0026-895X/95/010140-10$3.00/0

MOLECULAR PHARMACOLOGY, 48:140–149 (1995).

Type 4a Metabotropic Glutamate Receptor: Identification of New Potent Agonists and Differentiation from the L-(+)-2-Amino-4-phosphonobutanoic Acid-Sensitive Receptor in the Lateral Perforant Pathway in Rats

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Received December 16, 1994; Accepted April 3, 1995

SUMMARY

Before the discovery of the metabotropic glutamate receptors (mGluRs), the glutamate analogue L-2-amino-4-phosphonobutanoic acid (L-AP4) was identified as a potent presynaptic inhibitor of evoked synaptic transmission in the lateral perforant pathway in rats. The localization and L-AP4 sensitivity of the mGluR4a subtype of mGluRs were consistent with the hypothesis that this receptor mediates the synaptic depressant effects of L-AP4 in the lateral perforant pathway. In the present study, the pharmacology of mGluR4a expressed in baby hamster kidney 570 cells was characterized and compared with that previously reported for the lateral perforant pathway responses. The endogenous excitatory amino acid L-aspartate was inactive at mGluR4a, whereas L-homocysteic acid was only 5-fold less potent than L-glutamate. These data suggest that L-homocysteic acid may be an endogenous agonist at mGluR4a. Of the 30 L-AP4 analogues examined, several compounds were identified as agonists at mGluR4a. The cyclopropyl-AP4 analogue (*Z*)-(±)-2-amino-2,3-methano-4-phosphonobutanoic acid inhibited forskolin-stimulated cAMP production with an EC_{50} of 0.58 μM, which is comparable to that of L-AP4 (EC_{50} = 0.43 μM). Two other cyclic analogues of L-AP4 were approximately 10-fold less potent as agonists at mGluR4a, i.e., (±)-1-amino-3-(phosphonomethylene)cyclobutanecarboxylic acid (EC_{50} = 4.4 μM) and (*E*)-(±)-2-amino-2,3-methano-4-phosphonobutanoic acid (EC_{50} = 7.9 μM). Comparison of the potencies of the compounds for activation of mGluR4a with their potencies for inhibition of lateral perforant pathway responses demonstrates that some compounds have comparable activities in the two systems, whereas several compounds are at least 10-fold more potent in one of the systems. In addition, although the mGluR antagonist (+)-α-methyl-4-carboxyphenylglycine blocked the effects of L-AP4 in the lateral perforant pathway, it did not block the effects of L-AP4 at the cloned receptor. These data provide evidence that mGluR4a does not mediate the effects of L-AP4 in the lateral perforant pathway, they provide new tools to identify the function of these receptors in the mammalian central nervous system, and they indicate that the effects of L-AP4 in the lateral perforant pathway are mediated by a (+)-α-methyl-4-carboxyphenylglycine-sensitive receptor.

The acidic amino acids are the predominant excitatory neurotransmitters in the mammalian central nervous system. These EAAs, including L-Glu, L-Asp, L-HCA, and possibly other EAA analogues, activate two classes of receptors, the ionotropic receptors and the mGluRs. The ionotropic L-Glu receptors are ligand-gated ion channels and have been classified into three subtypes, which are named for the selective agonists NMDA, AMPA, and kainate (1, 2). The mGluRs are coupled to second messenger systems via G proteins and were first discovered in 1985 when Sladeczek *et al.* (3) demonstrated that L-Glu and quisqualic acid stimulate PI hydrolysis, in rat striatal neurons grown in culture, by a mechanism independent of the activation of ionotropic receptors.

Before the discovery of the mGluRs, Koerner and Cotman (4) reported that a structural analogue of L-Glu, L-AP4, in-

This work was supported by National Institutes of Health Grants NS29868 and GM34781 (M.B.R.) and NS17944 (J.F.K). P.A.J. was supported by National Institutes of Health Training Grant MH14654.

ABBREVIATIONS: EAA, excitatory amino acid; AP3, 2-amino-3-phosphonopropionic acid; AP4, 2-amino-4-phosphonobutanoic acid; AP5, 2-amino-5-phosphonopentanoic acid; AP6, 2-amino-6-phosphonohexanoic acid; AP7, 2-amino-7-phosphonoheptanoic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; L-Asp, L-aspartic acid; DMEM, Dulbecco's modified Eagle's medium; L-Glu, L-glutamic acid; HCA, homocysteic acid; IBMX, isobutylmethylxanthine; mGluR, metabotropic glutamate receptor; (+)-MCPG, (+)-α-methyl-4-carboxyphenylglycine; NMDA, *N*-methyl-D-aspartic acid; PI, phosphatidylinositol; cyclobutylene-AP5, (±)-1-amino-3-(phosphonomethylene)cyclobutanecarboxylic acid; *trans*-ACPD, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid; cyclopentyl-AP4, (±)-1-amino-3-phosphonocyclopentanecarboxylic acid; cyclohexyl-AP4, (±)-1-amino-3-phosphonocyclohexanecarboxylic acid; cyclopropyl-AP4, (±)-2-amino-2,3-methano-4-phosphonobutanoic acid; AMPB, 2-amino-4-(methylphosphino)butanoic acid; β-methyl-AP4, 2(*RS*)-amino-3(*RS*)-methyl-4-phosphonobutanoic acid; CHO, Chinese hamster ovary; BHK, baby hamster kidney.

hibits evoked synaptic transmission at low micromolar concentrations in the lateral perforant pathway in rats. Inhibition by L-AP4 is selective for the lateral perforant pathway, compared with several other hippocampal pathways (4, 5). In addition, L-AP4 inhibits synaptic transmission in several other systems, including the spinal cord (6), the lateral olfactory tract (7, 8), and the mossy fiber-CA3 hippocampal pathway in guinea pigs (9, 10) and rats (11). L-AP4 does not block the ionotropic receptors at concentrations that inhibit lateral perforant pathway neurotransmission, suggesting that these inhibitory effects of L-AP4 are not mediated by the identified ionotropic receptors (2). By comparing the effects of L-AP4 on paired pulse potentiation with those of low extracellular Ca^{2+} concentrations and with those of the postsynaptic antagonist kynurenate, Harris and Cotman (12) provided evidence that the effect of L-AP4 is mediated by a presynaptic receptor. Also consistent with this hypothesis is the observation that L-AP4 does not decrease the frequency or the amplitude of miniature excitatory postsynaptic currents (13). Finally, the observation that L-AP4 reduces the release of L-Glu from mossy fiber synaptosomes prepared from guinea pig provides additional evidence for a presynaptic mechanism of action (14). Taken together, these observations provide evidence for a novel "L-AP4 receptor" that presynaptically regulates synaptic transmission. L-AP4 is still one of the most potent inhibitors of excitatory synaptic transmission in the mammalian central nervous system. Therefore, identification of the receptor mediating this effect may provide novel strategies to regulate excitatory synaptic transmission.

