Br. J. Pharmac. (1983), **80**, 355–364

Characterization of the binding of DL-[^{3}H]-2-amino-4-phosphonobutyrate to L-glutamate-sensitive sites on rat brain synaptic membranes

S.P. Butcher, J.F. Collins* & P.J. Roberts

Department of Physiology and Pharmacology, Medical and Biological Sciences Building, University of Southampton, Southampton, SO9 3TU and Department of Chemistry*, City of London Polytechnic, 31 Jewry Street, London EC3N 2EY

1 The binding of DL-[3,4-^{3}H] 2-amino-4-phosphonobutyric acid DL[^{3}H]-APB to rat whole brain synaptic membranes was investigated.

2 Binding was linear with membrane protein concentration, and optimal at physiological pH and temperature. The association rate was rapid, achieving equilibrium within 10 min. Prolonged incubations (> 20 min) revealed additional sites, which apparently possessed identical binding characteristics to those detected with 10 min incubations.

3 Binding of DL-[^{3}H]-APB was enriched in synaptic membrane fractions, and assessment of the regional distribution, indicated greatest binding in those areas with a rich glutamatergic innervation.

4 The binding of DL-[^{3}H]-APB in HEPES-KOH buffer exhibited an absolute requirement for Cl^-. The addition of Ca^{2+} resulted in a further enhancement of binding.

5 Saturation analysis revealed the presence of specific glutamate-sensitive DL-[^{3}H]-APB binding sites, with a $K_D = 1.26\,\mu M$ and $B_{max} = 12.08$ pmol mg^{-1} protein. A Hill plot revealed a slope slightly greater than unity, which could possibly be a reflection of a contribution to binding of a further site which is relatively insensitive to glutamate. Analysis of 60 min incubation data indicated an approximately 3 fold increase in the capacity of the system, but a relatively unchanged K_D.

6 Examination of the pharmacological specificity of binding, showed that for both agonist and antagonist molecules, the L-enantiomers were invariably more active than the D-forms. For example, the L-(+)-2-amino-4-phosphonobutyrate isomer was 15 times more active than the D-(−)-form in inhibiting the binding of DL-[^{3}H]-APB. This is in close agreement with the ability of these compounds to produce depression of synaptic transmission. The most potent inhibitor of binding was quisqualate. It is suggested that APB may interact with a quisqualate-preferring class of excitatory amino acid receptors, possibly localised predominantly on presynaptic terminals.

Introduction

Systematic neuropharmacological studies have indicated the presence of at least three types of receptor with which the neurotransmitters glutamate, aspartate and other endogenous dicarboxylic amino acids may interact. These receptors are activated preferentially by the analogues N-methyl-D-aspartate (NMDA), kainate and quisqualate (Watkins, 1981a). While the NMDA site has been well characterized pharmacologically, there is a lack of suitable chemical probes for investigating the kainate and quisqualate receptors. Indeed, it is still not clear how many receptor types, or subclasses fall within the non-NMDA category. L-Glutamate diethylester (GDEE) has been reported to antagonize quisqualate and glutamate, but not kainate responses in cat spinal neurones (Davies & Watkins, 1979; McLennan & Lodge, 1979). However, this compound is of low potency and is often considered to be a rather unreliable antagonist of uncertain mode of action. The compound γ-D-glutamyl-glycine is able to discriminate between quisqualate and kainate receptors in the cat spinal cord, depressing spinal responses elicited by the latter compound (Davies & Watkins, 1981). The most potent antagonist at quisqualate receptors is *cis*-2,3-piperidinedicarboxylate, although this substance also blocks NMDA and kainate receptors.

Highly potent and specific antagonists for the

NMDA receptor have been revealed amongst a series of ω-phosphono-α-carboxylic amino acids, notably, 2-amino-5-phosphonopentanoate and the corresponding heptanoate analogue (Evans & Watkins, 1981; Watkins, 1981a; Evans, Francis, Jones, Smith & Watkins, 1982). A lower homologue, DL-2-amino-4-phosphonobutyrate (APB) (in which the ω-carboxylate group of glutamate is replaced by phosphonate) was originally reported to possess weak glutamate-like agonist activity (Curtis & Watkins, 1965; Watkins, Curtis & Brand, 1977), while in several invertebrate species, APB depressed glutamate-induced depolarizations (Cull-Candy, Donnellan, James & Lunt, 1976; Dudel, 1977). More recent studies have demonstrated antagonism by APB at vertebrate excitatory amino acid receptors (Davies & Watkins, 1979; Hori, Auker, Braitman & Carpenter, 1981). However, this appeared to be rather non-selective, since APB was effective in blocking responses elicited by glutamate, aspartate, kainate, quisqualate and NMDA. This effect at postsynaptic amino acid receptors seems to be a property of the D(−)-isomer (Davies & Watkins, 1982; Evans *et al.*, 1982). In contrast, L(+)-APB has a potent and stereoselective synaptic depressant action (Davies & Watkins, 1982; Evans *et al.*, 1982; Koerner & Cotman, 1981) which does not appear to be associated with blockade of postsynaptic amino acid receptors. At somewhat higher concentrations than those necessary for synaptic depressant effects, the L-isomer elicits 2-amino-5-phosphonopentanoate-sensitive depolarization, indicating some NMDA receptor agonist activity (Evans *et al.*, 1982).

With the recent availability of highly-labelled DL-APB (Monaghan, Fagg, Mena, Nieto-Sampedro, McMills, Chamberlin & Cotman, 1982), we have investigated the characteristics of the binding sites for this substance on rat brain synaptic membranes, in an attempt to gain additional information concerning non-NMDA type excitatory amino acid receptors. A preliminary account of this work has appeared as a short note (Butcher, Roberts & Collins, 1983).

