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[^{3}H]-L-2-amino-4-phosphonobutyrate labels a metabotropic glutamate receptor, mGluR4a

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1 The ligand binding site of subtype mGluR4a of the metabotropic glutamate receptor family was characterized by using [^{3}H]-L-2-amino-4-phosphonobutyrate ([^{3}H]-L-AP4) binding.

2 Specific [^{3}H]-L-AP4 binding to membranes prepared from baby hamster kidney (BHK) cells transfected with a vector encoding mGluR4a accounted for 60–70% of the total binding whereas no specific binding of [^{3}H]-L-AP4 was observed to membranes prepared from BHK cells expressing the vector only.

3 Specific binding of [^{3}H]-L-AP4 to mGluR4a was detectable at 0°C, was saturated within 10 min and enhanced by Cl^--ions but not by divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}).

4 [^{3}H]-L-AP4 binding showed a maximal binding density (B_{max}) of 3.0±0.5 pmol mg^{-1} protein and an affinity (K_D) of 441 nM. A modest decrease in affinity was observed in the presence of 0.1 mM guanosine-5′-O-(3-thio)trisphosphate-γ-S, the K_D being 761 nM and the B_{max} 3.4±0.6 pmol mg^{-1} protein.

5 The following rank order of affinity for mGluR4a was observed: L-AP4 = L-serine-O-phosphate > glutamate = (2S,1S,2S)-2-(carboxycyclopropyl)-glycine > 1-amino-3-(phosphonomethylene)cyclobitanecarboxylate > > (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate = quisqualate > ibotenate.

6 A highly significant correlation was observed between the potencies of the compounds to inhibit forskolin-stimulated cyclic AMP-formation in BHK cells expressing mGluR4a and the affinity for displacement of [^{3}H]-L-AP4 binding from mGluR4a suggesting that this binding site is functionally relevant.

7 In conclusion, [^{3}H]-L-AP4 is a suitable radioligand for characterizing mGluR4a when expressed in BHK cells. Interestingly, a significant correlation was found between the ability of various compounds to displace [^{3}H]-L-AP4 binding from mGluR4a and the previously observed potencies for inhibition of synaptic transmission via L-AP4 sensitive glutamatergic pathways. These data support the hypothesis that the L-AP4 receptor is contained within the mGluR family.

Keywords: Metabotropic glutamate receptor binding; [^{3}H]-L-2-amino-4-phosphonobutyrate; L-2-amino-4-phosphonobutyrate receptor; cyclic AMP; (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate; guanine nucleotide shift

Introduction

Receptors for the excitatory amino acid, glutamate have been classified into three major groups consisting of ionotropic glutamate receptors, metabotropic glutamate receptors and L-2-amino-4-phosphonobutyrate (L-AP4) receptors (Monaghan *et al.*, 1989). Ionotropic glutamate receptors, which form ligand-gated ion channels, have been further subclassified according to their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (Monaghan *et al.*, 1989). Metabotropic glutamate receptors (mGluR) are selectively activated by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate ((1S,3R)-ACPD) and are linked to their effectors via guanosine triphosphate (GTP)-binding proteins. Molecular cloning of the metabotropic glutamate receptors has revealed the existence of at least eight subtypes termed mGluR1 through mGluR8, and of these subtypes, mGluR4a, mGluR6, mGluR7 and mGluR8 are preferentially activated by L-AP4 and L-serine-O-phosphate (L-SOP) (Suzdak *et al.*, 1994; Duvoisin *et al.*, 1995). The diversity within the mGluR family is further increased by the existence of alternative spliced variants of mGluR1, mGluR4 and mGluR5 with regard to the C-terminal domains (Suzdak *et al.*, 1994). In the case of mGluR4, a stop codon is deleted in mGluR4a leading to a protein termed mGluR4b with a longer and different C-terminal domain as compared to mGluR4a (Simoncini *et al.*, 1993).

The existence of L-AP4 receptors has been proposed in order to explain the depressant actions of L-AP4 on various neuronal glutamatergic pathways (Koerner & Cotman, 1981; Forsythe & Clements, 1990; Rainnie & Shinnick-Gallagher, 1992; Kahle & Cotman, 1993). Based on their agonist selectivities and the regional distribution of rat brain mRNA for mGluR4a (Thomsen *et al.*, 1992; Kristensen *et al.*, 1993; Tanabe *et al.*, 1993), mGluR6 (Nakajima *et al.*, 1993) and mGluR7 (Okamoto *et al.*, 1994; Saugstad *et al.*, 1994), these subtypes have been proposed as candidates for the L-AP4 receptor (Thomsen *et al.*, 1992; Tanabe *et al.*, 1993; Okamoto *et al.*, 1994).

However, when the L-AP4 receptor is labelled with [^{3}H]-AP4, the ligand selectivity of [^{3}H]-AP4 binding to membrane preparations from rat brain does not correspond to the electrophysiologically defined L-AP4 receptor (Fagg & Lanthorn, 1985; Robinson *et al.*, 1985; Butcher *et al.*, 1987) or to mGluR4a-types of mGluRs (Kristensen *et al.*, 1993; Tanabe *et al.*, 1993). Indeed, [^{3}H]-AP4 'binding' is currently believed to represent Ca^{2+}/Cl^--dependent sequestration of glutamate into synaptic vesicles (Monaghan *et al.*, 1983; Pin *et al.*, 1984; Butcher *et al.*, 1987) and may not reflect binding to mGluR4a-types of mGluRs (Thomsen *et al.*, 1994a).

In the present study, it was shown that [^{3}H]-L-AP4 binding to mGluR4a is different from that of [^{3}H]-AP4 binding to brain membranes in terms of its kinetics, temperature requirements and ligand selectivity. On the other hand, significant correlations were found between the ability of several analogues of L-AP4 and glutamate to inhibit [^{3}H]-L-AP4 binding to mGluR4a and the potency of these compounds for inducing depression of synaptic transmission via the lateral or medial perforant

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pathways (for a review see: Koerner & Johnson, 1992). This observation further suggests that the receptor which mediates the presynaptic depressant effects of L-AP4 on synaptic transmission is contained within the mGluR family.