A combination of expression cloning and cloning based on sequence homology led to the identification of 11 different mGluR cDNAs, including variants that result from alternative splicing of the mRNAs. These receptors have been organized into three groups, based on sequence similarity, agonist sensitivity, and second messenger coupling (for review, see Ref. 15). The third group of these receptors, including mGluR4a (16), mGluR6 (17), and mGluR7 (18, 19), are coupled to the inhibition of adenylate cyclase activity in heterologous expression systems (15). Although L-AP4 is an agonist at all three receptors, only mGluR4 and mGluR6 are activated by low micromolar concentrations of L-AP4. These data, combined with the restricted distribution of mGluR6 mRNA in the retina (17), prompted the hypothesis that mGluR4a was the receptor mediating the synaptic depressant effects of L-AP4 (16). Consistent with this hypothesis is the observation that mGluR4 mRNA is expressed in the entorhinal cortex, the area that contains the cell bodies of the neurons that project to the hippocampus via the perforant pathway (16).

The goal of the present study was to characterize the pharmacology of mGluR4a expressed in a non-neuronal cell line that does not express endogenous mGluRs. This pharmacology was compared with that previously published for the inhibition of lateral perforant pathway neurotransmission (20–25). In addition, we compared the sensitivity of both systems to the mGluR antagonist (+)-MCPG (26–28).

Experimental Procedures

Materials. BHK 570 cells stably expressing mGluR4a subcloned into the pZEM219b expression vector were generously provided by Zymogenetics, Inc. (Seattle, WA). Cell culture media were purchased from Gibco. Characterized fetal bovine serum was purchased from Hyclone. IBMX, L-Glu, L-Asp, and L-HCA were purchased from Sigma Chemical Co. (St. Louis, MO). The following phosphonate analogues were purchased from Tocris: D- and L-AP3, D- and L-AP4, D- and L-AP5, D- and L-AP6, D- and L-AP7, cyclobutylene-AP5, *trans*-ACPD, (*E*)- and (*Z*)-cyclopentyl-AP4, (*E*)- and (*Z*)-cyclohexyl-AP4, (+)-MCPG, and L-serine-*O*-phosphate. All other AP4 analogues were synthesized as described previously (see Table 1 for references). All compounds except IBMX were dissolved in deionized distilled water at a concentration of either 1 mM or 10 mM and were neutralized with 1 N NaOH. IBMX stock was made at a concentration of 50 mM in 0.1 N NaOH. For assays, dilutions were made from these stocks into DMEM containing IBMX (100 μM).

Cell culture. BHK 570 cells were grown in DMEM supplemented with 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin, in an atmosphere of 5% CO_2. Stable expression of mGluR4 was maintained by the inclusion of methotrexate (250 nM) to take advantage of the methotrexate resistance gene in the expression vector.

Adenylate cyclase assay. cAMP accumulation was measured according to the method of Shimizu *et al.* (29). BHK 570 cells expressing mGluR4a were grown to 60–90% confluence in 12-well tissue culture plates. On the day of the experiment, cells were incubated with 1 ml of DMEM containing 1 μCi/ml [^{3}H]adenine to label endogenous stores of ATP. After 2 hr, excess tritium was rinsed away and the cells were preincubated for 10 min at 37° in DMEM containing 100 μM IBMX to inhibit the breakdown of cAMP. Assays were initiated by the addition of forskolin (10 μM final concentration) in the absence or presence of EAA analogues. The final assay volume was 500 μl, and drug additions were 50 μl. Assays were stopped after 10 min by the addition of 500 μl of 10% trichloroacetic acid and cooling on ice. Cells were sonicated, transferred to tubes, and centrifuged at 29,000 × *g* for 15 min to remove protein. cAMP was separated from ATP/ADP using sequential Dowex and alumina chromatography, according to the method of Salomon *et al.* (30), and was quantitated using scintillation counting. We have confirmed that under these conditions ATP, ADP, and cAMP co-chromatograph with authentic standards. Both Dowex and alumina columns were recycled after each experiment, according to the method of Salomon *et al.* (30), and were reused. Data from experiments in which the basal activity was more than twice the average value were not included in the data analysis. The columns were replaced with fresh Dowex and alumina resin when basal activity was elevated, and the basal levels then returned to normal.

Lateral perforant pathway electrophysiology. Transverse hippocampal slices were prepared from 30–100-day-old male Sprague-Dawley rats as described previously (4). The slices were equilibrated in a buffer that contained 124 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 26.4 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 2.4 mM $MgSO_4$, and 10 mM D-glucose and that had been pre-equilibrated with 95% O_2/5% CO_2 (with the pH adjusted to 7.4). Bipolar stimulating electrodes were placed in the outer one third of the molecular layer of the dentate gyrus. Stimulation (0.1 msec, 10–40 V, 0.1 Hz) was delivered below the threshold necessary to evoke a population spike, and field potentials were recorded with a glass microelectrode (2–14-MΩ impedance, filled with NaCl), which was also placed in the outer one third of the molecular layer of the dentate gyrus. The peak amplitude of the field potential was recorded on a chart recorder. Sensitivity to a single concentration of L-AP4 (10 μM) was first examined, to confirm electrode placement (5). Cumulative concentration-response curves for inhibition of lateral perforant path responses by L-AP4 were obtained in the absence and in the presence of (+)-MCPG (0.5, 1.0, 2.0, and 4.0 mM). Slices were equilibrated for 20 min with (+)-MCPG before the addition of L-AP4 and were re-equilibrated with buffer for 20 min during (+)-MCPG washout. The stability of synaptic responses and desensitization of the L-AP4 receptor were assessed by comparison of the inhibition produced by 8 μM L-AP4 before (+)-MCPG addition and after washout of (+)-MCPG. In addition, the reversibility of the effect of (+)-MCPG was assessed by

TABLE 1

Comparison of the potencies of EAA analogues for mGluR4a-mediated inhibition of forskolin-stimulated cAMP production and inhibition of lateral perforant pathway responses

Numbers in parentheses indicate the number of observations, each done in triplicate. The values presented are the mean ± standard error. For the five compounds for which the highest concentration did not permit calculation of the maximal response, the EC_{50} value was determined assuming no insensitive component, to permit a comparison of the relative potencies.