Methods

Synaptic membranes

Albino Wistar rats (250–300 g, either sex) were killed by decapitation and the brains removed rapidly. Synaptic membranes were prepared by a modification of our procedure (Sharif & Roberts, 1980). Briefly, brains were homogenised in a Teflon-glass homogeniser (0.1 mm clearance, 5 strokes, approx. 2,000 r.p.m.), in 20 vol 0.32 M sucrose, buffered with 5 mM HEPES-KOH (pH 7.4). The homogenate was centrifuged for 10 min at 1,000 *g* in a Beckman J2-21 centrifuge, and the pellet discarded. The supernatant was centrifuged again at 17,000 *g* for 20 min, and the resulting P_2 pellet was resuspended in 5 mM HEPES-KOH buffer (pH 7.4) with a tight-fitting glass-glass homogeniser, and the suspension placed on ice for 10 min. This procedure was followed by centrifugation at 17,000 *g* for 20 min, resuspension of the pellet; in fresh buffer and further centrifugation. The fluffy 'buffy coat' was carefully removed from the surface of the pellet; resuspended in 5 mM HEPES buffer and centrifuged at 50,000 *g* for 20 min. This washing procedure was repeated a further twice, and the synaptic membrane-enriched pellet finally resuspended in 50 mM HEPES-KOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$.

Subcellular fractionation

In some experiments, whole rat brains were homogenised with a Teflon-glass homogeniser in 20 vol 0.32 M sucrose, buffered with 5 mM HEPES (pH 7.4), and a P_2 pellet prepared as described above. This was resuspended in a small volume (less than 1 ml) of sucrose, and layered onto a biphasic gradient consisting of 7 ml 0.8 M sucrose, and 7 ml 1.2 M sucrose, buffered with 5 mM HEPES-KOH (pH 7.4). After centrifugation for 60 min at 100,000 *g* in a Beckman SW 27 swing-out rotor, crude myelin, synaptosomal, and mitochondrial fractions were harvested, and lysed in 5 mM HEPES-KOH buffer, using a glass-glass homogeniser. After standing on ice for 10 min, membranes were pelleted by centrifugation at 50,000 *g* for 20 min. Pellets were resuspended in buffer, and recentrifuged 3 times prior to final resuspension in buffer, and assay for binding of APB.

DL-[^{3}H]-2-amino-4-phosphonobutyrate binding assay

Assays were carried out in 1.9 ml polypropylene microfuge tubes (Elkay Inc., MA, U.S.A.). To each tube was added 25 μl of DL-[3,4-^{3}H]-2-amino-4-phosphonobutyric acid (26.6 Ci/mmol) plus, in inhibitor studies, 25 μl of a solution of the substance under test, or buffer control. Non-specific binding was defined by total radioactivity bound minus radioactivity bound in the presence of 1 mM L-glutamate in the medium. In experiments where a single, fixed DL-[^{3}H]-APB concentration was required, this was usually 30 nM (10 nM label, diluted with 20 nM unlabelled DL-APB). The binding assay was initiated by addition of 0.5 ml of membrane suspension (in 50 mM HEPES-KOH, pH 7.4, with 2.5 mM $CaCl_2$) and continued, usually for 10 min at 37°C in a shaking water bath. Tubes were then centrifuged for 30 s in a Beckman Microfuge B, and the

supernatant aspirated immediately. Pellets were rinsed carefully with 1 ml buffer, the tube tips cut off, and the membranes solubilised overnight in scintillation vials containing 2% SDS. Bound radioactivity was determined by liquid scintillation counting following the addition of 8 ml scintillant (xylene, 5.34 ml; synperonic NXP, 2.66 ml; PPO, 32 mg and dimethyl POPOP, 4 mg) in a Beckman LS 7500 liquid scintillation spectrometer with external standardisation and automatic quench correction.

Compounds and chemicals

DL-[^{3}H]-APB was supplied by New England Nuclear, Boston, MA, U.S.A. Scintillation fluors were from Koch-Light; synperonic NXP from ICI plc and standard reagents from BDH Ltd., Poole, Dorset. N-methyl-D-aspartate, γ-D-glutamylglycine, D-α-aminoadipate, L-α-aminoadipate, DL-α-aminosuberate and DL-α-aminopimelate were gifts from Dr J.C. Watkins (Bristol). α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) was from Dr P. Krosgaard-Larsen (Copenhagen); (±)-ibotenate from Prof C.H. Eugster (Zurich) and 1-hydroxy-3-aminopyrrolid-2-one (HA-966) from Dr H.J. Broxterman (Leiden). All other amino acid analogues were synthesised by ourselves, or purchased from Sigma U.K., or Cambridge Research Biochemicals. The isomers of APB had the following $[\alpha]_{546}$ values (determined in water): D-(−)-APB, −11.8° and L-(+)-APB, +10.8°.

Results

Dependence of binding on membrane protein concentration

The specific binding of DL-[^{3}H]-APB (30 nM), which represented approximately 30% of the total binding, was found to be linear with membrane protein concentration over a wide range (16–780 μg protein). At the highest protein concentration, this represented approximately 230 fmol DL-[^{3}H]-APB specifically bound. In all routine experiments, protein concentrations were maintained in the range 200–600 μg per assay.

Effects of varying pH and temperature

Specific binding of DL-[^{3}H]-APB was evident over the pH range 6.0–9.0. However, it exhibited a distinct optimum between pH 7.0 and 8.0 (Table 1).

The incubation of membranes with 30 nM DL-[^{3}H]-APB for 10 min at various temperatures, indicated greatly reduced binding at 0°C and 50°C, compared with 22°C and 37°C. The decreased binding at 50°C is almost certainly a consequence of binding site denaturation. The non-specific binding component was essentially constant under each of these conditions. It is worth noting, that, in common with L-glutamate binding (Foster & Roberts, 1978), specific binding of DL-[^{3}H]-APB was substantially reduced following freezing of the synaptic membranes.

Table 1 Effects of pH and temperature on specific DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding to whole brain synaptic membranes

Treatment		*Specific binding* (fmol mg^{-1} protein)
pH	6.0	105 ± 17
	7.0	178 ± 36
	7.4	251 ± 27
	8.0	154 ± 11
	9.0	68 ± 19
Temperature (°C)	0	39 ± 61
	22	256 ± 79
	37	299 ± 28
	50	3 ± 28

Freshly-prepared, whole rat brain synaptic membranes were incubated for 10 min with DL-[^{3}H]-APB (30 nM) in the absence, or presence of L-glutamate (1 mM) as described in the text. Results are means ± s.e.mean from duplicated experiments performed in quadruplicate.

