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Cl^-/Ca^{2+}-dependent L-glutamate binding sites do not correspond to 2-amino-4-phosphonobutanoate-sensitive excitatory amino acid receptors

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1 A series of phosphono and phosphino analogues of glutamate were used to compare the pharmacological properties of (a) Cl^-/Ca^{2+}-dependent, 2-amino-4-phosphonobutanoate (AP4)-sensitive L-[^{3}H]-glutamate binding sites in rat brain synaptic plasma membranes (SPMs) and (b) AP4-sensitive excitatory synaptic responses by use of electrophysiological techniques.

2 In the presence of Cl^- and Ca^{2+}, L-[^{3}H]-glutamate bound to SPMs with K_d 804 nM and B_{max} 53 pmol mg^{-1} protein. The AP4-sensitive (K_i 7.3 μM) population of binding sites represented 61% of L-glutamate specifically bound. ω-Substituted analogues of AP4 were potent inhibitors of L-[^{3}H]-glutamate binding (K_i values 2.4–38 μM), whereas N-substituted compounds or propionic acid derivatives were inactive. Experiments with AP4 alone and in combination with other analogues demonstrated that the primary target of all substances was the AP4-sensitive population of L-glutamate binding sites.

3 In the hippocampal slice *in vitro*, AP4 antagonized lateral perforant path-evoked field potentials with an IC_{50} of 2.7 μM. In contrast to their actions at AP4-sensitive L-glutamate binding sites, all other compounds (except for the ω-carboxymethylphosphino analogue, IC_{50} 19 μM) were weak or inactive as antagonists of this synaptic response (IC_{50} values > 100 μM). Inactive compounds which exhibited activity in the binding assay did not reverse the synaptic depressant effects of AP4, indicating that they were neither agonists nor antagonists at AP4-sensitive synapses.

4 The lack of correspondence between (a) the Cl^-/Ca^{2+}-dependent, AP4-sensitive population of L-[^{3}H]-glutamate binding sites and (b) AP4-sensitive synaptic responses indicates that these binding sites are not the receptors through which AP4 exerts its neuropharmacological effects. The possibility that Cl^-/Ca^{2+}-dependent 'binding sites' represent transport into resealed SPM vesicles is discussed.

5 Electrophysiological data demonstrate that AP4-sensitive synaptic receptors display a high degree of ligand selectivity. High antagonist potency is shown only by glutamate analogues with unmodified α-amino and α-carboxyl groups, and with a bifunctional (dianionic) ω-terminal.

Introduction

L-Glutamate is thought to be a major excitatory neurotransmitter in the vertebrate central nervous system (Watkins & Evans, 1981; Fagg & Foster, 1983). Electrophysiological studies indicate that the synaptic responses evoked by this amino acid are mediated by several receptor sub-types. Three of these are characterized by their preference for the excitants, N-methyl-D-aspartate (NMDA), quisqualate and kainate (receptor categories A1, A2 and A3, respectively, see reviews by Watkins & Evans, 1981; Foster & Fagg, 1984; Fagg, 1985), while a fourth sub-type has been proposed on the basis of the potent antagonist actions of L-2-amino-4-phosphonobutanoate (L-AP4, a glutamate analogue) at specific sub-populations of acidic amino acid-using synapses (Koerner & Cotman, 1981; Slaughter & Miller, 1981; Evans *et al.*, 1982; Davies & Watkins, 1982; Collins, 1982; Lanthorn *et al.*, 1984). The mechanism by which AP4 suppresses synaptic excitation, however, has not yet been elucidated (see Butcher *et al* 1983; Harris & Cotman, 1983; Foster & Fagg, 1984).

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Radioligand binding techniques have formed the basis of many investigations of the nature and properties of neurotransmitter receptors. In the case of excitatory amino acids, recent studies in which membrane binding and autoradiographic procedures were used have resolved three radioligand binding sites with characteristics of receptors A1, A2 and A3 (see Fagg, 1985). However, the largest population of L-glutamate binding sites detected in synaptic plasma membranes (SPMs) is dependent on Cl^- and Ca^{2+} and exhibits a distinct ligand selectivity (Fagg *et al.*, 1982b, 1983a; also see Butcher *et al.*, 1983, 1984). Comparative pharmacological analyses led to the suggestion that these Cl^-/Ca^{2+}-dependent binding sites might represent the fourth, L-AP4-sensitive synaptic receptor proposed on the basis of electrophysiological experiments (see Foster & Fagg, 1984).