Compound	Inhibition of forskolin-stimulated cAMP production		Inhibition of lateral perforant pathway IC_{50}	Lateral perforant pathway IC_{50}/cAMP EC_{50} ratio
	EC_{50}	Maximal inhibition or inhibition at 100 μM or 1 mM		
	μM	%		
Endogenous EAAs				
L-Asp		11 ± 11 at 1 mM (4)	>1 mM	
L-Glu	10 ± 1 (4)	83 ± 6	12 mM[a]	
L-HCA	49 ± 15 (4)	82 ± 5	400 μM (depol.)[a,b]	
Linear phosphonate analogues				
L-AP3		−4 ± 10 at 1 mM (3)	>1 mM[c]	
D-AP3		10 ± 5 at 1 mM (4)	>1 mM[c]	
L-AP4	0.43 ± 0.2 (3)	85 ± 0.03	2.5 μM[a,d]	5.8
D-AP4	5.0 ± 0.3 (3)	85 ± 2	100 μM[d]	20
L-AP5	84 ± 17 (4)	85 ± 2	190 μM[c]	2.3
D-AP5	763 ± 80 (3)	64 ± 5 at 1 mM	660 μM[c]	0.9
L-AP6	344 ± 113 (3)	67 ± 4 at 1 mM	780 μM[c]	2.3
D-AP6		14 ± 4 at 1 mM (3)	>1 mM[c]	
L-AP7		51 ± 14 at 1 mM (5)	>1 mM[c]	
D-AP7		16 ± 3 at 1 mM (3)	>1 mM[c]	
Cyclic analogues of AP4				
Cyclobutylene-AP5	4.4 ± 1.7 (4)	82 ± 4	41 μM[e]	9.3
(*Z*)-Cyclopropyl-AP4	0.58 ± 0.17 (3)	81 ± 3	18 μM[f]	31
(*E*)-Cyclopropyl-AP4	7.9 ± 0.8 (3)	93 ± 3	17 μM[f]	2.2
(*E*)-Cyclopentyl-AP4	27 ± 4 (4)	91 ± 3	960 μM[g]	36
(*Z*)-Cyclopentyl-AP4	86 ± 42 (5)	77 ± 7	130 μM[g]	1.5
trans-ACPD	448 ± 87 (3)	66 ± 5 at 1 mM	NA[h]	
(*E*)-Cyclohexyl-AP4		−18 ± 3 at 1 mM (3)	>1 mM[g]	
(*Z*)-Cyclohexyl-AP4		6 ± 10 at 1 mM (3)	>1 mM[g]	
cis-4-Phosphonoxy-L-proline		−9 ± 22 at 100 μM (3)	>1 mM[i]	
trans-4-Phosphonoxy-L-proline		−5 ± 9 at 100 μM (3)	>1 mM[i]	
Substituted analogues of AP4 and other EAA analogues				
N-methyl-2-amino-4-phosphonobutanoic acid		32 at 100 μM (3)	>1 mM[j]	
β-Methyl-AP4	86 ± 30 (3)	70 ± 2 at 100 μM	500 μM[j]	5.8
3-Amino-5-phosphonopentanoic acid		−7 ± 8 at 100 μM (3)	>1 mM	
DL-AMPB	1200 (2)	51 ± 5 at 1 mM (3)	110 μM[k]	0.1
O-Methylphosphonyl-L-serine		21 ± 8 at 100 μM (3)	>1 mM[k]	
γ-Benzyl-2-amino-4-phosphonobutanoate		1 ± 14 at 100 μM (3)	>1 mM[i]	
L-2-Amino-4-(5-tetrazolyl)butanoate		0 ± 13 at 100 μM (3)	200 μM (depol.)[e]	
L-Serine-*O*-phosphate	1.13 ± 0.12 (3)	83 ± 2	23 μM[e]	20
D-HCA		9 ± 7 at 1 mM (3)	130 μM (depol.)[e]	

[a] Data from Koerner *et al.* (5).
[b] depol., compound depolarized the dentate granule cells at a lower concentration than it activated the presynaptic L-AP4 receptors.
[c] Data from Schulte *et al.* (25).
[d] Data from Koerner and Cotman (4).
[e] Data from Peterson *et al.* (24).
[f] Data from Kroona *et al.* (23).
[g] Data from Crooks *et al.* (22).
[h] NA, data not available.
[i] Previously unpublished data.
[j] Data from Crooks *et al.* (21).
[k] Data from Freund *et al.* (20).

alternating one-point L-AP4 additions (8 μM) with and without 2 mM (+)-MCPG present.

Data analysis. Data are expressed as a percentage of the radioactivity in the cAMP fraction divided by the total radioactivity in the cAMP fraction plus the ATP fraction (percentage conversion). Data for the inhibition of forskolin-stimulated cAMP production are expressed as a fraction of control (no inhibitor). Experiments were performed in triplicate and repeated a minimum of three times. In every experiment, 100 μM L-AP4 was included as a control. Commercially available compounds were initially tested at 1 mM. Compounds that were not commercially available were initially tested at 100 μM. Those compounds that inhibited forskolin-stimulated cAMP production by >50% were examined further using a full range of concentrations. Except for some compounds that were weak agonists (EC_{50} > 100 μM) or that were not available commercially, the range of concentrations was to at least 10 times the EC_{50}. Concentration-response curves were analyzed by nonlinear regression analysis according to an equation for a simple bimolecular interaction with a component of forskolin-stimulated cAMP production that is insensitive to agonist (see below).

$$F = a\left(\frac{1}{1+\frac{C}{EC_{50}}}\right) + b$$

In this equation, F equals the fraction of forskolin-stimulated cAMP production, a equals the maximal inhibition by the agonist of forskolin stimulation, b equals the fraction of forskolin- stimulated

cAMP production that is insensitive to inhibition by the agonist (the sum of a and b was held to 1), C equals the concentration of agonist, and EC_{50} equals the concentration of agonist that inhibits 50% of the sensitive component. The data for inhibition of lateral perforant pathway responses were fit assuming no insensitive component (b=0). Curves were fit to the data from each experiment, and the EC_{50} values and maximal inhibitory effects from each experiment were used to determine the average EC_{50} values and average maximal responses presented in Table 1. To generate the theoretical curves for each compound shown in the figures, data from all experiments were averaged and curves were fit through these averages.

Results

Basal cAMP production. In cells transfected with vector containing the mGluR4a cDNA, the average basal percentage conversion was 0.20 ± 0.01% (33 experiments) and the percentage conversion in response to 10 μM forskolin was 1.02 ± 0.03% (33 experiments), corresponding to 5-fold stimulation over basal levels. L-AP4 (100 μM) caused 80 ± 5% inhibition of forskolin-stimulated cAMP production. In cells stably transfected with vector that did not contain the mGluR4a cDNA, the basal and forskolin-stimulated cAMP production levels were similar to those of cells that express mGluR4a, but L-AP4 had no effect (three experiments, data not shown).

Endogenous EAAs. The effects of endogenous EAAs on inhibition of forskolin-stimulated cAMP production in BHK 570 cells expressing mGluR4a are shown in Fig. 1A. L-Glu was the most potent of the endogenous amino acids tested for agonist activity at this receptor. The EC_{50} of L-Glu for the inhibition of forskolin-stimulated cAMP production was 10 ± 1 μM, with the maximal effect being 83 ± 6% inhibition. L-HCA was also a fairly potent agonist at mGluR4a, with an EC_{50} of 49 ± 15 μM. In contrast, L-Asp was virtually inactive at this receptor.

