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Stereoselectivity and Mode of Inhibition of Phosphoinositide-Coupled Excitatory Amino Acid Receptors by 2-Amino-3-phosphonopropionic Acid

DARRYLE D. SCHOEPP, BRYAN G. JOHNSON, EDWARD C. R. SMITH, and LORETTA A. MCQUAID

Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285

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SUMMARY

DL-2-Amino-3-phosphonopropionic acid, a phosphonate-substituted derivative of aspartic acid, has been shown to be an inhibitor of excitatory amino acid-stimulated phosphoinositide hydrolysis in rat brain slices. In this study, the enantiomers of 2-amino-3-phosphonopropionic acid were synthesized and used to further characterize the stereoselectivity and mechanism of interaction of this compound for inhibiting phosphoinositide-coupled (metabotropic) excitatory amino acid receptors. L-2-Amino-3-phosphonopropionic acid was 3–5 times more potent than D-2-amino-3-phosphonopropionic acid as an inhibitor of ibotenate-stimulated [^{3}H]inositol monophosphate formation in slices of the rat hippocampus or quisqualate-stimulated [^{3}H]inositol monophosphate formation in neonatal rat cerebral cortical slices. Carbachol-stimulated phosphoinositide hydrolysis was not inhibited by L-2-amino-3-phosphonopropionic acid, and L-2-amino-3-phosphonopropionic acid had no appreciable affinity for ionotropic excitatory amino acid receptors at concentrations required to inhibit metabotropic excitatory amino acid responses. The inhibitory effects of L-2-amino-3-phosphonopropionic acid or L-2-amino-4-phosphonobutyric acid on phosphoinositide hydrolysis were not competitive, because they could not be surmounted by increasing concentrations of ibotenate or quisqualate. L-2-Amino-3-phosphonopropionic acid inhibition also could not be prevented by washing the tissue before incubation with ibotenate. Thus, L-2-amino-3-phosphonopropionic acid is a stereoselective inhibitor of metabotropic excitatory amino acid receptors with little affinity for ionotropic receptors. However, the inhibitory effects of L-2-amino-3-phosphonopropionic acid or L-2-amino-4-phosphonobutyric acid were not readily reversed, and the site at which they act to inhibit metabotropic excitatory amino acid receptors remains to be determined.

It has been well established that EAA neurotransmitters such as glutamate and aspartate mediate cellular effects via multiple receptors types in the central nervous system. EAA receptor subtypes can be classified into two groups, based on the transduction mechanisms to which they are linked. These are "ionotropic" receptors, which upon activation are directly coupled to the opening of cation channels, and the "metabotropic" receptor, which acts through a GTP-binding protein-dependent mechanism to elicit phosphoinositide hydrolysis (1). There are at least three ionotropic EAA receptor subtypes, which are distinguished by selective activation with the agonists NMDA (NMDA receptor), kainate (kainate receptor), and quisqualate or AMPA (quisqualate or AMPA receptor).

The metabotropic EAA receptor is pharmacologically distinguished from ionotropic receptors. In rat brain slices (2, 3) or synaptoneurosomes (4), phosphoinositide hydrolysis can be elicited by quisqualate but not the more selective ionotropic quisqualate receptor agonist AMPA. Phosphoinositide hydrolysis can also be enhanced by ibotenate, a compound that also has affinity for NMDA ionotropic EAA receptors (5). However, NMDA does not mimic this effect of ibotenate, and selective NMDA antagonists do not attenuate ibotenate stimulation in the rat hippocampus (2, 6). Thus, ibotenate does not stimulate phosphoinositide hydrolysis via activation of ionotropic NMDA receptors but may act on metabotropic EAA receptors that are also activated by quisqualate.

In slices of the rat hippocampus, ibotenate- or quisqualate-stimulated phosphoinositide hydrolysis can be antagonized by the γ-phosphono derivative of glutamic acid, DL-AP4 (6). This inhibitory effect of DL-AP4 has been shown to reside in the L-isomer of this compound (2). However, L-AP4 has other actions that suggest that it may not be a selective inhibitor of phosphoinositide-coupled EAA receptors. For example, L-AP4 has also been shown to block synaptic transmission in hippocampal slices following lateral perforant path stimulation (7), antagonize lateral olfactory tract evoked potentials in slices of the

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AP3, 2-amino-3-phosphonopropionic acid; AP4, 2-amino-4-phosphonobutyric acid; AP5, 2-amino-5-phosphonopentanoic acid; AP7, 2-amino-5-phosphonoheptanoic acid; EAA, excitatory amino acid; IP, inositol monophosphate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid.

olfactory cortex (8), and prevent mossy fiber-induced excitation of CA3 neurons in slices of guinea pig hippocampus (9, 10). Thus, it is thought that L-AP4 also interacts at this inhibitory "L-AP4 receptor" (1).

More recently, we found the β-phosphono derivative of aspartic acid, DL-AP3, to be a more potent inhibitor of ibotenate- and quisqualate-stimulated phosphoinositide hydrolysis in brain slices than L-AP4. Moreover, DL-AP3 does not mimic the effects of L-AP4 at electrophysiologically defined inhibitory L-AP4 receptors (7). This more selective antagonist effect of DL-AP3 when compared with L-AP4 further illustrates the unique pharmacology of the metabotropic EAA receptor. However, the mechanism by which these compounds selectively inhibit phosphoinositide-coupled EAA receptors in brain slices has not been investigated.

