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A quantitative study of the actions of excitatory amino acids and antagonists in rat hippocampal slices

J.F. Blake, M.W. Brown & *G.L. Collingridge

Departments of Anatomy and *Pharmacology, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD

1 A quantitative pharmacological investigation of the actions of excitatory amino acids on hippocampal CA1 neurones has been made using a new slice preparation developed for grease gap recording; d.c. potential was measured across a grease barrier placed between alvear fibres and the bathing medium.

2 In Mg^{2+}-free perfusate, N-methyl-D-aspartate (NMDA, 1–100 μM), quisqualate (1–500 μM), kainate (1–200 μM) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 1–100 μM) caused dose-dependent depolarizations.

3 The dose-response relationships were fitted to logistic expressions. The maximum responses to AMPA, NMDA and kainate were similar; their respective EC_{50} values were 5, 13 and 23 μM. Quisqualate had a smaller maximum; its EC_{50} value was 10 μM. The slopes of the dose-response relationships were different for the 4 agonists; the order of steepness of the slopes was NMDA > AMPA > kainate > quisqualate.

4 Similar amino acid-induced depolarizations were observed in slices of just the CA1 region or in whole slices bathed in tetrodotoxin. Isolated alvear fibres, however, were insensitive to the excitatory amino acids.

5 D-2-Amino-5-phosphonovalerate (APV, 50 μM) selectively and reversibly antagonized responses induced by NMDA (apparent $pA_2 = 5.21$).

6 Kynurenic acid (1 mM) reversibly depressed responses to the three agonists tested. The dose-ratios for antagonism of AMPA, kainate and quisqualate were 6.9, 5.6 and 4.6 respectively.

7 This preparation has a different sensitivity profile to agonists from those of previously reported preparations of spinal cord, neocortex and cerebellum. The greater sensitivity to NMDA may be due to the higher density of NMDA receptors in the hippocampus. The effects of the antagonists, APV and kynurenate, are similar to those found in other brain areas.

Introduction

Three types of postsynaptic excitatory amino acid receptor, named after the agonists N-methyl-D-aspartate (NMDA), kainate and quisqualate, have been identified in the mammalian central nervous system (Watkins & Evans, 1981). Another potent excitant α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Krogsgaard-Larsen *et al.*, 1980) is thought to act at quisqualate receptors and has been used in radioligand studies for this purpose (Cotman *et al.*, 1987).

Subfield CA1 of the hippocampus has the highest density of NMDA receptors in the brain (Cotman *et al.*, 1987) and these receptors may be involved in synaptic plasticity (Collingridge & Bliss, 1987). They have also been implicated in epilepsy and neuronal cell death (Meldrum, 1985), pathologies to which the hippocampus is extremely vulnerable. It is thought that receptors of the non-NMDA type may be involved in fast synaptic transmission in this region (Collingridge *et al.*, 1983b; Ganong *et al.*, 1983).

To obtain more quantitative pharmacological information on the actions of excitatory amino acids in the CA1 region of the hippocampus, grease gap recordings have been obtained from a modified slice preparation. We describe the dose-response relationships of the selective agonists NMDA, kainate,

AMPA and quisqualate, and the selectivity of the antagonists D-2-amino-5-phosphonovalerate (APV) and kynurenic acid, at doses used in previous studies of synaptic transmission in the hippocampus (e.g. Ganong *et al.*, 1983; Collingridge *et al.*, 1988).

Some of these results have appeared in a preliminary form (Blake *et al.*, 1986).

Methods

Experiments were performed on 77 slices obtained on separate days from different rats.

Preparation of slices

Adult female rats, weighing 150–250 g, were anaesthetized with halothane and then decapitated. The brain was rapidly removed into gassed (95% O_2, 5% CO_2) medium at room temperature and transverse slices (400 μm thick) were prepared with a McIlwain tissue chopper. Slices were kept at the interface of the medium and an oxygen-enriched, humidified atmosphere in a storage chamber at room temperature for 1–3 h. The medium contained (mM): NaCl 124, $NaHCO_3$ 26, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, D-glucose 10.

Prior to recording, a scalpel cut was made to separate partially the alveus from the slice. This cut extended from the subiculum into the CA1 subfield (Figure 1). In some experiments, subfield CA1 was isolated from the rest of the slice by further scalpel cuts (Figure 1). In other experiments the alveus was completely separated from the grey matter.