Linear phosphonate analogues. Linear phosphonate analogues were tested for agonist activity at mGluR4a. L-AP4 was the most potent agonist of these linear analogues, with an EC_{50} of 0.43 ± 0.2 μM and a maximal effect of 85 ± 0.03% inhibition of forskolin-stimulated cAMP production (Fig. 1B). The EC_{50} values and maximal responses for the other linear phosphonate analogues are summarized in Table 1. The D-isomer of AP4 was approximately 10-fold less potent than L-AP4, with an EC_{50} of 5.0 ± 0.3 μM. L-AP5, D-AP5, L-AP6, and L-AP7 were all agonists but were much less potent than L-AP4. D-AP3, L-AP3, D-AP6, and D-AP7 were essentially inactive at a concentration of 1 mM.

Cyclic AP4 analogues. Of the 10 cyclic analogues of AP4 tested for agonist activity at mGluR4a, one compound, (*Z*)-cyclopropyl-AP4, had an EC_{50} (0.58 ± 0.17 μM) comparable to that of L-AP4 (Fig. 1B; Table 1). Two other compounds, cyclobutylene-AP5 and (*E*)-cyclopropyl-AP4, were also potent agonists at mGluR4a, with EC_{50} values of 4.4 ± 1.7 μM and 7.9 ± 0.8 μM, respectively. (*E*)-Cyclopentyl-AP4 was less potent (EC_{50} = 27 ± 4 μM), but it was 3-fold more potent than (*Z*)-cyclopentyl-AP4 (EC_{50} = 86 ± 42 μM). *trans*-ACPD was a very weak agonist at mGluR4a (EC_{50} = 448 ± 87 μM). (*E*)- and (*Z*)-Cyclohexyl-AP4 and *cis*- and *trans*-4-phosphonoxy-L-proline were essentially inactive at mGluR4a at the highest concentrations tested (Table 1).

Substituted analogues of AP4. Only one substituted analogue of AP4, β-methyl-AP4, was an agonist (EC_{50} = 86 ± 30 μM). All of the other substituted AP4 analogues were essentially inactive at a concentration of 100 μM (Table 1).

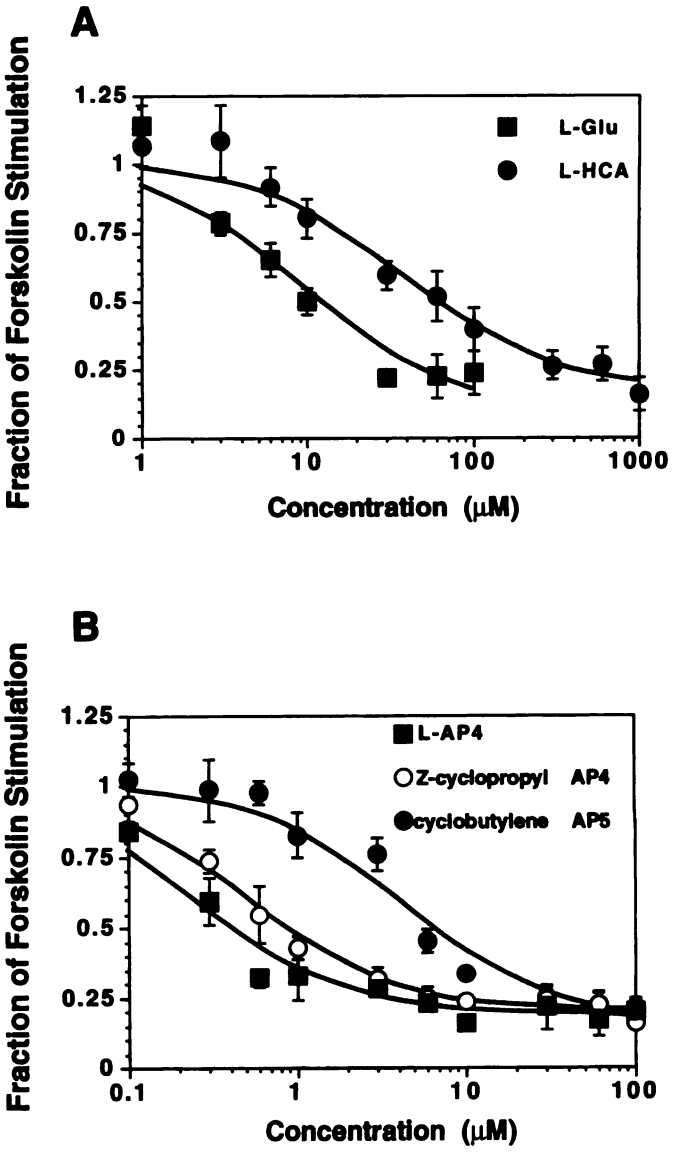

Fig. 1. Effect of endogenous EAAs and phosphonate analogues on mGluR4a-mediated inhibition of forskolin-stimulated adenylate cyclase activity. BHK 570 cells stably expressing mGluR4a were assayed for adenylate cyclase activity as described in Experimental Procedures. Data are expressed as a fraction of the cAMP production observed with forskolin alone. A, Endogenous EAAs. *Lines through the data*, theoretical curves with EC_{50} values and maximal inhibitory responses as follows: L-Glu, 9.7 μM and 91%; L-HCA, 40.4 μM and 84%, respectively. B, Phosphonate analogues. *Lines through the data*, theoretical curves with EC_{50} values and maximal responses as follows: L-AP4, 0.26 μM and 82%; cyclobutylene-AP5, 4.6 μM and 86%; (*Z*)-cyclopropyl-AP4, 0.53 μM and 81%, respectively. Data in A represent the mean ± standard error of four experiments, each performed in triplicate. Data in B represent the mean ± standard error of three experiments, each performed in triplicate.

Other EAA analogues. L-Serine-*O*-phosphate was a potent agonist at mGluR4a (EC_{50} = 1.13 ± 0.12 μM). In contrast to L-HCA, D-HCA was essentially inactive at 1 mM (Table 1).

Antagonist activity. All of the commercially available compounds that exhibited essentially no agonist activity at 1 mM were tested for antagonist activity. At a concentration of

1 mM, none of these compounds blocked the effects of 1 μM L-AP4 (three independent observations, data not shown). Similarly, the non-commercially available compounds that exhibited essentially no agonist activity at 100 μM were tested for antagonist activity. At a concentration of 100 μM, none of these compounds blocked the effects of 0.3 μM L-AP4 (three independent observations, data not shown). In addition to the AP4 analogues, (+)-MCPG was tested for antagonist activity at mGluR4a. At a concentration of 1 mM, (+)-MCPG had no effect on L-AP4-mediated inhibition of forskolin-stimulated cAMP production (Fig. 2A).