Time course of binding: association and dissociation

The time dependency of specific DL-[^{3}H]-APB binding was biphasic. Initially, binding was extremely rapid, attaining an equilibrium value of approximately 240 fmol bound mg^{-1} protein, within 10 min. This equilibrium value was maintained for at least a further 10 min, after which, binding increased again sharply, reaching a new equilibrium value of approximately 600 fmol mg^{-1} protein after 50–60 min incubation (Figure 1). With these longer incubations, some clumping and precipitation of membrane particles was evident. This was never observed during the time period over which the initial equilibrium was established.

The dissociation of bound DL-[^{3}H]-APB was rapid and followed a typical exponential decay curve, with a half-life of approximately 90 s (Figure 2). After 20 min however, there was still significant detectable residual binding, which may reflect the changes occurring in the synaptic membranes during long incubations.

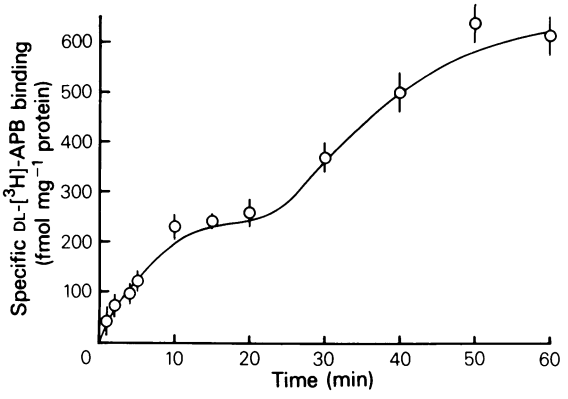

Figure 1 Time course of association of DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) to whole rat brain synaptic membranes. Synaptic membranes (200–600 μg protein) were incubated for various times at 37°C in HEPES-KOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ and 30 nM DL-[^{3}H]-APB as described in the text. Specific APB binding was defined as that displaced by 1 mM L-glutamate. Results are means with s.e.mean of quadruplicate determinations from 4 independent experiments. Note the biphasic nature of the curve.

Effects of Ca^{2+}, Cl^- and other ions on DL-[^{3}H]-APB binding

Incubation of synaptic membranes for 10 min with DL-[^{3}H]-APB, in the absence of any added ions other than K^+ (50 mM HEPES-KOH buffer, pH 7.4), resulted in minimal or non-detectable levels of specific binding (Table 2). In the presence of both Ca^{2+} and

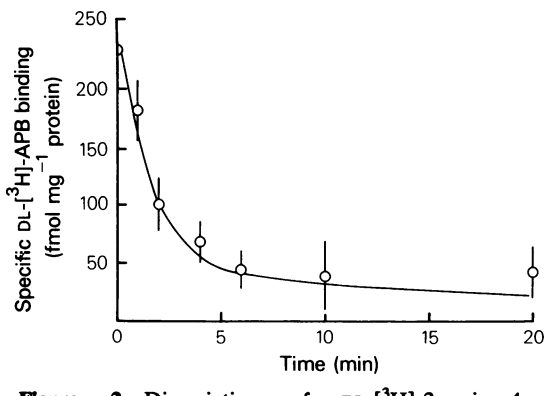

Figure 2 Dissociation of DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) from specific binding sites on synaptic membranes. Synaptic membranes were incubated at 37°C with 30 nM DL-[^{3}H]-APB as described in the text. After a 10 min incubation, excess unlabelled (1 mM) L-glutamate was added and, at various times, specific binding was determined. Results are means with s.e.mean of quadruplicate determinations from 2 independent experiments.

Table 2 Effects of ions on specific DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding binding

Ions added (2.5 mM)	*Specific binding* (fmol mg^{-1} protein)
None	16 ± 19
Calcium chloride	251 ± 18
Calcium acetate	33 ± 6
Ammonium chloride	102 ± 18
Ammonium acetate	18 ± 33
Magnesium chloride	127 ± 32
Manganese chloride	185 ± 25

Whole rat brain synaptic membranes were incubated in HEPES-KOH buffer (pH 7.4) for 10 min with DL-[^{3}H]-APB (30 nM), in the absence or presence of added ions. Specific binding was determined as described in the text. Results are means ± s.e.mean from at least 3 experiments, performed in quadruplicate.

Cl^- (as $CaCl_2$, 2.5 mM), specific binding of 30 nM DL-[^{3}H]-APB was approximately 250 fmol mg^{-1} protein. In view of the report that Cl^- stimulates L-glutamate binding to synaptic plasma membranes, and that Ca^{2+} acts only in the presence of Cl^- to enhance this response further (Mena, Fagg & Cotman, 1981), we investigated the binding of DL[^{3}H]-APB to synaptic membranes in the presence of a number of ionic species (Table 2). Indeed, Cl^- alone (NH_4Cl compared with CH_3COONH_4, both at 2.5 mM) was able to enhance binding, and thus appears to promote the further enhancement seen in the presence of Ca^{2+}. Calcium acetate had a minimal stimulatory action. Magnesium, and especially manganese were able partially to mimic the effects of calcium (all as the chloride salt).

Regional specificity of binding

Synaptic membranes from a number of regions of the rat central nervous system were prepared as described for whole brain, and the specific binding of DL-[^{3}H]-APB determined (Table 3). Compared with binding to whole brain membranes, specific binding was enriched particularly in striatum and hippocampus, and was least in the pons/medulla, where it was approximately one fifth of that occurring in the former two regions.

Subcellular distribution of DL-[^{3}H]-APB binding sites

Subcellular fractionation of whole rat brain homogenates, resulted in an initial enrichment in the P_2 fraction, which was further increased in the synaptic membrane fraction. Binding in the P_1, myelin and

Table 3 Regional specificity of DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding

Neural-tissue	*Specific binding* (fmol mg^{-1} protein)
Striatum	559±78
Hippocampus	574±90
Cortex	467±83
Cerebellum	203±79
Pons/medulla	129±17
Spinal cord	44±67
Whole brain	279±30

Following killing of the animals, the above regions were rapidly dissected out and homogenized in 20 vol buffered 0.32 M sucrose. Synaptic membranes were prepared simultaneously for each of the regions as described in the text, and the specific binding of 30 nM DL-[^{3}H]-APB determined during a 10 min incubation. Results are means ± s.e.mean from 2 experiments performed in quadruplicate.

mitochondrial fractions was variable between preparations, but was essentially minimal (Table 4).