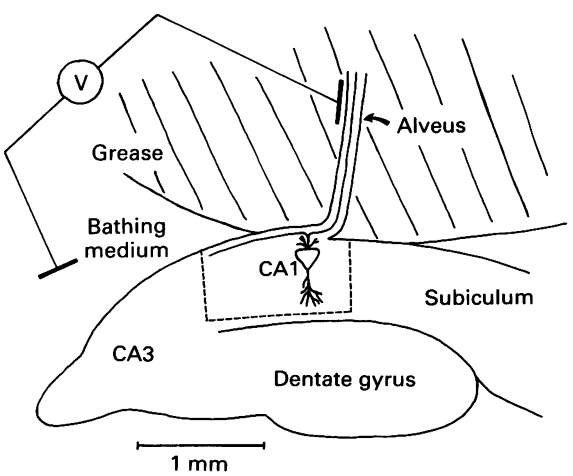

Figure 1 Schematic diagram of a hippocampal slice set up for recording. Potential changes were measured between the distal end of the alveus (CA1 axons) and the bathing medium. The dotted line surrounds the CA1 area from which the rest of the slice was removed in some experiments.

Electrical recording techniques

The preparation was then transferred to a recording system similar to that described for hemisected spinal cord preparations (Evans, 1978). The slice was placed on an inclined, greased (liquid paraffin/petroleum jelly; 50%/50%, w/w), temperature regulated (26°C) block and the wick of a Ag/AgCl electrode was placed in contact with the separated distal end of the alvear bundle. The fibre tract was then covered with the grease mixture to form an electrically-insulating barrier between the fibre tract and the bathing medium, and to prevent dessication of the alvear fibres. The preparation was covered with absorbent paper and was perfused with gassed (95% O_2/5% CO_2) medium at a flow rate of 1.5 ml min^{-1}; d.c. potentials were recorded differentially between the electrode in contact with the alveus and an identical electrode in contact with the perfusion medium (Figure 1).

After approximately 30 min, slice viability was tested with agonists. Providing the slice responded with a depolarization of at least 0.2 mV then the perfusing medium was changed to one containing no $MgSO_4$ for the remainder of the experiment. Agonists were added in volumes of 2 ml at intervals of between 10 and 20 min. In some experiments tetrodotoxin (TTX) was added to the perfusion medium (0.1 μM, following an initial 2 ml application of 2 μM). Such treatment rapidly abolishes action potentials in rat hippocampal slices.

Drugs

Drugs were stored frozen as the sodium salt in stock solutions of 3 mM KCl. N-methyl-D-aspartate, quisqualate, kainate, γ-aminobutyric acid and kynurenic acid were obtained from Sigma. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and D-2-amino-5-phosphonovalerate (specific optical rotation -24.8 in 6 M HCl at 589 nm) were kindly provided by Dr J.C. Watkins (Dept. Pharmacology, University of Bristol).

Statistical analysis

For construction of full dose-response curves lines were fitted to a logistic expression by a least squares method (Barlow, 1983). Data are presented as mean $\pm$ s.e.mean and the number of slices is given in parentheses. Data were inspected for normality of distribution and as appropriate, two-tailed parametric or non-parametric tests were performed as indicated.

Results

Dose-response relationships

Dose-response curves were constructed after slices had been perfused with Mg^{2+}-free medium for at least 30 min, at a time when sensitivity to NMDA had stabilized. Since preliminary experiments had shown that repeated applications of high doses of agonist were detrimental to slice viability (see also Collins & Surtees, 1986; Garthwaite *et al.*, 1986) 'full' dose-response curves (4–8 doses) were constructed with only one, or occasionally two, agonists per slice (Figure 2). A logistic expression was fitted to the data from each experiment by use of a least squares method (Figure 3). The maxima obtained for NMDA, kainate and AMPA were similar; their relative order of potency in terms of EC_{50} was AMPA > NMDA > kainate (Table 1). Quisqualate produced a smaller maximum than the other three agonists and had an EC_{50} similar to NMDA (Table 1). The slopes of the dose-response curves were different for the four agonists; the order in terms of steepness was NMDA > AMPA > kainate > quisqualate (Table 1).

In 23 experiments, within slice comparisons were obtained where 3 or 4 agonists were compared over a narrower, submaximal dose range. Similar differences in the dose-response relationships were observed under these conditions (Figure 4).