This study is focused on the Cl^-/Ca^{2+}-dependent, AP4-sensitive L-glutamate binding site in SPMs. It was prompted by the finding that L-α-glycerophosphorylserine, an ω-substituted AP4 analogue, inhibits L-glutamate binding to these sites with a potency similar to that of AP4 itself (Foster *et al.*, 1982). Hence, phosphino derivatives of similar structure might provide a basis for the synthesis of affinity ligands, or for the design of lipophilic glutamate antagonists which better traverse the blood-brain barrier. Our data show that a wide range of ω-substituted AP4 analogues (1) retain activity at Cl^-/Ca^{2+}-dependent L-glutamate binding sites but (2) are of low potency in electrophysiological analyses. These observations question the hypothesis that Cl^-/Ca^{2+}-dependent L-glutamate binding sites are the receptors through which L-AP4 exerts its neuropharmacological actions.

Methods

Subcellular fractionation and L-[^{3}H]-glutamate binding assay

SPM fractions were isolated from the fresh brains (excluding brain stem) of adult male albino rats (180–260 g) essentially as described by Jones & Matus (1974). Fractions were washed 4 times by resuspension and centrifugation (40,000 g_{av}, 30 min) in 0.5 mM HEPES-KOH buffer (pH 7.2) before assaying the protein content (Lowry *et al.*, 1951) and L-[^{3}H]-glutamate binding activity.

The specific Na^+-independent binding of L-[^{3}H]-glutamate was determined by a microcentrifugation procedure (Foster & Roberts, 1978). Aliquots of SPM (0.2 mg protein) were incubated in triplicate in a final volume of 1.0 ml with 50 nM L-[^{3}H]-glutamate (25–1200 nM for K_d determinations) and other compounds as indicated in the Results. The assay buffer was 50 mM Tris containing 40 mM Cl^- and 1 mM Ca^{2+} (adjusted to pH 6.8 at 32°C with acetic acid); these ionic conditions are optimal for the assay of Cl^-/Ca^{2+}-dependent binding sites (Mena *et al.*, 1982; Fagg *et al.*, 1983a; Monaghan *et al.*, 1983; Butcher *et al.*, 1983; 1984). After 30 min at 32°C, tubes were centrifuged for 3 min in an Eppendorf microcentrifuge and the supernatant was aspirated. Radioactivity in the pellets was determined by liquid scintillation spectrometry after dissolving in 2% sodium dodecyl sulphate and addition of 8 ml scintillant. Specific binding was defined as that which was inhibited by an excess (0.5 mM) of L-glutamate.

Hippocampal slice preparation and electrophysiological analyses

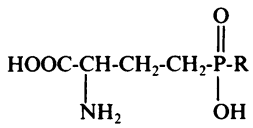
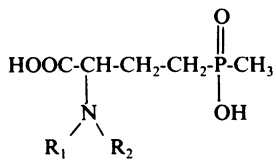
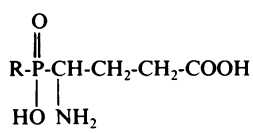
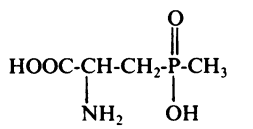
Hippocampal slices were prepared from male albino Sprague-Dawley rats as described previously (Lanthorn & Cotman, 1981; Lanthorn, 1984). Slices were maintained in oxygenated medium (composition (mM): NaCl 124, KCl 3.3, KH_2PO_4 1.2, $NaHCO_3$ 26.4, $CaCl_2$ 2.5, $MgCl_2$ 2.4, and D-glucose 10) at 33–35°C, and were transferred to a small chamber for testing the various glutamate analogues. For the analysis of drug-induced focal potentials, slices were maintained at the surface of the bathing medium and compounds were applied from coarse micropipettes (Lanthorn & Cotman, 1983). The synaptic antagonist properties of analogues were evaluated with submerged and perfused (1–1.5 ml min^{-1}) slices, in this case compounds being applied in the bathing medium.