Methods

[^{3}H]-L-AP4 binding experiments

BHK cells stably expressing mGluR4a or vector only (control cells) were cultured in Dulbecco's modified Eagles media supplemented with 5% dialysed foetal bovine serum, 2 mM glutamine, 0.05 mg ml^{-1} gentamycin, 0.1 mg ml^{-1} neomycin and 10 μM methotrexate in a humidified atmosphere (95% air, 5% CO_2) at 37°C. Confluent BHK cells were harvested with phosphate buffered saline/EDTA (Mg^{2+} and Ca^{2+}-free, pH 7.4) and centrifuged (2000 r.p.m., 10 min, 25°C). The cells were suspended in 20 ml lysis buffer at 0°C (the composition was: HEPES-Na 30 mM, pH 7.4, EDTA 1 mM, $MgCl_2$ 5 mM, phenylmethylsulphonyl fluoride (PMSF) 0.1 mM) and sonicated for 20 s (VibraCell probe sonicator, setting 80 at 20 kHz, Sonics and Materials, U.S.A.) followed by centrifugation (40000 *g*, 20 min, 0°C). The pellets were resuspended by use of an UltraTurrax homogenizer (Janke & Kunkel, Germany) for 5 s in 15 ml lysis buffer supplemented with 0.08% Triton X-100 and incubated at 37°C for 10 min. The tissue was placed on ice, an additional 25 ml of lysis buffer was added and the membranes were centrifuged (40000 *g*, 20 min, 0°C). The pellets were resuspended in assay buffer at 0°C and the membranes were centrifuged (40000 *g*, 20 min 0°C). Unless otherwise indicated, the buffer composition was HEPES-Na 30 mM, pH 8.0, NaCl 110 mM, $MgCl_2$ 1.2 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, PMSF 0.1 mM. The resulting pellets were resuspended in assay buffer (0.5–0.9 mg protein/assay) and added to test tubes containing [^{3}H]-L-AP4, buffer and test compounds in a volume of 0.5 ml. For displacement studies, the final concentration of [^{3}H]-L-AP4 was 30 nM. After 30 min incubation at 0°C (unless otherwise indicated), the samples were centrifuged (40000 *g*, 3 min, 0°C) and the pellets were quickly rinsed with 2 x 2 ml ice cold wash buffer (the buffer composition was: HEPES-Na 30 mM, pH 8.0, NaCl 110 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.2 mM). The pellets were solubilized in 0.5 ml NaOH (2 M) and transferred to scintillation vials with 0.5 ml water and membrane-bound radioactivity was quantified by scintillation counting. Non-specific binding was defined as the binding in the presence of 0.1 mM L-SOP.

Measurements of cyclic AMP-formation

Measurements of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-formation in BHK cells expressing mGluR4a were performed as previously described (Thomsen *et al.*, 1992).

Materials

[^{3}H]-L-2-amino-4-phosphonobutyrate ([^{3}H]-L-AP4) was purchased from Tocris Cookson (Bristol, U.K.) and the specific activity was 50 Ci mmol^{-1}. The compounds used in this study were obtained from Tocris Cookson, Research Biochemicals Int. (Natick, U.S.A.) or Sigma (St. Louis, U.S.A.).

Data analysis

IC_{50} values were calculated from competition binding experiments by a non-linear regression analysis by use of the GraphPad Prism program (GraphPad Software, U.S.A.). Saturation binding experiments were analysed by an iterative curve fitting program (EBDA-program from G.A. McPherson, Elsevier-Biosoft, U.K.) to calculate K_D, B_{max} and Hill coefficients. Statistical analysis was performed were appropriate by the InStat program (GraphPad Software, U.S.A.).

Results

Discrimination of specific [^{3}H]-L-AP4 binding to mGluR4a from binding to BHK cell membranes

Sequestering of glutamate into membrane vesicles by Na^+- and Ca^{2+}/Cl^--dependent transport mechanisms is not restricted to cells of neuronal origins, but is also evident in cell lines derived from peripheral organs (Thomsen *et al.*, 1994c).

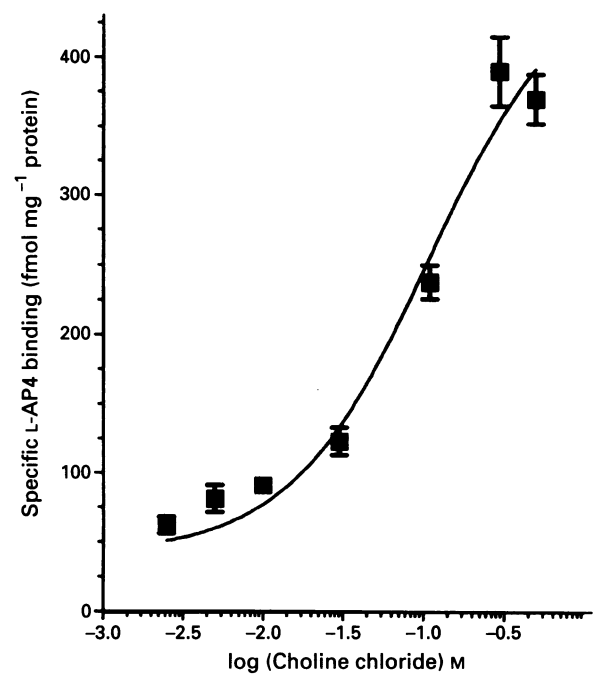

Figure 1 The effects of Cl^--ions (in the form of choline chloride) on specific [^{3}H]-L-AP4 binding to membranes prepared from BHK cells expressing mGluR4a. For comparison the fit of a sigmoidal curve is shown with a Hill number set to unity. The EC_{50} for enhancement of [^{3}H]-L-AP4 binding by choline chloride was 0.1 M. The experiments were conducted as described in Methods and the results are mean ± s.e.mean of 3 experiments, which were performed in triplicate. Assay buffer (30 mM HEPES-KPH, pH 8.0 + 0.1 mM PMSF) was supplemented with choline chloride in the concentrations indicated without correcting for osmolarity. Non-specific binding was defined as the binding in the presence of 0.1 mM L-SOP.

Table 1 Ionic requirements of [^{3}H]-L-2-amino-4-phosphonobutyrate ([^{3}H]-L-AP4) binding

	mM	*Specific [^{3}H]-L-AP4 binding* (fmol mg^{-1} protein)
No ions added	–	41 ± 5
NaCl	110	214 ± 12
NH_4Cl	2.5	66 ± 5
$CaCl_2$	2.5	93 ± 6
$MgCl_2$	1.2	66 ± 13
$MnCl_2$	2.5	83 ± 10
KCl	5	55 ± 2
NaCl +	110	243 ± 10
$CaCl_2$ +	2.5	
$MgCl_2$ +	1.2	
KCl	5	

The experiments were performed as described in Methods, except that an assay buffer composed of 30 mM HEPES-KOH, pH 8.0 + 0.1 mM PMSF was used with the ions added as indicated. Non-specific binding was defined as the binding in the presence of 0.1 mM L-serine-O-phosphate.