Lateral perforant pathway electrophysiology. The effects of (+)-MCPG on the inhibition of lateral perforant pathway responses by L-AP4 were examined. Concentration-response curves for L-AP4 were obtained in the absence or presence of increasing concentrations of (+)-MCPG. As previously observed (4), L-AP4 caused a concentration-dependent inhibition of the evoked synaptic responses when the recording electrode was positioned in the lateral perforant pathway. (+)-MCPG shifted the L-AP4 concentration-response curve to the right. The IC_{50} value for L-AP4 was 10 ± 2 μM in the absence of (+)-MCPG, 18 ± 4 μM in the presence of 0.5 mM (+)-MCPG, 30 ± 3 μM in the presence of 1 mM (+)-MCPG, 42 ± 8 μM in the presence of 2 mM (+)-MCPG, and 125 ± 33 μM in the presence of 4.0 mM (+)-MCPG. Data represent the mean ± standard error of at least four determinations (Fig. 2B). These data were examined by Schild analysis (Fig. 2B, *inset*). The K_i value obtained for (+)-MCPG was 290 μM, and the slope of this line was nearly 1 (0.905). (+)-MCPG (500 μM) alone had no effect on the size of the synaptic response (fraction of control, 0.98 ± 0.02). Although data from previous studies have suggested that the lateral perforant path response does not desensitize to the effects of L-AP4 (20–25), this was directly examined in the present study by using two approaches. In several experiments, the effects of 8 μM L-AP4 were compared before and after the concentration-response curves were obtained in the presence of (+)-MCPG. The percentage inhibition at the beginning of the experiment (43 ± 5% inhibition) was not significantly different (p = 0.45, paired t test) from that

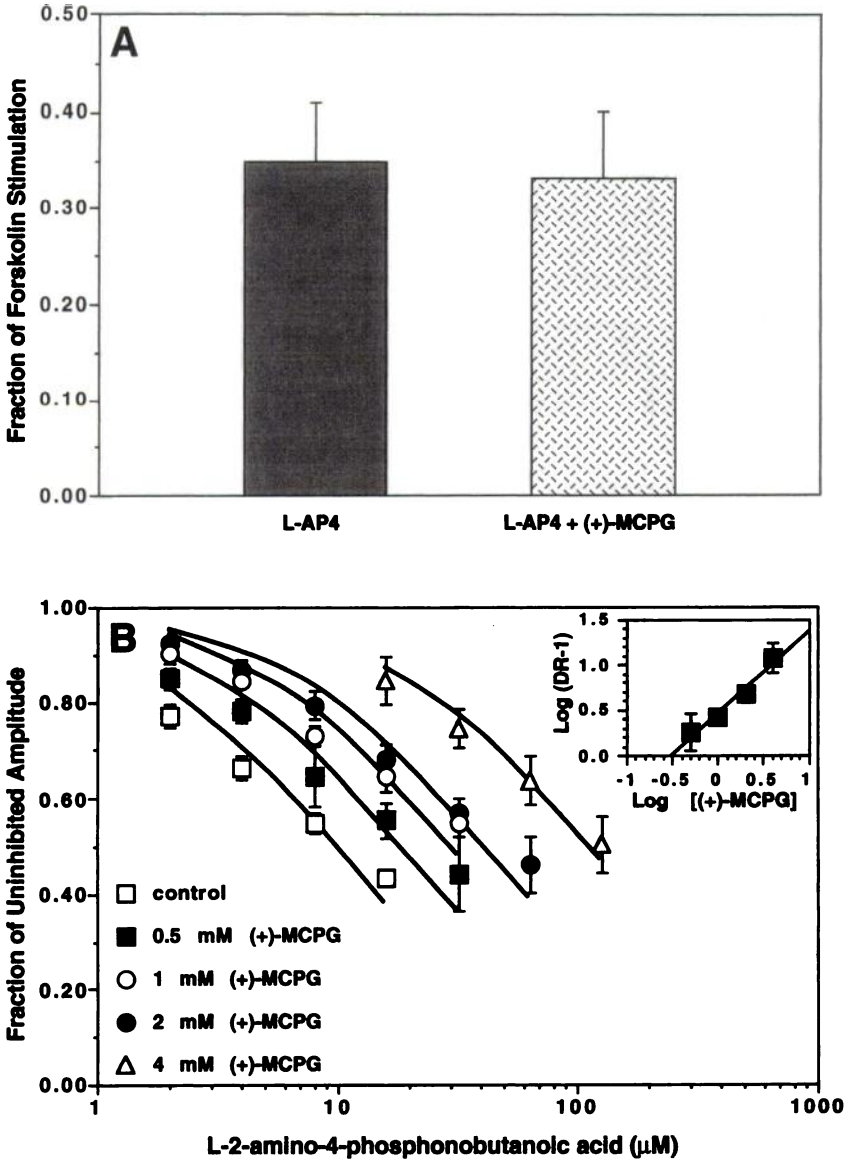

Fig. 2. Effect of the mGluR antagonist (+)-MCPG on the effects of L-AP4. A, BHK 570 cells stably expressing mGluR4a were assayed for adenylate cyclase activity as described in Experimental Procedures. The forskolin concentration was 10 μM, and data are expressed as a fraction of forskolin stimulation. Data for forskolin with L-AP4 (1 μM) and for forskolin with L-AP4 (1 μM) and (+)-MCPG (1 mM) represent the mean ± standard error of three experiments done in triplicate. B, Transverse slices of rat hippocampus were prepared and recorded from as described in Experimental Procedures. Cumulative concentration-response curves for L-AP4 were generated in the absence and presence of (+)-MCPG (0.5, 1.0, 2.0, and 4.0 mM). Data are expressed as a fraction of the maximal response of the field potential in the absence of L-AP4. Data represent the mean ± standard error of four experiments. Data were fit as described in Experimental Procedures. The IC_{50} for L-AP4 in the absence of (+)-MCPG was 10 μM, and the inhibitory response to L-AP4 was dose-dependently shifted to the right in the presence of (+)-MCPG. *Inset*, Schild plot of these data. The dose ratio (*DR*) − 1 is plotted as a function of log [(+)-MCPG]. The x-intercept (−log K_i) gave a K_i for (+)-MCPG of 290 μM, and the slope was 0.905.

observed at the end of the experiment (41 ± 5% inhibition). These data are the mean ± standard error of six observations. In addition, the effects of 8 μM L-AP4 were examined in the absence and presence of 2 mM (+)-MCPG (Fig. 3). In these experiments, L-AP4 repeatedly caused the same level of inhibition in the absence of (+)-MCPG, and this inhibition was repeatedly blocked by (+)-MCPG. The inhibition by (+)-MCPG of the effects of L-AP4 on synaptic transmission was completely reversible (Fig. 3).

Discussion

In the present study, the agonist and antagonist sensitivities of the mGluR4a subtype of mGluR were examined. In addition, the sensitivity to (+)-MCPG of the L-AP4-mediated inhibition of the lateral perforant pathway was examined.