All recordings were made extracellularly by use of glass microelectrodes filled with 2 M NaCl. Lateral perforant path-evoked field potentials were elicited with a bipolar stimulating electrode and were recorded in the outer molecular layer of the infrapyramidal blade of the dentate gyrus in order to avoid contamination by the tempero-ammonic tract response. Stimuli (0.1 Hz) were subthreshold for eliciting a population spike. The identity of the lateral perforant path-evoked response was confirmed by the effects of paired pulse stimulation at 40 ms and 400 ms interpulse intervals; all responses exhibited facilitation at the 40 ms interval and slight facilitation or no change at the 400 ms interval (see Lanthorn, 1984). Contamination by the medial perforant path-evoked response was reduced by including 8 μM (−)-baclofen in the bathing medium (Lanthorn & Cotman, 1981).

Chemicals

L-[3,4-^{3}H]-glutamic acid of specific activity 45.8 Ci $mmol^{-1}$ was purchased from New England Nuclear (Boston, U.S.A.), and nonradiolabelled L-glutamic acid was from Merck (Darmstadt, FRG). All phosphorus-containing analogues of L-glutamate were generous gifts from Ciba-Geigy (Basel, Switzerland):

Table 1 The potencies of some phosphono and phosphino analogues of glutamate on Cl^-/Ca^{2+}-dependent L-glutamate binding and on lateral perforant path-evoked synaptic responses

Compound Number	*Structure*	*L-Glutamate binding* I_{max} (%)	K_i (μM)	*Synaptic response* IC_{50} (μM)
				
AP4	R = OH	61 ± 7	7.3 ± 1.4 (7)	2.7 (5)
1	= H	62 ± 6	3.1 ± 0.3 (7)*	> 100 (4)
2	= CH_3	60 ± 7	3.4 ± 0.3 (7)*	> 100 (4)
3	= CH_2COOH	68 ± 17	7.5 ± 1.2 (3)	19 (3)
4	= $CH_2CH_2CH(NH_2)COOH$	64 ± 7	38 ± 6 (4)*	NT
5	= $CH_2.C_6H_4Br$	59 ± 7	2.4 ± 0.5 (7)*	> 100 (4)
6	= $CH_2.C_6H_3Cl_2$	59 ± 9	3.0 ± 1.2 (4)	NT
				
7	$R_1 = H$, $R_2 = CH_3$	—	> 100 (3)	NT
8	$R_1 - R_2 = -(CH_2)_6-$	—	> 100 (3)	> 100 (5)
				
9	R = H	59 ± 8	4.0 ± 1.3 (3)	> 100 (3)
10	= OH	—	> 100 (3)	NT
				
11		—	> 100 (3)	> 100 (3)

The binding of L-[^{3}H]-glutamate to synaptic plasma membranes and electrophysiological analyses using the hippocampal slice preparation were performed as described in the text. K_i and I_{max} values were derived by Scatchard analyses of inhibition data (Figure 1b). The K_i values were calculated using the relationship: $K_i' = K_i(1 + F/K_d)$, where K_i' is the apparent K_i value determined from Scatchard plots (Figure 1b), F is the free molar concentration of L-[^{3}H]-glutamate and K_d its equilibrium dissociation constant. I_{max} is the maximum percentage inhibition of specific L-glutamate binding. Values are means ± s.e.mean. IC_{50} values were measured from composite log concentration-response curves of the synaptic response (Figure 2b). The number of independent experiments is shown in parentheses. NT, not tested. *$P < 0.02$ for significance of difference between test compound and AP4 (paired t test). AP4, 2-amino-4-phosphonobutanoate.

ω-phosphino and ω-phosphono derivatives were provided by Dr L. Maier, and the α-phosphino and α-phosphono compounds by Drs J. Dingwall and P. Diel, respectively (see Maier & Lea, 1983; Maier & Rist, 1983; Baylis *et al.*, 1985). All compounds were racemates. Structures are shown in Table 1.

Results

L-[^{3}H]-glutamate binding

Kinetics The specific binding of L-[^{3}H]-glutamate to SPMs exhibited saturation kinetics, and Scatchard

