Synthesis and Biology of the Conformationally Restricted ACPD Analogue, 2-Aminobicyclo[2.1.1]hexane-2,5-dicarboxylic Acid-I, a Potent mGluR Agonist

Alan P. Kozikowski,*,† Darryl Steensma,† Gian Luca Araldi,† Werner Tückmantel,† Shaomeng Wang,† Sergey Pshenichkin,‡ Elena Surina,‡ and Jarda T. Wroblewski‡

Drug Discovery Program, Institute of Cognitive and Computational Sciences, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, D.C. 20007-2197, and Department of Pharmacology, Georgetown University Medical Center, 3900 Reservoir Road, NW, Washington, D.C. 20007

Received October 21, 1997

To better characterize the roles of metabotropic glutamate receptors (mGluRs) in physiological and pathophysiological processes, there is an important need to learn more about the structural features relevant to the design of novel, high-affinity ligands that are family and subtype specific. To date, many of the biological studies that have been conducted in the area of mGluR research have made use of the agonist (1*S*,3*R*)-ACPD. This compound has been shown to act as an agonist at both the group I and group II receptors while showing little selectivity among the four subtypes belonging to these two groups. Moreover, (1*S*,3*S*)-ACPD, the cis isomer, shows negligible activity at group I receptors and is a good agonist of mGluR2. Since ACPD is itself somewhat flexible, with four distinctive conformations being identified from molecular modeling studies for the trans isomer and five conformations for the cis isomer, we believed that it would be of interest to examine the activity of an ACPD analogue that has been constrained through the introduction of a single carbon atom bridge. Accordingly, we have prepared an aminobicyclo[2.1.1]hexanedicarboxylic acid (ABHxD-I) analogue of ACPD. The synthesis of this compound was accomplished by use of an intramolecular [2 + 2] photocycloaddition reaction, in which four distinct isomers were isolated. Of these four compounds, only a single isomer, ABHxD-I (**6a**), was found to be a potent agonist of the mGluRs. This compound, which expresses the fully extended glutamate conformation, was found to be more potent than ACPD at all six of the eight mGluR subtypes that were investigated and to be comparable to or more potent than the endogenous ligand, glutamate, for these receptors. Interestingly, despite its fixed conformation, ABHxD-I, like glutamate, shows little subtype selectivity. Through modeling studies of ABHxD-I (**6a**), ABHD-VI, LY354740, (1*S*,3*R*)-ACPD, (1*S*,3*S*)-ACPD, and L-glutamate, we conclude that the aa conformation of L-glutamate is the active conformation for both group I and group II mGluRs. Moreover, the modeling-based comparisons of these ligands suggest that the selectivity exhibited by LY354740 between the group I and group II mGluRs is not a consequence of different conformations of L-glutamate being required for recognition at these mGluRs but rather is related to certain structural elements within certain regions having a very different impact on the group I and group II mGluR activity. The enhanced potency of ABHxD-I relative to *trans*-ACPD commends it as a useful starting point in the design of subtype selective mGluR ligands.

Introduction

The amino acid glutamate (Glu) plays a pivotal role in biological processes ranging from memory and learning to neuronal degeneration. This major excitatory amino acid (EAA) acts through disparate Glu receptors, which can be categorized into two distinct types, the ionotropic glutamate receptors and the metabotropic glutamate receptors. The ionotropic Glu receptors, or iGluRs, are associated with integral cation-specific ion channels and include the NMDA [*N*-methyl-D-aspartic acid], AMPA [2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid], and KA (kainic acid) subtypes.[1] On the other hand, the metabotropic Glu receptors (mGluR) are coupled to cellular effectors through GTP-binding proteins.[2] The mGluRs have been distinguished pharmacologically from the iGluRs by the use of the mGluR-selective agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD] generally through measurements involving phosphoinositide hydrolysis or Ca^{2+} mobilization. To date the use of expression cloning techniques has led to the identification of eight mGluR subtypes which have been placed into three major categories based on their molecular structure, signal transduction mechanisms, and pharmacological properties.[3] Group I mGluRs (mGluR1 and 5) are coupled to phosphoinositide (PI) hydrolysis, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) are negatively linked to adenylyl cyclase activity. The group I receptors are more sensitive to quisqualic acid than they are to ACPD, the group II receptors are more sensitive to ACPD than quisqualic acid, and the group III receptors are most sensitive to 2-amino-4-phosphonobutyric acid (L-AP4). While the mGluRs have been shown to play a fundamental role in the modulation of synaptic transmission, considerable attention has also

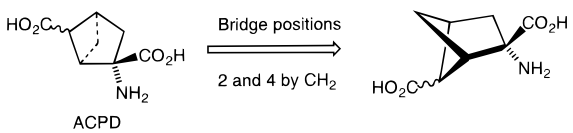
† Drug Discovery Program.
‡ Department of Pharmacology.

S0022-2623(97)00719-X CCC: $15.00
Published on Web 04/11/1998

been given to their role in neurodegenerative diseases including Alzheimer's disease, cerebral ischemia, Parkinsonism, spinal cord injury, and epilepsy.[4]

To better characterize the roles of GluRs in physiological and pathophysiological processes, there is an important need to learn more about the structural features relevant to the design of novel, high-affinity ligands that are family and subtype specific. To date, many of the biological studies that have been conducted in the area of mGluR research have made use of the agonist (1*S*,3*R*)-ACPD. This compound has been shown to act as an agonist at both the group I and group II receptors while showing little selectivity among the four subtypes belonging to these two groups. Moreover, (1*S*,3*S*)-ACPD, the cis isomer, shows negligible activity at group I receptors and is a good agonist of mGluR2.[5] Since ACPD is itself somewhat flexible, with four distinctive conformations being identified from molecular modeling studies for the trans isomer, and five conformations for the cis isomer,[6] we believed that it would be of interest to examine the activity of ACPD analogues that have been constrained through the introduction of a single carbon atom bridge. Specifically, we envisioned adding the methylene group so as to bridge carbon atoms 2 and 4 of the cyclopentane ring. In pursuit of this idea, it was of interest to find a literature report describing the synthesis of several norbornane analogues of ACPD.[7] However, none of these compounds were found to be particularly promising because of their low potency, with the best compound acting as an antagonist at mGluR1a with a K_B value of 300 μM. Also, this series of molecules was prepared only in racemic form. As will be seen below, the biological activity found for our one carbon lower homologues stands in stark contrast to the activity reported for the norbornanes.

Within the field of new compound development for the mGluRs, notable recent achievements include the identification of 2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) as a potent group II agonist (EC_{50} = 5.1 nM at mGluR2),[8] and reports of the enhanced potency of (2*S*,1′*S*,2′*S*,3′*R*)-2-(2′-carboxy-3′-phenylcyclopropyl)glycine (PCCG-4)[9] and (2*S*,1′*S*,2′*S*)-2-(2′-carboxycyclopropyl)-3-(9-xanthenyl)alanine (LY341495)[10] as mGluR2 antagonists. (2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylic acid (2*R*,4*R*-APDC), a structural analogue of (1*S*,3*R*)-ACPD, is selective for human mGluR2 and mGluR3 and is inactive at other mGluR subtypes.[11] The 1-benzyl derivative of (2*R*,4*R*)-APDC, on the other hand, shows some selectivity for mGluR6.[6] 3,5-Dihydroxyphenylglycine is a selective agonist for group I mGluRs with no activity at the group II and group III mGluRs.[12] Last, a recent article provides an account of the mGluR activity of ACPD analogues containing an additional carboxyl group.[13]



Chemical Synthesis of the ABHxDs

As an obvious synthetic approach to the ABHxDs, we considered carrying out an intramolecular photochemical [2 + 2] cycloaddition reaction of an appropriately functionalized dehydroglutamate. One compelling virtue of this approach was that we could employ L-serine as a chiral educt, thus allowing us to access the required amino diacids in optically pure form. Accordingly, by employing the procedure first developed by Seebach,[14] the oxazolidine **1** was generated in good yield and in excellent optical purity (Scheme 1). Next, treatment of **1** with HCl in methanol afforded α-allylserine methyl ester which was protected with benzyl chloroformate to give compound **2**. The alcohol was oxidized under Swern conditions, and the resulting aldehyde was treated with methyl (triphenylphosphoranylidene)acetate to produce the α,β-unsaturated ester **3**, the required precursor for the [2 + 2] photocycloaddition chemistry.[15] Diene **3** was then irradiated in the presence of acetophenone in benzene as solvent using either a quartz or Pyrex apparatus to afford a mixture of all four possible bicyclic products **4a**−**d**.

