Cyclobutane Quisqualic Acid Analogues as Selective mGluR5a Metabotropic Glutamic Acid Receptor Ligands

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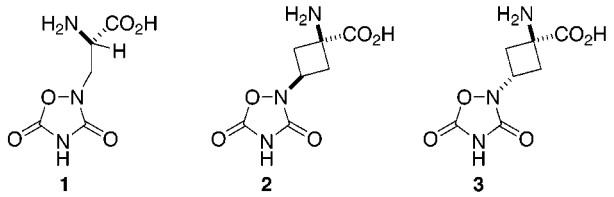
The conformationally constrained cyclobutane analogues of quisqualic acid (*Z*)- and (*E*)-1-amino-3-[2′-(3′,5′-dioxo-1′,2′,4′-oxadiazolidinyl)]cyclobutane-1-carboxylic acid, compounds **2** and **3**, respectively, were synthesized. Both **2** and **3** stimulated phosphoinositide (PI) hydrolysis in the hippocampus with EC_{50} values of 18 ± 6 and 53 ± 19 μM, respectively. Neither analogue stimulated PI hydrolysis in the cerebellum. The effects of **2** and **3** were also examined in BHK cells which expressed either mGluR1a or mGluR5a receptors. Compounds **2** and **3** stimulated PI hydrolysis in cells expressing mGluR5a but not in those cells expressing mGluR1a. The EC_{50} value for **2** was 11 ± 4 μM, while that for **3** was 49 ± 25 μM. Both **2** and **3** did not show any significant effect on cells expressing the mGluR2 and mGluR4a receptors. In addition, neither compound blocked [^{3}H]glutamic acid uptake into synaptosomal membranes, and neither compound was able to produce the QUIS effect as does quisqualic acid. This pharmacological profile indicates that **2** and **3** are selective ligands for the mGluR5a metabotropic glutamic acid receptor.

Introduction

The acidic amino acids L-glutamic acid and L-aspartic acid are the major excitatory neurotransmitters in the mammalian central nervous system (CNS). These excitatory amino acids (EAAs) activate both receptors coupled to ion channels (ionotropic) and receptors coupled to second-messenger systems (metabotropic).[1] The ionotropic glutamic acid receptors have been divided into the following subtypes based on agonist specificity: *N*-methyl-D-aspartate (NMDA), kainic acid, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. These receptors have been implicated in mediating fast synaptic events, are involved in long-term potentiation, and can cause neuronal degeneration upon excessive activation.[1−3]

To date, eight distinct metabotropic glutamic acid receptors (mGluRs) have been cloned (mGluR1−mGluR8) with multiple splice variants existing for several of these receptor subtypes.[1,4,5] The mGluRs have been categorized into three groups based on agonist specificity and second-messenger coupling. Group 1 receptors (mGluR1a−d, mGluR5a,b) are coupled to phosphoinositide (PI) hydrolysis and are stimulated by the EAA analogues quisqualic acid (**1**) and (1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid [(1*S*,3*R*)-ACPD].[6,7] Group 2 receptors (mGluR2, mGluR3) are coupled to inhibition of cyclic AMP (cAMP) formation and are stimulated by (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740)[8] and (2*S*,2′*R*,3′*R*)-2-(2′3′-dicarboxycyclopropyl)glycine (DCG-IV).[9] Group 3 receptors (mGluR4a,b, mGluR6, mGluR7, mGluR8) are also coupled to inhibition of cAMP formation but are stimulated by L-2-amino-4-phosphonobutanoic acid (L-AP4).[10] The mGluRs have been implicated in mediating development of the nervous system,[11,12] long-term potentiation,[13,14] modulation of neuronal activity,[15,16] and regulation of glutamic acid release.[17,18]

Great success, in the past, has been achieved in the synthesis of selective metabotropic receptor ligands by synthesizing conformationally constricted analogues of glutamic acid and L-AP4. Since quisqualic acid is one of the most potent agonists for group 1 mGluRs, the synthesis of conformationally constrained analogues of **1** has been undertaken in hopes of developing high-affinity ligands which can be used to define the functions of these receptors in the mammalian CNS. In the present study, we describe the synthesis and selective mGluR5a receptor agonist activity of the cyclobutyl analogues of quisqualic acid, compounds **2** and **3**.