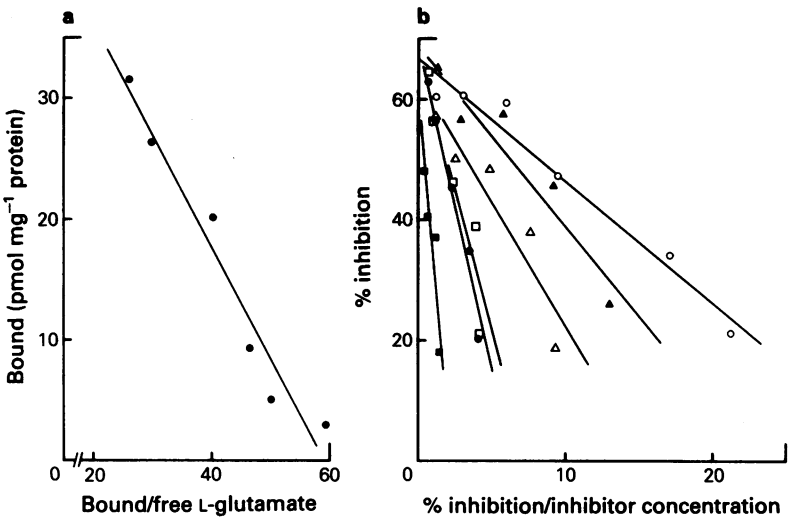


Figure 1 (a) Scatchard plot describing the specific binding of L-[^{3}H]-glutamate to synaptic plasma membranes isolated from the rat brain. Membranes were incubated with 25–1200 nM L-[^{3}H]-glutamate in the presence of 40 mM Cl^- and 1 mM Ca^{2+}, with 0.5 mM L-glutamate to define non-specific binding. Points are means of triplicate determinations, and were fitted by linear regression analysis. Three such experiments were conducted, and the mean K_d and B_{max} values derived are given in the text. (b) Scatchard plots of the inhibition of specific L-[^{3}H]-glutamate binding by 2-amino-4-phosphonobutanoate (AP4, ●), and by compounds 1 (▲), 2 (△), 3 (□), 4 (■) and 5 (○). Membranes were incubated with 50 nM L-[^{3}H]-glutamate in the presence of Cl^- and Ca^{2+}, and the percentage inhibition by 0.5–100 μM of each analogue was determined. Points are means of triplicate observations, and lines of best fit were determined by linear regression analysis. The number of experiments for each compound, and the mean K_i and I_{max} (maximum inhibition) values are shown in Table 1, as are the structures of all compounds.

analyses (Figure 1a) revealed an apparently homogeneous population of binding sites with K_d 804 ± 143 nM and B_{max} 53 ± 10 pmol mg^{-1} protein (means ± s.e.mean, $n = 3$). These values are in agreement with those previously reported for the binding of L-glutamate to freshly-prepared rat brain SPM preparations in the presence of $Cl^- \pm Ca^{2+}$ (Foster & Fagg, 1984).

Inhibition studies Twelve compounds, including AP4, were examined for their ability to inhibit the binding of L-glutamate to SPMs (see Table 1). All are glutamate analogues except compound 11, which is an aspartate analogue. Compounds 1–6 form a series of ω-phosphino derivatives with substituents ranging in size from H (the phosphonous acid, compound 1) to *p*-bromobenzyl and *m*, *p*-dichlorobenzyl (compounds 5 and 6). Compounds 7 and 8, in addition to an ω-methylphosphino group, are amino substituted. Compounds 9 and 10 represent two analogues in which the α-carboxylic acid moiety of glutamate is replaced, either with a phosphonous (compound 9) or a phosphonic acid group (compound 10).

Scatchard plots (Figure 1b) demonstrated that, over the concentration-range tested, compounds inhibited L-glutamate binding to a sub-population of sites which represented 59–68% of the L-glutamate specifically

Table 2 Inhibition of L-glutamate binding by phosphino analogues: lack of additivity with 2-amino-4-phosphonobutanoate (AP4)

Compound number	*Concentration* (μM)	*% inhibition* Alone	+ 200 μM AP4
AP4	200	66 ± 6	
1	50	66 ± 7	75 ± 4
2	50	68 ± 4	64 ± 8
3	50	58 ± 9	64 ± 8
4	125	54 ± 6	63 ± 9
5	50	61 ± 6	62 ± 9
6	50	62 ± 4	68 ± 5
9	50	64 ± 5	67 ± 8

Synaptic membranes were incubated with 50 nM L-[^{3}H]-glutamate and the percentage inhibition of specific binding by each compound alone and in combination with 200 μM AP4 was determined. Values are means ± s.e.mean of data from 3 independent experiments. $P > 0.05$ for significance of difference between inhibition by AP4 alone and that by AP4 plus any other compound (paired *t* test).