As previously reported using the BHK 570 cell line (31, 32) as well as CHO cell lines (33), activation of this receptor inhibited forskolin-stimulated cAMP production. The agonist effects of some of the linear analogues of L-AP4 have been examined using these heterologous expression systems. Although there is general agreement that L-AP4 is the most potent of these linear analogues, Kristensen *et al.* (32) reported that DL-AP6 and D-AP5 were without activity at 1 mM. In the present study, these compounds were weak agonists; D-AP5 had an EC_{50} of 760 μM, and the individual isomer L-AP6 had an EC_{50} value of 340 μM. L-AP5, which was not previously studied, also was an agonist at this receptor, with an EC_{50} value of 84 μM. *trans*-ACPD, a L-Glu analogue that preferentially activates metabotropic EAA receptors and not ionotropic EAA receptors (34), was a relatively weak agonist (EC_{50} = 450 μM) in the present study. This is in contrast to the data of Kristensen *et al.* (32), who reported an EC_{50} value of 39 μM. Our results with *trans*-ACPD do agree with those previously reported by Tanabe *et al.* (33), who found that 100 μM *trans*-ACPD caused slight inhibition of forskolin-stimulated cAMP production in CHO cells expressing mGluR4a.

L-Glu and L-Asp are generally accepted to be the primary EAA neurotransmitters (35, 36), but additional compounds, including HCA and L-cysteine sulfinic acid, fulfill many of the criteria for neurotransmitter status (37–40). Therefore, by analogy to the close structural homologues glycine and γ-aminobutyric acid, or dopamine and norepinephrine, which activate distinct receptors, it is possible that subtypes of EAA receptors are activated by distinct EAAs. For example, Boss *et al.* (41) presented evidence that L-cysteine sulfinic acid preferentially activates a mGluR coupled to phospholipase D activity. In the present study, the sensitivity of mGluR4a to activation by three endogenous EAAs was examined. As reported previously, L-Asp was essentially inactive at this receptor and L-Glu was an agonist at this receptor, with an EC_{50} of approximately 10 μM (32, 33). In contrast to the data of Kristensen *et al.* (32), we found that L-HCA was a relatively potent agonist, with an EC_{50} of 49 μM, compared with their published EC_{50} value of 432 μM. Therefore, it appears that mGluR4a was preferentially activated by L-Glu and L-HCA and not by L-Asp. Consistent with a transmitter role for L-HCA, calcium-dependent, depolarization-evoked release of L-HCA is observed in cerebellum (39), the brain region that, based on *in situ* hybridization, is enriched with mGluR4 mRNA. In addition, L-HCA preferentially interacts with the subtype of sodium-dependent, high affinity, EAA transporters that is expressed in cerebellum (42). Therefore, although L-HCA was 5-fold less potent than L-Glu, it is possible that L-HCA activates this receptor *in vivo*.

In the present study, three novel agonists for mGluR4a that exhibit EC_{50} values of <10 μM were identified, i.e., (*Z*)-cyclopropyl-AP4 (EC_{50} = 0.58 μM), cyclobutylene-AP5 (EC_{50} = 4.4 μM), and (*E*)-cyclopropyl-AP4 (EC_{50} = 7.9 μM). These are all cyclic analogues of the prototypic mGluR4 ag-

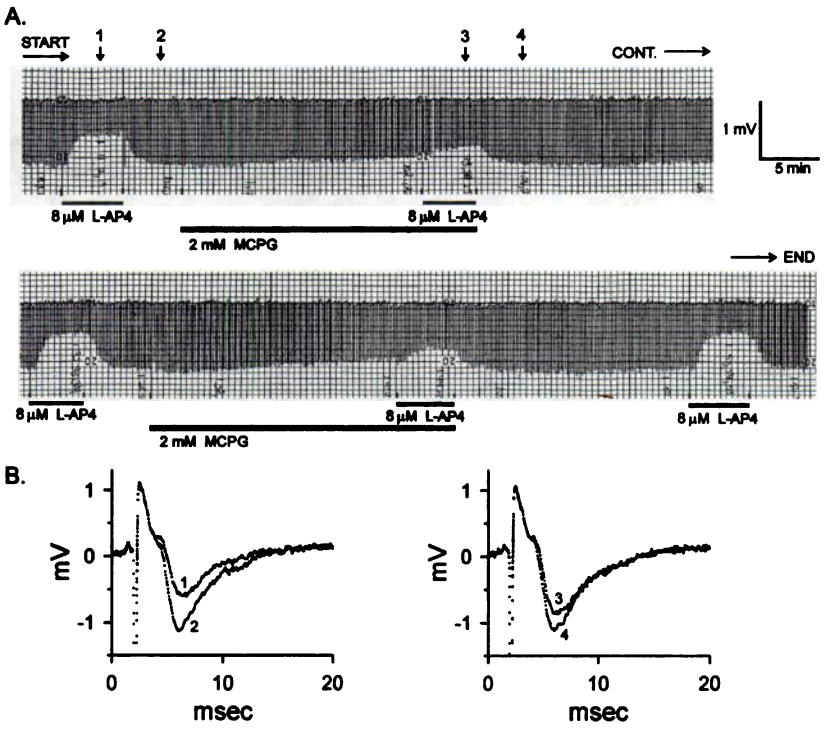

Fig. 3. Antagonizing effect of (+)-MCPG on L-AP4-mediated inhibition of lateral perforant path synaptic transmission. L-AP4 (8 μM) was applied to transverse hippocampal slices, in the absence and presence of 2 mM (+)-MCPG, while extracellular synaptic potentials in the lateral perforant pathway were recorded as described in Experimental Procedures. Slices were equilibrated for 20 min after the addition and washout of (+)-MCPG. A, An example of a typical strip recording from these experiments is shown. The maximal amplitude of each response was recorded over time (minutes). B, The actual extracellular synaptic potentials at specific times are shown. L-AP4 caused a 47% decrease in the synaptic response (*1*), and after washout the synaptic potential amplitude returned to 1.14 mV (*2*). In the presence of (+)-MCPG, the synaptic potential was inhibited by only 29% by 8 μM L-AP4 (*3*). After washout of both (+)-MCPG and L-AP4, the synaptic response returned to 1.14 mV (*4*). This protocol of adding 8 μM L-AP4, with and without 2 mM (+)-MCPG present, was repeated two additional times. These data are from a single experiment that was repeated three times.

onist L-AP4. Another cyclic analogue, (*E*)-cyclopentyl-AP4, was also an agonist at mGluR4a, with an EC_{50} value of 27 μM. Only one substituted AP4 analogue, β-methyl-AP4, exhibited significant potency at mGluR4a (EC_{50} = 86 μM).