Thus, parallel experiments were performed with control BHK cells expressing vector only and BHK cells expressing mGluR4a. By using sonication and Triton-X-100-treatment of the membranes, 60–70% specific [^{3}H]-L-AP4 binding was observed to membranes from mGluR4a-expressing cells (331 ± 36 and 114 ± 12 fmol mg^{-1} protein in the presence and absence of 0.1 mM L-SOP, respectively), but not to membranes from control BHK cells (73 ± 8 and 61 ± 6 fmol mg^{-1} protein, respectively). With these assay conditions, the specific binding of [^{3}H]-L-AP4 to membranes from mGluR4a-expressing cells was linear with the amounts of protein added in the range of 0.2 to 3 mg protein/assay (data not shown).

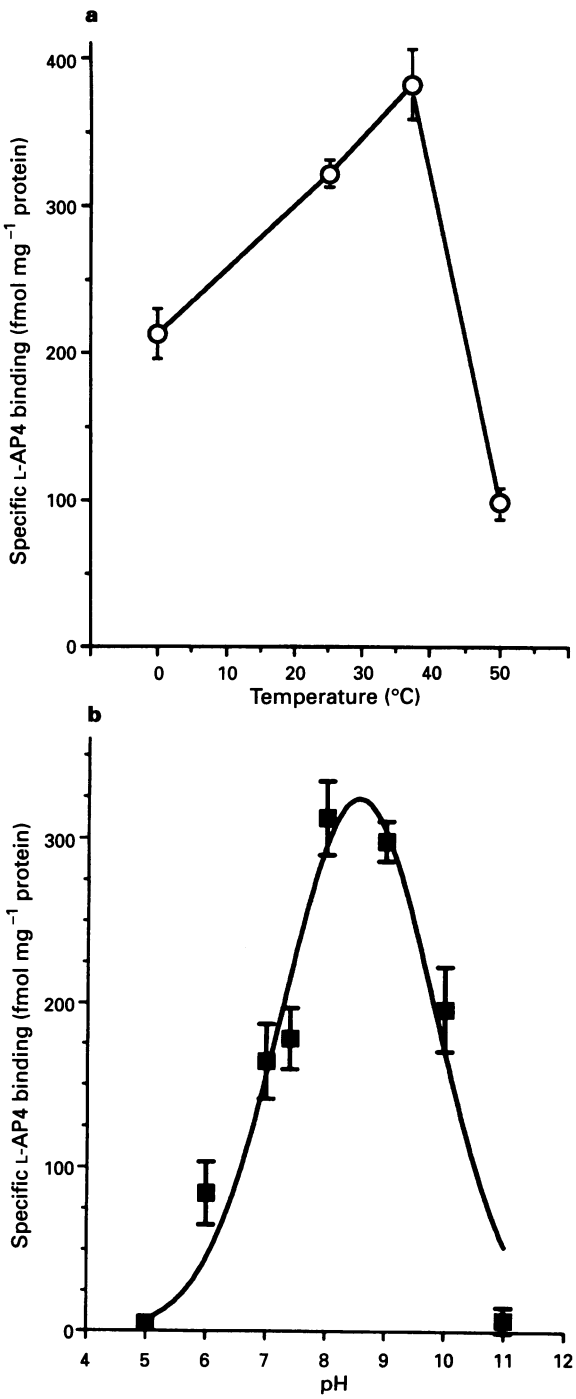

Figure 2 Sensitivity of [^{3}H]-L-AP4 binding to (a) incubation temperature and (b) pH of the incubation buffer. When the temperature was varied, the pH was held constant (8.0), whereas the temperature was 0°C in pH-sensitive experiments. Specific binding (mean ± s.e.mean of 3 triplicate experiments) accounted for 60–67% of total binding in these experiments. Non-specific binding was defined as the binding in the presence of 0.1 mM L-SOP, and the amount of non-specific binding (fmol mg^{-1} protein) was unaffected by varying the pH or temperature.

Ionic requirements for [^{3}H]-L-AP4 binding to mGluR4a

Chloride ions, added as choline chloride, increased specific binding of [^{3}H]-L-AP4 to mGluR4a by 8–10 fold (Figure 1). The dose-response curves for Cl^--induced enhancement of [^{3}H]-L-AP4 binding to mGluR4a followed a sigmoidal curve with a Hill number close to unity (Figure 1), suggesting that one Cl^--ion interacts with the binding site for L-AP4. Similar amounts of binding were obtained when Cl^--ions were added in the form of NaCl suggesting that [^{3}H]-L-AP4 binding is not affected by Na^+ (Table 1). Divalent cations in the form of chloride salts ($CaCl_2$, $MnCl_2$, $MgCl_2$) did not increase binding above the levels which were observed by the addition of equimolar amounts of choline chloride (Table 1). No specific binding of [^{3}H]-L-AP4 to control BHK cell membranes was

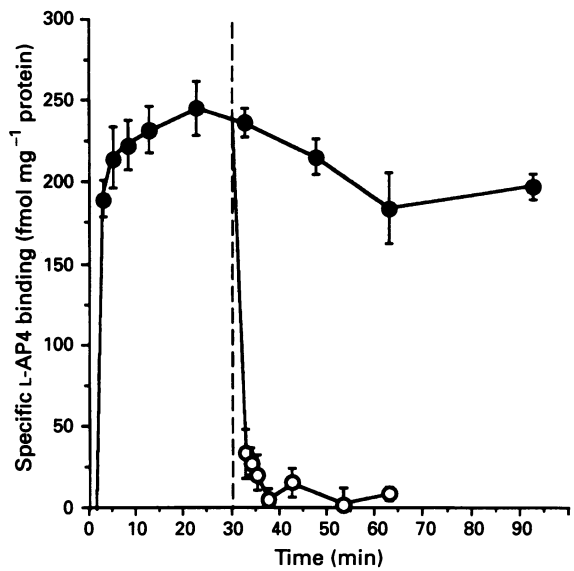

Figure 3 Time-courses for association (●) and dissociation (○) of [^{3}H]-L-AP4 binding to mGluR4a at 0°C. Association was initiated by adding [^{3}H]-L-AP4 to membranes and the samples were centrifuged for 3 min following 0; 2; 5; 10; 20; 30; 45; 60 and 90 min of incubation. Non-specific binding was determined by adding 0.1 mM L-SOP to one set of samples. In dissociation experiments, samples were incubated for 30 min at 0°C and buffer or L-SOP (0.1 mM) was added for the time indicated with immediate centrifugation corresponding to 3 min of incubation. Results are shown as specific [^{3}H]-L-AP4 binding and are mean ± s.e.mean of 2–3 experiments, which were performed in triplicate. Specific binding accounted for 65–70% of total binding in these experiments.