Source of the depolarization

To determine the source of the recorded potentials three separate experiments were performed. Firstly, NMDA, kainate and quisqualate (5–50 μM) were applied to 11 isolated CA1 subfields (Figure 5a). All three agonists had effects similar to those seen with intact slices.

In the second series of experiments, TTX was added to the perfusate. This agent had little or no effect on either the baseline potential or on the agonist-induced depolarizations (Figure 5b). Thus, the size of the responses expressed as a percentage of those recorded in the absence of TTX (for 10 or 20 μM of the agonist) were for NMDA, 102 ± 9 (9); AMPA, 90 ± 23 (3); kainate, 100 ± 10 (6) and quisqualate, 92 ± 5 (7).

The third set of experiments was designed to investigate the possibility that the excitants were acting directly on the alvear fibres to cause the depolarizations. The alveus was completely separated from the remainder of the slice in 3 preparations, and recordings obtained between the distal insulated end and the part of the alveus still in contact with the bathing medium. In none of these preparations did NMDA, quisqualate or kainate, in doses of up to 50 μM, have any effect (AMPA was not examined). The fibres were depolarized, however, by increasing the potassium concentration or by the addition of γ-aminobutyric acid (GABA, 500 μM) ($n = 2$). The effects on an isolated alveus are illustrated in Figure 5c. In this example, responses to NMDA and quisqualate were recorded from the fibre tract before the alveus was separated from the grey matter (Figure 5b).

Effects of antagonists

Experiments were performed in Mg^{2+}-free medium since Mg^{2+} is a potent NMDA antagonist (Davies *et al.*, 1979). Slices were prepared and allowed to stabilize in Mg^{2+}-containing medium to prevent excessive activation of NMDA receptors and possible consequent neurotoxicity during this period. To determine the effects of Mg^{2+} on responses to the agonists, submaximal responses were obtained before and

Table 1 Dose-response relationships of excitatory amino acids

Agonist	*Maximum* (mV)	*Slope*	EC_{50} (μM)
AMPA	0.74 ± 0.08*	1.87 ± 0.12*†	5.0 ± 1.1†‡
Kainate	0.85 ± 0.20	1.49 ± 0.23†	22.8 ± 2.8
NMDA	0.75 ± 0.08*	2.98 ± 0.30	12.8 ± 2.3‡
Quisqualate	0.40 ± 0.07	1.08 ± 0.08†	10.5 ± 3.0‡

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA = N-methyl-D-aspartate.
Log dose-response plots were fitted to a logistic expression by a least squares method. Data for each agonist are presented as means ± s.e.mean for 5 slices. Analyses of variance yielded the following results for differences between the 4 agonists: *Maximum*: $F(3,16) = 2.56$, $P < 0.10$ (Kruskal-Wallis $H = 7.61$, $P < 0.05$); *Slope*: $F(3,16) = 16.55$, $P < 0.0001$; *log* EC_{50}: $F(3,16) = 12.22$, $P < 0.001$. Individual comparisons using t tests (confirmed by Mann-Whitney U-tests) are shown in the table as:
* Significantly different from corresponding value obtained for quisqualate ($P < 0.02$).
† Significantly different from corresponding value obtained for NMDA (slopes, $P < 0.01$; EC_{50}, $P < 0.02$).
‡ Significantly different from corresponding value obtained for kainate (AMPA, $P < 0.01$; NMDA, $P < 0.05$; quisqualate, $P < 0.02$).

