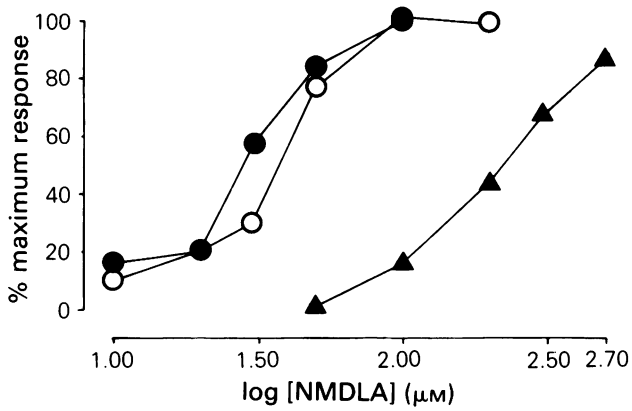

Figure 2 Semi-logarithmic plot of normalized response amplitude against concentration of N-methyl-DL-aspartate in artificial cerebrospinal fluid nominally free of magnesium in control (●) and recovery (○) periods, and in the presence of 50 M 2-amino-5-phosphonovalerate (APV) (△). The extent of the rightward parallel shift gave an estimate of the dose-ratio for 50 μM APV.

Treated as a group the pA_2 for APV was 4.99 ± 0.03 (mean ± s.e. mean); none of the estimates lay outside the 95% confidence interval though ibotenate is near the boundary. However, if the data for quinolinate, NMDLA and NMDA are pooled, the position of the regression line for ibotenate is statistically different ($P < 0.01$) from the regression line for the other pooled data. This manoeuvre seems reasonable since there were no significant differences between the positions of the regression lines for NMDA, NMDLA and quinolinate; the NMDA line was significantly and the quinolinate line very nearly significantly different from that of ibotenate using the conservative Fisher rule, and in light of the kynurenate data. The apparent pA_2 for APV using the pooled group of 3 agonists was 4.96 ± 0.02 (mean ± s.e. mean).

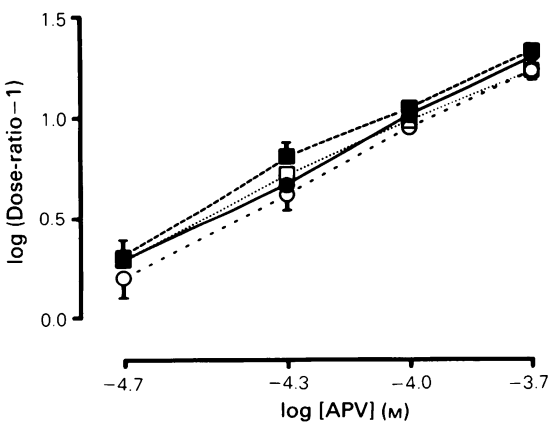

Figure 3 Schild plots for 2-amino-5-phosphonovalerate (APV) using quinolinate (□), ibotenate (■), N-methyl-D-aspartate (○) and N-methyl-DL-aspartate (●) as agonists. Points are means with vertical lines indicating s.e mean (not shown where smaller than symbol).

Effect of kynurenate on response to agonists

Data were collected in a similar way to those for APV, using 1–4 incremental concentrations of kynurenate. The dose-ratios obtained are presented in Table 3, as geometric means and 95% confidence intervals.

Schild plots for kynurenate

The data in Table 3 were re-analysed and Schild plots of log (dose-ratio − 1) versus log (kynurenate concentration, M) were made for each of quinolinate, ibotenate, NMDA and NMDLA. These are illustrated in Figure 4. Regression lines were fitted to each data group over the comparable range 200 μM–2000 μM kynurenate; the difficulty of obtaining precise dose-ratios at 50 μM and 100 μM kynurenate precluded their inclusion in the analysis. Each regression line was submitted to an analysis of

Table 2 Schild slopes and apparent pA_2 values for 2-amino-5-phosphonovalerate (APV)

Agonist	*Schild slope* (±s.e.)	*Apparent* pA_2
Quinolinate	0.9353 ± 0.0445 ($n = 28$)	4.98
Ibotenate	0.9843 ± 0.0578 ($n = 20$)	5.05
NMDA	1.0233 ± 0.0792 ($n = 20$)	4.92
NMDLA	1.0084 ± 0.0906 ($n = 16$)	5.00
Pooled slope	0.9818 ± 0.0325	

For key to abbreviations used see Table 1.