We have recently synthesized the D- and L-isomers AP3. In this study we compared the relative potencies of D- and L-AP3 for inhibition of metabotropic versus ionotropic (NMDA, kainate, and AMPA) EAA receptors and further investigated the mechanism(s) by which L-AP3 and L-AP4 inhibit EAA-stimulated phosphoinositide hydrolysis.

Materials and Methods

Phosphoinositide hydrolysis in rat cortical brain slices. Experiments of phosphoinositide hydrolysis in slices of the rat brain were performed as described previously, using the rat hippocampus (11) or the neonatal rat cortex (12). Briefly, hippocampal tissue was obtained from 35–40-day-old male Sprague-Dawley rats and cortical slices were prepared from 7-day-old neonatal (male and female) Sprague-Dawley rats. Animals were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and provided free access to food and water before use. Rats were decapitated and the brain was dissected while being bathed in ice-cold Kreb's-bicarbonate buffer (equilibrated with 95% O_2/5% CO_2, pH 7.4). Cortical tissue was chopped twice in perpendicular directions using a McIlwain mechanical tissue chopper at 0.3-mm intervals. Tissue was suspended in cold buffer and centrifuged. Washed slices were resuspended in buffer and incubated with *myo*-[^{3}H]inositol (10 μCi/ml of tissue suspension; 0.7 μM) for 60 min at 37° to label ^{3}H-phosphoinositides. Labeled tissue was washed twice and then resuspended in a modified Kreb's bicarbonate buffer, which contained 10 mM LiCl and 10 mM nonlabeled *myo*-inositol. Aliquots of this tissue suspension (240 μl, 1.0–2.0 mg of tissue protein) were preincubated at 37° for 20 min. A 5-μl aliquot of antagonist compound or 5 μl of water vehicle were added to the sample before this preincubation. Phosphoinositide hydrolysis was initiated by addition of a 5-μl aliquot of the EAA agonist or water vehicle and incubation for an additional 60 minutes at 37°. The incubation was terminated by addition of 1 ml of ice-cold 10 mM LiCl solution and placement of the samples in an ice bath.

Samples were homogenized to extract [^{3}H]inositol phosphates and then centrifuged. The pellet from each sample was solubilized in 3% NaOH and analyzed for protein (13). [^{3}H]IP was isolated from the supernatant using anion exchange column chromatography. Accell QMA Sep-Pak columns (formate form) were attached to an Amersham Superseparator apparatus that was modified to allow collection into 20-ml glass scintillation vials. The sample was passed over the cartridge, followed by 10 ml of water ([^{3}H]inositol fraction) and 10 ml of 5 mM sodium borate ([^{3}H]glycerol inositol phosphate fraction). [^{3}H]IP was then eluted directly into a scintillation vial with 4 ml of 0.1 mM ammonium formate/0.01 mM formic acid/5 mM sodium borate. Separation of [^{3}H]IP from [^{3}H]inositol and other [^{3}H]inositol phosphates was verified using radioactive standards.

Data were calculated as dpm of [^{3}H]IP/mg of protein and then were converted to a percentage of the basal value (water vehicle only) in each experiment. Statistical significance was determined using either Student's *t* test or analysis of variance in conjunction with Duncan's multiple range test procedure for comparison of multiple means (14). A probability of <0.05 was considered significant. Concentrations of compounds producing 50% inhibition of agonist-stimulated phosphoinositide hydrolysis over the basal value (IC_{50}) were calculated using the median-effect plot of Chou and Talalay (15).

Radioligand binding. For all binding assays, male Sprague-Dawley rats (150–175 g) were used. Displacement of the specific binding of [^{3}H]CGS19755 (10 nM) to Triton-X-treated synaptosomal membranes of rat forebrain was used to determine NMDA receptor affinity (16). Nonspecific binding was determined using 10 μM L-glutamate. Samples were incubated in an ice bath for 30 min, and bound ligand was separated from the free ligand by rapid filtration through Whatman GF/B glass fiber filters. [^{3}H]Kainate binding was performed using washed synaptosomal membranes from the rat forebrain (17). [^{3}H]Kainate (5 nM) was added to 50 mM Tris-HCl buffer (pH 7.4 at 4°) containing 200–300 μg/ml of tissue protein. Samples were incubated for 30 min in an ice bath and then rapidly filtered using a Brandel cell harvester and Whatman GF/C filters. Filters were washed twice with 3 ml of cold buffer. Nonspecific binding was determined using 100 μM nonlabeled kainate. The binding of [^{3}H]AMPA (5 nM) was conducted with crude membranes of rat forebrain in the presence of 100 mM KSCN, as described by Nielsen *et al.* (18). Nonspecific binding was determined with 10 μM nonlabeled AMPA.