Table 2 Binding kinetics of [^{3}H]-L-AP4: effects of GTP-γ-S

	K_D (nM)	B_{max} (pmol mg^{-1} protein)	*Hill coefficient*
Control	441 ± 66	3.0 ± 0.5	0.90 ± 0.03
GTP-γ-S	761 ± 125*	3.4 ± 0.6	0.98 ± 0.04

* A significant increase in the K_D in presence as compared to the absence of GTP-γ-S ($P < 0.05$, by a non-parametric test, paired Wilcoxon test, $n = 5$, one-tailed). The results are expressed as mean ± s.e.mean. For further information see legend to Figure 4.

observed in the presence of these ions (data not shown). However, an insoluble complex was apparently formed with [^{3}H]-L-AP4 and Zn^{2+}-ions (0.5–5 mM) since high levels of radioactivity were observed also in the absence of membranes (data not shown). This precluded examination of the effects of Zn^{2+}-ions on [^{3}H]-L-AP4 binding to mGluR4a. The further characterization of [^{3}H]-L-AP4 binding was performed with a buffer composition which resembled that of the buffer used for measurements of cyclic AMP-formation in transfected cell lines (see Methods).

Optimal temperature and pH for [^{3}H]-L-AP4 binding to mGluR4a

Increasing the temperature from 0°C to 37°C resulted in an enhancement of specific [^{3}H]-L-AP4-binding, whereas little specific binding was observed at a denaturating temperature (50°C) (Figure 2a). Although a somewhat higher degree of binding was observed at 37°C as compared to 0°C, experiments were performed at 0°C due to the rapid binding kinetics (see below) and in order to eliminate potential uptake of [^{3}H]-L-AP4 into resealed membrane vesicles. Specific binding of [^{3}H]-L-AP4 to mGluR4a membranes showed a pH optimum between pH 8 and pH 9 (Figure 2b) while non-specific binding was insensitive to pH within the range examined.

Time-course of [^{3}H]-L-AP4 binding to mGluR4a

Association of [^{3}H]-L-AP4 to mGluR4a was rapid and near maximal specific binding was reached within the 3 min period that was necessary for separating bound from free radioligand by centrifugation (Figure 3). Also the rapid binding kinetics of [^{3}H]-L-AP4 did not allow for accurate determinations of the off rate of radioligand from mGluR4a since very little specific binding was observed when 0.1 mM L-SOP was added immediately before centrifugation (Figure 3).

Saturation binding experiments with [^{3}H]-L-AP4

Binding of [^{3}H]-L-AP4 to membranes from mGluR4a expressing cells was saturable (Figure 4) and the binding parameters were estimated by Scatchard analysis and shown in Table 2. The Hill numbers were slightly lower than unity indicating some degree of heterogeneity in receptor affinity. In the presence of a non-hydrolysable analogue of guanosine trisphosphate, guanosine-5'-O-(3-thio)trisphosphate-γ-S (GTP-γ-S) a decrease in the affinity was observed as shown in Figure 4, and in these experiments the Hill number was close to one (Table 2).

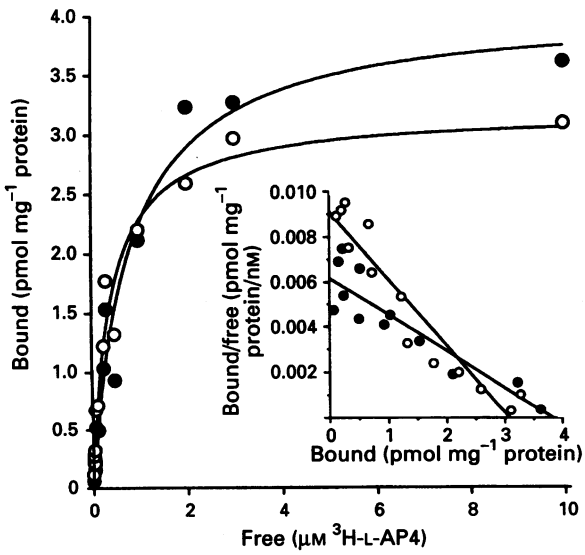

Figure 4 Saturation binding experiments with [^{3}H]-L-AP4 to membranes prepared from mGluR4a cells in the absence (○) or presence (●) of 0.1 mM GTP-γ-S. Insert show Scatchard transformations of the saturation curves. [^{3}H]-L-AP4 (30 nM) was diluted with non-labelled L-AP4 at 12 concentrations ranging from 0.01 μM to 10 μM, and the experiments ($n = 5-8$) were performed in triplicate as described in Methods. Non-specific binding was defined as the binding in the presence of 0.1 mM L-SOP.

Ligand selectivity of mGluR4a

The pharmacological specificity of the mGluR4a was examined by measuring the ability of several compounds to

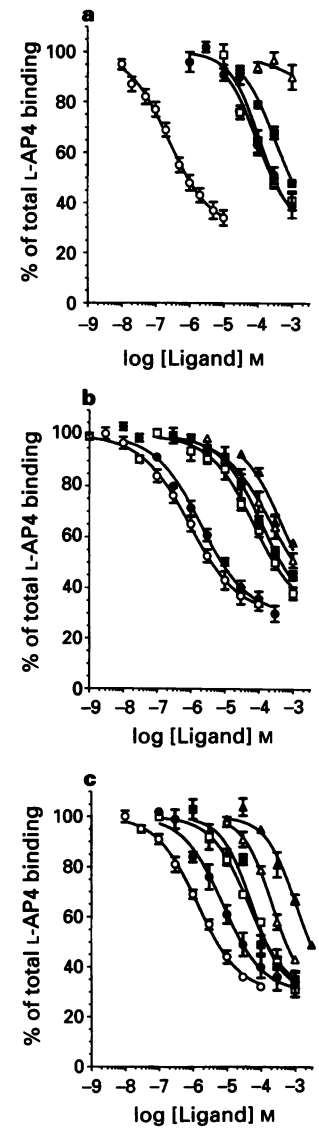

Figure 5 Displacement of [^{3}H]-L-AP4 binding by various analogues of glutamate and L-AP4. (a) L-AP3 (□), L-AP4 (○), L-AP5 (●), DL-AP6 (■) and L-AP7 (△); (b) L-SOP (○), L-glutamate (●), (1S,3R)-ACPD (□), quisqualate (■), ibotenate (△) and L-α-aminoadipate (▲); (c) L-CCG-I (○), cyclobutylene-AP5 (●), (±)-trans-1-amino-3-phosphonocylcopentanecarboxylate (□), (±)-*cis*-1-amino-3-phosphonocyclopentanecarboxylate (■), (±)-1-amino-*cis*-1,3-cyclohexanedicarboxylate (△) and (±)-1-amino-*trans*-1,3-cyclohexane dicarboxylate (▲). The displacement curves were fitted to sigmoidal dose-response curves by use of the GraphPad Prism program (GraphPad Software, U.S.A.), and IC_{50}s and Hill coefficients were determined. The data are expressed as % of total [^{3}H]-L-AP4 binding and are mean ± s.e.mean of 3–5 experiments, which were performed in triplicate.