Compounds **4a**−**c** were isolated as a chromatographically inseparable mixture while **4d** was isolated as a single compound. The mixture of compounds **4a**−**c** was deprotected with Pd/C under an atmosphere of H_2 to afford compounds **5a**−**c** as three chromatographically separable isomers. Compound **4d** was also deprotected using H_2 and Pd/C. The diesters **5a**−**d** were individually treated with 6 N HCl at reflux for 1 h to afford the final products **6a**−**d**.

Stereochemical Assignments

The assignment of stereochemistry to each of the four diastereoisomeric amino acids **6a**−**d** was based on high-field NMR experiments of the methyl esters **5a**−**d**. From the 1D ^{1}H NMR spectrum, the diagnostic W-coupling constant J_{1-4} for the bicyclo[2.1.1]hexanes **5a**−**d** was determined to be 6.9 Hz, a number which is typical for the coupling of bridgehead protons. This value is also in agreement with that reported in the literature for 2-azabicyclo[2.1.1]hexane.[16] A remarkably large W-coupling of about 8 Hz was observed between H_5 and $H_{6\alpha}$ for both compounds **5a** and **5b**. Also characteristic for these two derivatives is the W-coupling between $H_{3\beta}$ and $H_{6\beta}$ of 2.4 and 2.7 Hz. 2D-NOESY experiments on compound **5a** show correlations between H_5 and $H_{3\beta}$, H_5 and NH_2, and $H_{6\alpha}$ and $H_{3\alpha}$ (Scheme 2). These results suggest that the carboxylate groups in **5a** have a cis relationship. The 2D NOESY experiments on **5b** revealed correlations between $H_{6\alpha}$ and $H_{3\alpha}$ and between $H_{6\alpha}$ and NH_2. These results define a trans relationship for the two carboxylate groups (Scheme 2).

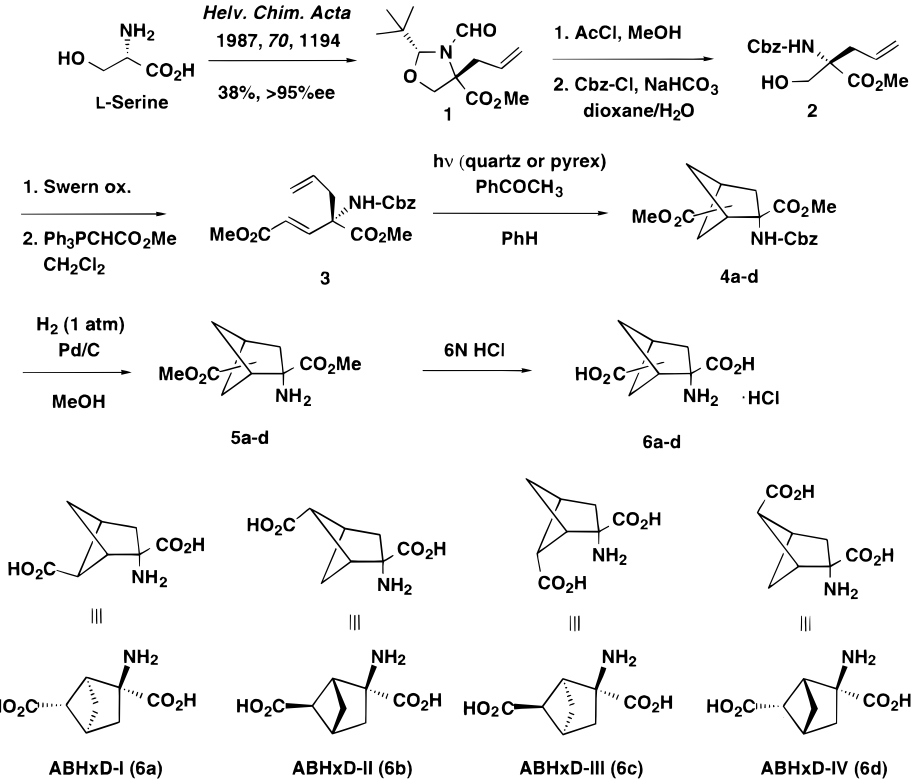
The 1D ^{1}H NMR spectra for compounds **5c** and **5d** show no coupling between H_5 and $H_{6\alpha}$ and exhibit small coupling constants (2.1 Hz) between $H_{3\beta}$ and $H_{6\beta}$. The depicted stereochemistry of **5c** follows from the observation that this compound undergoes a facile intramolecular cyclization reaction to afford the corresponding five-membered ring lactam.

The above spectral and chemical information thus provides strong support for our stereochemical assignments.

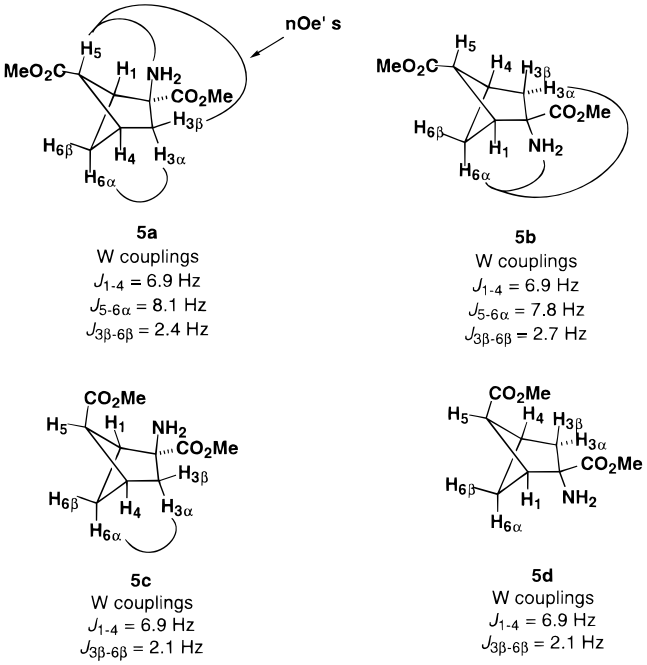
Pharmacological Results

The activity of ABHxD isomers was tested in cell lines expressing the individual mGluRs. Group I receptors

Scheme 1. Synthetic Route to the ABHxD Analogues of ACPD



Scheme 2. Structural Elucidation of Compounds **5a**−**d**



(mGluR1a and mGluR5a) are coupled to phospholipase C, and their activity was determined by accumulation of inositol phosphates. Group II receptors included the cAMP-coupled mGluR2, as well as the chimeric receptor mGluR3/1a which, as shown previously,[17] combines the pharmacological properties of mGluR3 with the activation of phospholipase C. Group III receptors were represented by mGluR4a and mGluR6, both coupled to the inhibition of cAMP formation.[18] The agonist dose−response curves for all four ABHxD isomers are shown in Figure 1 (group I mGluRs), Figure 2 (group II mGluRs), and Figure 3 (group III mGluRs). These studies are summarized in Table 1, which shows the EC_{50} values at all six mGluR subtypes. Among the tested compounds, ABHxD-I is the most potent in activating all six mGluR subtypes, with potencies in most cases comparable to or higher than that of the endogenous agonist glutamate. In contrast, ABHxD-IV shows little activity at any of the tested mGluRs. In all cases in which agonist activity was not observed at 1 mM concentrations, compounds were also tested for possible antagonist activity. However, none of the ABHxD isomers showed antagonist action at any of the six mGluRs (data not shown).