Table 3 Dose-ratios for kynurenate using amino acid agonists

	Quinolinate	*Ibotenate*	*NMDA*	*NMDLA*
Kyn 50	ND	1.49 0.76–2.90 $n = 3$	ND	1.28 0.10–15.6 $n = 2$
Kyn 100	ND	1.85 0.76–4.50 $n = 2$	ND	ND
Kyn 200	2.08 1.85–2.33 $n = 6$	3.00 2.74–3.28 $n = 7$	1.83 1.35–2.47 $n = 3$	2.01 1.58–2.56 $n = 6$
Kyn 400	3.12 2.62–3.72 $n = 6$	5.16 4.08–6.52 $n = 4$	3.08 2.47–3.83 $n = 8$	3.59 3.28–3.59 $n = 10$
Kyn 1000	8.24 6.84–9.91 $n = 6$	16.71 15.35–18.18 $n = 4$	8.61 7.24–10.25 $n = 8$	10.11 8.07–12.6 $n = 10$
Kyn 2000	19.04 13.03–27.83 $n = 6$	44.40 38.56–51.13 $n = 4$	21.60 16.19–28.84 $n = 8$	25.77 20.38–32.5 $n = 7$

Each entry from top downwards contains the geometric mean of the dose-ratio, the lower and upper 95% confidence limits, and the number of data points. The concentrations of kynurenate (Kyn) are in μM. For key to abbreviations used see Table 1.

variance with a test for linearity, and this indicated that there was no reason to doubt the basic assumption of linearity for any of the lines. An analysis of variance for differences between regression slopes did not indicate any significant difference between the individual slopes ($F_{3,92} = 1.4512$, NS). The individual and pooled regression slopes are presented in Table 4. In each case the regression slope was significantly greater than the predicted Schild slope of 1 ($P < 0.01$). Presumably this reflects some effect at a site(s) other than the receptor since the slopes of the log dose-response curves were independent of the concentration of kynurenate. An analysis of covariance indicated that there was reason to reject the null hypothesis of coincident regression lines ($F_{3,95} = 38.6754$, $P \ll 0.01$). By use of Fisher's rule for simultaneous inference, the significant contrasts were between ibotenate and any of quinolinate, NMDA and NMDLA ($P < 0.05$). If the regression lines can be validly extended into the range 100–200 μM kynurenate, which seems reasonable, it follows that the apparent pA_2 for kynurenate obtained using ibotenate is significantly different from the apparent pA_2 obtained using quinolinate, NMDA and NMDLA. The pA_2 value is obtained when log (dose-ratio − 1) = 0; when the slope b is not unity, the pA_2 is given by a/b where a is a regression coefficient. These apparent pA_2 values are given in Table 4. The apparent pA_2 for kynurenate estimated from the group of 3 excitatory amino acids was 3.668 ± 0.019 (mean ± s.e. mean), and the estimated pA_2 obtained using ibotenate lies outside the 95% confidence interval.

Table 4 Schild slopes and apparent pA_2 values for kynurenate.

Agonist	*Schild slope* (± s.e.)	*Apparent* pA_2
Quinolinate	1.2587 ± 0.0437 ($n = 24$)	3.65
Ibotenate	1.3373 ± 0.0374 ($n = 19$)	3.89
NMDA	1.4171 ± 0.0792 ($n = 29$)	3.65
NMDLA	1.4059 ± 0.0658 ($n = 33$)	3.71
Pooled slope	1.3556 ± 0.0309	

For key to abbreviations used see Table 1.

Effect of co-application of APV and kynurenate on responses to agonists

For these experiments, dose-response curves for ibotenate and quinolinate were constructed in control and recovery periods, and during co-application of 100 μM APV and 400 μM kynurenate. The dose-ratios obtained are given in Table 5 as the appropriate geometric mean and 95% confidence interval. Table 5 also shows the calculated dose-ratios for each agonist at 100 μM APV and 400 μM kynurenate if the interaction is multiplicative (DR(APV) × DR(KYN)) or additive (DR(APV) + DR(KYN) − 1). These data indicated that an additive interaction described the effects on responses to quinolin-