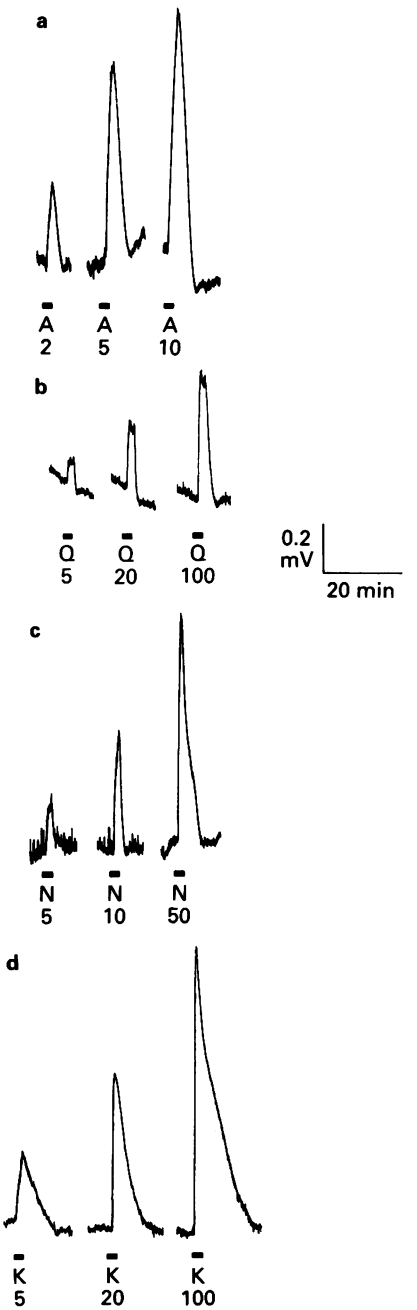

Figure 2 Responses of hippocampal neurones to the four agonists (a) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, A), (b) quisqualate (Q), (c) N-methyl-D-aspartate (N) and (d) kainate (K). The four examples are from different preparations and show dose-dependent responses to 2 ml applications of the agonists. In this and subsequent records all concentrations are shown in μM.

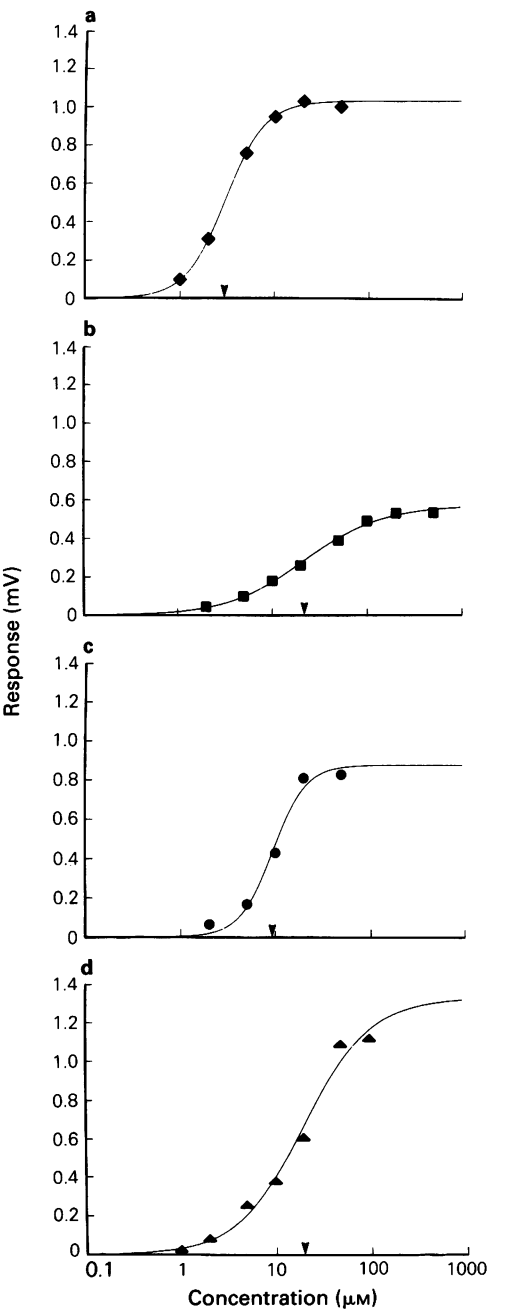

Figure 3 Log dose-response plots for the corresponding four experiments in Figure 2, (a) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (b) quisqualate, (c) N-methyl-D-aspartate and (d) kainate. The points are fitted to the logistic expression by a least-squares method. The EC_{50} values are indicated by arrowheads. For ease of comparison the calculated logistic curves have been drawn to 1000 μM.