Materials. Ibotenic acid, quisqualic acid, NMDA, and kainic acid were purchased from Sigma Chemical Company (St. Louis, MO). L-AP4 and AMPA were purchased from Tocris Neuramin (Essex, England). D-AP3 (LY231311) and L-AP3 (LY231267) were prepared by exhaustive acid hydrolysis of the corresponding D- and L-isomers of methyl *N*-(*tert*-butoxycarbonyl)-2-amino-3-dimethylphosphonopropionate, which were obtained by the enantioselective addition of trimethylphosphite to (*R*)- and (*S*)-*N*-(*tert*-butoxycarbonyl)-3-amino-2-oxetanone (19). D- and L-AP3 were obtained in greater than 97% enantiomeric purity, as determined by ^{1}H NMR spectroscopy. These compounds were dissolved in water and a sodium hydroxide solution was used to facilitate dissolution and to neutralize the stock solutions to pH 7–8. [*piperidinyl*-^{3}H]CGS19755 (30 Ci/mmol), DL-α-[5-methyl-^{3}H]AMPA (27.6 Ci/mmol), and [*vinylidene*-^{3}H]kainic acid were from New England Nuclear (Boston, MA). *myo*-[2-^{3}H]Inositol (14–15 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Results

Phosphoinositide hydrolysis that is stimulated in slices of the rat hippocampus or cerebral cortex by ibotenate or quisqualate can be selectively inhibited by DL-AP3 (2, 12). However, the potency of metabotropic EAA antagonists and the efficacy of metabotropic EAA agonists are greatly influenced by the age of the animal used. Specifically, as animals age from the early postnatal period through adulthood, α-amino-ω-phosphonocarboxylate antagonists such as DL-AP3 and L-AP4 become more potent, while agonists such as ibotenate and quisqualate become less potent and less efficacious (20). Quisqualate is particularly difficult to study in more mature animals, because it appears as a partial agonist in comparison with ibotenate (2). With this in mind, we characterized two tissues in this study. Ibotenate was used as an agonist in slices of the hippocampus from 35–40-day-old rats, and quisqualate effects were examined using cortical slices from neonatal (7-day-old) rats.

When hippocampal slices from 35–40-day-old rats were used, 10^{-3} M ibotenate stimulated [^{3}H]IP formation by 742 ± 34% over the basal values (see legend to Fig. 1). The ability of L- and D-AP3 to inhibit ibotenate (10^{-3} M) stimulation is shown in Fig. 1. L-AP3 was about 3 times more potent than the corresponding D-isomer. IC_{50} values for L-AP3 and D-AP3 in

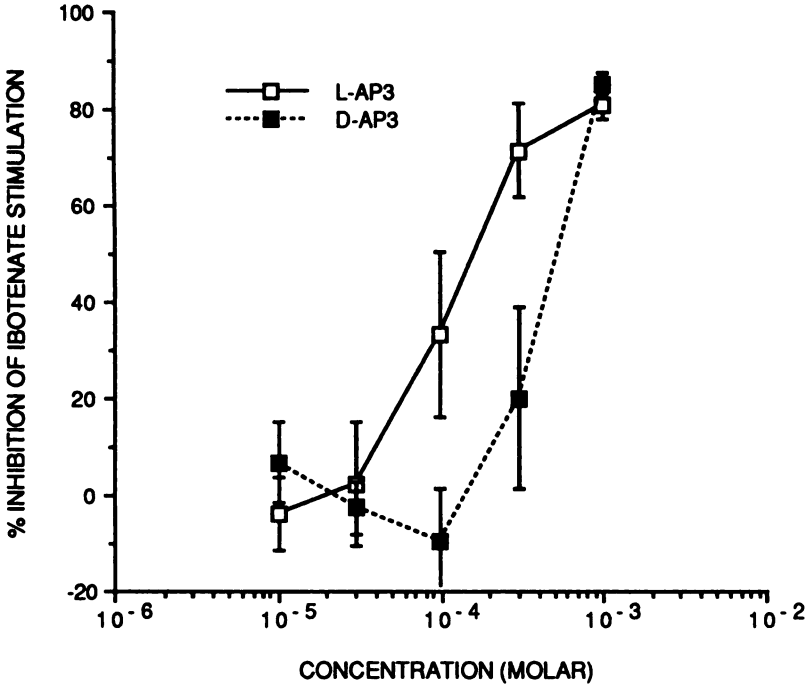

Fig. 1. Concentration-effect inhibition of ibotenate-stimulated ^{3}H-phosphoinositide hydrolysis by the L- and D-isomers of AP3. Hippocampal slices from 35–40-day-old rats were incubated with *myo*-[^{3}H]inositol for 60 min to prelabel ^{3}H-phosphoinositides. Tissue was washed and then further incubated for 20 min in buffer with nonlabeled *myo*-inositol (10 mM) and LiCl (10 mM), in the absence (controls) and presence of varying concentrations of L- or D-AP3. An aliquot of the agonist (ibotenate, 10^{-3} M final concentration) or water vehicle was then added and the incubation was continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange (QMA Sep-Pak) column chromatography. Data (mean ± standard error of seven experiments) calculated as dpm of [^{3}H]IP/mg of protein were expressed as percentage of inhibition of ibotenate-stimulated [^{3}H]IP above the basal value in each experiment. The absolute values (mean ± standard error) for basal and ibotenate-stimulated [^{3}H]IP were 2,241 ± 147 and 16,473 ± 888 dpm/mg of protein, respectively.