Chemistry

The synthesis of **2** and **3** is outlined in Scheme 1. The synthetic strategy for the two cyclobutyl quisqualic acid analogues was centered around the construction of the oxadiazolidinedione ring on a suitable cyclobutane precursor and employed methodology similar to that used in the synthesis of L-quisqualic acid.[19,20] In our approach, condensation of cyclobutanone **4**[21] with *O*-benzylhydroxylamine in the presence of sodium cyanoborohydride gave the *N*-benzyloxyamino derivative as a

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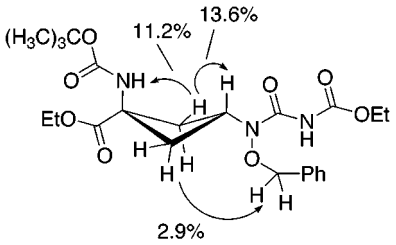
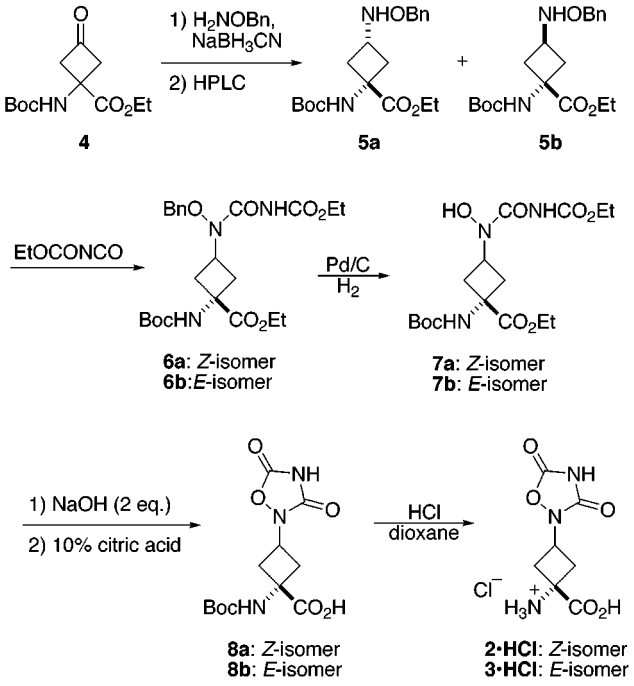


Figure 1. NOEs observed for **6b**.

Scheme 1



mixture of *Z* (**5a)** and *E* (**5b**) isomers in a 1:1 ratio. The isomers were separated by HPLC, and each isomer then was acylated with ethoxycarbonyl isocyanate to yield **6a** and **6b**, respectively. Removal of the benzyl protecting group from each isomer of **6** by hydrogenolysis gave the *N*-hydroxy esters **7a** and **7b**. Treatment of each of these under basic conditions led to formation of the oxadiazolidinedione derivatives **8a** and **8b**. Removal of the *tert*-butoxycarbonyl protecting group with acid provided **2** and **3** as their HCl salts.

Assignment of the geometrical configuration of **2** and **3** was accomplished through a combination of NOE experiments and X-ray crystallography. Initially, an NOE experiment was carried out on **6b** involving selective irradiation of the two individual cyclobutane methylene envelopes. By virtue of the symmetry within **6b**, one envelope was assumed to be the axial-like protons on C-2 and C-4 and the other to be the equatorial-like protons. As illustrated in Figure 1, enhancement in the benzyl protons was observed upon irradiation of the cyclobutane ring protons at 2.94−3.01 ppm, while enhancements in the carbamate NH and methine 3-H protons were observed upon irradiation of the cyclobutane ring protons at 2.31−2.42 ppm. These results were consistent with a geometry in which the *tert*-butoxycarbonylamino and the *N*-(benzyloxy)-*N*-(ethoxycarbonyl) groups were in an *E* relationship to one another.

Subsequently, a crystal structure of **7a** was achieved. This compound was found to crystallize with two independent molecules plus one THF molecule per asymmetric unit. Both independent molecules showed a *Z* relationship between the *tert*-butoxycarbonylamino moiety and the *N*-(benzyloxy)-*N*-(ethoxycarbonyl) group. The molecular structure of one independent molecule of **7a** is shown in Figure 2. On the basis of these results, the geometrical configuration of cyclobutyl quisqualic acid analogues **2** and **3** was assigned to be *Z* and *E*, respectively.