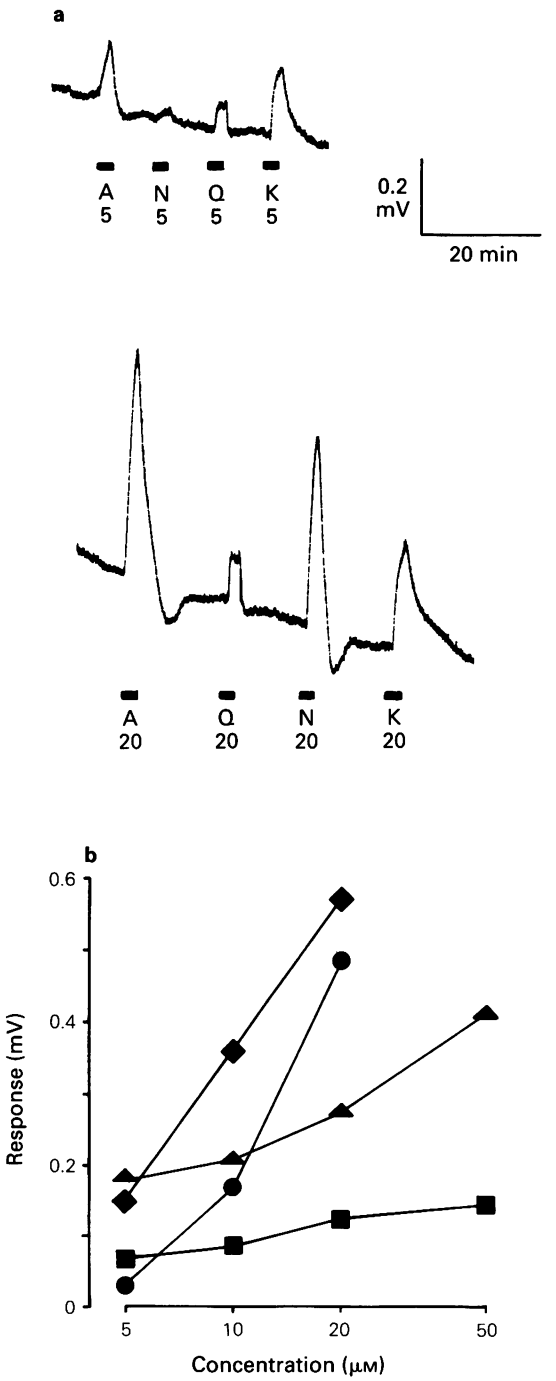

Figure 4 An example of depolarizations (a) and the corresponding plot (b) of responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (A, ◆), quisqualate (Q, ■), N-methyl-D-aspartate (N, ●), and kainate (K, ▲) on the same preparation.

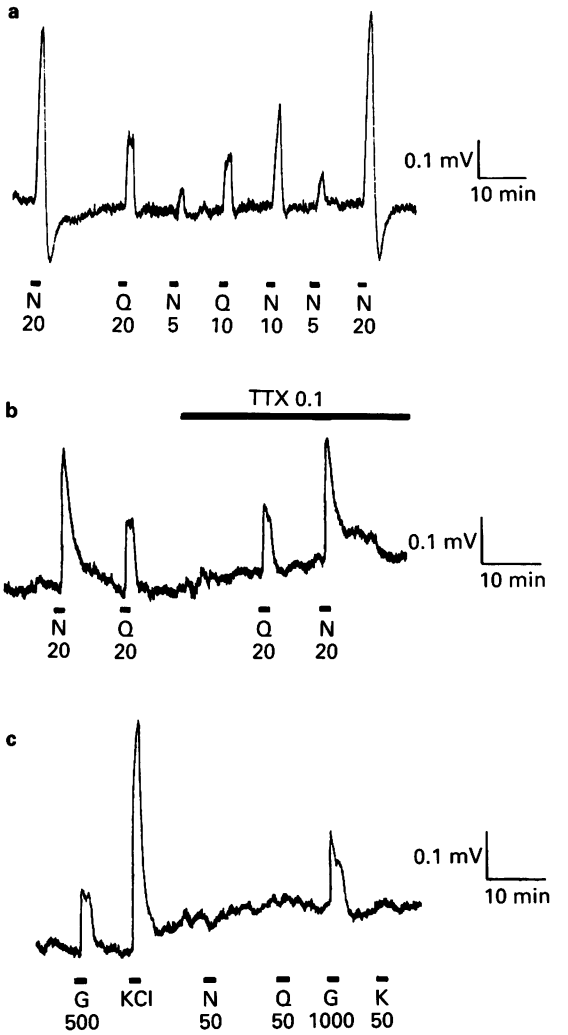

Figure 5 Source of the depolarizations: (a) depolarizations produced by N-methyl-D-aspartate (NMDA, N) and quisqualate (Q) in an isolated CA1 region slice (see Figure 1). (b) Effect of tetrodotoxin (TTX) on depolarizations induced by NMDA and quisqualate. (c) Absence of effects of NMDA, quisqualate and kainate (K) on an isolated alvear fibre tract. Depolarizations induced by γ-aminobutyric acid (G) and by KCl (increase of 5 mM) are shown. Record (b) was obtained before and record (c) after separation of the alveus from the same slice.