these experiments, calculated from the average values, were 168 and 515 μM, respectively. In slices from 7-day-old neonatal rats, quisqualate (100 μM) stimulated phosphoinositide hydrolysis by 517 ± 7% (see legend to Fig. 2). L-AP3 was about 5 times more potent than D-AP3 in inhibiting quisqualate stimulations (Fig. 2). The IC_{50} values for L-AP3 and D-AP3 for inhibiting quisqualate were 369 and 1722 μM, respectively. As noted previously for DL-AP3 (11), maximum inhibition by L or D-AP3 was about 80%. As before, this is due to the modest stimulatory effects of L- and D-AP3 alone, which prevent inhibition of agonist stimulation back down to the 100% inhibited basal value (see Figs. 3–5). As shown in Fig. 3, L-AP3 was a selective inhibitor of EAA-stimulated phosphoinositide hydrolysis, and this compound did not inhibit [^{3}H]IP formation stimulated by the cholinoceptor agonist carbachol.

Because AP3 is a phosphonate-substituted analog of aspartic acid and aspartic acid is a substance that stimulates ionotropic EAA receptors, we examined L- and D-AP3 for affinity at ionotropic EAA receptors, using the selective ligands [^{3}H]AMPA (quisqualate receptor), [^{3}H]kainate (kainate receptor), and [^{3}H]CGS19755 (NMDA receptor). L-AP3 affinity for these ionotropic sites was relatively modest. At quisqualate and kainate sites there was <20% displacement by L-AP3 at concentrations up to 1000 μM (Table 1). L-AP3 significantly displaced [^{3}H]CGS19755 binding, but only by 33% at 1000 μM. In contrast, D-AP3 exhibited appreciable activity against [^{3}H]CGS19755. The IC_{50} value for D-AP3 versus [^{3}H]CGS19755

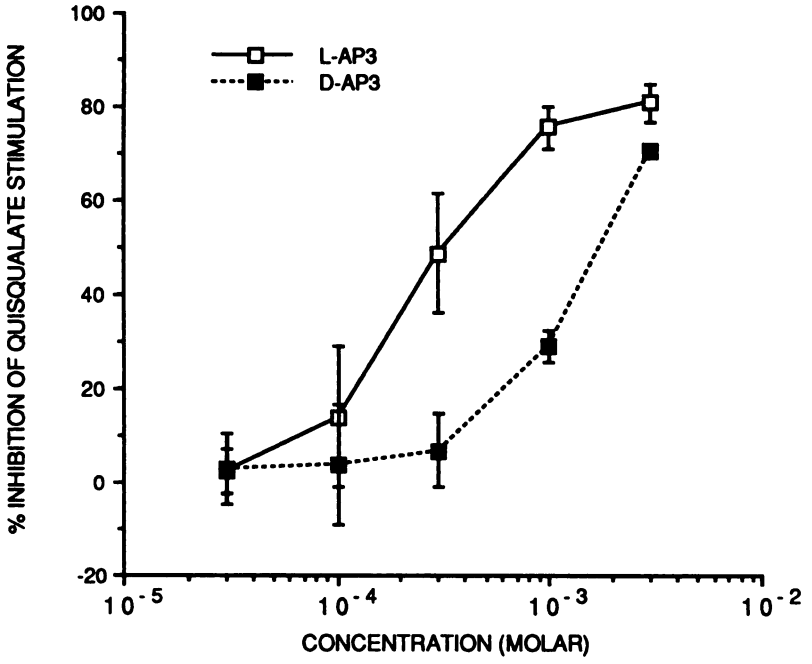

Fig. 2. Concentration-effect inhibition of quisqualate-stimulated ^{3}H-phosphoinositide hydrolysis in cerebral cortical slices from 7-day-old rats by the L- and D-isomers of AP3. See legend to Fig. 1 for experimental details. Data (mean ± standard error of four experiments) calculated as dpm of [^{3}H]IP/mg of protein were expressed as percentage of inhibition of quisqualate (10^{-4} M)-stimulated [^{3}H]IP above the basal value in each experiment. The absolute values (mean ± standard error) for basal and quisqualate-stimulated [^{3}H]IP were 3,740 ± 441 and 19,396 ± 2,471 dpm/mg of protein, respectively.