To further establish their selectivity, the ABHxD isomers were tested for possible activity at ionotropic glutamate receptors by measuring $^{45}Ca^{2+}$ influx in primary cultures of cerebellar neurons.[19] As shown in Table 2, in conditions where agonists of all ionotropic glutamate receptors are active, none of the ABHxD isomers stimulated $^{45}Ca^{2+}$ influx. Tests of possible antagonistic action showed that several ABHxD isomers used at 1 mM concentrations partially inhibited NMDA responses. The strongest inhibition was produced by ABHxD-IV, which was inactive at mGluRs. The responses induced by kainate and AMPA were not substantially inhibited by any of the ABHxD isomers. ABHxD-I, the most potent mGluR agonist, produced a slight inhibition of NMDA and kainate responses; however, this effect appeared at a 1 mM concentration, which is 3 orders of magnitude higher than the potency of ABHxD-I to activate most of the mGluRs.

Molecular Modeling Studies

To gain further insights into the structural/conformational requirements for ligand potency and selectivity among the mGluR groups, we have performed confor-

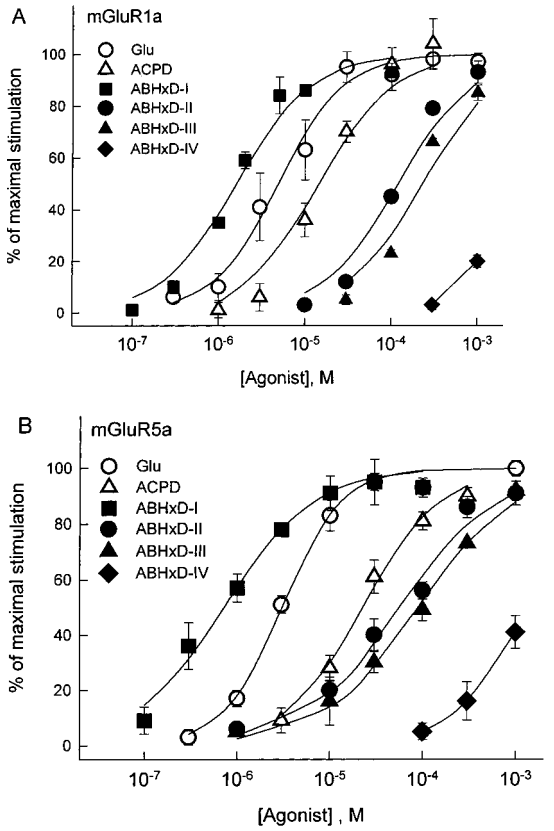


Figure 1. Agonist dose−response curves of the different ABHxD isomers at group I mGluRs. IP formation was measured in cells expressing mGluR1a (A) and mGluR5a (B) receptors after a 30 min incubation with the indicated concentration of respective agonists. Data are expressed as the percent of maximal response obtained with 1 mM glutamate after subtracting the basal IP formation. Values are means ± SEM of four to six independent experiments performed in duplicate.

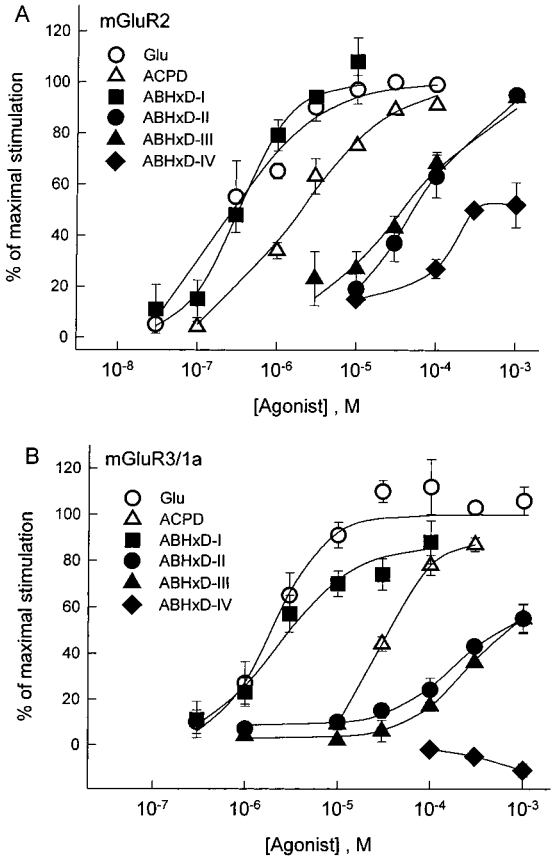


Figure 2. Agonist dose−response curves of the different ABHxD isomers at group II mGluRs. In cells expressing mGluR2 (A), agonist activity was measured as the decrease of forskolin-induced cAMP accumulation during a 10 min incubation with the indicated concentrations of agonists. In cells expressing the chimeric mGluR3/1a (B), the IP formation was measured after a 30 min incubation with agonists. Data are expressed as the percent of maximal response obtained with 1 mM glutamate after subtracting the basal values. Values are means ± SEM of four to six independent experiments performed in duplicate.

mational analyses on all four ABHxD isomers using high-temperature molecular dynamics simulations followed by energy minimization of each conformation generated by the simulations. These studies confirmed the expectation that the ABHxD isomers are rather inflexible. The superposition of the energy-minimized conformations of each of these isomers using all heavy atoms, excluding the four terminal oxygens, gave a root-mean-square value of less than 0.1 Å, indicating that each isomer essentially adopts only one low-energy conformation.

The low-energy conformation of each compound was then compared to that of L-glutamate, the endogenous ligand, and LY354740 (structure shown at the bottom of Table 3), a potent and selective group II mGluR agonist. L-glutamate adopts nine low-energy, distinct conformations. The aa conformation of L-glutamate is believed to be the biologically active conformation in binding to mGluRs.[6,9] We have shown previously that LY354740 can adopt two slightly different, low-energy conformations, both of which have excellent overlap (RMS = 0.17 Å) with L-glutamate's aa conformation.[6] LY354740 is a potent group II mGluR agonist with an EC_{50} value of 5.1 nM at human mGluR2 and 24.3 nM at human mGluR3, while it has no activity at cells expressing human mGluR4 or mGluR7 (group III) and fails to activate or inhibit human mGluR1a or mGluR5a when tested at concentrations up to 1 μM.[8] Consistent with the findings of others,[8,9,21] we have therefore concluded that the aa conformation of L-glutamate is the active conformation for the group II mGluRs.

Among the four ABHxD isomers, ABHxD-II (**6b**), ABHxD-III (**6c**), and ABHxD-IV (**6d**) have very poor overlap with the aa conformation of L-glutamate (Table 3 and Figure 4), and their RMS values range from 0.58 Å (**6b**) to 0.89 Å (**6d**). These results are consistent with the poor activity of **6b**−**d** at the group II mGluRs (Table 1). On the other hand, ABHxD-I (**6a**) has an excellent overlap with the aa conformation of L-glutamate, with an RMS value of 0.18 Å (Table 3 and Figure 4). This result is again consistent with the activity of **6a** at group II mGluRs (Table 1).

ABHxD-I (**6a**) also shows fairly potent activity at the group I mGluRs (Table 1). Although both ABHxD-I and LY354740 are rigid structures and exhibit excellent overlap with the aa conformation of L-glutamate (RMS value 0.17 vs 0.18 Å), it is intriguing that LY354740 is a very selective group II mGluR agonist, while ABHxD-I (**6a**) exhibits little selectivity among group I, group II, and group III mGluRs. Accordingly, it is clear that structural elements other than conformation must play an important role in determining the selectivity of these compounds for the mGluR subtypes.