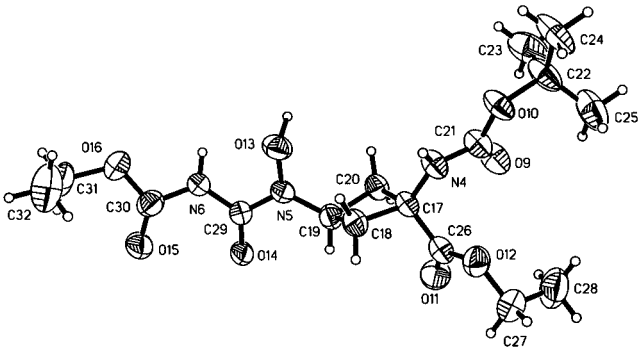


Figure 2. Molecular structure of one of the two independent molecules of **7a** with atomic numbering scheme.

Biological Results

The two cyclobutyl quisqualic acid analogues **2** and **3**, along with L-glutamic acid, (1*SR*,3*RS*)-ACPD, and quisqualic acid, were tested for their ability to stimulate PI hydrolysis in either neonatal rat hippocampus or cerebellum (Figure 3). Quisqualic acid (100 μM), L-glutamic acid (1 mM), and (1*SR*,3*RS*)-ACPD (300 μM) stimulated PI hydrolysis in both hippocampus ($p < 0.001$, $p < 0.01$, and $p < 0.01$ for difference from baseline, respectively) and cerebellum ($p < 0.01$, $p < 0.001$, and $p < 0.01$ for difference from baseline, respectively). Interestingly, compounds **2** (100 μM) and **3** (100 μM) stimulated PI hydrolysis in hippocampus ($p < 0.001$ and $p < 0.01$ for difference from baseline, respectively) but not in cerebellum.

The effects of increasing concentrations of **2** and **3** on PI hydrolysis in hippocampus were examined (Figure 4). In hippocampus, we previously demonstrated that data for the stimulation of PI hydrolysis by L-glutamic acid or (1*SR*,3*RS*)-ACPD were best fit to curves having a Hill coefficient of 1 with EC_{50} values of 450 ± 120 and 51 ± 5 μM, respectively, while data for quisqualic acid (see Figure 4) were best fit to curves having two components ($p < 0.01$) with the high-affinity component ($EC_{50} = 0.43$ μM) accounting for 70% of the total effect and the low-affinity component ($EC_{50} = 44$ μM) accounting for 30% of the total effect.[22,23] In the current study, the EC_{50} values for **2** and **3** were 18 ± 6 and 53 ± 19 μM, respectively. The maximal effects (in DPM/100 000 DPM incorporated) were 30 200 ± 7 900 for **2** and 31 800 ± 8 400 for **3**. The maximal effects of these compounds were comparable, in parallel assays, to the effect caused by a concentration of (1*SR*,3*RS*)-ACPD (300 μM) that induces a maximal effect in the hippocampus.

Of the metabotropic receptor subtypes that are coupled to PI hydrolysis (mGluR1 and mGluR5), mGluR1a is expressed at high levels in both cerebellum and hippocampus while mGluR5a is expressed at high levels in hippocampus but at low levels in cerebellum.[24] The