bound (Table 1). The K_i value determined for AP4 (7.3 μM, Table 1) is similar to that described by a number of authors studying the Cl^-/Ca^{2+}-dependent population of binding sites (Fagg *et al.*, 1982b; Monaghan *et al.*, 1983; Butcher *et al.*, 1983). The ω-phosphonous analogue (compound 1), together with the methyl (compound 2) and *p*-bromobenzyl (compound 5)-substituted derivatives, were significantly more potent than AP4, whereas the remaining ω-phosphino derivatives were of similar potency or slightly weaker (Table 1). Amino group substitutions in the ω-methylphosphino analogue abolished inhibitory activity (compounds 7 and 8), as did a reduction of carbon chain length (compound 11). Replacement of the α-carboxyl group of glutamate led to a compound of similar potency to AP4 in the case of the phosphonous analogue (compound 9), and one devoid of activity in the case of the phosphonic substitution (compound 10).

Experiments with AP4 and other analogues alone and in combination indicated that their effects were not additive. This was shown by using a high concentration of each analogue (higher than the K_i value, Table 1) either with or without sufficient AP4 (200 μM) to essentially saturate Cl^-/Ca^{2+}-dependent binding sites. In no case were the combined effects of AP4 plus another compound significantly greater than that observed with AP4 alone (Table 2). For compound 1, there was some suggestion of partial additivity with AP4 and, although this did not quite achieve statistical significance ($P < 0.1$), subsequent experiments (not shown) have demonstrated that this analogue is alone amongst those studied here to show inhibitory activity at L-glutamate binding sites in isolated postsynaptic densities (Fagg & Matus, 1984). However, the data demonstrate that the primary target of these compounds in SPM fractions is, like AP4 (Fagg *et al.*, 1982b; 1983b), the Cl^-/Ca^{2+}-dependent population of L-glutamate binding sites.

Electrophysiological analyses

Antagonism of synaptic responses Figure 2a illustrates chart recordings of the peak amplitudes of lateral perforant path-evoked field potentials and their antagonism by some AP4 analogues. AP4 itself was the most potent antagonist tested, with an IC_{50} (2.7 μM, see Figure 2b and Table 1) in good agreement with that reported in earlier studies (Koerner & Cotman, 1981; Fagg *et al.*, 1982a). Based on the effects of AP4 and using the criterion elaborated by Koerner *et al.* (1983), 70–100% of the evoked potential routinely could be ascribed to activation of lateral perforant path-granule cell synapses.

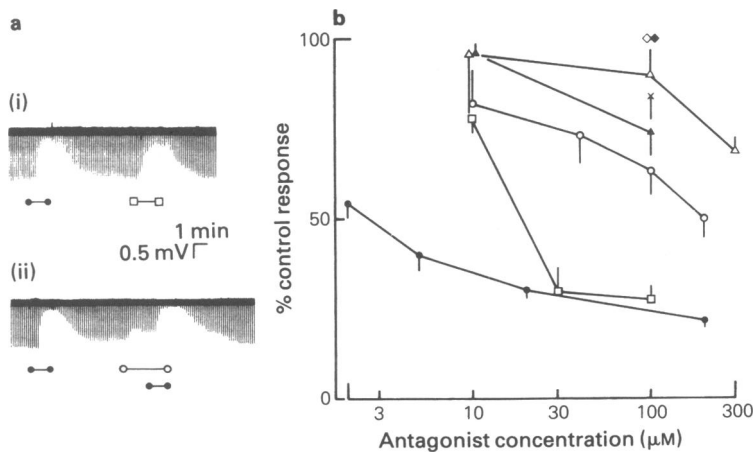


Figure 2 (a) Typical polygraph records showing peak amplitudes (downward deflections) of extracellularly recorded synaptic field potentials evoked by stimulation of the lateral perforant path. Hippocampal slices were maintained in oxygenated medium at 33–35°C, and compounds were applied in the bathing medium for the duration of the horizontal bars: (●—●) 20 μM 2-amino-4-phosphonobutanoate (AP4); (□—□) 30 μM compound 3; (○—○), 40 μM compound 5. (b) Log concentration-response curves showing the antagonism of lateral perforant path-evoked field potentials by AP4 (●), and by compounds 1 (▲), 2 (△), 3 (□), 5 (○), 8 (×), 9 (◆) and 11 (◇). The amplitude of the field potential in the presence of each compound is expressed as a percentage of the control response (measured in the absence of drug). Points are means of data from 3–5 separate experiments; s.e.means shown by vertical lines. IC_{50} values determined from these plots are recorded in Table 1, together with the structures of all compounds.