displace [^{3}H]-L-AP4 binding from mGluR4a. Displacement curves for various glutamate analogues are shown in Figure 5, and the compounds are listed according to their affinities in Table 3. L-AP4 and the close structural analogue, L-SOP showed the highest affinities fore mGluR4a while shorter (L-2-amino-3-phosphonopropionic acid, L-AP3) and longer (L-2-amino-5-phosphonopentanoic acid (L-AP5), DL-2-amino-6-phosphonohexanoic acid (DL-AP6) and L-2-amino-7-phosphonoheptanoic acid (L-AP7)) phosphonic acid analogues displayed lower affinities as compared to L-AP4 (Figure 5a). Similarly, L-α-aminoadipate was 280 fold less potent than L-glutamate (Figure 5b). Quisqualate, ibotenate and the selective mGluR agonist, (1S,3**R**)-ACPD showed low affinities for mGluR4a (Figure 5b) in agreement with their weak functional activities at mGluR4a (Kristensen *et al.*, 1993; Tanabe *et al.*, 1993). Since (1**R**,3S)-ACPD was completely inactive at mGluR4a (Table 3) the affinity of (±)-trans-ACPD is presumably half of that of (1S,3**R**)-ACPD (74 μM). Thus, replacing the distal carboxylic group with a phosphonic acid group (eg. (±)-trans-1-amino-3-phosphonocyclopentanecarboxylate) slightly increased the affinity at mGluR4a (Figure 5c). With a cyclohexyl ring in place of the cyclopentyl ring of (1S,3**R**)-ACPD (e.g. 1-amino-3-cyclohexanedicarboxylate), a marked decrease in affinity for mGluR4a was observed (Figure 5c), and this was accompanied by a shift in the preference for the *trans*-isomers over the *cis*-isomers (Table 3). A conformational restricted analogue of an extended form of L-AP4, (±)-1-amino-3-(phosphonomethylene)cyclobutane-1-carboxylate (cyclobutylene-AP5) showed a relatively high affinity for mGluR4a (see Figure 5c and Table 3). Similarly, an analogue of the extended form of glutamate, (2S,1S,2S)-2-(carboxycyclopropyl)-glycine (L-CCG-I) displaced [^{3}H]-L-AP4 binding from mGluR4a with

Table 3 Affinities of excitatory amino acids for mGluR4a

	[^{3}H]-L-AP4 binding to mGluR4a			*Inh. of cyclic AMP*	
Compound	*IC_{50}* (μM) (% inhibition)		*Hill no.*	*IC_{50}* (μM) (% of max)	
L-SOP	0.67±0.11	(=100%)	0.75±0.05	4.4±1.4[b]	(=100%)
L-CCG-I	1.4±0.1	(99±2%)	0.74±0.06	3.0±0.7	(98±4%)
L-Glutamate	1.6±0.3	(104±3%)	0.70±0.05	3.2±0.6[a]	(102%)
Cyclobutylene-AP5	6.3±1.5	(97±4%)	0.82±0.08	4±1	(98±2%)
D-AP4	10.1±1.9	(94±3%)	0.84±0.14	73±15[a]	(100%)
L-Serine-O-sulphate	46±6	(91±4%)	0.80±0.05	324±47[d]	(88±6%)
(±)-*trans*-1-Amino-3-phosphono cyclopentanecarboxylate	47±3	(96±1%)	0.77±0.03	31±6	(91±3%)
L-Homocysteate	56±2	(93±7%)	0.83±0.12	432±43[a]	(100%)
(±)-*cis*-1-Amino-3-phosphono cyclopentanecarboxylate	60±11	(89±39%)	0.83±0.13	95±18	(81±3%)
(1S,3**R**)-ACPD	74±15	(98±3%)	0.74±0.08	39±8[a]	(80±12)
L-AP5	105±5	(88±3%)	1.1±0.1	101±6	(99±2%)
Quisqualate	112±29	(87±5%)	0.82±0.05	129±21[a]	(90±8%)
L-AP3	117±22	(89±7%)	0.86±0.09	>1000[a]	(42±5%)
(±)-1-Amino-*cis*-1,3-cyclohexane dicarboxylate	236±21	(80±2%)	1.00±0.01	1145±174	(55±4%)
Ibotenate	285±30	(95±6%)	0.70±0.05	590±62[a]	(88%)
(±)-*cis*-1-Amino-3-phospono cyclohexanecarboxylate	313±38	(64±8%)	0.70±0.05	>1000	(29±7%)
DL-AP6	341±35	(74±4%)	0.85±0.06	>1000[a]	(48±6%)
(1S,3**R**)-ACPD	345±18	(82±8%)	1.00±0.03	567±96[d]	(74±5%)
L-α-Aminoadipate	462±42	(60±4%)	0.73±0.02	>1000[d]	(43±5%)
L-(*trans*)-Pyrrolidine-2,4-dicarboxylate	524±59	(63±5%)	1.05±0.04	>1000[d]	(10±4%)
(**R**)-3-Hydroxyphenylglycine	623±75	(58±10%)	0.92±0.04	>1000[c]	(36±6%)
(±)-*cis*-2,4-Piperidine dicarboxylate	639±80	(65±8%)	1.09±0.05	>1000	(19±5%)
(±)-*trans*-2,4-Piperidine dicarboxylate	818±92	(63±9%)	0.96±0.07	>1000	(16±4%)
(S)-4-carboxyphenylglycine	838±24	(58±7%)	0.91±0.11	>1000[c]	(21±10%)
(S)-3-carboxy-4-hydrophenylglycine	999±97	(52±4%)	1.14±0.06	>1000[c]	(47±2%)
(±)-1-Amino-trans-1,3-cyclohexane dicarboxylate	1012±28	(48±5%)	1.05±0.13	>1000	(23±4%)
(S)-4-carboxy-3-hydroxyphenylglycine	1380±120	(52±5%)	0.92±0.12	>1000[c]	(11±4%)
(±)-*trans*-1-Amino-3-phosphono cyclohexanecarboxylate	>1000	(33±6%)	–	>1000	(27±5%)