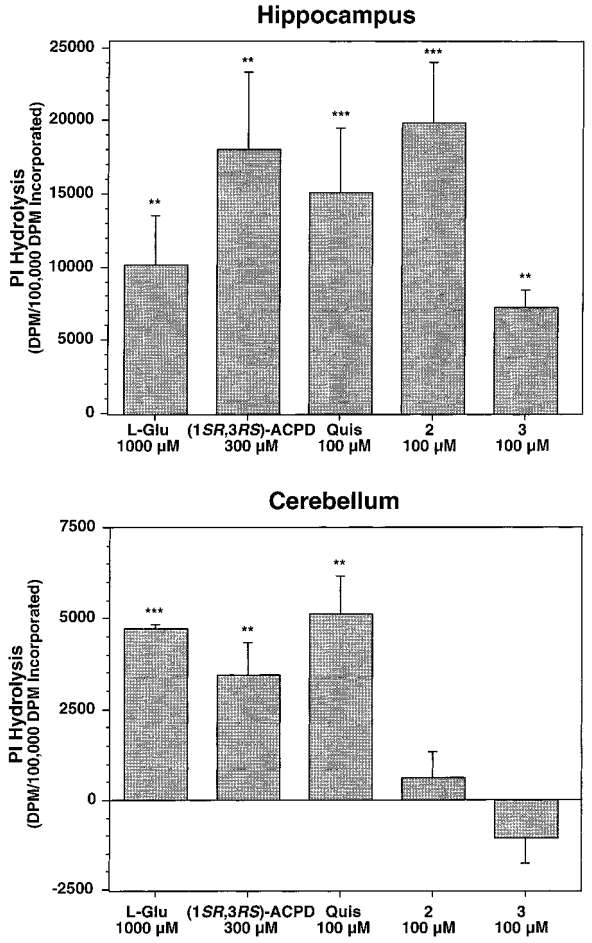


Figure 3. Effects of quisqualic acid analogues on PI hydrolysis in neonatal rat hippocampus and cerebellum. The effects of L-glutamic acid, (1*SR*,3*RS*)-ACPD, quisqualic acid, **2**, or **3** on PI hydrolysis were examined at the concentrations indicated in neonatal rat hippocampus (top panel) or cerebellum (bottom panel). Data represent the mean ± SEM of three experiments performed in parallel. Basal levels of PI hydrolysis (in DPM/100 000 DPM incorporated) were 6890 ± 490 in hippocampus and 7170 ± 1310 in cerebellum. $^{**}p < 0.01$ for difference from control; $^{***}p < 0.001$ for difference from control.

observation that compounds **2** and **3** activate PI hydrolysis in hippocampus but not in cerebellum suggests that these compounds activate mGluR5. To further explore this possibility, the effects of these compounds on PI hydrolysis in BHK cells which express either mGluR1a or mGluR5a were examined. In BHK cells expressing mGluR1a (Figure 5A−C, open circles), EC_{50} values were 1.0 ± 0.4 μM for quisqualic acid, 5.9 ± 2.4 μM for L-glutamic acid, and 60 ± 20 μM for (1*SR*,3*RS*)-ACPD; the maximal effects (in DPM/100 000 DPM incorporated) were 14 200 ± 4 660 for quisqualic acid, 15 200 ± 2 730 for L-glutamic acid, and 12 170 ± 1 900 for (1*SR*,3*RS*)-ACPD (data not shown, $n \geq 3$). In BHK cells expressing mGluR5a (Figure 5A−C, solid circles), EC_{50} values were 0.03 ± 0.004 μM for quisqualic acid, 1.3 ± 0.3 μM for L-glutamic acid, and 12.5 ± 1.6 μM for (1*SR*,3*RS*)-ACPD; the maximal effects (in DPM/100 000 DPM incorporated) were 36 830 ± 4 540 for quisqualic acid, 31 800 ± 3 330 for L-glutamic acid, and 32 900 ± 2 460 for (1*SR*,3*RS*)-ACPD. Compounds **2** and **3** stimulated PI hydrolysis in cells expressing mGluR5a (Figure 5D,E, solid circles) with EC_{50} values of 11 ± 4 μM for **2** and 49 ± 25 μM for **3**; the maximal effects (in DPM/100 000 DPM incorporated) were 26 700 ± 3 700 for **2** and 40 200 ± 11 600 for **3**. At concentrations up to 1 mM, neither compound **2** nor **3** stimulated PI hydrolysis in cells expressing mGluR1a (Figure 5D,E, open circles).