Examination of the potencies of this family of phosphonate analogues provides insights into the structural features of L-AP4 required for interaction with this receptor. Of the linear compounds, the optimal length of the side chain for interaction with mGluR4a is two methylene carbons. Shortening the chain length to more closely mimic the separation predicted for a folded conformation of L-AP4 abolishes activity. On the other hand, increasing the length of the side chain decreases agonist potency, but to a much lesser extent than does shortening the length of the side chain. As observed in the inhibition of the lateral perforant path, the nature of the side chain acidic function has a significant effect on the inhibition of forskolin-stimulated cAMP production. The best activity is observed when the side chain functionality is a phosphonate moiety, as in AP4, or a phosphate group, as in L-serine-*O*-phosphate. Replacement of these functional acidic groups with such acidic moieties as tetrazole, phosphinic acid, or sulfonic acid leads to compounds with much lower activity.

Like L-Glu, L-AP4 can exist in a number of conformations in solution. It is likely, however, that a specific conformation of AP4 is recognized by mGluR4a. In an attempt to gain some insight into the bioactive conformation of AP4, the previously synthesized, conformationally constrained pyrrolidinyl, cyclohexyl, cyclopentyl, cyclobutyl, and cyclopropyl analogues of AP4 (Fig. 4) were tested on mGluR4a. These conformationally constrained analogues were designed to mimic a variety of extended and folded conformations of AP4. The three most potent conformationally constrained AP4 analogues were the two cyclopropyl analogues [(*E*)- and (*Z*)-cyclopropyl-AP4] and the cyclobutyl analogue (cyclobutylene-AP5). The activity profile of these three compounds suggests that mGluR4a recognizes an extended form of L-AP4, as has been predicted for the receptor that mediates the effects of L-AP4 in the lateral perforant pathway. The poor activity of the cyclopentyl and cyclohexyl analogues of AP4 suggests that mGluR4a is sensitive to steric bulk, because the bridging unit used to introduce the conformational constraint in these molecules is relatively larger than that for the cyclopropyl and cyclobutyl analogues.

L-AP4 inhibits synaptic transmission in several systems in the mammalian central nervous system (see the introduction). There is evidence that these effects are mediated presynaptically by a G protein-coupled receptor (43). The expression of mGluR4 mRNA in cells that comprise the perforant pathway and the sensitivity of mGluR4a to L-AP4 suggested that mGluR4a may mediate presynaptic inhibition by L-AP4 of synaptic transmission in the lateral perforant pathway. However, the pharmacology of these two systems had not been examined with many of the AP4 analogues that are active in the lateral perforant pathway. With the pharmacological data obtained in the present study, these comparisons are now possible (Table 1). In Fig. 5, the log (EC_{50}) values for inhibition of forskolin-stimulated cAMP production are compared with the log (IC_{50}) values for inhibition of lateral perforant pathway responses. Although there is a reasonable correlation between the potencies of these compounds in the two systems (r = 0.79), the y-intercept is 1.265, indicating that the average compound is 18-fold more potent as an agonist at mGluR4a than as an inhibitor in the lateral perforant pathway. There were, however, some specific differences. Some of the compounds examined, including AP5, (*E*)-cyclopropyl-AP4, and (*Z*)-cyclopentyl-AP4, were approximately equipotent in the two systems. D-AP4, (*Z*)-cyclopropyl-AP4, and (*E*)-cyclopentyl-AP4 were 20–36-fold more potent as agonists at mGluR4a than as inhibitors of synaptic transmission in the lateral perforant pathway. In contrast, the effects of 1 mM DL-AMPB were equivalent to the effects obtained with 100 μM in the lateral perforant pathway. Because the lower potency of DL-AMPB at mGluR4a could also be explained by partial agonist effects, a concentration-response curve (1–1000 μM) was examined and confirmed

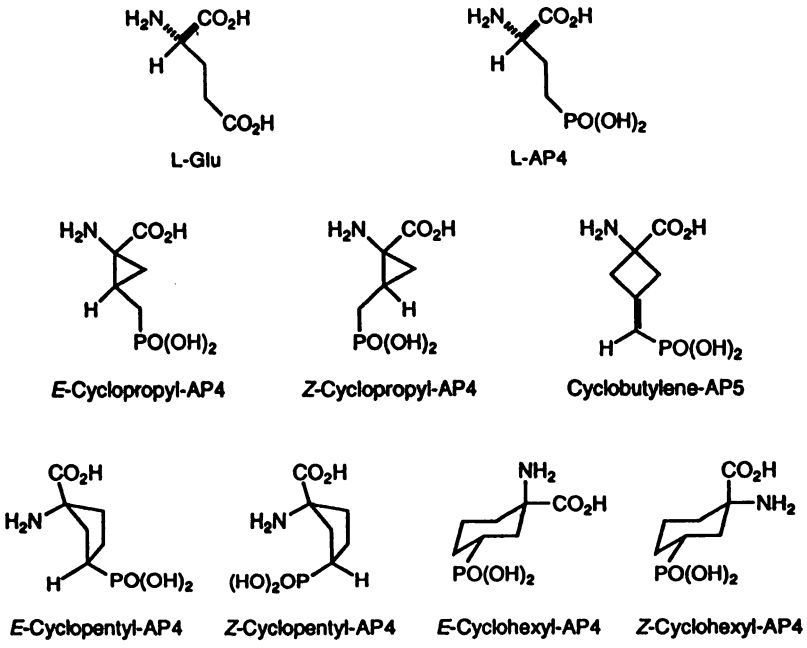

Fig. 4. Structures of L-AP4, L-Glu, and the cyclic analogues of AP4.