Saturability of DL-[^{3}H]-APB binding

In view of the earlier observation that the time course of DL-[^{3}H]-APB binding exhibited biphasic kinetics with an initial rapid establishment of equilibrium, followed by a further increase in binding, studies were carried out with incubations of both 10 and 60 min duration. Analysis of untransformed specific binding data obtained over the concentration range

Table 4 Subcellular distribution of specific DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB)

Tissue fraction	*Specific binding* (fmol mg^{-1} protein)
P1 pellet	—
P1 supernatant	67±22
P2 supernatant	23±11
P2 pellet	118±29
Crude myelin	64± 8
Mitochondrial	23±17
Synaptic membrane	257±24
Washed synaptic membranes	283±96

Whole rat brains were fractionated as described in the text, and the binding of DL-[^{3}H]-APB (30 nM) determined. Results are means ± s.e.mean of 2 experiments (performed in quadruplicate) from 5 or 6 pooled brains.

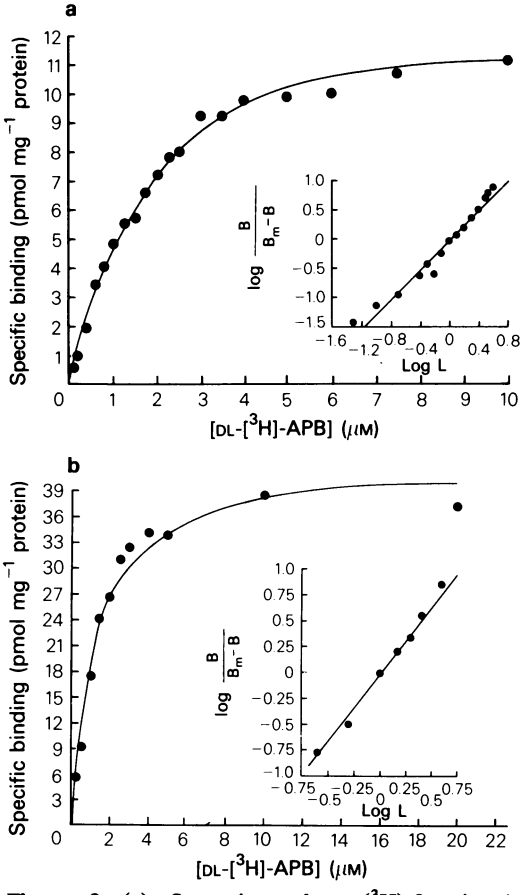

Figure 3 (a) Saturation of DL-(^{3}H)-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding to whole rat brain synaptic membranes. Synaptic membranes were incubated at 37°C for 10 min with a range of concentrations of DL-[^{3}H]-APB. Specific APB binding was defined using 1 mM L-glutamate. Results are means of quadruplicate determinations from 2 independent experiments. K_D and B_{max} were calculated using a computer-derived curve fitting process, based on that described by Wilkinson (1961). The inset shows a Hill plot of the binding data. (b) Saturation of DL-[^{3}H]-APB binding to whole rat brain synaptic membranes incubated at 37° for 60 min. Otherwise, conditions were identical to those described for (a).

10 nM–20 μM DL-[^{3}H]-APB revealed the apparent presence of a single, saturable population of binding sites (Figure 3a and b). For the 10 min incubation, sites were detected with a $K_D = 1.26 \pm 0.07$ μM and $B_{max} = 12.08 \pm 0.51$ pmol mg^{-1} protein. After 60 min incubation, the values found were $K_D = 1.09 \pm 0.06$ μM and $B_{max} = 39.35 \pm 0.945$ pmol mg^{-1} protein (means ± s.e.mean of quadruplicate determinations performed in duplicate). Thus, the increased binding observed following prolonged incubation of the