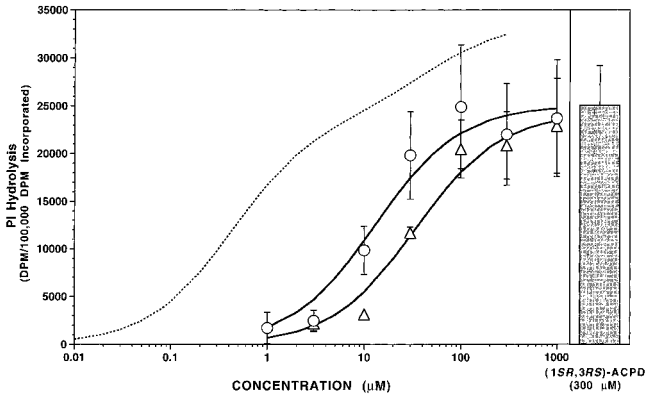


Figure 4. Stimulation of PI hydrolysis by quisqualic acid analogues in neonatal rat hippocampus. The effects of increasing concentrations of **2** (○) or **3** (△) on PI hydrolysis were measured. Data for **2** or **3** were best fit to curves having a Hill coefficient of 1. The effect of a maximal concentration of (1*SR*,3*RS*)-ACPD (300 μM), measured in parallel assays, is shown for comparison. These data represent the mean ± SEM of four experiments. Basal levels of PI hydrolysis in these experiments were 12 270 ± 800 DPM/100 000 DPM incorporated. The effect of quisqualic acid (dotted line), measured in previous experiments,[22] is shown for comparison. Data for quisqualic acid were best fit to curves having two components. In two experiments on the effects of increasing concentrations of quisqualic acid performed in parallel with the current studies (data not shown), data were also best fit to two components with EC_{50} values of 0.14 μM (82% of the total response) and 24 μM (18% of the total response); the maximal effect was 19 760 DPM/100 000 DPM incorporated.

The effect of increasing concentrations of (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), a compound that has been identified previously as a specific agonist of mGluR5a relative to mGluR1a,[25] also was examined in cells expressing mGluR5a and was compared with the results obtained for **2** and **3** (Figure 6). The EC_{50} value for CHPG was 400 ± 70 μM, and the maximal effect was 22 000 ± 2 700 DPM/100 000 DPM incorporated.

To further explore the specificity of these compounds for mGluR5a, the effects of **2** and **3** on the inhibition of cAMP production stimulated by forskolin were examined in BHK 570 cells stably expressing either a group 2 (mGluR2) or group 3 (mGluR4a) metabotropic receptor subtype. In addition, since several EAA analogues, including quisqualic acid, block L-[^{3}H]glutamic acid uptake,[26,27] the effects of **2** and **3** on L-[^{3}H]glutamic acid uptake into synaptosomal membrane preparations were measured. The results of these studies are depicted in Figure 7. L-Glutamic acid (100 μM) and (1*SR*,3*RS*)-ACPD (100 μM) blocked cAMP formation in cells expressing mGluR2 ($p < 0.001$ for difference from control). L-AP4 (10 μM), an agonist of group 3 metabotropic receptors, blocked cAMP formation in cells expressing mGluR4a ($p < 0.001$ for difference from control). Compounds **2** and **3**, however, were without significant effect in either of these systems at concentrations up to 100 μM. Interestingly, CHPG (1 mM) reduced forskolin-stimulated cAMP formation in cells expressing mGluR2 to 58 ± 8% of control ($p < 0.01$ for difference from