In contrast to their effects at Cl^-/Ca^{2+}-dependent L-glutamate binding sites, most analogues examined were weak antagonists of the synaptically-evoked field potentials (IC_{50} values $> 100\,\mu M$, Figure 2b and Table 1). With the exception of AP4, the only compound displaying high potency was the ω-carboxymethyl-phosphino analogue (compound 3), with an IC_{50} of $19\,\mu M$. The selectivity of this substance in the hippocampal slice was examined by testing it against Schaffer collateral-evoked field potentials in area CA1. Like AP4 (Koerner & Cotman, 1982), compound 3 had no effect on this response in concentrations as high as 0.3 mM (data not shown).

Focal potentials. In order to determine whether their synaptic inhibitory activity was due to 'classical' antagonism or to depolarization of the postsynaptic neurone, compounds were analyzed by the 'focal potential' technique (Lanthorn & Cotman, 1983). As a reference substance in this test, L-glutamate produced a negative d.c. potential shift of peak amplitude 5.1 mV (10 mM solution, Table 3). AP4 showed weak depolarizing activity (also see Evans *et al.*, 1982; Lanthorn & Cotman, 1983), while the ω-phosphonous analogue (compound 1) was a more powerful excitant than L-glutamate. The α-phosphonous derivative (compound 9) also exhibited weak excitatory properties, whereas the other compounds tested were essentially devoid of such activity. Thus, there appears to be no relationship between the depolarizing effects of these substances and their ability to antagonize lateral perforant path-evoked synaptic responses in low micromolar concentrations.

Analogue interactions The data presented above indicate that there is no correlation between (1) the inhibitory potencies of these analogues at AP4-sensitive L-glutamate binding sites and (2) their antagonist activity at an AP4-sensitive synapse (see Table 1). One explanation of this finding is that AP4-sensitive binding sites are not the receptors through which AP4 antagonizes synaptic responses. However, the mechanism by which AP4 suppresses excitatory synaptic transmission is not known with certainty (Davies & Watkins, 1982; Harris & Cotman, 1983; Butcher *et al.*, 1983, Foster & Fagg, 1984). Hence, an alternative possibility is that AP4 functions not as a receptor antagonist, but as an agonist to reduce the synaptic excitatory response (e.g. by regulating Cl^- channels, Slaughter & Miller, 1981). The low activity of AP4 analogues in our physiological experiments and their high potency in the binding assay might then be explained if they act primarily as antagonists at this 'AP4 receptor'.

This hypothesis was tested by determining whether compound 5 was able to reduce or abolish the depressant effect of AP4 on lateral perforant path-evoked field potentials. Compound 5 was chosen because (1) it was the most potent inhibitor of Cl^-/Ca^{2+}-dependent L-glutamate binding, with an affinity 3 fold greater than AP4 itself, (2) it exhibited no depolarizing properties and (3) it showed only weak effects on the synaptic response (see Tables 1 and 3). However, perfusion of hippocampal slices with $40\,\mu M$ of this analogue (16 times its K_i value at L-glutamate binding sites) failed to modify the antagonist effect of AP4 (Figure 2a). Similar results were obtained by use of AP4 in conjunction with $200\,\mu M$ DL-2-amino-6-phosphonohexanoate, which is also a potent inhibitor at AP4-sensitive binding sites (K_i 3–15 μM, Fagg *et al.*, 1983a; Monaghan *et al.*, 1983; Butcher *et al.*, 1983) and is devoid of activity on lateral perforant path-evoked field potentials (data not shown). Hence, it seems likely that, with the exception of compound 3, the ω-phosphino analogues studied here do not interact to any significant extent with the hippocampal 'receptor' through which AP4 exerts its potent neuropharmacological effects.

Table 3 Focal potentials elicited by phosphono and phosphino analogues of glutamate

Compound number	*Focal potential* (mV)
L-Glutamate	5.1 ± 0.8 (6)
AP4	0.3 ± 0.04 (6)
1	8.5 ± 0.8 (4)
2	0 (3)
3	0.2 ± 0.02 (4)
5	0 0 (3)
9	1.2 ± 0.2 (5)

Hippocampal slices were maintained at the surface of oxygenated bathing medium. Compounds (10 mM solutions) were applied by pressure ejection from coarse micropipettes and the maximum shift in d.c. potential (negative) was recorded by use of extracellular microelectrodes. Values are means ± s.e.mean of data from the number of experiments in parentheses. AP4, 2-amino-4-phosphonobutanoate.