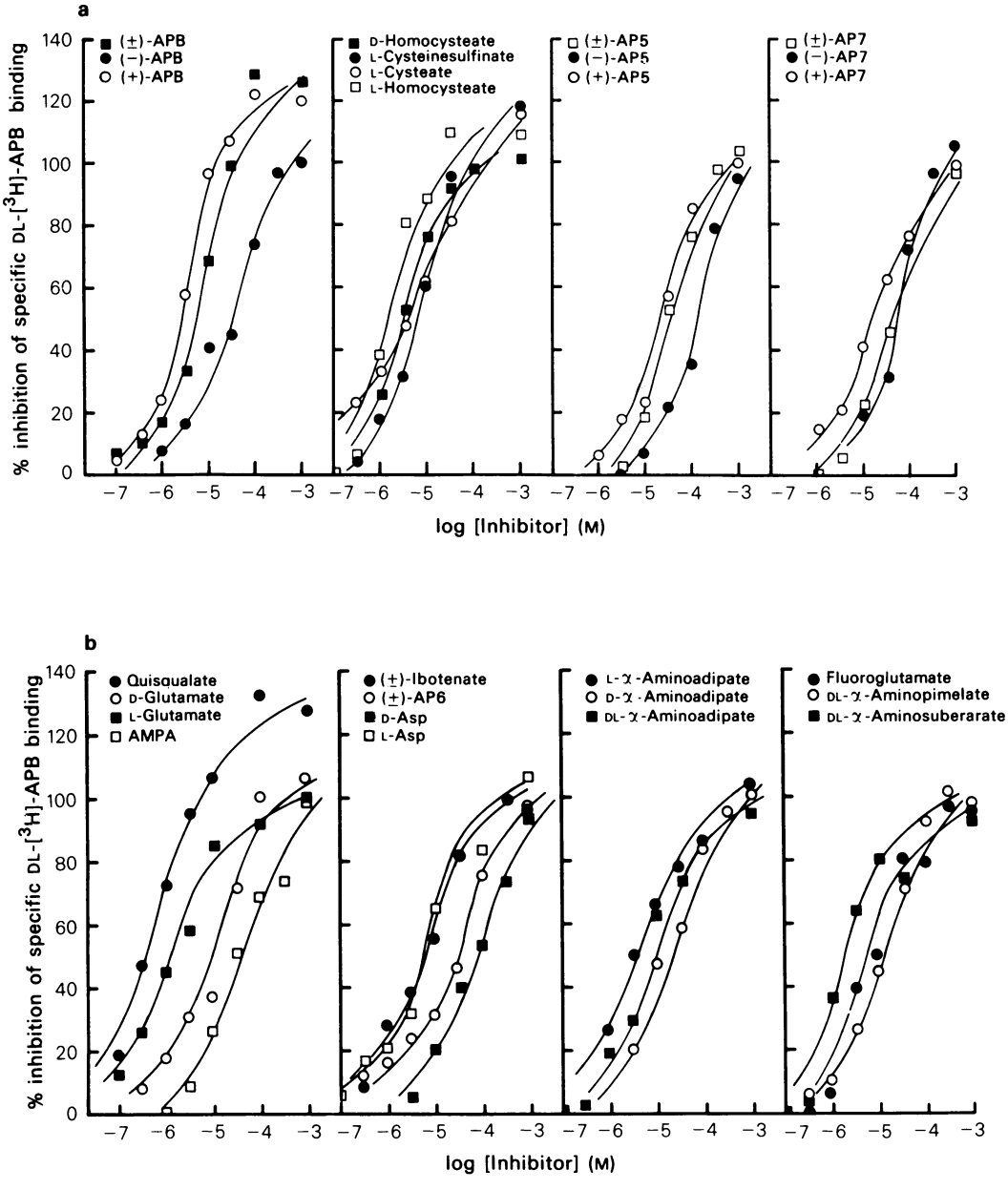

Figure 4 (a and b) The inhibition of DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding to rat brain synaptic membranes by excitatory amino acid analogues. Synaptic membranes were incubated for 10 min at 37°C with 30 nM DL-[^{3}H]-APB in the presence of a wide range of inhibitory compounds as described in the text. The compound under test was added simultaneously with the labelled ligand. Results are means of quadruplicate determinations from at least two independent experiments. IC_{50} values (that concentration of inhibitor that reduced binding by 50%) were read directly from the plots.

AP5: 2-amino-5-phosphonopentanoate; AP6: 2-amino-6-phosphonohexanoate; AP7: 2-amino-7-phosphonoheptanoate; AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionate.

synaptic membranes, appears to be wholly due to an increase in the number of binding sites exposed to the ligand, with no change in their affinity. Hill plots for both sets of data (Figure 3a and b insets) revealed slopes of 1.26 ± 0.01 and 1.35 ± 0.03 for the 10 and 60 min incubations respectively. Although these values were greater than unity, they were insufficiently different to be confident that there is site heterogeneity, or interactions between binding sites. Of interest was the finding that a 10 min incubation of DL-[^{3}H]-APB in the presence of Cl^- but not Ca^{2+} (binding assay medium of HEPES-KOH + 2.5 mM NH_4Cl) gave a K_D of approximately 1.44 μM, and a $B_{max} = 7.60$ pmol mg^{-1} protein (data not shown), indicating that the effect of the further addition of Ca^{2+} is to increase the number of sites available for interaction with the ligand.

Pharmacological specificity of DL-[^{3}H]-APB binding

The pharmacological specificity of the binding of DL-[^{3}H]-APB was investigated by incubating synaptic membranes with ligand (30 nM) for 10 min at 37°C in the presence of a range of concentrations (0.3 μM–1.0 mM) of the inhibitory compound under test. Log concentration-% inhibition curves were constructed (Figure 4a and b) and IC_{50} values determined (Table 5).

The L-isomer of APB was approximately 15 times more potent than the D-form in inhibiting binding. For a number of agonists such as homocysteate, glutamate, and aspartate, L/D-isomer activity ratios of around 6, 11 and 9 respectively, were obtained. The binding site was not sensitive to kainate or to a number of specific ligands for other receptors, viz. carbachol, glycine, γ-aminobutyric acid (GABA) and noradrenaline. N-methyl-D-aspartate (or N-methyl-DL-aspartate) and quinolinic acid, were very weakly active in inhibiting DL-[^{3}H]-APB binding.

With specific excitatory amino acid receptor antagonists, the pattern of binding was confusing. L-(+)-APB was highly active, and in common with the L-isomers of sulphur-containing amino acids, and with quisqualate, inhibited [^{3}H]-APB binding to a substantially greater extent than the 100% defined by 1 mM L-glutamate (Figure 4a and b). The isomers of other phosphono amino acid derivatives, such as 2-amino-5-phosphonopentanoate, and 2-amino-7-phosphonoheptanoate, all exhibited some activity. Again, it is striking that the L-(+)-isomers were the more active. A similar pattern emerged for α-aminoadipate and α-aminosuberate. A number of other antagonists were also investigated, and were found to possess either little (L-glutamate diethylester) or no (*cis*-2,3 piperidine dicarboxylate and γ-D-glutamylglycine) activity.

Table 5 Inhibition of DL-[^{3}H]-2-amino-4-phosphonobutyrate (DL-[^{3}H]-APB) binding to rat brain synaptic membranes by excitatory amino acid receptor agonists and antagonists

Agonist	IC_{50} (μM)
Quisqualate	0.398
L-Homocysteate	1.21
L-Glutamate	1.70
L-Cysteate	4.00
D-Homocysteate	6.30
L-Aspartate	6.97
L-Cysteine sulphinate	7.08
DL-Fluoroglutamate	7.90
(±)-Ibotenate	10.00
D-Glutamate	18.10
α-Amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)	25.24
D-Aspartate	56.20
Antagonist	
DL-α-Aminosuberate	1.58
L(+)-APB	2.24
L-α-Aminoadipate	3.00
DL(±)-APB	3.61
DL-α-Aminoadipate	6.13
DL(±)-2-Amino-6-phosphonohexanoate	14.40
D-α-Aminoadipate	16.18
DL-α-Aminopimelate	16.51
L(+)2-Amino-7-phosphonoheptanoate	18.96
L(+)-2-Amino-5-phosphonopentanoate	20.00
D(−)-APB	30.06
DL(±)-2-Amino-7-phosphonoheptanoate	32.60
DL(±)-2-Amino-5-phosphonopentanoate	39.80
D(−)-2-Amino-7-phosphonoheptanoate	100
D(−)-2-Amino-5-phosphonopentanoate	126

Weakly active ($IC_{50} > 100$ μM)
N-methyl-D-aspartate, quinolinic acid, (±)-2-amino-3-phosphonopropionate, *cis*-2,3-piperidinedicarboxylate, DL-α, ε-diaminopropionate, γ-D-glutamyl glycine.