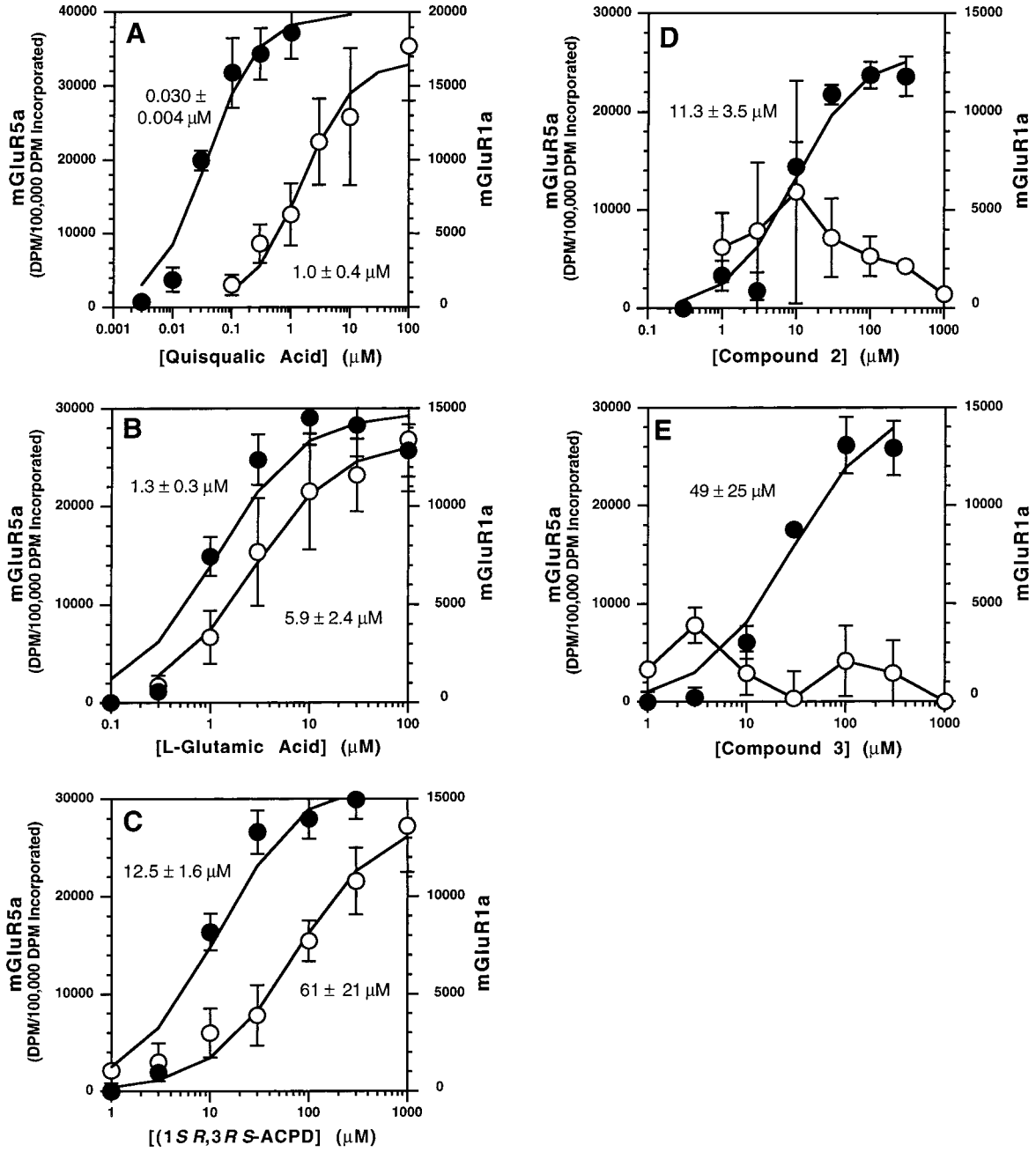


Figure 5. Effects of quisqualic acid analogues on PI hydrolysis in BHK cells which express either mGluR1a or mGluR5a. The effects of increasing concentrations of quisqualic acid (A), L-glutamic acid (B), (1*SR*,3*RS*)-ACPD (C), **2** (D), or **3** (E) on PI hydrolysis were examined in cells which express either mGluR1a (open circles, right axis) or mGluR5a (filled circles, left axis). These data represent the mean ± SEM of four to six experiments unless otherwise indicated. Due to the limited supply of **2** and **3**, the effects of a high concentration (1000 μM) of these compounds on cells expressing mGluR1a were only examined in one experiment. Basal levels of PI hydrolysis (in DPM/100 000 DPM incorporated) were 7840 ± 2280 in cells expressing mGluR1a and 6090 ± 1030 in cells expressing mGluR5a. The EC_{50} values (mean ± SEM) for each compound are given.

control, data not shown). In addition, compounds **2** and **3** did not block L-[^{3}H]glutamic acid uptake in synaptosomal membranes.

Since quisqualic acid is also a prototypic agonist for the ionotropic glutamate receptors now termed AMPA receptors, analogues **2** and **3** were examined for their effects on these receptors. As shown in Table 1, analogues **2** and **3** are 10 and 58 times less potent, respectively, than **1** in depolarizing rat hippocampal CA1 pyramidal neurons, an effect which is presumably mediated by their interaction with AMPA receptors.