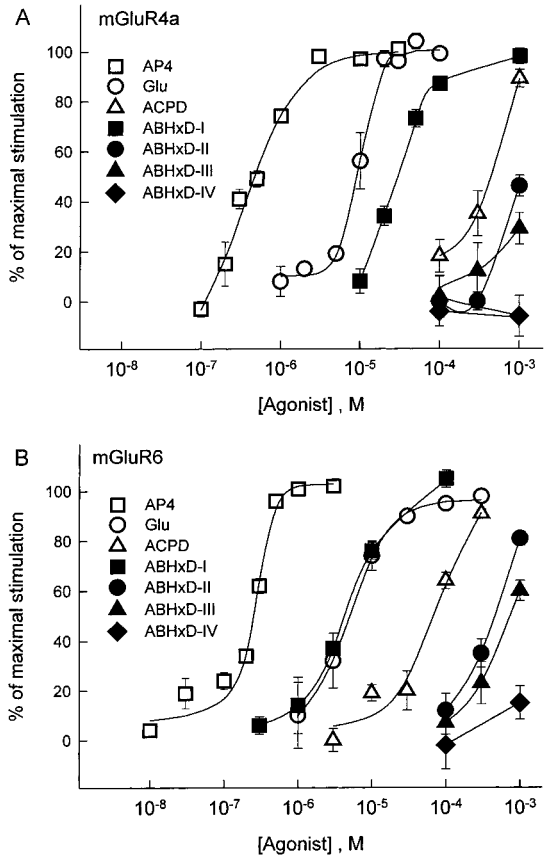


Figure 3. Agonist dose−response curves of the different ABHxD isomers at group III mGluRs. In cells expressing mGluR4a (A) and mGluR6 (B), agonist activity was measured as the decrease of forskolin-induced cAMP accumulation during a 10 min incubation with the indicated concentrations of agonists. Data are expressed as the percent of maximal response obtained with 1 mM glutamate for mGluR4a and with 1 mM AP4 for mGluR6 after subtracting the basal values. Values are means ± SEM of four to six independent experiments performed in duplicate.

To shed more light on the possible structural elements that may be important to the selectivity and/or potency of these compounds, we have compared LY354740, ABHxD-I (**6a**), and the aa conformation of L-glutamate. In addition, we have included one of the aminobicyclo-[2.2.1]heptanedicarboxylic acids (ABHD-VI), which can be viewed as an analogue of *cis*-ACPD that has been rigidified by the incorporation of a two-carbon bridge (see bottom of Table 3). ABHD-VI was found to have only weak activity at group I (antagonist at mGluR1a) and group II (agonist at mGluR2) mGluRs when tested at a concentration of 3 mM.[7] The superposition of these compounds on the aa conformation of L-glutamate is shown in Figure 5. Like LY354740 and ABHxD-I (**6a**), ABHD-VI was also found to show excellent overlap with the aa conformation of L-glutamate (RMS = 0.17 Å, Table 3 and Figure 5). This result clearly suggests that good overlap with the aa conformation of L-glutamate is a necessary but not a sufficient condition to achieve potent activity at group I and group II mGluRs. As can be observed in Figure 5, the comparison of these compounds reveals two regions where significant structural differences occur among these compounds. The crucial structural difference between LY354740 and ABHxD-I (**6a**) occurs in region A, while in all other regions these two compounds occupy similar three-dimensional space. Because LY354740 is a potent and selective group II mGluR ligand and ABHxD-I (**6a**) is a fairly potent but nonselective mGluR ligand, occupancy of region A appears to be beneficial to the activity at group II mGluRs and/or to be detrimental to activity at group I and group III mGluRs. The crucial structural difference between ABHD-VI and ABHxD-I (**6a**) occurs in region B. The very low potency of ABHD-VI at both group I and group II mGluRs clearly suggests that the occupancy of region B by a ligand may be detrimental to its activity at both group I and group II mGluRs.

(1*S*,3*R*)-ACPD has been used as a mGluR ligand although it has little selectivity between group I and group II mGluRs. On the other hand, (1*S*,3*S*)-ACPD is a selective ligand for group II mGluRs. However, both ligands are not very potent. Conformational analysis showed that (1*S*,3*R*)-ACPD can adopt four different conformations and (1*S*,3*S*)-ACPD can adopt five different conformations using an RMS equal to 0.1 Å as the cutoff value.[20] Superposition of these low-energy, distant conformations of (1*S*,3*R*)-ACPD on the aa conformation of L-glutamate showed that none of these conformations displays substantially good overlap, with an average RMS value of 0.59 Å being observed. The previously proposed mGluR active conformation for (1*S*,3*R*)-ACPD has an RMS value of 0.62 Å when superimposed on the aa conformation of L-glutamate (Figure 6).[7] The modeling results for (1*S*,3*R*)-ACPD are consistent with the experimental results that (1*S*,3*R*)-ACPD is not a very potent mGluR ligand.

For (1*S*,3*S*)-ACPD, one of the low-energy conformations has an excellent overlap with the aa conformation of L-glutamate (Figure 6), with an RMS value of 0.20 Å, while the other four conformations overlap poorly with the aa conformation of L-glutamate (the RMS values range from 0.47 to 0.78 Å). Based solely upon the excellent overlap of (1*S*,3*S*)-ACPD on the aa conformation of L-glutamate, one might predict (1*S*,3*S*)-ACPD to be a potent mGluR ligand. Experimentally, (1*S*,3*S*)-ACPD is a modestly active ligand at group II (13 μM at mGluR2[2] which compares to an EC_{50} of ~2 μM in our own system) and group III (50 μM at mGluR4a)[2] receptors as compared to L-glutamate, while its activity at group I is >300 μM. In comparison with ABHD-VI (Figure 6), one of the carbon atoms of (1*S*,3*S*)-ACPD was found to occupy region B. In view of the fact that (1*S*,3*S*)-ACPD, like ABHD-VI, is poorly active at group I receptors, one may conclude that occupancy of region B is detrimental to group I activity, but that occupancy of this region does not preclude group II activity. These results once again reveal that an appropriate ligand conformation, while necessary for potent mGluR activity, is not in and of itself sufficient for achieving such activity.

In summary, through modeling studies of ABHxD-I (**6a**), ABHD-VI, LY354740, (1*S*,3*R*)-ACPD, (1*S*,3*S*)-ACPD, and L-glutamate, we conclude that the aa conformation of L-glutamate is the active conformation for both group I and group II mGluRs. The selectivity exhibited by LY354740 between the group I and group II mGluRs is not a consequence of different conformations of L-glutamate being required for recognition at

Table 1. EC_{50} Values Obtained for the ABHxDs at Six of the mGluR Subtypes[a]

	EC_{50} values, μM					
	group I mGluRs		group II mGluRs		group III mGluRs	
agonist	mGluR1a	mGluR5a	mGluR2	mGluR3/1a	mGluR4a	mGluR6
Glu	4.9 ± 0.21	3.1 ± 0.23	0.29 ± 0.07	1.9 ± 0.31	9.8 ± 0.81	4.9 ± 0.37
(±)-*trans*-ACPD	15 ± 2.2	23 ± 2.6	2.0± 0.3	40 ± 5.8	~800	82 ± 6.2
(1*S*,3*S*)-ACPD	>300[b]	>300[b]	13[b]	30 (mGluR3)[b]	50[b]	
AP4					0.33 ± 0.09	0.28 ± 0.03
ABHxD-I (**6a**)	1.6 ± 0.14	0.72 ± 0.11	0.33 ± 0.06	2.2 ± 1.5	23 ± 7.1	5.3 ± 0.93
ABHxD-II (**6b**)	121 ± 10	57 ± 6	54 ± 9	~1000[c]	~1000	~800
ABHxD-III (**6c**)	232 ± 23	91 ± 11	38 ± 10	~1000[c]	>1000	>1000
ABHxD-IV (**6d**)	>1000	>1000	>300[c]	>1000	>1000	>1000

[a] EC_{50} values were calculated by fitting the normalized data (shown in Figures 1−3) to the logistic equation by nonlinear regression. Values represent means ± SEM from four to six experiments performed in duplicate as described in the Experimental Section. [b] Data taken from ref 2. [c] These EC_{50} values were estimated assuming that the compounds act as full agonists.