Inactive ($IC_{50} > 1$ mM)
Kainate, dihydrokainate, 1-hydroxy-3-aminopyrrolid-2-one, carbachol, glycine, GABA, noradrenaline, phenobarbitone.

Synaptic membranes were incubated with 30 nM DL-[^{3}H]-APB in the absence or presence of a wide range of concentrations (0.3 μM–1.0 mM) of the compound under test, as described in the legend to Figure 4. IC_{50}s (the concentration required to produce 50% inhibition for that compound) were read directly from the log concentration/percentage inhibition curves.

Discussion

The mechanism of action of DL-APB has been the subject of debate since the compound was first shown

to be an antagonist at the locust neuromuscular junction (Cull-Candy *et al.*, 1976). Early experiments with vertebrate preparations indicated that on cerebral and cerebellar neurones in the rat, DL-APB was a glutamate-like agonist (Bioulac, De Tinguy-Moreaud, Vincent & Neuzil, 1979), while ionophoretic application of DL-APB to cat spinal neurones failed to reduce excitation by glutamate (Watkins *et al.*, 1977). Recent resolution of the D-(−)- and L-(+)-isomers of APB has helped to clarify the situation, and it is now generally accepted that the D-(−)-isomer is indeed a rather weak and probably non-selective antagonist at postsynaptic excitatory amino acid receptors (Davies & Watkins, 1982; Hori *et al.*, 1981). The actions of the L-(+)-isomer remain enigmatic: it has been reported by several groups that the compound possesses a potent and stereoselective synaptic depressant action (Koerner & Cotman, 1981; Davies & Watkins, 1982). It has been proposed that L-(+)-APB may act by specifically inhibiting the release of an excitatory amino acid transmitter, acting at non-NMDA receptors, or alternatively, by antagonizing the postsynaptic effects of an as yet unidentified transmitter (Davies & Watkins, 1982). Collingridge, Kehl & McLennan (1983a) have found that on rat hippocampal CA1 neurones, the effect of L-APB was typically to enhance amino acid-induced responses, and occasionally to excite cells by itself. In accordance with the excitatory effects reported in the frog and rat spinal cords (Evans *et al.*, 1981), the agonist effects were sensitive to both D-APB and 2-amino-5-phosphonopentanoate, implicating an involvement of NMDA receptors. Similar results were found at the Schaffer collateral-commissural pathway, with both isomers of APB being weakly active in depressing the e.p.s.p. (Collingridge, Kehl & McLennan, 1983b). Neurochemical studies have shown that DL-APB is without effect on high-affinity glutamate uptake (Balcar & Johnston, 1972; Roberts & Watkins, 1975) (but see Vincent & McGeer, 1980). Thus, this is unlikely to be the mechanism by which responses to excitatory amino acids are potentiated by APB.

In terms of neurochemically- (as distinct from electrophysiologically) defined receptor sites, considerable information has accumulated. Foster & Roberts (1978) reported that DL-APB inhibited the Na^+-independent binding of L-[^{3}H]-glutamate to rat cerebellar synaptic membranes, in a Cl^--containing medium. In a later study, where a postsynaptic response was measured (the ability of excitatory amino acids to increase tissue levels of cyclic GMP), DL-APB was an effective, though weak antagonist of glutamate and aspartate, and had rather less effect on the responses elicited by NMDA and kainate (Roberts, Foster, Sharif & Collins, 1982). With regard to glutamate binding studies, an important observation has been that chloride and calcium ions apparently separate distinct receptor populations (Fagg, Foster, Mena & Cotman, 1982; 1983; Mena *et al.*, 1981). The Cl^-/Ca^{2+}-dependent glutamate binding was inhibited potently by L-APB, which was some 15 times more active than the D-isomer in this respect. This latter binding site does not appear to conform to the current model for excitatory amino acid receptors (Watkins, 1981a). It is insensitive to both NMDA and kainate, while quisqualate and (the proposed NMDA receptor-preferring agonist) ibotenate, exhibited somewhat surprisingly similar potencies, and yielded non-linear Scatchard plots in the presence of $CaCl_2$. Maximally, 70–75% of glutamate binding was inhibited with K_is of 0.1–0.5 μM, and the remaining 25% with much lower affinity (40–80 mM).

Because L-glutamate is a 'mixed agonist' (Watkins & Evans, 1981), interacting with several subpopulations of receptors in binding experiments, attempts to investigate the sites of action of APB are most likely to be resolved using labelled APB itself, and preferably, the individual isomers. In this study, we have shown that DL-[^{3}H]-APB binds specifically to L-glutamate-sensitive sites on rat whole brain synaptic membranes, with a $K_D = 1.3$ μM. This value corresponds well with the K_i of 16 μM for APB on glutamate binding (Fagg *et al.*, 1982). DL-[^{3}H]-APB binding was optimal at physiological pH and temperature and was totally dependent on Cl^- and Ca^{2+}, and in the absence of these ions, only minimal binding (possibly attributable to residual ions) was detectable. Binding assays performed in full physiological medium (Krebs-Ringer, buffered with HEPES, pH 7.4) revealed no binding in excess of that observed with added Cl^-/Ca^{2+}.

The distribution of binding, both regional and subcellular, was wholly consistent with its being associated with membranes of synaptic origin derived from neurones involved in excitatory amino acid transmission. For example, the high level of binding observed in the striatum and hippocampus is compatible with the rich glutamatergic innervation of these areas of the brain.