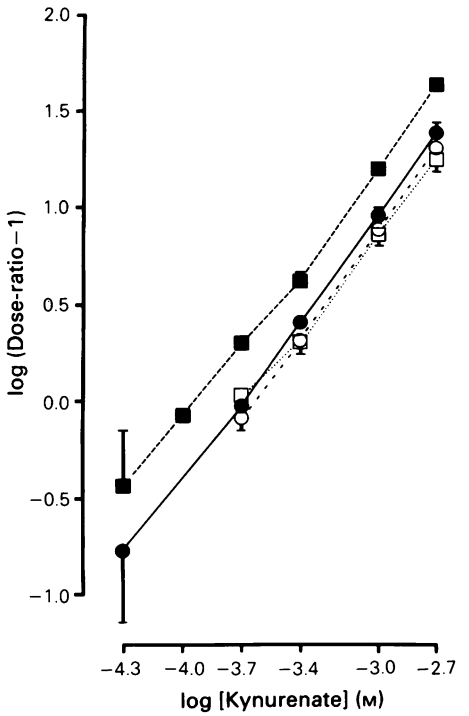

Figure 4 Schild plots for kynurenate using quinolinate (□), ibotenate (■), N-methyl-D-aspartate (○) and N-methyl-DL-aspartate (●) as agonists. Points are means with vertical lines indicating s.e. mean (not shown where smaller than symbol).

ate and ibotenate, since the confidence intervals obtained experimentally covered the predicted values for additive but not multiplicative dose-ratios.

Discussion

The experimental preparation relies upon the wedge containing sufficient neurones projecting via the corpus callosum to allow somatic depolarization to be measured. This necessarily limits observations to this kind of neurone; cells that do not project via the callosal fibres but bear a receptor subtype for example could not be detected. Indirect effects on projecting neurones in these wedges mediated by neurones using action potentials must be very small, since inclusion of tetrodotoxin (TTX) in the ACSF had no measurable effect on the responses evoked by a variety of excitatory amino acid agonists (Harrison & Simmonds, 1985; Burton *et al.*, 1987); though the contribution if any of direct TTX resistant effects on presynaptic terminals cannot be assessed. These experiments also assume firstly that drug effects at receptors are well described by the single site model (see Barlow, 1980), and secondly that the data obtained (after logarithmic transformation) are normally distributed.

Effects of APV

The data obtained in this study using APV and the excitatory amino acid agonists showed a significant difference in the position of regression lines for ibotenate and NMDA in the Schild plots, with suspicion, reinforced by the kynurenate data, that the line for ibotenate differed from those for quinolinate and NMDLA also. If the data for these agonists were pooled, the increased statistical resolution allowed a clear distinction ($P < 0.01$) between regression lines for ibotenate and the regression line for the pooled data for NMDA, quinolinate and NMDLA. Provided that the regressions are valid outside the regions where data were collected, this implies that the apparent pA_2 for APV (5.05) using ibotenate is significantly different from that for NMDA, NMDLA and quinolinate (4.96 ± 0.02, group mean $\pm$ s.e. mean), and therefore that ibotenate acts on a distinct receptor. The apparent pA_2 values for APV obtained using NMDA and quinolinate in this study agree well with those obtained using NMDA (4.9 ± 0.1) and quinolinate (4.8 ± 0.1) in rat neocortex (Lodge & Martin, 1986).

Table 5 Comparisons of experimental and predicted dose-ratios for competitive and non-competitive behaviour

Agonist	*Calculated* $DR(APV) \times DR(Kyn)$	*Calculated* $DR(APV) + DR(Kyn) - 1$	*Experimental* $DR(APV + Kyn)$
Quinolinate	32.72	12.60	9.23 6.70–12.70 $n = 4$
Ibotenate	63.10	16.39	18.98 12.70–28.38 $n = 8$

Additive or multiplicative dose-ratios (DR) were obtained using appropriate data from Table 1 and Table 3. 2-Amino-5-phosphonovalerate (APV) was used at 100 μM, together with kynurenate (Kyn) at 400 μM.

Regional variations in neuronal sensitivity to quinolinate compared with NMDA (Perkins & Stone, 1983a,b; McLennan, 1984) led to the speculation that there may be a subdivision of NMDA receptors into two classes, NMDA 1 activated by NMDA but not quinolinate in the spinal cord, and NMDA 2 activated by NMDA or quinolinate and present in cortex, striatum and hippocampus. A regional difference in pA_2 would therefore be predicted. However, Wheatley & Collins (1986) found an apparent pA_2 value of 5.0 for APV in neonatal rat spinal cord, using NMDA as the agonist. The apparent pA_2 of APV using quinolinate as agonist remains to be determined in the mammalian spinal cord.