after the change from dissection-(1 mM Mg^{2+}) to experimental-(0 mM Mg^{2+}) medium in 13 slices. Only responses to NMDA were significantly affected (Table 2). These were potentiated in Mg^{2+}-free medium; however, small depolarizations were often increased to a greater extent, such that there was a

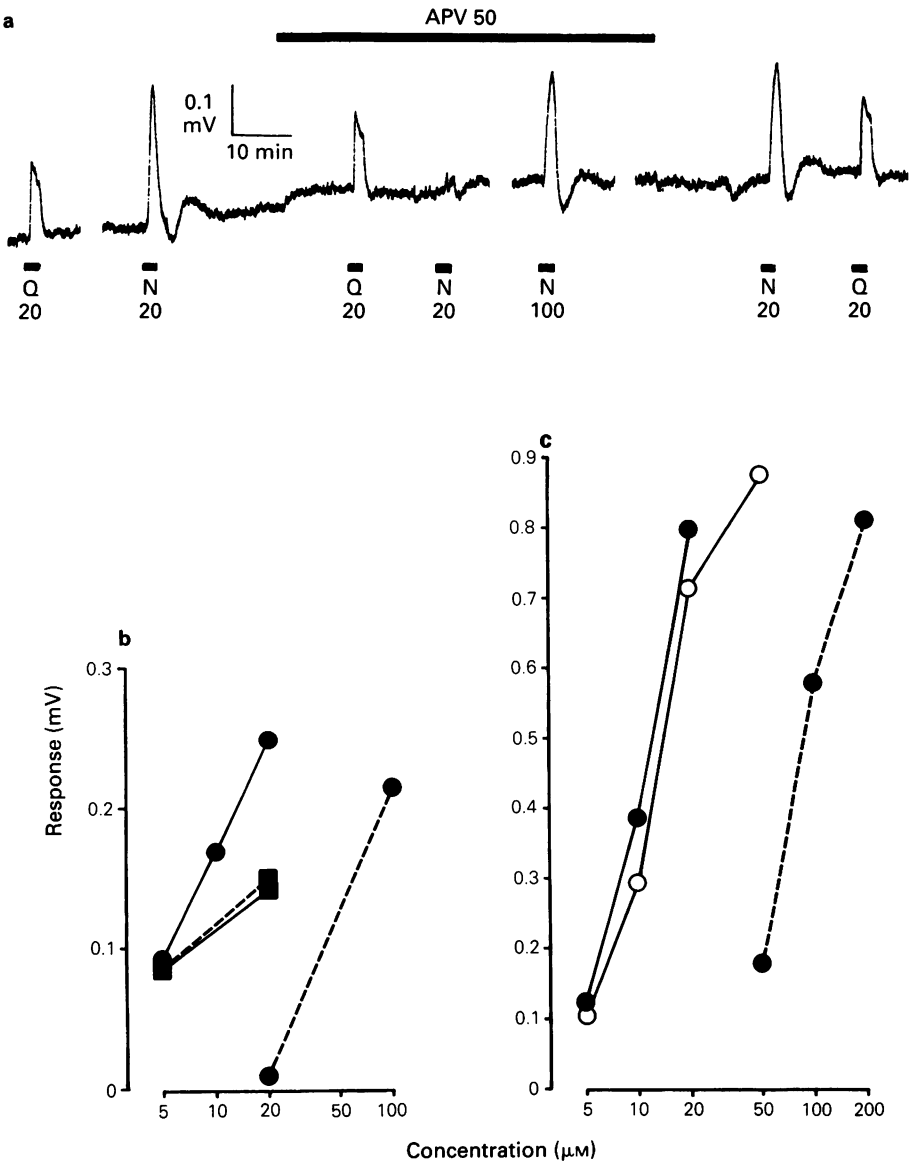

Figure 6 The effect of 50 μM D-2-amino-5-phosphonovalerate (APV) on depolarizations induced by N-methyl-D-aspartate (N) and quisqualate (Q). Depolarizations in (a) are plotted in (b). The plot in (c) is from another slice. Gaps in the record represent 20 min. Effects of NMDA in control medium (●——●), in the presence of 50 μM APV (●- - -●) and following washout of APV (○——○) and for quisqualate in control medium (■——■) and in the presence of APV (■- - -■).

non-parallel shift in the dose-response plot for NMDA. Hence the dose-ratio was measured at 10 μM NMDA which is approximately the EC_{50} for this agonist.