A most striking observation, was the sharp biphasic nature of the time course of binding, whereby a second binding component appeared, resulting in a second binding equilibrium with approximately 30 min, or longer incubations. This was due entirely to an increase in binding site capacity. Recent work indicates that the pharmacological characteristics of these sites are identical. This 'unveiling' of APB binding sites is mediated by a calcium-dependent process, and may involve activation of a calcium-dependent cysteine proteinase, since *p*-chloromercuribenzoyl-sulphonic acid and leupeptin were effective in preventing the second phase of

binding (Butcher, Roberts & Collins, unpublished). Interestingly, a similar observation has been made for the regulation of hippocampal glutamate receptors (Baudry & Lynch, 1980; Vargas, Greenbaum & Costa, 1980; Baudry, Bundman, Smith & Lynch, 1981) and has been proposed as a possible mechanism involved in long-term synaptic potentiation.

The pharmacology of DL-[^{3}H]-APB binding generally corresponds closely with that for APB-sensitive glutamate binding (Fagg *et al.*, 1983). In particular, L-APB was 15 times the activity of D-APB in both studies, and excitants such as kainate and NMDA were essentially inactive. Quisqualate was the most active substance investigated and, in contrast to the previous study (Fagg *et al.*, 1983), was much more potent than ibotenate. Since L-APB, quisqualate, and the sulphur-containing excitatory amino acids were able to inhibit binding to a greater degree than L-glutamate, it follows that the DL-[^{3}H]-APB binding sites are not homogeneous (notwithstanding the observed Hill slopes of close to unity for these compounds). The L-(+)-isomers of agonists were also more active than the D-(−)-forms in inhibiting DL-[^{3}H]-APB binding, and so also were the L(+)-isomers of a number of NMDA-receptor antagonists (where the D-(−)-isomer carries most, if not all of the pharmacological activity), such as 2-amino-5-phosphonopentanoate and 2-amino-7-phosphonoheptanoate.

Thus, the binding site for DL-APB recognizes primarily L-isomers, and one might be tempted to propose that it is a quisqualate class of glutamate receptor. A cautionary note here though, is that α-amino-3-hydroxy-5-meythylisoxazole-4-propionate (AMPA), a proposed agonist at quisqualate-preferring receptors, is a much weaker inhibitor of binding than expected. When AMPA binding itself was determined (Honoré, Krogsgaard-Larsen, Hansen & Lauridsen, 1981), the two agonists were equipotent. Additionally, the quisqualate-type antagonist, L-glutamate diethylester was of low potency, although it is pertinent that the usefulness of this compound is uncertain.

Finally, it must be stated that this study has not provided any direct information as to the localisation of DL-[^{3}H]-APB binding sites, or concerning the mechanism of the ionic specificity of binding. For the latter, Mena *et al.*, (1982) proposed that these binding sites may be linked to a membrane Cl^- ion channel, or alternatively, that the Cl^-/Ca^{2+}-dependent site might represent a desensitized form of the receptor. In view of the general observation that the L-conformations of both agonist and antagonist molecules are significantly more potent in inhibiting DL-[^{3}H]-APB binding than the D-isomers, we consider it likely that the site is agonist-preferring, and possibly located presynaptically. In hippocampal slices, DL-APB behaves as an antagonist at glutamate autoreceptors, by preventing the auto-inhibition by glutamate of its own release (McBean & Roberts, 1981). However, since in that study only the racemate was examined, a composite effect was probably being observed. In the retina, L-APB was found to inhibit both acetylcholine release and the ERG b-wave some 15 times more effectively than the D-isomer (Neal, Cunningham, James, Joseph & Collins, 1981). This potency ratio is in accord with our binding data. However, here at least, both D- and L-APB appear to be acting primarily as agonists at a post-synaptic excitatory receptor. Current experiments in our laboratory are aimed at resolving the D- and L-[^{3}H]-APB binding components, and the elucidation of the cellular localisation of these sites.

This work was supported by a research project grant to P.J.R. from the Science and Engineering Research Council. We thank Dr Dick Young (New England Nuclear) for his interest and help, and Dr J.C. Watkins (Bristol), Dr P. Krogsgaard-Larsen (Copenhagen) and Professor C.H. Eugster (Zurich) for generous gifts of compounds, and their continued interest in this research. Correspondence to P.J.R., please.

References

BALCAR, V.J. & JOHNSTON, G.A.R. (1972). Glutamate uptake by brain slices and its relation to the depolarisation of neurones by acidic amino acids. *J. Neurobiol.*, **3**, 295–301.

BAUDRY, M. & LYNCH, G. (1980). Regulation of hippocampal glutamate receptors: evidence for the involvement of a calcium-activated protease. *Proc. natn. Acad. Sci., U.S.A.*, **77**, 2298–2303.

BAUDRY, M., BUNDMAN, M., SMITH, E. & LYNCH, G. (1981). Micromolar levels of calcium stimulate proteolytic activity and glutamate receptor binding in rat brain synaptic membranes. *Science*, **212**, 937–938.

BIOULAC, B., DE TINGUY-MOREAUD, E., VINCENT, J.-D. & NEUZIL, E. (1979). Neuroactive properties of phosphonic amino acids. *Gen. Pharmac.*, **10**, 121–125.

BUTCHER, S.P., ROBERTS, P.J. & COLLINS, J.F. (1983). DL-[^{3}H] 2-amino-4-phosphonobutyrate binding to L-glutamate-sensitive sites on rat brain synaptic membranes. *IRCS Med. Sci.*, **11**, 42–43.

COLLINGRIDGE, G.L., KEHL, S.J. & MCLENNAN, H. (1983a). The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. *J. Physiol.*, **334**, 19–31.

COLLINGRIDGE, G.L., KEHL, S.J. & MCLENNAN, H.

(1983b). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol.*, **334,** 33–46.

CULL-CANDY, S.G., DONNELLAN, J.F., JAMES, R.W. & LUNT, G.G. (1976). 2-amino-4-phosphonobutyric acid as a glutamate antagonist on locust muscle. *Nature, Lond.*, **262,** 402–409.

CURTIS, D.R. & WATKINS, J.C. (1965). The pharmacology of amino acids related to γ-aminobutyric acid. *Pharmac. Rev.*, **17,** 347–391.