Table 2. Effects of ABHxD Compounds on Ionotropic Glutamate Receptors in Cerebellar Neurons[a]

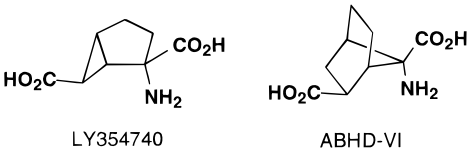
			antagonist activity[c]		
additions		agonist activity[b] % of basal activity	% of NMDA effect	% of kainate effect	% of AMPA effect
NMDA	100 μM	245 ± 24	100 ± 9.8		
kainate	50 μM	243 ± 14		100 ± 5.9	
AMPA	30 μM	389 ± 23			100 ± 6.0
MK-801	1 μM	62 ± 14	0 ± 0.0		
NBQX	10 μM	79 ± 5.9		3 ± 0.3	11 ± 0.6
ABHxD-I	1 mM	102 ± 15	70 ± 3.9	77 ± 4.9	95 ± 9.9
ABHxD-II	1 mM	82 ± 7.2	58 ± 2.5	94 ± 8.1	88 ± 3.2
ABHxD-III	1 mM	93 ± 4.5	96 ± 4.4	97 ± 3.4	100 ± 2.0
ABHxD-IV	1 mM	69 ± 11	17 ± 0.9	91 ± 3.5	86 ± 2.8

[a] Activity at ionotropic glutamate receptors was measured as $^{45}Ca^{2+}$ influx in cerebellar granule neurons as described in the Experimental Section. All values represent means ± SEM from six measurements performed on two separate preparations of neuronal cultures. [b] Agonist activity at all ionotropic glutamate receptors was measured in Mg^{2+}-free medium supplemented with 1 μM glycine to allow for activity at NMDA receptors, and with 10 μM cyclothiazide to inhibit desensitization of AMPA receptors, and is expressed as percent of basal $^{45}Ca^{2+}$ influx. [c] Antagonist activity at NMDA receptors was measured in a Mg^{2+}-free medium in the presence of 100 μM NMDA, 1 μM glycine, and 10 μM NBQX to inhibit non-NMDA receptors. Antagonism of kainate action was tested in the presence of 1 mM Mg^{2+}, 50 μM kainate, and 1 μM MK-801 to inhibit NMDA receptors, while the effects on AMPA action were measured in the presence of 1 mM Mg^{2+}, 30 μM AMPA, 10 μM cyclothiazide, and 1 μM MK-801. All antagonist effects are expressed as percent of $^{45}Ca^{2+}$ influx induced by agonist alone.

Table 3. Calculated RMS Deviations for Selected mGluR Ligands in Comparison to the aa Conformation of L-Glutamate[a]

ligand	RMS (Å)	ligand	RMS (Å)
ABHxD-I (**6a**)	0.18	LY354740	0.17
ABHxD-II (**6b**)	0.58	ABHD-VI	0.17
ABHxD-III (**6c**)	0.77	(1*S*,3*R*)-ACPD	0.62
ABHxD-IV (**6d**)	0.89	(1*S*,3*S*)-ACPD	0.20

[a]



LY354740 ABHD-VI

the group I and group II mGluRs, but rather is related to certain structural elements within a certain region (region A) having a very different impact on the group I and group II mGluR activity. We have determined that the occupancy of region A by a ligand may enhance its activity at group II mGluRs, while possibly being detrimental to its group I and group III mGluR activity. Modeling comparisons of ABHD-VI also suggests that the occupancy of region B is prohibitive to achieving good activity at group I mGluRs. Last, on the basis of the fairly good activity of ABHxD-I (**6a**) at group III mGluRs, we propose that the active conformation for the group III mGluRs should be one that resembles the aa conformation of L-glutamate. The current modeling studies thus offer additional insights into the design of potent, group specific mGluR ligands.

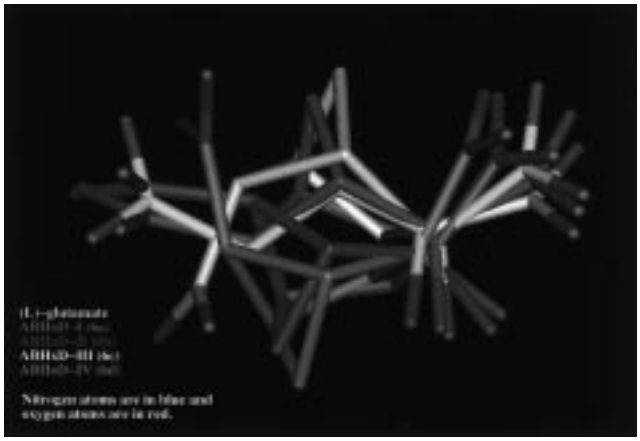


Figure 4. Overlay of the minimized conformations of the ABHxD isomers **6a**−**d** with the aa conformation of L-glutamate.

Discussion

As is apparent from the data presented in Table 1, the bicycle ABHxD-I (**6a**) is more potent than ACPD as an agonist at all six of the eight subtypes tested. The increase in potency ranges from 6- to 34-fold. The other three isomers show much poorer or little activity. It is interesting to note that the rank order of potency of *trans*-ACPD at the mGluRs is mGluR2 > 1a > 5a > 3 > 6 > 4a, while for the bicycle **6a** the rank order is mGluR2 > 5a > 1a > 3 > 6 > 4a. These potency orders

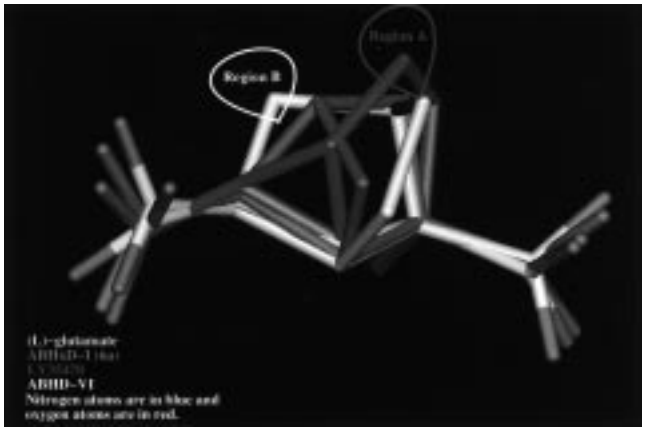


Figure 5. Overlay of ABHxD-I, LY354740, and ABHD-VI with the aa conformation of L-glutamate. Region A highlights the extra space occupied by LY354740, while region B demarcates the extra steric volume of ABHD-VI.