In 11 slices, APV (50 μM) was found to block selectively and reversibly responses to NMDA (Figure 6a,b) producing a parallel shift in the dose-response curve (Figure 6c). The values for the dose-ratios for antagonism by 50 μM APV of the four agonists are presented in Table 2. The apparent pA_2 value was calculated individually for each experiment, assuming a Schild slope of unity (Evans *et al.*, 1982;

Table 2 Dose-ratios of excitatory amino acid antagonists

Antagonist	*AMPA*	*Kainate*	*Quis*	*NMDA*
Mg^{2+} 1 mM	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	3.0 ± 0.2*
APV 50 μM	1.1 ± 0.8	1.2 ± 0.2	1.1 ± 0.1	9.4 ± 0.9*
Kynu 1 mM	6.9 ± 0.4*	5.6 ± 0.3*†	4.6 ± 0.5*†	NT

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Quis = quisqualate; NMDA = N-methyl-D-aspartate; Kynu = kynurenic acid.
Data are presented as means ± s.e.mean for 5–13 slices.
Analyses of variance for the log dose-ratios for the different antagonists yielded the following results: Mg^{2+}: $F(3,35) = 33.37$, $P < 0.0001$; APV: $F(3,26) = 137.44$, $P < 0.0001$; Kynu: $F(2,17) = 9.00$, $P < 0.01$. Individual means were tested using *t* tests.
* Significantly different from unity ($P < 0.0001$).
† Significantly different from AMPA (kainate, $P < 0.05$; quisqualate, $P < 0.01$).
NT = not tested.

Harrison & Simmonds, 1985; Martin & Lodge, 1985; Wheatley & Collins, 1986); the mean apparant pA_2 value was 5.21 ± 0.04 (11).

Kynurenic acid (1 mM) reversibly blocked responses induced by AMPA, kainate and quisqualate. (NMDA was not examined). These data are presented in Table 2.

Spontaneous activity

In 8 of 77 preparations spontaneous fluctuations in the baseline potential developed after slices had been in Mg^{2+}-free medium for at least 1 h. This spontaneous activity was always blocked by 1 mM Mg^{2+} (2), 50 μM APV (3) or TTX (2).

Discussion

In the present investigation, a new preparation has been used to investigate the excitatory amino acid pharmacology of hippocampal neurones. The method is based on that originally developed to study the pharmacology of spinal cord preparations (Curtis *et al.*, 1961; Evans, 1978) and has recently been adapted for use with cortical tissues (Wheatley, 1986). It is similar in principle to the grease gap method which has been used to study the excitatory pharmacology of neocortical tissue (Harrison & Simmonds, 1985; Wong *et al.*, 1986; Burton *et al.*, 1987); Martin & Lodge, 1987) and cerebellar tissue (Garthwaite *et al.*, 1986). Quantitative pharmacological data of a similar type have also been obtained from olfactory cortex by use of a wick electrode technique (e.g. Surtees & Collins, 1985).

The CA1 region of the hippocampal slice was chosen in view of the well characterized electrophysiology and established role of excitatory amino acid receptors, particularly those of the NMDA subtype, in synaptic events in this area (see Collingridge & Bliss, 1987). The observations that similar responses were obtained from slices further dissected to leave only the CA1 region, and from slices in the presence of TTX, but not from isolated alvear fibres, demonstrate that neuropil in the CA1 region is the source of the depolarizations. Since in the CA1 region, pyramidal neurones are the main cell type and are the major source of axons in the alveus (Swanson *et al.*, 1978) it is likely that the recordings originate primarily from CA1 pyramidal neurones. Theoretically, this preparation could be adapted for similar studies in the CA3 region or the dentate gyrus. Garthwaite *et al.* (1986) have exploited this principle in the cerebellum to differentiate between the pharmacology of responses of Purkinje and granule cells.