DAVIES, J. & WATKINS, J.C. (1979). Selective antagonism of amino acid-induced and synaptic excitation in the cat spinal cord. *J. Physiol.*, **297,** 621–635.

DAVIES, J. & WATKINS, J.C. (1981). Differentiation of kainate and quisqualate receptors in the cat spinal cord by selective antagonism with γ-D (and L)-glutamyl glycine. *Brain Res.*, **206,** 172–177.

DAVIES, J. & WATKINS, J.C. (1982). Actions of D and L forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal cord. *Brain Res.*, **235,** 378–386.

DUDEL, J. (1977). Aspartate and other inhibitors of excitatory synaptic transmission in crayfish muscle, *Pflugers Arch.*, **369,** 7–16.

EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effects of a series of ω-phosphonic α-carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmac.*, **75,** 65–76.

EVANS, R.H. & WATKINS, J.C. (1981). Pharmacological antagonists of excitatory amino acids. *Life Sci.*, **28,** 1303–1308.

FAGG, G.E., FOSTER, A.C., MENA, E.E. & COTMAN, C.W. (1982). Chloride and calcium ions reveal a pharmacologically-distinct population of L-glutamate binding sites in synaptic membranes: correspondence between biochemical and electrophysiological data. *J. Neurosci.*, **2,** 958–964.

FAGG, G.E., FOSTER, A.C., MENA, E.E. & COTMAN, C.W. (1983). Chloride and calcium ions separate L-glutamate receptor populations in synaptic membranes. *Eur. J. Pharmac.*, **88,** 105–110.

FOSTER, A.C. & ROBERTS, P.J. (1978). High-affinity L-[^{3}H] glutamate binding to postsynaptic receptor sites in rat cerebellar membranes. *J. Neurochem.*, **31,** 1467–1477.

HONORÉ, T., KROGSGAARD-LARSEN, P., HANSEN, J.J. & LAURIDSEN, J. (1981). Glutamate and aspartate agonists structurally related to ibotenic acid. *Molec. cell. Biochem.*, **38,** 123–128.

HORI, N., AUKER, C.R., BRAITMAN, D.J. & CARPENTER, D.O. (1981). Lateral olfactory tract transmitter: glutamate, aspartate or neither? *Cell molec. Neurobiol.*, **1,** 115–120.

KOERNER, J.F. & COTMAN, C.W. (1981). Micromolar L-2-amino-4-phosphonobutyric acid selectively inhibits perforant path synapses from lateral entorhinal cortex. *Brain Res.*, **216,** 192–198.

MCBEAN, G.J. & ROBERTS, P.J. (1981). Glutamate-preferring receptors regulate the release of D-[^{3}H] aspartate from rat hippocampal slices. *Nature*, **291,** 593–594.

McLENNAN, H. & LODGE, D. (1979). The antagonism of amino acid-induced excitation of spinal neurones in the cat. *Brain Res.*, **169,** 83–90.

MENA, E.E., FAGG, G.E. & COTMAN, C.W. 1981). Chloride ions enhance L-glutamate binding to rat brain synaptic membranes, *Brain Res.*, **243,** 378–382.

MONAGHAN, D.T., FAGG, G.E., MENA, E.E., NIETO-SAMPEDRO, M.C., McMILLS, A.R., CHAMBERLIN, A.R. & COTMAN, C.W. (1982). New ligands for studying acidic amino acid receptors: [^{3}H] 2-amino-4-phosphonobutyric acid and [^{3}H] L-serine-0-sulphate. *Soc. Neurosci. Abst.*, **8,** 403.

NEAL, M.J., CUNNINGHAM, J.R., JAMES, T.A., JOSEPH, M. & COLLINS, J.F. (1981). The effect of 2-amino-4-phosphonobutyrate (APB) on acetylcholine release from the rabbit retina: evidence for ON-channel input to cholinergic amacrine cells. *Neurosci. Lett.*, **26,** 301–305.

ROBERTS, P.J., FOSTER, G.A., SHARIF, N.A. & COLLINS, J.F. (1982). Phosphonate analogues of acidic amino acids: inhibition of excitatory amino acid transmitter binding to cerebellar membranes and of the stimulation of cerebellar cyclic GMP Levels. *Brain Res.*, **238,** 475–479.

ROBERTS, P.J. & WATKINS, J.C. (1975). Structural requirements for the inhibition of glutamate uptake into glia and nerve endings. *Brain Res.*, **85,** 120–125.

SHARIF, N.A. & ROBERTS, P.J. (1980). Problems associated with the binding of L-glutamic acid to synaptic membranes: methodological aspects. *J. Neurochem.*, **34,** 779–784.

VARGAS, F., GREENBAUM, L. & COSTA, E. (1980). Participation of cystein proteinase in the high affinity Ca^{2+}-dependent binding of glutamate to hippocampal synaptic membranes. *Neuropharmac.*, **19,** 791–794.

VINCENT, S.R. & McGEER, E.G. (1980). A comparison of sodium-dependent glutamate binding with high-affinity glutamate uptake in rat striatum. *Brain Res.*, **184,** 99–108.

WATKINS, J.C. (1981a). Pharmacology of excitatory amino acid transmitters. In *Amino Acid Neurotransmitters.* ed. De Feudis, F.V., & Mandel, P. pp. 205–212. New York: Raven Press.

WATKINS, J.C. (1981b). Pharmacology of excitatory amino acid receptors. In *Glutamate: Transmitter in the Central Nervous System*, ed. Roberts, P.J., Storm-Mathisen, J. & Johnston, G.A.R. pp. 1–24. Chichester: John Wiley & Sons.

WATKINS, J.C., CURTIS, D.R. & BRAND, S.S. (1977). Phosphonic analogues as antagonists of amino acid excitants. *J. Pharm. Pharmac.*, **29,** 324.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *A. Rev. Pharmac. Tox.*, **21,** 165–204.

WILKINSON, G.N. (1961). Statistical estimations in enzyme kinetics. *Biochem. J.*, **80,** 324–332.

(Received April 27, 1983.
Revised June 15, 1983.)