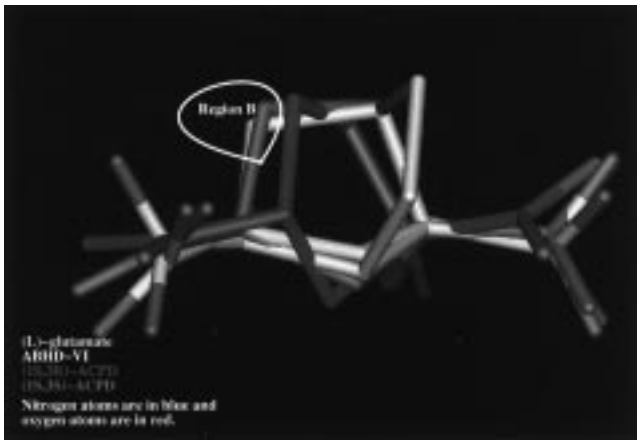


Figure 6. Overlay comparisons of (1*S*,3*R*)-ACPD, (1*S*,3*S*)-ACPD, ABHD-VI, and L-glutamate, which emphasizes partial occupation of region B by (1*S*,3*S*)-ACPD.

are basically the same, except for the switch in activity at 1 and 5. The value of **6a** as a research tool is further enhanced by its general lack of activity for members of the ionotropic receptor family.

Previously it has been proposed by Pellicciari that the fully extended conformation of glutamate represents the bioactive conformation at the group II mGluRs.[8,9,21] Due to the rigidity of the bicycle **6a**, it is clear that its embedded glutamate moiety may adopt only the fully extended (aa) conformation. This structural preference is supported by the modeling studies, as detailed in the previous section. The excellent potency of **6a** for mGluR2 therefore provides further experimental support for this proposed structural preference. In view of the more or less parallel activity of *trans*-ACPD and **6a** at each of the six subtypes tested, it would also be reasonable to conclude that the active conformation of ACPD at each of these receptor subtypes must be that which best mimics an extended conformation.

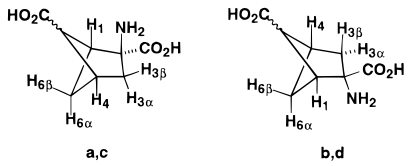
In light of the fact that ABHxD-I shows little real selectivity for any of the mGluRs, with the exception of mGluR4a at which it is the least potent, one could argue that the extended glutamate conformation is readily accommodated by the majority of the mGluR subtypes.[22] In fact, it is rather intriguing that a rigid molecule like this shows so little difference in its ability to interact with and to activate the individual mGluRs in comparison to the readily mobile ligand L-glutamate. Therefore, to achieve any real differences in subtype selectivity with ABHxD-I or related molecules will require the addition of other functionality capable of either preventing or enhancing receptor interactions through a combination of steric and electronic effects. In particular, as already delineated in the modeling section, occupancy of region A (Figure 5) by a ligand appears to enhance group II activity, while possibly being detrimental to group I and group III activity. Moreover, ligand occupancy of region B appears to be prohibitive to group I activity.

In light of the higher potency of **6a** relative to ACPD, we would suggest the former to be the preferred agent for use in future biological studies of mGluRs in cases where subtype selectivity is not required. In fact, studies of a ligand capable of interacting much like glutamate with the individual mGluRs while being devoid of ionotropic activity may prove intriguing. ABHxD-I (**6a**) is a possible candidate for future drug design work, as it bears an additional carbon atom, relative to ACPD, to which diverse substituents may be appended in order to obtain compounds that are subtype selective.

Experimental Section

General. Starting materials were obtained from Aldrich Chemical Co. or from other commercial suppliers. Solvents were purified as follows: diethyl ether was distilled from phosphorus pentoxide; THF was freshly distilled under nitrogen from sodium benzophenone.

IR spectra were recorded on an ATI Mattson Genesis spectrometer. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Unity Inova instrument at 300 and 75.46 MHz, respectively. ^{1}H chemical shifts (δ) are reported in ppm downfield from internal TMS. ^{13}C chemical shifts are referenced to $CDCl_3$ (central peak, δ = 77.0 ppm), benzene-d_6 (central peak, δ = 128.0 ppm), or DMSO-d_6 (central peak, δ = 39.7 ppm). NMR assignments were made with the help of COSY, DEPT, HETCOR, and NOESY experiments. The subscript numbers used to identify the ABHxD protons are defined according to the following numbering scheme:



Melting points were determined in Pyrex capillaries with a Thomas-Hoover Unimelt apparatus and are uncorrected. Mass spectra were measured in the EI mode at an ionization potential of 70 eV. TLC was performed on Merck silica gel $60F_{254}$ glass plates; column chromatography was performed using Merck silica gel (60−200 mesh). The following abbreviations are used: DMSO = dimethyl sulfoxide, ether = diethyl ether; THF = tetrahydrofuran; DCM = dichloromethane.

(2*S*)-Methyl 2-[(Benzyloxycarbonyl)amino]-2-(hydroxymethyl)-4-pentenoate (2). Acetyl chloride (460 mL, 6.47 mol) was added via addition funnel to methanol (1.08 L) at 0 °C. A solution of **1** (18.3 g, 71.8 mmol) in methanol (10 mL) was added and the reaction mixture refluxed for 3 h. Evaporation of the volatiles in vacuo produced a white solid which was dissolved in dioxane (150 mL) and H_2O (50 mL). $NaHCO_3$ (12.1 g, 143.7 mmol) and benzyl chloroformate (20.5 mL, 143.67 mmol) were added, and the reaction mixture was stirred overnight. The reaction mixture was extracted with EtOAc (3 × 200 mL), and the combined organic phases were washed

with brine, dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2, 50% EtOAc/hexane) afforded **2** (12.3 g, 58%) as a colorless oil: $[\alpha]^{25}_D$ −0.4° (*c* 2.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.52 (dd, J = 14.1 and 7.2 Hz, 1H), 2.78 (dd, J = 13.8 and 7.8 Hz, 1H), 3.51 (br s, 1H), 3.75 (s, 3H), 3.88−3.76 (m, 1H), 4.18−4.07 (m, 1H), 5.15−5.01 (m, 4H), 5.68−5.52 (m, 1H), 5.79 (br s, 1H), 7.37−7.29 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 36.7, 52.7, 64.9, 65.1, 66.7, 119.9, 127.9, 128.0, 128.4, 131.0, 136.0, 155.2, 172.3; IR (film) 3407, 1724 cm^{-1}.

(2*S*)-Methyl 4-Allyl-4-[(benzyloxycarbonyl)amino]glutaconate (3). A solution of DMSO (0.57 mL, 7.8 mmol) in CH_2Cl_2 (8 mL) was added to a solution of oxalyl chloride (0.51 mL, 5.9 mmol) in CH_2Cl_2 (4 mL) over 15 min. Alcohol **2** (1.15 g, 3.95 mmol) in CH_2Cl_2 (20 mL) was added over 10 min. After an additional 10 min of stirring, Et_3N (2.73 mL, 19.6 mmol) in CH_2Cl_2 (15 mL) was added over 10 min and the temperature was maintained between −60 and −70 °C for 30 min. The reaction mixture was quenched with a saturated solution of NH_4Cl (30 mL) and extracted with CH_2Cl_2 (5 × 40 mL). The combined organic fractions were dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in CH_2Cl_2 (45 mL), methyl (triphenylphosphoranylidene)acetate (1.97 g, 5.88 mmol) was added, and the mixture was stirred at room temperature for 4 h. The reaction mixture was then poured into H_2O and extracted with CH_2Cl_2 (5 × 40 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated. Purification of the residue by column chromatography (SiO_2, 33% EtOAc/hexane) afforded compound **3** (1.36 g, quant) as a colorless oil: $[\alpha]^{25}_D$ −2.2° (*c* 1.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.67 (dd, J = 13.5, 7.5 Hz, 1H), 2.89 (dd, J = 13.2, 6.6 Hz, 1H), 3.78 (s, 3H), 3.74 (s, 3H), 5.20−5.00 (m, 4H), 5.65−5.50 (m, 1H), 5.73 (br s, 1H), 5.98 (d, J = 15.9 Hz, 1H), 7.16 (d, J = 15.9 Hz, 1H), 7.41−7.27 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 40.3, 51.7, 53.3, 63.2, 66.9, 120.9, 121.5, 128.1, 128.2, 128.5, 130.4, 136.0, 145.3, 154.2, 166.2, 170.8; IR (film) 3352, 1724 cm^{-1}.