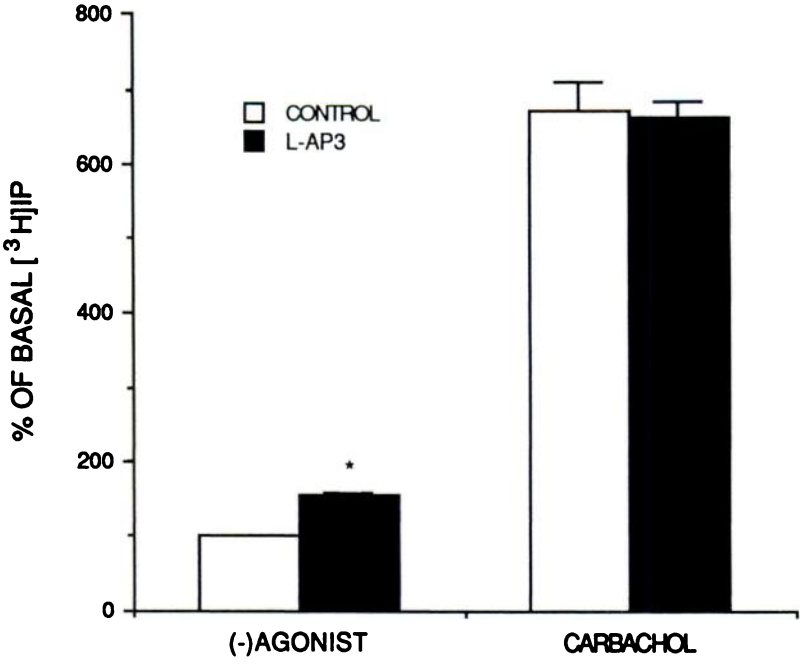

Fig. 3. Effect of L-AP3 (1000 μM) on carbachol (1000 μM)-stimulated ^{3}H-phosphoinositide hydrolysis in cerebral cortical slices from 7-day-old rats. See legend to Fig. 1 for experimental details. Data (mean ± standard error of four experiments) are expressed as a percentage of the basal value in each experiment. The absolute basal value (mean ± standard error) from all experiments was 2036 ± 80 dpm of [^{3}H]IP/mg of protein. *, $p < 0.05$ when compared with the corresponding control.

TABLE 1

Displacement of ionotropic EAA receptor binding by the stereoisomers of AP3

Values are mean ± standard error of three experiments.

	Displacement of specific binding		
	[^{3}H]AMPA[a]	[^{3}H]Kainate[b]	[^{3}H]CGS19755[c]
	%		
L-AP3			
100 μM	4.9 ± 0.8	6.8 ± 4.3	14.8 ± 2.2
300 μM	8.3 ± 3.4	9.1 ± 2.9	25.4 ± 1.8
1000 μM	12.2 ± 3.1	18.6 ± 4.7	32.5 ± 7.8
D-AP3			
100 μM	4.4 ± 2.5	6.8 ± 3.9	30.2 ± 4.5
300 μM	6.1 ± 2.5	9.0 ± 2.0	49.1 ± 1.1
1000 μM	9.7 ± 4.6	18.0 ± 1.1	73.7 ± 2.3

[a] Binding was performed using washed crude membranes from rat forebrain in the presence of 100 mM KSCN and 5 nM [^{3}H]AMPA. Nonspecific binding was determined with 10 μM nonlabeled AMPA and was <10% of total binding. Specific binding in the absence of AP3 was 261 ± 7 fmol/mg of protein. In saturation experiments, the K_D value for [^{3}H]AMPA binding was 34.8 nM.

[b] Binding was performed using washed synaptosomal membranes from rat forebrain and 5 nM [^{3}H]kainate. Nonspecific binding was determined with 100 μM nonlabeled kainate. Specific binding in the absence of AP3 was 244 ± 10 fmol/mg of protein. In saturation experiments, the K_D value for [^{3}H]kainate binding was 8.4 nM.

[c] Binding was performed using Triton X-100-treated synaptosomal membranes from rat forebrain and 10 nM [^{3}H]CGS-19755. Nonspecific binding was determined with 10 μM L-glutamate. Nonspecific binding was determined with 10 μM nonlabeled AMPA and was <10% of total binding. Specific binding in the absence of AP3 was 149 ± 13 fmol/mg of protein. The reported K_D value for [^{3}H]CGS19755 binding (16) under these conditions was 24 nM.

binding, calculated using the mean data from Table 1, was 292 μM. D-AP3 did not appreciably displace [^{3}H]AMPA or [^{3}H] kainate binding, even at 1000 μM (<20% displacement).

To study the mechanism by which L-AP3 inhibits metabotropic EAA receptor responses, concentration-effect curves for ibotenate- and quisqualate-stimulated [^{3}H]IP formation were examined in the presence of fixed inhibitory concentrations of L-AP3. If L-AP3 competitively inhibits this receptor by acting on the glutamate (or aspartate) recognition site in a reversible manner, then its inhibitory effects should be overcome by increasing concentrations of the agonist. However, in the case of both ibotenate (Fig. 4) and quisqualate (Fig. 5), inhibitory concentrations of L-AP3 could not be overcome at higher agonist concentrations. The absence of a competitive type of inhibition was also observed for L-AP4 inhibition of ibotenate in hippocampal slices (Fig. 6). Further experiments examined whether the inhibitory effect of L-AP3 was irreversible or reversible in hippocampal slices. It was found that inhibition by a maximally effective concentration of L-AP3 (300 μM) was not reversed by washing L-AP3 out of the tissue before addition of ibotenate (Fig. 7).

Discussion

In previous work, we have shown in slices of the mature rat hippocampus (11), neonatal rat hippocampus (20), and the neonatal rat cortex (12) that DL-AP3 is the most potent inhibitor of EAA-stimulated phosphoinositide hydrolysis yet described. This study further addressed several aspects of the inhibitory effects of AP3 on phosphoinositide-coupled EAA receptors. These included stereoselectivity, relative affinity for ionotropic EAA receptors at concentrations shown to inhibit metabotropic responses, and the mechanism by which AP3 produces inhibitory effects.