Footnotes in Table 3 and below indicate that the cyclic AMP data are from: [a]Kristensen *et al.*, 1993; [b]Thomsen & Suzdak, 1993; [c]Thomsen *et al.*, 1994b and [d]Thomsen *et al.*, 1994c. For displacement binding experiments with [^{3}H]-L-AP4 the affinity (IC_{50}) is listed. In parentheses, the % inhibition of the specific binding at 1 mM is shown. Non-specific binding is defined as in Figure 4. For measurements of cyclic AMP-formation in BHK cells expressing mGluR4 the agonist potencies (EC_{50}s) are shown. In parentheses, the % inhibition of forskolin-induced cyclic AMP-formation is listed relative to the response evoked by 0.1 mM L-SOP which was 53±3% inhibition of induced cyclic AMP-levels. For further information see legend to Figure 6. Compounds which weakly displaced [^{3}H]-L-AP4 binding included (% inhibition at 1 mM in parentheses): DL-aspartate-β-hydroxamate[d] (34±5%) and (±)-β-*p*-chlorophenylglutamate (31±6%). Inactive compounds, showing less than 20% inhibition of [^{3}H]-L-AP4 binding and of forskolin-stimulated cyclic AMP-formation in BHK cells expressing mGluR4a included (all at 1 mM): 4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid (SITS); γ-aminobutyric acid-pentin; (±)-1-amino-cis-1,3-cyclobutanedicarboxylate[d]; (±)-1-amino-trans-1,3-cyclobutanedicarboxylate[d]; (1**R**,3S)-ACPD[d]; (1**R**,3**R**)-ACPD[d]; AMPA[a]; D-AP5[a]; L-AP7[a]; D-AP7[a]; D-aspartate[d]; L-aspartate[a]; (**R**)-4-carboxy-3-hydroxyphenylglycine[c]; (**R**)-3-carboxy-4-hydroxyphenylglycine[c]; (**R**)-4-carboxyphenylglycine[c]; (**RS**)-3-carboxyphenylalanine; 3-((**RS**)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP); L-cysteine; L-cysteine sulphinate[d]; 1,4-dicarboxymethyl-3-hydroxypiperadine; dihydrokainate[a]; D-glutamate; γ-D-glutamylamino-ethylphosphonate; γ-D-glutamylaminomethylphosphonate[a]; DL-homocysteine; (S)-3-hydroxyphenylglycine[c]; L-(+)-*p*-hydroxyphenylglycine; kainate[a]; kynurenic acid; β-N-methyl-amino-L-alanine[a]; (**RS**)-α-methyl-4-carboxyphenylglycine[c]; NMDA[a] (±)-*cis*-2,5-piperidine dicarboxylate; (±)-trans-2,5-piperidine dicarboxylate; (±)-trans-2,3-piperidine dicarboxylate; (±)-*cis*-2,3-piperidine dicarboxylate; 2,6-piperidine dicarboxylate; L-serine; L-threo-3-hydroxy aspartate[d]; D-threo-3-hydroxy aspartate[d]; DL-threo-3-methylaspartate[d]. For further information see legends to Figures 5 and 6.

high affinity (Figure 5c). Finally, derivatives of phenylglycine, which have previously been shown to have little or no effect on mGluR4a-mediated cyclic AMP-formation (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994b) weakly displaced [^{3}H]-L-AP4 binding at mM concentrations.

Measurements of cyclic AMP-formation in BHK cells expressing mGluR4a

Compounds were tested for their ability to inhibit forskolin-stimulated cyclic AMP-formation in BHK cells expressing mGluR4a. The potencies of compounds which were found to be active at mGluR4a in the present study are listed in Table 3. In addition, values from previous studies (Kristensen *et al.*, 1993; Thomsen & Suzdak, 1993; Thomsen *et al.*, 1994b,c) are included in Table 3 for the sake of clarity. Potent agonists at mGluR4a included (±)-1-amino-3-phosphonocyclopentanecarboxylate (*trans* and *cis* isomers), L-CCG-I and cyclobutylene-AP5 and the dose-response curves are shown in Figure 6. A highly significant correlation was observed between the potencies for inhibition of forskolin-induced cyclic AMP-formation in mGluR4a cells and the affinities for displacement of [^{3}H]-L-AP4 binding from mGluR4a ($P < 0.0001$, two tailed test for linear correlation (Spearman's non-parametric test, $n = 19$)).

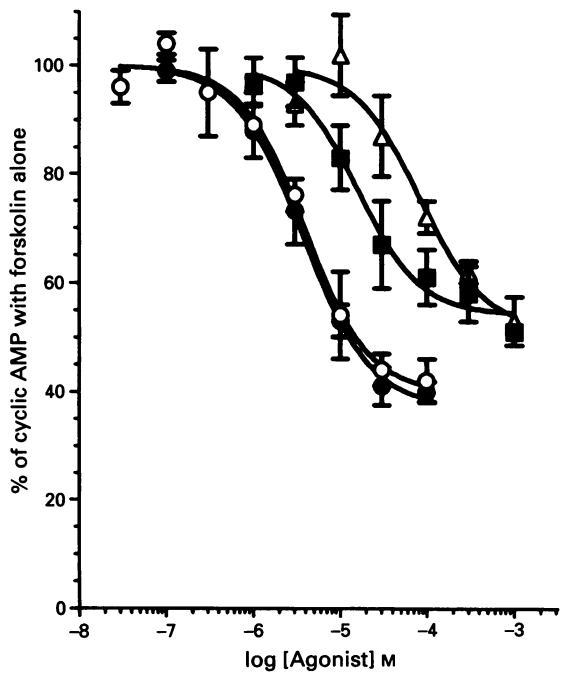

Figure 6 Dose-response curves for inhibition of forskolin-stimulated cyclic AMP-formation in BHK cells expressing mGluR4a by L-CCG-I (○), cyclobutylene-AP5 (●), (±)-*trans*-1-amino-3-phosphonocyclopentanecarboxylate (■) and (±)-*cis*-1-amino-3-phosphonocyclopentanecarboxylate (△). Experiments ($n = 3-4$) were performed in duplicate as described in Methods, and the data are expressed as % of the levels of cyclic AMP with forskolin alone. Agonists were added 2 min before incubation with 10 μM forskolin at 37°C. The basal levels of cyclic AMP-formation were 2.0 ± 0.3 pmol mg^{-1} protein, which were stimulated 9–14 fold by forskolin.

Discussion

The present study was undertaken in order to obtain more detailed information on the pharmacology of a subtype of the mGluR family, mGluR4a. Since mGluR4a is potently activated by L-AP4, [^{3}H]-L-AP4 binding seemed to be a suitable radioligand for characterizing this subtype in place of [^{3}H]-glutamate which is not (Kristensen *et al.*, 1993; Tanabe *et al.*, 1993). Previously, [^{3}H]-AP4 receptor binding to brain membranes from rats have been characterized (Butcher *et al.*, 1983; Monaghan *et al.*, 1983; Robinson *et al.*, 1985). However, when comparing the pharmacology of [^{3}H]-AP4 binding to rat cortical membranes with that of [^{3}H]-L-AP4 binding to mGluR4a, several differences may be noticed (Table 4). First of all, [^{3}H]-L-AP4 binding to mGluR4a is dependent upon the presence of Cl^--ions, but is not enhanced by Ca^{2+}-ions or by other divalent cations such as Mn^{2+} and Mg^{2+}, and this is in contrast to [^{3}H]-AP4 binding to cortical membranes (Butcher *et al.*, 1983; Monaghan *et al.*, 1983). The apparent optimum pH for [^{3}H]-L-AP4 binding to mGluR4a (between pH 8 and 9) was higher than the physiological pH (7.4) at which [^{3}H]-AP4 binding to cortical membranes is maximal (Monaghan *et al.*, 1983). Although the physiological relevance of this pH optimum may be questionable, a pH optimum above pH 7.4 has previously been observed with other recombinant G-protein coupled receptors such as the A_1-adenosine receptors (Jockers *et al.*, 1994). While [^{3}H]-L-AP4 binding to mGluR4a increased with temperature up to 37°C this was also accompanied by a modest increase in apparent binding to membranes prepared from control BHK cells. Thus, in order to avoid possible [^{3}H]-L-AP4 accumulation into resealed vesicles, binding experiments were performed at 0°C at which no specific binding to control BHK cells was observed. Association and dissociation of [^{3}H]-L-AP4 binding to mGluR4a at 0°C were almost complete within the 3 min time period, which is required to separate bound from free radioligand. While this is in contrast to the 50–60 min time