Ffrench-Mullen *et al.* (1986) demonstrated that bath application of APV could discriminate between responses to iontophoretically applied NMDA, quinolinate and aspartate. These authors claimed that they selected doses of each agonist to evoke comparable neuronal responses to 3–5 action potentials, though in their Figure 1 not only were many more action potentials evoked but also the firing frequencies were not the same, having rank order aspartate > quinolinate > NMDA. Then it would not be surprising for APV to have an apparently selective effect, successively abolishing neuronal responses to NMDA, quinolinate and aspartate as the concentration of APV was increased from 1 to 100 μM. Therefore, their claim that APV can distinguish between responses evoked by NMDA and quinolinate must be viewed with caution.

Effects of kynurenate

The data obtained in this study using kynurenate and the excitatory amino acid agonists showed a clear and statistically significant difference in the positions of the regression lines for ibotenate and the other agonists in the Schild plots. Assuming that the regression lines are still valid outside the range where data were compared (200 μM–2000 μM), which seems reasonable since the regression lines for ibotenate and NMDA still appear linear with the same slope at 50–100 μM, then the apparent pA_2 for kynurenate using ibotenate as agonist (3.90) is statistically significantly different ($P < 0.01$) from the apparent pA_2 values for kynurenate using quinolinate, NMDA and NMDLA (3.67) $\pm$ 0.02, mean $\pm$ s.e. mean). This implies that ibotenate acts at a receptor distinct from that at which quinolinate, NMDA and NMDLA act.

This interpretation must be regarded cautiously. All the individual, and the pooled, regression slopes in the Schild plots were greater than unity, implying some violation of the assumptions on which the plots are based. There was no indication of non-competitive behaviour since the slopes of the log dose-response curves were independent of kynurenate concentration. An apparently non-competitive action of kynurenate at NMDA receptors in the rat spinal cord was described by Evans *et al.* (1987). However, their Gaddum-Schild plot was concave upward and their data for dose-ratios, using combinations of APV and kynurenate, fell between the predictions for competitive and non-competitive behaviour. Hence, the validity of their conclusion is unclear. However, kynurenate acts competitively at NMDA receptors in hippocampus (Ganong & Cotman, 1986). Presumably, therefore, kynurenate has some other and as yet unidentified effect whereby responses to these excitatory amino acids are depressed when used at millimolar concentrations. It would be expected that this effect would become progressively smaller with progressive dilution of kynurenate, so that the apparent pA_2 estimates may have validity.

The data of Ganong *et al.* (1983), using iontophoretic ejection of NMDLA and quinolinate to evoke focal d.c. potentials in hippocampal slices, indicated that 100 μM kynurenate depressed responses to NMDLA more than to quinolinate though at 500 μM the difference was much smaller. If the regressions in this study may be validly extended into the 100 μM region no such difference would be predicted; however, the lack of precision in estimating dose-ratios less than two for ibotenate and NMDA, and presumably for quinolinate and NMDLA, at 50 and 100 μM made collection of data at these doses unrewarding.

Combination experiments

Both APV (Olverman *et al.*, 1984) and kynurenate (Ganong & Cotman, 1986) behave as NMDA receptor antagonists. The dose-ratio produced by a combination of two antagonists can be used as a test for competitive behaviour. By using relatively low concentrations of kynurenate (400 μM) (to minimize any non-specific depression) producing a dose-ratio of 3–5 and APV (100 μM) producing dose-ratios of 10–12, the nature of the antagonism could be inferred. Assuming that APV is a competitive antagonist at these receptors, the results indicated that kynurenate is also a competitive antagonist at ibotenate and quinolinate receptor sites.

The receptor model

The receptor model proposed by Stone *et al.* (1987) may require modification. Though the prediction that APV will reduce neuronal responses to ibotenate more than those to NMDA has been confirmed, these data do not support the prediction that APV

would reduce responses to NMDA more than to quinolinate. Also, its prediction of a receptor activated by NMDA and ibotenate but not quinolinate, and blocked by APV but not kynurenate has not been substantiated. The data in this study suggest the existence of an independent ibotenate receptor more readily blocked by APV and kynurenate than the receptor type activated by quinolinate, NMDA and NMDLA and support the data of Greenamyre *et al.* (1985), which demonstrated different regional profiles for NMDA and ibotenate receptors.

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