Discussion

The present data demonstrate that the AP4-sensitive, Cl^-/Ca^{2+}-dependent population of L-glutamate binding sites in rat brain SPMs are not the receptors through which AP4 exerts its neuropharmacological actions. Consideration of the phosphorus-containing analogues tested here reveals no correlation between their inhibitory activity at Cl^-/Ca^{2+}-dependent binding sites and their antagonist activity at an identified L-AP4-sensitive synapse. Moreover, ω-phosphino

analogues which were active in the binding assay did not reverse the synaptic antagonist effect of AP4, indicating that these compounds acted neither as agonists nor as antagonists at AP4 receptive sites. Similar observations, but with a different series of glutamate analogues, recently have been made in comparative studies of [^{3}H]-AP4 binding in SPMs and perforant path-evoked field potentials in the rat hippocampal slice preparation (Freund *et al.*, 1984; Robinson *et al.*, 1985).

If Cl^-/Ca^{2+}-dependent, AP4-sensitive L-glutamate binding sites are not AP4-sensitive synaptic receptors, what is their function? A recent report suggests that the 'binding' observed in SPM fractions may in fact be a Cl^--driven uptake of L-glutamate into resealed plasma membrane vesicles (Pin *et al.*, 1984), and several lines of evidence support this hypothesis. (1) A number of membrane transport systems (Kanner, 1978; Kuhar & Zarbin, 1978; Ross, 1980; Nelson & Rudnick, 1982), including those for glutamate and aspartate in neural tissue (Kuhar & Zarbin, 1978; Marvizon *et al.*, 1981; Waniewski & Martin, 1984), exhibit a Cl^--requirement. Moreover, the anion specificity of these systems is similar to that for AP4-sensitive L-glutamate binding (Mena *et al.*, 1982; Fagg *et al.*, 1983a; Butcher *et al.*, 1984), and corresponds generally to those anions known to permeate the plasma membrane. (2) The high density of AP4-sensitive L-glutamate binding sites in SPMs (see Foster & Fagg, 1984; also Monaghan *et al.*, 1983; Butcher *et al.*, 1983; Robinson *et al.*, 1985; this study), and their temperature and freezing sensitivity (Foster & Roberts, 1978; Fagg *et al.*, 1983b; Monaghan *et al.*, 1983; Butcher *et al.*, 1983), are characteristics generally associated with membrane transport processes. The number of binding sites, in particular, are greatly in excess of those determined for other neurotransmitter receptors in SPMs, including receptors expected to be present in abundance, such as those for γ-aminobutyrate (see Olsen, 1982). Further evaluation of the hypothesis that Cl^-/Ca^{2+}-dependent L-glutamate 'binding' does indeed correspond to vesicular uptake will require confirmation (1) that membrane vesicles are present in SPM preparations (e.g. using extracellular space markers) and (2) that the observed accumulation of radiolabelled L-glutamate is dependent upon a transmembrane Cl^- gradient.

L-AP4 is one of the most potent substances currently known to antagonize transmission at a sub-population of excitatory amino acid synapses in the brain. As such, its mechanism of action and structure-activity relationships are of obvious interest. Structurally, both increases and decreases in carbon chain length have been shown to result in a reduction of antagonist potency (Koerner & Cotman, 1981), as have N-alkylation and substitution in the carbon chain (Fagg *et al.*, 1982a; Freund *et al.*, 1984; Crooks *et al.*, 1985). Our previous work indicated that a bifunctional ω-terminal (e.g. phosphonate) is necessary for antagonist activity at AP4-sensitive synapses (Fagg *et al.*, 1982a), and the present findings provide additional support for this hypothesis. Thus, the only compounds showing high antagonist potency at lateral perforant path-granule cell synapses were AP4 itself and the ω-carboxymethylphosphino analogue (compound 3). Both possess an ω-terminal with two anionic groups in close proximity. All other ω-phosphino analogues were of low potency and were essentially devoid of AP4-like antagonist activity (also see Freund *et al.*, 1984). The synthesis of AP4 analogues with modified, but dianionic ω-terminals may represent a strategy for affinity labelling AP4 recognition sites in brain tissue and ultimately for determining their physiological function.

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