Table 4 Differences in characteristics of [^{3}H]-L-AP4 binding to mGluR4a as compared to [^{3}H]-AP4 binding to rat brain membranes

Characteristics	*[^{3}H]-L-AP4 binding to mGluR4a*	*[^{3}H]-AP4 binding to rat brain membranes*
Ionic requirements	Enhanced by Cl^- No effect of Ca^{2+}	Dependent upon Cl^- Enhanced by Ca^{2+}
pH optimum	8–9	6.8–7.4
Temperature	Highest at 37°C Medium at 0°C	Highest at 37°C Very low at 0°C
Association time	< 5 min at 0°C	50–60 min at 37°C
K_D	0.4 μM	2–6 μM
B_{max}	3 pmol mg^{-1} protein	60–420 pmol mg^{-1} protein
Sensitive to SITS	No	Yes
Ligand affinity	L-AP4 > > L-homocysteate > quisqualate > L-aminoadipate	Quisqualate > L-homocysteate > L-aminoadipate = L-AP4

The information on the characteristics of [^{3}H]-AP4 biding to rat brain membranes was from: Monaghan *et al.*, 1983; Butcher *et al.*, 1993; Robinson *et al.*, 1985. * Note that despite the extensive procedure used to disrupt the cell membranes some binding was observed to control BHK cell membranes at 37°C.

period required for complete association of [^{3}H]-AP4 binding to cortical membranes (Butcher *et al.*, 1983; Monaghan *et al.*, 1983; Robinson *et al.*, 1985), a similar rapid time-course has been obtained for (1S,3S)-ACPD-sensitive [^{3}H]-glutamate binding to rat brain membranes (Schoepp & True, 1992). Finally, [^{3}H]-L-AP4 binding to mGluR4a was not affected by an inhibitor of Cl^--transport, SITS in contrast to L-AP4-sensitive Ca^{2+}/Cl^--dependent [^{3}H]-glutamate binding to cortical membranes (Recasens *et al.*, 1987). As summarized in Table 4, the characteristics of [^{3}H]-L-AP4 binding to mGluR4a are clearly distinct from the pharmacology of [^{3}H]-AP4 binding to rat cortical membranes, which share characteristics with a high capacity Ca^{2+}/Cl^--dependent vesicular glutamate transporter (Pin *et al.*, 1984; Fagg & Lanthorn, 1985; Zaczek *et al.*, 1987).

Saturation binding experiments with radiolabelled agonists to recombinant G-protein coupled receptors often generate biphasic Scatchard plots owing to the presence of coupled as well as uncoupled populations of receptors. In the present study, saturation binding of [^{3}H]-L-AP4 to mGluR4a was preferentially fitted to a model involving a single affinity site, although the Hill numbers were somewhat below one. Uncoupling the receptors by including GTP-γ-S resulted in an increase in the K_D for [^{3}H]-L-AP4 binding to mGluR4a and a Hill coefficient close to unity. Compared to the G-protein coupled receptors such as adenosine receptors the effect of GTP-γ-S was rather weak (Mahan *et al.*, 1991). However, the approximately 2 fold increase in K_D observed was similar to that which has been obtained when measuring (1S,3R)-ACPD-sensitive [^{3}H]-glutamate binding to rat cortical membranes (Catania *et al.*, 1994). The variations in GTP-sensitivity among these G-protein coupled receptors may be related to (a) the about 100 fold difference in the affinity of the available radioligands, which in the case of mGluRs complicates the detection of lower affinity sites and (b) that actual differences in the mechanisms of G-protein coupling between mGluRs and other G-protein coupled receptors (Pin *et al.*, 1994) may explain the relative insensitivity of mGluR agonist binding towards GTP. In this context, it is interesting that [^{3}H]-glutamate binding to membranes prepared from BHK cells expressing mGluR1a is insensitive to GTP-γ-S (Thomsen, unpublished observations). Since the ligand binding site at mGluR1a is believed to be located at the extracellular domain (O'Hara *et al.*, 1993) and not in the seven transmembrane domain (Dohlman *et al.*, 1992) the allosteric mechanisms of G-protein activation via mGluRs may be different from other G-protein coupled receptors.

Displacement of [^{3}H]-L-AP4 binding from mGluR4a by compounds which showed a high affinity for mGluR4a followed sigmoidal curves with Hill coefficients ranging from 0.7 to 0.9. As discussed above this apparent heterogeneity is most likely related to the presence of G-protein coupled and uncoupled populations of receptors. However, when comparing the IC_{50} values for displacement of [^{3}H]-L-AP4 binding to mGluR4a with the potencies for inducing decreases in cyclic AMP-formation in mGluR4a transfected cells a highly significant correlation was observed suggesting that this binding site is functionally relevant. Nevertheless, when mGluR4a is expressed *in situ* the functional coupling is likely to be different from the coupling of mGluR4a in transfected mammalian cell lines and, thus, the various agonists may show different affinities for mGluR4a in the brain. For clarification of this issue, [^{3}H]-L-AP4 binding experiments to brain membranes prepared from the cerebellum, which contain high levels of mGluR4a (Kristensen *et al.*, 1993; Tanabe *et al.*, 1993), may be useful if uptake of L-AP4 into resealed vesicles can be avoided. The high affinity ($IC_{50}=1.4$ μM) and the potent agonist effect ($EC_{50}=3$ μM) of L-CCG-I for mGluR4a was unexpected, since L-CCG-I is very potent at inhibiting forskolin-induced cyclic AMP-formation in CHO cells transfected with mGluR2 ($EC_{50}=0.3$ μM), but much weaker at mGluR4a when expressed in a similar cell line ($EC_{50}=50$ μM) (Hayashi *et al.*, 1992). It should be noted, though, that the relative potency of L-CCG-I and glutamate at mGluR4a has been shown to be almost similar in different cell lines with EC_{50} values of about 50 μM in CHO cells (Hayashi *et al.*, 1992) or 3 μM in BHK cells (Kristensen *et al.*, 1993; present study). Since a potency of glutamate of 5 μM in CHO cells expressing mGluR4a has also been obtained (Tanabe *et al.*, 1993), similar to that observed with mGluR4a expressed in BHK cells (Kristensen *et al.*, 1993), the apparent discrepancies between agonist potencies are likely to be related to differences in the relative amount of expression of mGluR4a (Hoyer & Boddeke, 1993) rather than the expression systems used.