The dose-response curves suggest that quisqualate could be a partial agonist. A similar conclusion has been reached recently in a neocortical preparation where it has been suggested that quisqualate is a partial agonist at receptors activated by AMPA (Horne & Simmonds, 1987). The much shallower slope of the quisqualate dose-response curve compared to the curves obtained with the other agonists means that comparisons in terms of equipotent molar ratios are highly dose-dependent.

There are data from several brain regions which are of a sufficiently quantitative nature to enable regional comparisons to be made with respect to excitatory amino acid pharmacology. The hippocampus is more sensitive to NMDA than the spinal cord (Martin & Lodge, 1985; Wheatley & Collins, 1986), cerebellum (Garthwaite *et al.*, 1986) and other cortical regions (Harrison & Simmonds, 1985; Surtees & Collins, 1985; Wong *et al.*, 1986; Martin & Lodge, 1987), whether expressed in terms of absolute potency or as potency relative to excitatory amino acids acting at non-NMDA receptors. This greater sensitivity may be due to the particularly high density of NMDA receptors in the CA1 region of the hippocampus (Cotman *et al.*, 1987). Such a differential distribution of excitatory amino acid

receptors might relate to the major involvement of the hippocampus in synaptic plasticity (Collingridge & Bliss, 1987) and may in part explain the greater susceptibility of this brain area to seizure and neuronal loss following ischaemia (Meldrum, 1985).

The observation that Mg^{2+} selectively blocked responses to NMDA is consistent with studies in other brain regions (e.g. Davies *et al.*, 1979; Harrison & Simmonds, 1985; Martin & Lodge, 1985). Due to the non-parallel shift of the dose-response curve sometimes observed following the removal of Mg^{2+}, the dose-ratio obtained was calculated at a fixed response size, corresponding to that produced by 10 μM NMDA. This method of measurement may account for the smaller value obtained in the present study compared to previous studies. The surmountable nature of the antagonism of NMDA-induced responses by Mg^{2+} may be explained on the basis of the voltage-dependent nature of the Mg^{2+} block of NMDA channels (Nowak *et al.*, 1984). Thus, with larger depolarizations the Mg^{2+} block may be reduced or eliminated.

Since APV is used widely in the study of synaptic plasticity in the hippocampus it seemed of interest to determine its potency and selectivity within this region in a quantitative manner. At 50 μM, a concentration that fully blocks those synaptic components in the hippocampus which are believed to be mediated by NMDA receptors (Collingridge *et al.*, 1988), APV selectively antagonized responses to NMDA. Other studies have indicated that APV is without effect on non-amino acid excitants (Davies *et al.*, 1981; Collingridge *et al.*, 1983a; Childs *et al.*, 1987). In spinal cord and cortex, APV has been found to be a competitive antagonist of NMDA with a Schild slope of 1.0 and a pA_2 value usually of between 5.0 and 5.4 (Evans *et al.*, 1982; Harrison & Simmonds, 1985; Wheatley & Collins, 1986). The apparent pA_2 of 5.21 obtained in the present experiments is consistent with these previous studies and suggests a similarity between the affinities of NMDA receptors present in different areas of the central nervous system (of the rat).

Kynurenic acid is a broad-spectrum excitatory amino acid antagonist (Perkins & Stone, 1982) and depresses fast synaptic excitation in the CA1 region of the hippocampus (Ganong *et al.*, 1983). Although it is most potent as an NMDA antagonist it is mainly used for its ability to block non-NMDA receptors. For this reason dose-ratios, at a concentration used in previous studies of synaptic transmission (Ganong *et al.*, 1983), were determined for agonists at non-NMDA receptors. These values are similar to those obtained recently in the spinal cord (Evans *et al.*, 1987) and neocortex (Kemp *et al.*, 1987; Horne & Simmonds, 1987). Although kynurenate differentially affected responses to AMPA, kainate and quisqualate, the differences were not sufficiently great to make it a particularly useful agent to determine the synaptic roles of the non-NMDA receptor subtypes.

In summary, the present data suggest that the pharmacology of excitatory amino acid receptors in the CA1 region of the rat hippocampus is similar to other brain regions examined. There are, however, differences in the potency of agonists compared to other areas. These might be explained on the basis of differential receptor density.

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