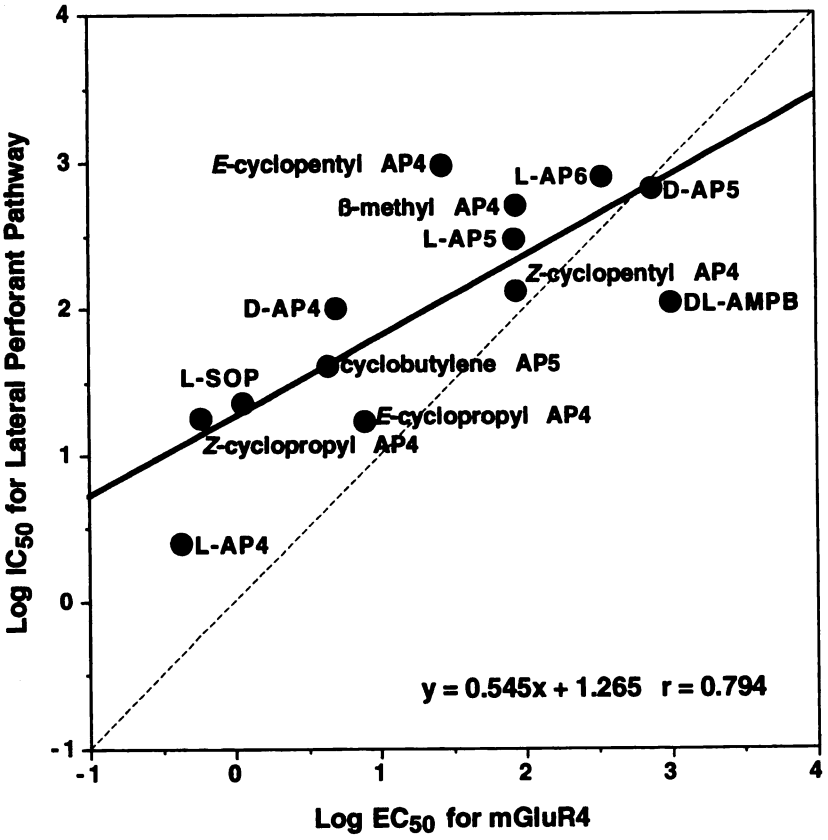

Fig. 5. Correlation between the EC_{50} of L-AP4 analogues at mGluR4a and the IC_{50} of the analogues for inhibition of the lateral perforant pathway. The log (EC_{50}) values for the inhibition of forskolin-stimulated cAMP production were plotted versus the log (IC_{50}) values for the inhibition of lateral perforant pathway responses. All of the compounds that have IC_{50}/EC_{50} ratios in Table 1 are plotted. *Solid line*, best fit through the data points. *Dashed line*, theoretical fit if all compounds were equipotent in both systems. The slope, *y*-intercept, and correlation coefficient are indicated. *L-SOP*, L-serine-*O*-phosphate.

that the EC_{50} of DL-AMPB at mGluR4a was approximately 1 mM and that this compound was not a partial agonist (data not shown).

Although the differences in agonist potency are interesting, there are several possible reasons why agonist potency in the lateral perforant pathway may not agree with agonist potency at cloned mGluR4a. The higher agonist potency for compounds activating mGluR4a could be related to high levels of expression of mGluR4a in BHK 570 cells, resulting in spare receptors. Conversely, it is possible that differences in diffusional barriers between brain slices and cells in culture could account for the lower potency of the compounds in the lateral perforant pathway. However, the observation that all of the compounds caused maximal inhibition of synaptic transmission within 2 min of their application to the slices suggests that substantial diffusional barriers are unlikely in our system (20–25). It is also possible that the effects of the phosphonate analogues on synaptic transmission in the lateral perforant pathway are due to interaction with more than one receptor. Although previous studies have shown that NMDA receptor-specific antagonists do not reduce synaptic transmission in the lateral perforant pathway (2), it is not possible to rule out effects mediated by subtypes of NMDA or non-NMDA receptors.

To develop direct evidence for distinct receptors, the effects of the mGluR antagonist (+)-MCPG were examined in both systems. As previously reported for mGluR4a expressed in CHO cells (27), (+)-MCPG did not block the agonist effects of L-AP4 at mGluR4a expressed in BHK 570 cells. However, (+)-MCPG did block the effects of L-AP4 in the lateral perforant pathway. (+)-MCPG produced a reversible, concentration-dependent increase in the IC_{50} value of L-AP4. The K_i for (+)-MCPG was 290 μM and the slope was nearly 1 (0.905), which is consistent with a competitive mechanism of action. The apparent affinity is similar to that previously reported for the effects of (+)-MCPG on both cloned mGluRs coupled to PI hydrolysis and increases in PI hydrolysis stimulated by EAAs in brain slices (26–28). These data indicate that, in contrast to mGluR4a, the receptor mediating the effects of L-AP4 in the lateral perforant pathway is sensitive to (+)-MCPG. In addition, our data provide evidence that the effect of (+)-MCPG in the lateral perforant pathway is not due to desensitization of the receptor. Desensitization was assessed in several experiments by the addition of 8 μM L-AP4 after the washout of (+)-MCPG. The inhibition caused by 8 μM L-AP4 in each case was not different from the level of inhibition caused by the same concentration of L-AP4 applied before the initial addition of (+)-MCPG [43 ± 5% inhibition before (+)-MCPG, 41 ± 5% inhibition after (+)-MCPG washout; $p = 0.45$, paired t test]. As shown in Fig. 3, repeated sequential additions of L-AP4 (8 μM) or L-AP4 with (+)-MCPG caused reproducible effects. These data provide strong evidence that mGluR4a does not mediate the effects of L-AP4 in the lateral perforant pathway. It has recently been reported that (+)-MCPG blocks the effects of L-AP4 on synaptic transmission in the spinal cord with an apparent K_d of 227 μM, which is very close to that observed in the present study (44). Thus, the present results provide evidence that mGluR4a also does not mediate the effects of L-AP4 in the spinal cord. This does not imply, however, that the L-AP4 receptor in the spinal cord is the same as the L-AP4 receptor in the lateral perforant pathway. It is interesting that (+)-MCPG alone had no effect on synaptic transmission in the lateral perforant pathway. This provides evidence that there is no en-

dogenous activation of this receptor when responses are evoked at relatively long interstimulus intervals (10 Hz) but raises the possibility that this receptor may be activated when the inputs are repeatedly activated at high frequency.

Of the cloned mGluRs, three are activated by L-AP4, i.e., mGluR4, mGluR6, and mGluR7. The present study provides direct evidence that mGluR4a does not mediate the presynaptic effects of L-AP4 in the lateral perforant pathway. The observation, using Northern analysis, that mGluR6 mRNA is restricted to retinal tissue provides evidence that this receptor does not mediate the presynaptic effects of L-AP4 in the brain. Although mRNA for mGluR7 is widely distributed, the low sensitivity of this receptor to L-AP4, combined with its insensitivity to inhibition by (+)-MCPG (19), suggests that mGluR7 also does not mediate the effects of L-AP4 in the lateral perforant pathway. There is a subtype of mGluR4, resulting from alternative splicing of the message, that has been referred to as mGluR4b (45). This receptor is reported to differ only in the carboxyl-terminal region following the seventh putative transmembrane domain (45). Although there is no published characterization of this splice variant, the fact that hydrophobicity plots predict that the region of the protein that is different in mGluR4b is intracellular suggests that the pharmacological properties of mGluR4b would be similar to those of mGluR4a. Therefore, at present, there is evidence to suggest that none of the mGluRs that have been cloned to date mediate the presynaptic effects of L-AP4 in the lateral perforant pathway.

In conclusion, the present study has identified three novel agonists at mGluR4a, i.e., (*Z*)-cyclopropyl-AP4, (*E*)-cyclopropyl-AP4, and cyclobutylene-AP5. In addition, we have developed direct evidence that mGluR4a does not mediate the effects of L-AP4 on lateral perforant pathway synapses. At this time, most of the data suggest that the synaptic depressant effects of L-AP4 in the lateral perforant pathway are mediated by a subtype of mGluR that has not yet been cloned. Further pharmacological characterization of the mGluR subtypes using these novel agonists will aid in the elucidation of the physiological role of mGluRs in normal and abnormal brain function.

Acknowledgments

We thank Betty Haldeman of Zymogenetics, Inc., for providing the BHK cell line stably expressing mGluR4a. We also thank Pamela Garcia for expert technical assistance and Dolan Pritchett and Louis Littman for their helpful discussions.

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