In a number of electrophysiological studies, an L-AP4 receptor has been characterized which mediates depression of synaptic transmission via a presynaptic mechanism (for a review see Koerner & Johnson, 1992). It has been proposed that mGluR4a may be involved in L-AP4-induced presynaptic depression of synaptic transmission in some glutamatergic pathways (Thomsen *et al.*, 1992; Tanabe *et al.*, 1993), but this remains to be proven. Since the potencies of a number of compounds have been shown for inhibition of synaptic transmission via the lateral or the medial perforant pathways (Crooks *et al.*, 1986; Koerner & Johnson, 1992; Peterson *et al.*, 1992) it was tempting to compare these values with the affinities for mGluR4a (Table 3). Interestingly, a highly significant correlation was observed between the affinity for mGluR4a and the potency for depression of synaptic transmission via L-AP4-sensitive pathways (Figure 7). In a similar comparison between the affinity for [^{3}H]-AP4 binding sites in rat brain membranes and the depressant actions of a number of L-AP4-analogues no such positive correlation was observed (Fagg & Lanthorn, 1985). It should, however, be noted that glutamate

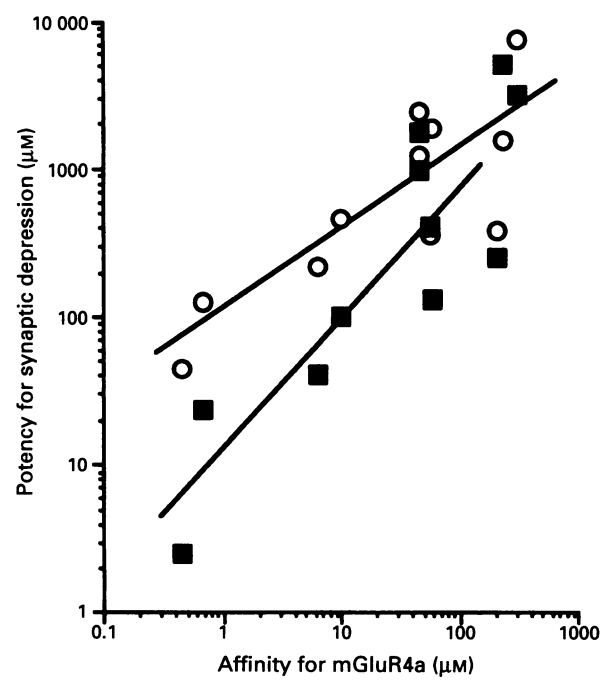

Figure 7 Comparison of binding affinities for mGluR4a with the potencies for inducing depression of synaptic transmission via the lateral perforant pathway (■) or medial perforant pathway (○) shown on a logarithmic scale. Affinities (IC_{50}s) for mGluR4a were from Table 3, and potencies for depression of synaptic transmission were from the literature (Crooks *et al.*, 1986; Koerner & Johnson, 1992; Peterson *et al.*, 1992). Linear correlation analysis revealed that the correlations were highly significant in both cases ($P<0.005$, two-tailed test for linear correlation (Spearman's non-parametric test, $n=11$) by use of the InStat program, GraphPad Software, U.S.A.). The figure was based on data for the following compounds: L-AP4, D-AP4, L-SOP, AP3, AP5, cyclobutylene-AP5, L-serine-O-sulphate, L-homocysteate, (±)-*trans*-1-amino-3-phosphonocyclopentanecarboxylate, (±)-cis-1-amino-3-phosphonocyclopentanecarboxylate, (±)-cis-1-amino-3-phosphonocyclohexanecarboxylate.

was not included in the present correlation (Figure 7), since there are several efficient uptake systems of L-glutamate in the brain which is very likely to lead to an underestimation of the potency of glutamate. A correlation of the binding affinities for mGluR4a with the potencies for depression of synaptic transmission via the lateral or the medial perforant pathways reveal slopes that are significantly different from unity (Figure 7). Thus, the concentration that is required to produce depression of synaptic transmission is several fold higher as compared to the binding affinity for mGluR4a (Figure 7). A similar discrepancy between binding affinity and functional potency has been observed with other G-protein coupled receptors such as the muscarinic receptors (Caulfield, 1993) or 5-hydroxytyrptamine receptors (Hoyer & Boddeke, 1993), and may be related to variations in the densities of receptors and/or G-proteins (Hoyer & Boddeke, 1993). Sequestration of L-AP4 and its structural analogues by a Ca^{2+}/Cl^{-}-dependent vesicular uptake mechanism (Butcher *et al.*, 1983; Monaghan *et al.*, 1983; Robinson *et al.*, 1985) may also contribute to the observed discrepancy, when comparing the potencies for synaptic depression with affinity for mGluR4a (Figure 7). Furthermore, additional L-AP4-sensitive members of the mGluR family are likely to participate in L-AP4-induced depression of synaptic transmission. Unfortunately, the pharmacology of subtypes mGluR6, mGluR7 and mGluR8 has been characterized using a limited number of agonists of which only glutamate and L-AP4 were found to be active (Nakajima *et al.*, 1993; Okamoto *et al.*, 1994; Saugstad *et al.*, 1994; Duvoisin *et al.*, 1995). Thus, it is not clear whether the agonist selectivity of L-AP4-sensitive mGluRs are identical or whether the pharmacology of mGluR6, mGluR7 or mGluR8 correspond to an L-AP4-sensitive receptor that is associated with synaptic depression. Differences in the regional distribution and pharmacology of L-AP4-sensitive mGluR subtypes may explain the different slopes when correlating the potency for synaptic depression via two different pathways with the affinity for mGluR4a (Figure 7). Although, the potencies of mGluR4a agonists to produce depression of synaptic transmission via the medial perforant path are lower as compared to the potencies for depression via the lateral path (Figure 7) this may also be related to variations in calcium buffering capacity and/or intensity of stimulation in the respective pathways (Duckles & Budai, 1990) rather than differences in mGluR subtype pharmacology. Finally, the [^{3}H]-L-AP4 binding assay characterized in the present study should be helpful for designing novel mGluR4a selective molecules, which may provide a clarification of the issue addressed above.

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