Dimethyl 2-[(Benzyloxycarbonyl)amino]bicyclo[2.1.1]hexane-2,5-dicarboxylate (4a−d). Diene **3** (3.84 g, 11.5 mmol) and acetophenone (0.40 mL) in benzene (400 mL) were first purged with argon and then irradiated with a Hanovia 450 W medium-pressure mercury lamp through a Pyrex filter. After 4.5 d, the reaction mixture was evaporated and the residue purified by column chromatography (SiO_2, 33% EtOAc/hexane) to afford a mixture of cis- and *trans*-**3** (R_f 0.8, 50% EtOAc/hexane, 1.16 g, 30%), **4a−c** (R_f 0.65, 50% EtOAc/hexane, 1.22 g, 32%), and **4d** (R_f 0.53, 50% EtOAc/hexane, 197 mg, 5%) as colorless oils: $[\alpha]^{25}_D$ +21° (*c* 0.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.43 (d, J = 7.2 Hz, $H_{6\alpha}$), 1.50−1.53 (m, $H_{6\beta}$), 1.77 (br d, J = 12.3 Hz, $H_{3\alpha}$), 2.67 (br s, H_5), 2.72−2.80 (m, H_4), 3.06 (dd, J = 1.8 and 12.3 Hz, $H_{3\beta}$), 3.20 (br s, H_1), 3.60 (br s, 6H), 5.07 (s, CH_2), 5.63 (br s, NH), 7.33−7.27 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 36.1, 37.1, 39.8, 49.6, 51.2, 51.3, 52.2, 63.3, 66.8, 128.0, 128.4, 136.1, 155.22, 170.5, 172.4.

Compounds 5a−c. Compounds **4a−c** (520 mg, 1.50 mmol) and 10% Pd/C (80 mg) were stirred in MeOH (25 mL) under an atmosphere of H_2. After 3 h, the reaction mixture was filtered and concentrated. Silica gel column chromatography (MeOH/EtOAc, 1/9) of the crude mixture led to the separation of the three products **5a−c**.

(1*R*,2*S*,4*R*,5*R*)-Dimethyl 2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylate (5a): yield 65 mg (20%); R_f 0.6 (EtOAc/MeOH, 8/1); ^{1}H NMR ($CDCl_3$) δ 1.29 (t, J = 8.1 Hz, $H_{6\alpha}$), 1.54 (dd, J = 2.4 and 11.7 Hz, $H_{3\beta}$), 1.78 (br s, NH_2), 2.20−2.30 (m, $H_{6\beta}$), 2.47 (d, J = 11.7 Hz, $H_{3\alpha}$), 2.64−2.74 (m, H_4), 2.77 (dd, J = 2.7 and 6.9 Hz, H_1), 3.01 (d, J = 7.8 Hz, H_5), 3.73 (s, 3H), 3.74 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 37.6 (CH_2), 39.9 (CH_2), 41.6 (CH), 51.6 (CH_3), 51.7 (CH_3), 52.1 (CH), 52.8 (CH), 63.3 (C), 173.7 (CO), 176.1 (CO).

(1*S*,2*S*,4*S*,5*R*)-Dimethyl 2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylate (5b): yield 156 mg (49%); R_f 0.49 (EtOAc/MeOH, 8/1); ^{1}H NMR (methanol-d_4) δ 1.54 (dd, J = 0.9 and 12.0 Hz, $H_{3\alpha}$), 1.72 (t, J = 7.8 Hz, $H_{6\alpha}$), 2.26 (ddd, J = 2.7, 5.4 and 8.1 Hz, $H_{6\beta}$), 2.48−2.56 (m, $H_{3\beta}$ and H_5), 2.62−2.68 (m, H_4), 2.72 (dd, J = 2.4 and 6.9 Hz, H_1), 3.68 (s, 3H), 3.76 (s, 3H), 4.82 (br s, NH_2); ^{13}C NMR (methanol-d_4) δ 36.9 (CH_2), 41.3 (CH_2), 42.9 (CH), 52.4 (CH_3), 53.0 (CH_3), 53.9 (CH), 55.5 (CH), 64.5 (C), 174.6 (CO), 176.9 (CO).

(1*S*,2*S*,4*S*,5*S*)-Dimethyl 2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylate (5c): yield 64 mg (20%); R_f 0.80 (EtOAc/MeOH, 8/1); ^{1}H NMR ($CDCl_3$) δ 1.07 (d, J = 7.5 Hz, $H_{6\alpha}$), 1.46−1.56 (m, $H_{6\beta}$), 1.67 (br s, NH_2), 1.97 (dd, J = 2.1 and 11.4 Hz, $H_{3\beta}$), 2.35 (d, J = 11.4 Hz, $H_{3\alpha}$), 2.61 (m, H_5), 2.64−2.74 (m, H_4), 2.9−3.0 (dt, J = 2.7 and 6.9 Hz, H_1), 3.60 (s, 3H), 3.71 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 36.4, 36.6, 39.7, 49.4, 50.9, 52.0, 52.6, 63.3, 172.4, 176.4.

(1*R*,2*S*,4*R*,5*S*)-Dimethyl 2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylate (5d). Compound **4d** (95 mg, 0.27 mmol) and 10% Pd/C (15 mg) were stirred in MeOH (5 mL) under an atmosphere of H_2. After 3 h, the reaction mixture was filtered and concentrated. Purification of the residue by silica gel column chromatography (MeOH/EtOAc, 1/9) afforded compound **5d** (58 mg, 100%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.46 (d, J = 11.7 Hz, 1H), 1.5−1.6 (m, 1H), 1.62 (d, J = 7.2 Hz, 1H), 1.85 (br s, 2H), 2.63−2.70 (m, 2H), 2.82 (dd, J = 1.8 and 11.7 Hz, 1H), 2.87−2.94 (m, 1H), 3.56 (s, 3H), 3.69 (s, 3H).

Compounds 6a−d. Compound **5a−d** were stirred with 6 N HCl at reflux for 1 h. Evaporation afforded compounds **6a−d**.

(1*S*,2*S*,4*S*,5*S*)-2-Aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid hydrochloride (6a): $[\alpha]^{25}_D$ +15° (*c* 0.5, MeOH); mp >220 °C; ^{1}H NMR (D_2O) δ 1.87 (t, J = 8.7 Hz, $H_{6\beta}$), 2.05 (br d, J = 12.6 Hz, $H_{3\beta}$), 2.30−2.40 (m, $H_{6\beta}$), 2.65 (d, J = 12.6 Hz, $H_{3\alpha}$), 2.80−2.90 (m, H_4), 2.92 (d, J = 8.7 Hz, H_5), 3.05−3.20 (dd, J = 2.1 and 6.9 Hz, H_1); ^{13}C NMR (D_2O) δ 36.8 (CH_2), 37.5 (CH_2), 41.6 (CH), 51.1 (CH), 53.7 (CH), 64.0 (C), 173.0 (CO), 175.6 (CO). Anal. ($C_8H_{11}NO_4 \cdot HCl \cdot 0.5H_2O$) C, H, N.