Other compounds that have been reported to inhibit ibotenate-stimulated phosphoinositide hydrolysis in brain slices include AP4 (2, 6), serine (21), phosphoserine (11, 21), and α-aminoadipic acid (11). The L-isomers of all these inhibitory compounds are more potent in this regard than the D-isomers (11, 21). We found here that the L-isomer of AP3 is also a more potent inhibitor of quisqualate- or ibotenate-stimulated phosphoinositide hydrolysis than the D-isomer. The similar stereoselectivity and the structural homology that these inhibitory compounds possess suggest that they may all act in the same manner to inhibit EAA stimulation of phosphoinositide hydrolysis.

In this study we have focused on AP3 because it is the most potent and selective of these inhibitory compounds. DL-AP3 has not been previously reported to interact at any other EAA

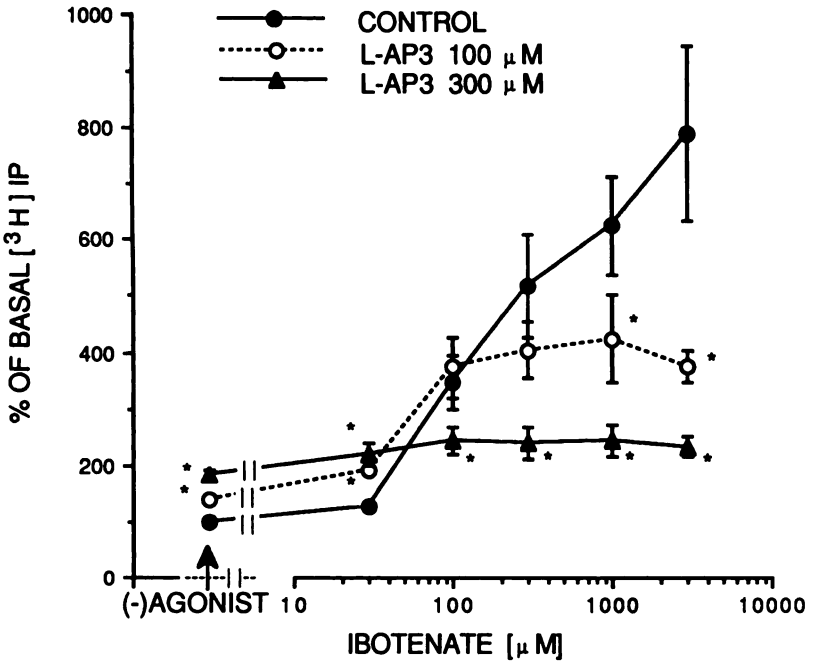

Fig. 4. Effect of L-AP3 on ibotenate concentration-effect stimulation of ^{3}H-phosphoinositide hydrolysis in hippocampal slices from 35–40-days-old rats. See legend to Fig. 1 for experimental details. Data (mean ± standard error of three experiments) are expressed as a percentage of the basal value in each experiment. The absolute basal value (mean ± standard error) from all experiments was 2175 ± 316 dpm of [^{3}H]IP/mg of protein. *, $p < 0.05$ when compared with the corresponding control.

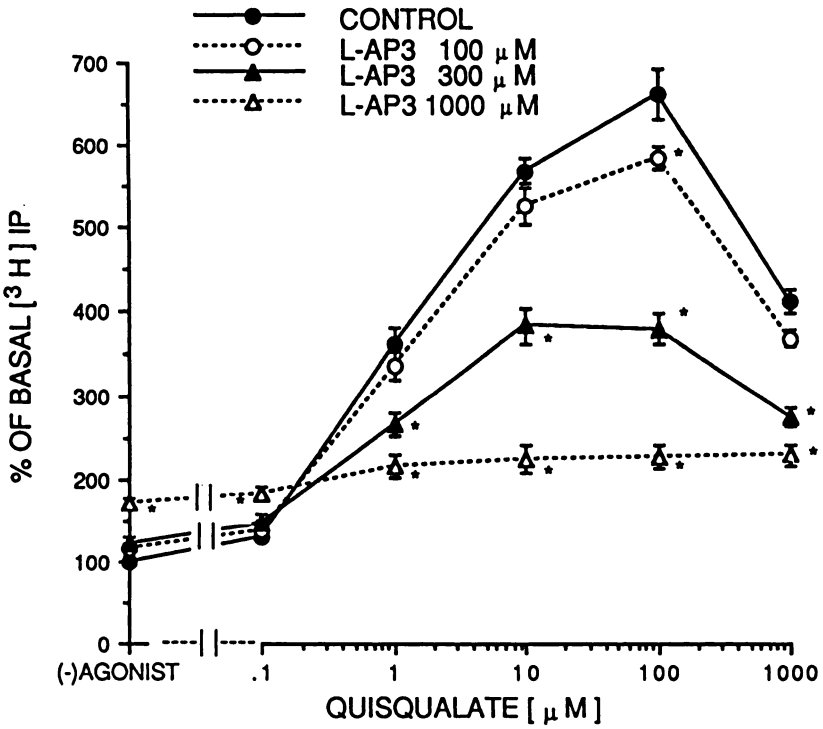

Fig. 5. Effect of L-AP3 on quisqualate concentration-effect stimulation of ^{3}H-phosphoinositide hydrolysis in cerebral cortical slices from 35–40-days-old rats. See legend to Fig. 1 for experimental details. Data (mean ± standard error of four experiments) are expressed as a percentage of the basal value in each experiment. The absolute basal value (mean ± standard error) from all experiments was 2981 ± 382 dpm of [^{3}H]IP/mg of protein. *, $p < 0.05$ when compared with the corresponding control.

receptor. It has been reported that up to 100 μM DL-AP3 does not appreciably displace [^{3}H]AP4 (22), [^{3}H]AMPA (23), [^{3}H] kainate (24), or NMDA-sensitive [^{3}H]glutamate (25) binding. Nevertheless, the concentrations of DL-AP3 required to inhibit phosphoinositide hydrolysis in mature rat brain slices (100 to 300 μM) are somewhat higher than those that have been studied at other EAA receptors. Furthermore, in neonatal brain slices there is a decreased sensitivity to α-amino-ω-phosphonocarboxylic acid antagonists such as AP4 and AP3. In neonatal hippocampal (20) and neonatal cerebral cortical (12) slices, 1000 μM DL-AP3 is required to maximally inhibit ibotenate-stimulated phosphoinositide hydrolysis. Longer chain α-amino-ω-phosphonocarboxylic acids such as AP5 or AP7 are very potent competitive inhibitors of the NMDA EAA receptor. NMDA receptor affinity for AP5 and AP7 is found in the D-isomers of these compounds at low micromolar concentrations (26). Thus, at the higher millimolar concentrations of AP3 that are needed to antagonize metabotropic EAA receptors, some NMDA affinity might be expected, particularly with the D-isomer. We showed here that D-AP3 is more potent in inhibiting NMDA receptor binding than it is in inhibiting EAA-stimulated phosphoinositide hydrolysis. However, L-AP3 was shown to be a selective metabotropic EAA receptor antagonist. At 1000 μM L-AP3, a concentration that maximally inhibits ibotenate or quisqualate stimulation in neonatal or mature brain slice preparations, there was little affinity for any ionotropic EAA receptor site.

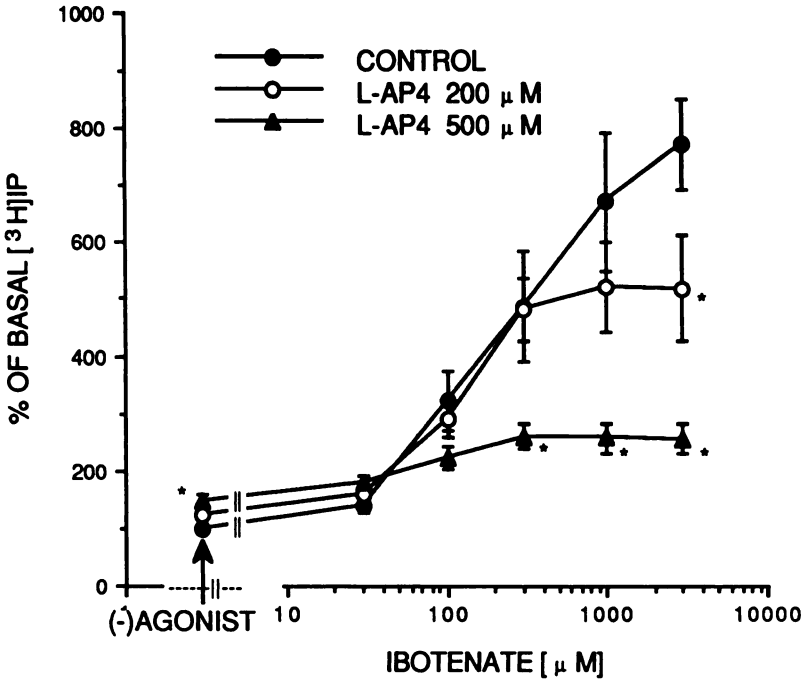

Fig. 6. Effect of L-AP4 on ibotenate concentration-effect stimulation of ^{3}H-phosphoinositide hydrolysis in hippocampal slices from 35–40-day-old rats. See legend to Fig. 1 for experimental details. Data (mean ± standard error of four experiments) are expressed as a percentage of the basal value in each experiment. The absolute basal value (mean ± standard error) from all experiments was 2560 ± 294 dpm of [^{3}H]IP/mg of protein. *, $p < 0.05$ when compared with the corresponding control.