(1*R*,2*S*,4*R*,5*R*)-2-Aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid hydrochloride (6b): $[\alpha]^{25}_D$ −7.0° (*c* 0.5, H_2O); mp >220 °C; ^{1}H NMR (D_2O) δ 1.65 (t, J = 9.0 Hz, $H_{6\alpha}$), 2.01 (d, J = 12.6 Hz, $H_{3\alpha}$), 2.46 (dt, J = 2.4 and 9.6 Hz, $H_{6\beta}$), 2.63 (br d, 12.9 Hz, $H_{3\beta}$), 2.80−2.90 (m, H_4), 3.08 (dd, J = 2.4 and 6.9 Hz, H_1), 3.15 (d, J = 8.7 Hz, H_5); ^{13}C NMR (D_2O) δ 36.6 (CH_2), 38.0 (CH_2), 41.5 (CH), 51.0 (CH), 53.7 (CH), 63.6 (C), 173.4 (CO), 176.0 (CO). Anal. ($C_8H_{11}NO_4 \cdot HCl \cdot 0.5H_2O$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-2-Aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid hydrochloride (6c): $[\alpha]^{25}_D$ −5.4° (*c* 0.25, H_2O); mp 198−200 °C; ^{1}H NMR (D_2O) δ 1.51 (d, J = 8.1 Hz, $H_{6\alpha}$), 1.65−1.75 (m, $H_{6\beta}$), 2.02 (dd, J = 2.7 and 13.2 Hz, $H_{3\beta}$), 2.70 (br d, J = 13.2 Hz, $H_{3\alpha}$), 2.90−3.00 (m, H_4), 3.06 (br s, H_5), 3.10−3.20 (m, H_1); ^{13}C NMR (D_2O) δ 34.3 (CH_2), 36.3 (CH_2), 43.2 (CH), 50.1 (CH), 52.0 (CH), 63.6 (C), 173.0 (CO), 177.0 (CO). Anal. ($C_8H_{11}NO_4 \cdot HCl \cdot 0.5H_2O$) C, H, N.

(1*R*,2*S*,4*R*,5*S*)-2-Aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid hydrochloride (6d): $[\alpha]^{25}_D$ +6° (*c* 0.25, H_2O); mp (dec) 205 °C; ^{1}H NMR (D_2O) δ 1.45 (d, J = 8.7 Hz, $H_{6\alpha}$), 1.80−1.90 (m, $H_{6\beta}$), 1.95 (br d, J = 13.2 Hz, $H_{3\alpha}$), 2.75−2.90 (m, H_4 and $H_{3\beta}$), 2.97 (br s, H_5), 3.20−3.30 (m, H_1). Anal. ($C_8H_{11}NO_4 \cdot HCl \cdot 0.5H_2O$) C, H, N.

Molecular Modeling. All molecular modeling studies were conducted using the QUANTA program,[23] and all molecular mechanics and molecular dynamics simulations were performed using the CHARMM program.[24] To access the different conformations that may be adopted by each of these ligands, high-temperature molecular dynamics simulations were performed as follows: (1) the temperature of the system was raised to 1000 from 0 K within 1 ps; (2) the system was then equilibrated for 30 ps at 1000 K; and (3) the system was finally simulated for 50 ps. Trajectories were recorded every 0.1 ps in the final simulation stage and subsequently minimized for 5000 steps, or until convergence, defined as an energy gradient tolerance of 0.001 kcal mol^{-1} $Å^{-1}$, using an adopted-basis Newton−Raphson algorithm, as implemented in the CHARMM program. The dielectric constant was set to 80 to simulate an aqueous environment. The carboxyl groups in these ligands was set to be deprotonated and the amine groups to be protonated, as expected under physiological conditions. These minimized structures were clustered using the Cluster Analy-

sis module in the QUANTA program using all non-hydrogen atoms as the reference atoms, unless otherwise stated, to obtain truly distinct low-energy conformations. In each conformational cluster, the lowest energy conformation was selected as the representative conformation for the cluster. The low-energy, distinct conformations for each ligand were compared to the aa conformation of L-glutamate employing the Molecular Similarity module in the QUANTA program using the five carbon atoms and one nitrogen atom in L-glutamate as the reference atoms. This comparison essentially provides the overall goodness of overlap between the low energy conformation of a mGluR ligand and the low energy conformation of L-glutamate. The root-mean-square (RMS) values were obtained for each ligand and provide a quantitative measure of the goodness of the overlap.

Biological Assays. Cell Cultures. Lines of Chinese hamster ovary (CHO) cells with stable expression of mGluR1a, mGluR2, mGluR5a, mGluR6, and chimeric mGluR3/1a receptors were prepared and cultured as described previously.[17] The baby hamster kidney (BHK) cell line expressing mGluR4a was a generous gift from Zymogenetics Inc. (Seattle). All cell lines were cultured in 96-well plates and passaged every 6−7 days. Primary cultures of neurons were prepared from cerebella of 7-day-old rats and cultured as described previously[18] in 96-well plates. Cerebellar neurons were used for experiments after 8−9 days in culture.

Assays of Inositol Phosphates (IPs) Accumulation. The activity of agonists at phospholipase C-coupled receptors (mGluR1a, mGluR5a, and mGluR3/1a) was determined by measurements of their ability to increase the hydrolysis of membrane phosphoinositides. CHO cells expressing mGluR1, mGluR5, or mGluR3/1a cultured in 96-well plates were incubated overnight in glutamine-free culture medium supplemented with 0.75 μCi *myo*-[^{3}H]inositol (Amersham) to label the cell membrane phosphoinositides. Incubations with agonists were carried out for 30 min at 37 °C in Locke's buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 20 mM Hepes, pH 7.4) containing 20 mM LiCl to block IP degradation. The reaction was terminated by aspiration, and IPs were extracted with 0.1 M HCl. The separation of [^{3}H]IPs was performed by anion exchange chromatography as described previously.[18] In each experiment, the stimulations induced by the various compounds were expressed as percent of maximal response induced by 1 mM glutamate.

Assay of cAMP Formation. The activity of agonists at receptors coupled negatively to adenylyl cyclase (mGluR2, mGluR4a, mGluR6) was determined by measurements of their ability to decrease the forskolin-induced elevation of cyclic AMP formation as described previously.[17] Cells cultured in 96-well culture plates were preincubated 10 min at 37 °C in Locke's medium containing 300 μM isobutylmethylxanthine to inhibit the activity of phosphodiesterases which degrade cAMP. Then 5 μM forskolin was added without or with mGluR agonists, and the incubation was continued for 10 min. After the incubation, the medium was rapidly aspirated and cAMP was extracted with 0.1 M HCl and measured by radioimmunoassay using a magnetic Amerlex RIA kit. In each experiment, the results were expressed as percent of the maximal response induced by 1 mM glutamate for mGluR2 and mGluR4, and by 1 mM AP4 for mGluR6.

Determination of Calcium Influx. Activity of ionotropic glutamate receptors was determined by measurements of $^{45}Ca^{2+}$ influx in cerebellar neurons, as described previously.[19] Cells cultured in 96-well plates were incubated for 15 min at room temperature in Locke's buffer containing 1 μCi/mL of $^{45}CaCl_2$ and the indicated additions. Agonist activity at all ionotropic glutamate receptors was measured in Mg^{2+}-free medium supplemented with 1 μM glycine to allow for activity at NMDA receptors and with 10 μM cyclothiazide to inhibit desensitization of AMPA receptors. Antagonist activity at NMDA receptors was measured in a Mg^{2+}-free medium in the presence of 100 μM NMDA, 1 μM glycine, and 10 μM NBQX to inhibit non-NMDA receptors. Antagonism of kainate action was tested in the presence of 1 mM Mg^{2+}, 50 μM kainate, and 1 μM MK-801 to inhibit NMDA receptors, while the effects on AMPA action were measured in the presence of 1 mM Mg^{2+}, 30 μM AMPA, 10 μM cyclothiazide, and 1 μM MK-801. Incubations were terminated by three washes with ice-cold buffer, and the cells were dissolved in 0.5 M NaOH for determination of accumulated radioactivity and protein content.

Acknowledgment. We are indebted in part to Pharmacia-Upjohn and the NIH (Grants NS28130 and NS01720) for their support of this research. We thank Dr. Francine Acher for a preprint of the paper cited in ref 7.

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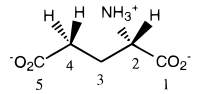

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JM970719Q