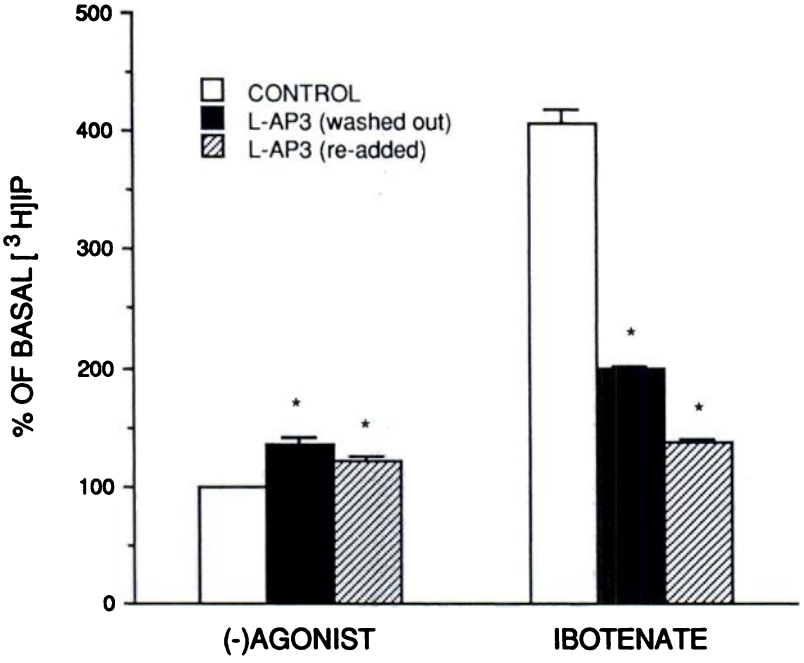

Fig. 7. Effect of washing out L-AP3 on inhibition of ibotenate-stimulated ^{3}H-phosphoinositide hydrolysis. Hippocampal slices from 35–40-day-old rats were incubated with *myo*-[^{3}H]inositol for 60 min to prelabel ^{3}H-phosphoinositides. Tissue was washed and then further incubated for 20 min in buffer with nonlabeled *myo*-inositol (10 mM) and LiCl (10 mM), in the absence and presence of L-AP3 (300 μM). All samples were then washed three times by resuspension in fresh buffer followed by centrifugation. An aliquot of ibotenate (10^{-3} M final concentration) or water vehicle (basal) was added to washed and resuspended tissue and incubated for 60 additional min. In the case of the L-AP3 (300 μM)-inhibited control, L-AP3 (300 μM) was added before washing and then it was "re-added," after washing, to the preincubated and washed tissue just before ibotenate. Data (mean ± standard error of four experiments) are expressed as a percentage of the basal value in each experiment. The absolute basal value (mean ± standard error) from all experiments was 6639 ± 390 dpm of [^{3}H]IP/mg of protein. *, $p < 0.05$ when compared with the corresponding control group.

L-AP3 is a β-phosphono-substituted derivative of L-aspartic acid, and aspartic acid acts as an agonist at metabotropic EAA receptors in brain slices (6, 20). This would suggest that L-AP3 and L-AP4 may be competitive antagonists at the aspartate or glutamate recognition site of the metabotropic receptor. However, in this study we could not overcome the inhibitory effects of either L-AP3 or L-AP4 with higher concentrations of EAA agonists. Our studies are consistent with a recent study of Godfrey and Taghavi (27), who showed that DL-AP4 is a noncompetitive inhibitor of quisqualate-stimulated phosphoinositide hydrolysis in slices of the adult rat cortex. In our study, the inhibitory effects of L-AP3 appeared irreversible, because they could not be prevented by washing L-AP3 out of the tissue before addition of the agonist. This suggests that these compounds may bind to the agonist site but they do not readily dissociate from the receptor. Alternatively, they may inhibit by acting at a site different from the receptor site that binds agonists such as quisqualate or ibotenate, and inhibition might occur by allosteric modulation of the agonist site or cell-to-cell interactions that might be occurring in the brain slice.

In the *Xenopus* oocyte expression system where the metabotropic receptor protein has been expressed from rat brain mRNA, it has been shown that metabotropic EAA responses are not sensitive to AP3 or AP4 inhibition (28). This suggests that L-AP3 and L-AP4 do not produce inhibitory effects by binding to the expressed receptor protein. The effects of L-AP3 and L-AP4 on selective ligand binding to the metabotropic EAA receptors would also be useful information in this regard.

However, there is currently no ligand that has been shown to bind selectively to the glutamate recognition site of the metabotropic EAA receptor.

It has been reported that NMDA ionotropic EAA receptor activation will inhibit stimulation of phosphoinositide hydrolysis by quisqualate (3). However, such an effect cannot explain the inhibitory effects of L-AP3 or L-AP4. L-AP3 and L-AP4 are phosphono-substituted derivatives of aspartate and glutamate, respectively, and ionotropic agonist-like effects at high concentrations could be hypothesized. However, as discussed above, only D-AP3 showed appreciable displacement of NMDA receptor binding at concentrations that inhibited EAA-stimulated phosphoinositide hydrolysis. L-AP3, which lacks affinity at ionotropic EAA receptors, as shown by binding here, was more potent than D-AP3 as an inhibitor of EAA-stimulated phosphoinositide hydrolysis. Thus, effects at ionotropic EAA receptors cannot explain L-AP3 or L-AP4 inhibitory effects. It is possible that these compounds produce cell death or dysfunction of a selective population of cells coupled to EAA-stimulated phosphoinositide hydrolysis by some other mechanism.

In summary, L-AP3 may be a useful pharmacological antagonist to further explore metabotropic EAA receptors. This compound, unlike DL-AP3 or L-AP4, inhibits metabotropic EAA receptors in brain slices at concentrations that do not affect other EAA receptors. However, further studies will be required to delineate the cellular mechanism by which L-AP3 and L-AP4 selectively inhibit EAA-stimulated phosphoinositide hydrolysis.

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Send reprint requests to: Dr. Darryle D. Schoepp, Central Nervous System Research, MC907, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285.