Analogues **2** and **3** also were examined for their ability to produce the QUIS effect.[28−30] This effect, which is widely distributed in the brain, results in a sensitization of neurons to depolarization by other excitatory amino acid analogues, in particular amino acid phosphonates, after exposure of the neurons to quisqualic acid.[28−30] As shown in Table 1, neither **2** nor **3** was able to induce the QUIS effect.

Discussion

Quisqualic acid (**1**) affects a number of systems within the CNS that are associated with excitatory amino acid neurotransmission. It is a potent agonist at several excitatory amino acid receptor subtypes including the kainate,[31] AMPA,[32] and metabotropic receptors.[33,34] It

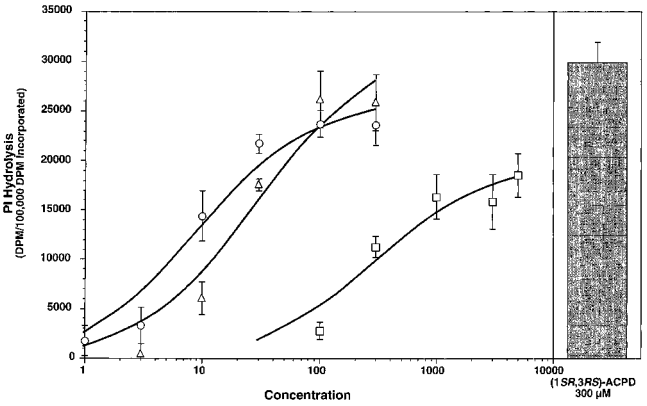


Figure 6. Stimulation of PI hydrolysis by quisqualic acid analogues in BHK cells which express mGluR5a. The effects of increasing concentrations of **2** (○), **3** (△), and (*RS*)-2-chloro-5-hydroxyphenylglycine (□) on PI hydrolysis were measured. These data represent the mean ± SEM of three to five experiments. Basal levels of PI hydrolysis in these experiments were 6880 ± 1450 DPM/100 000 DPM incorporated. The effect of (1*SR*,3*RS*)-ACPD at 300 μM is shown for comparison.

also inhibits the enzyme *N*-acetyl-α-linked acidic dipeptidase which hydrolyzes the brain dipeptide *N*-acetyl-L-aspartyl-L-glutamic acid[35] and the Ca^{2+}/Cl^{-}-dependent glutamic acid uptake system in brain synaptic plasma membrane preparations.[36] Furthermore, quisqualic acid produces a phenomenon known as the QUIS effect which entails the sensitization of neurons to depolarization by amino acid phosphonates such as AP4 after exposure of the neurons to quisqualic acid.[28−30]

The present study demonstrates that appending the three essential functionalities of quisqualic acid (the amino and carboxy groups and the oxadiazolidinedione ring) to a cyclobutane ring results in analogues of quisqualic acid that show selective pharmacological effects. Introduction of the cyclobutane constraint yielded analogues of quisqualic acid, compounds **2** and **3**, that were found to stimulate PI hydrolysis in the hippocampus but not in the cerebellum. In contrast, quisqualic acid stimulated PI hydrolysis in both areas. Interestingly, data for the stimulation of PI hydrolysis by quisqualic acid were best fit to two components in hippocampus but to only a single low-affinity component in cerebellum. Since mGluR1a is expressed in both the cerebellum and the hippocampus while mGluR5a is expressed in the hippocampus but not in the cerebellum,[24] these results indicate that **2** and **3** are selective for the group 1 metabotropic mGluR5a subtype of receptor. This result was further substantiated by the studies that were carried out with BHK cells which expressed either mGluR1a or mGluR5a metabotropic receptors. In these systems, quisqualic acid, L-glutamic acid, and (1*SR*,3*RS*)-ACPD were 33-, 4.5-, and 4.8-fold more potent for the stimulation of PI hydrolysis in cells expressing mGluR5a than for the stimulation of PI hydrolysis in cells expressing mGluR1a. Although **2** and **3** stimulated PI hydrolysis in cells expressing mGluR5a with EC_{50} values of 11 and 49 μM, respectively, neither **2** nor **3**, at concentrations up to 1000 μM, stimulated PI hydrolysis in cells expressing mGluR1a. Due to the limited supply of **2** and **3**, the effects of higher concentrations of these compounds were not examined, but the data from the current study suggests that the selectivities of these analogues for stimulation of mGluR5a

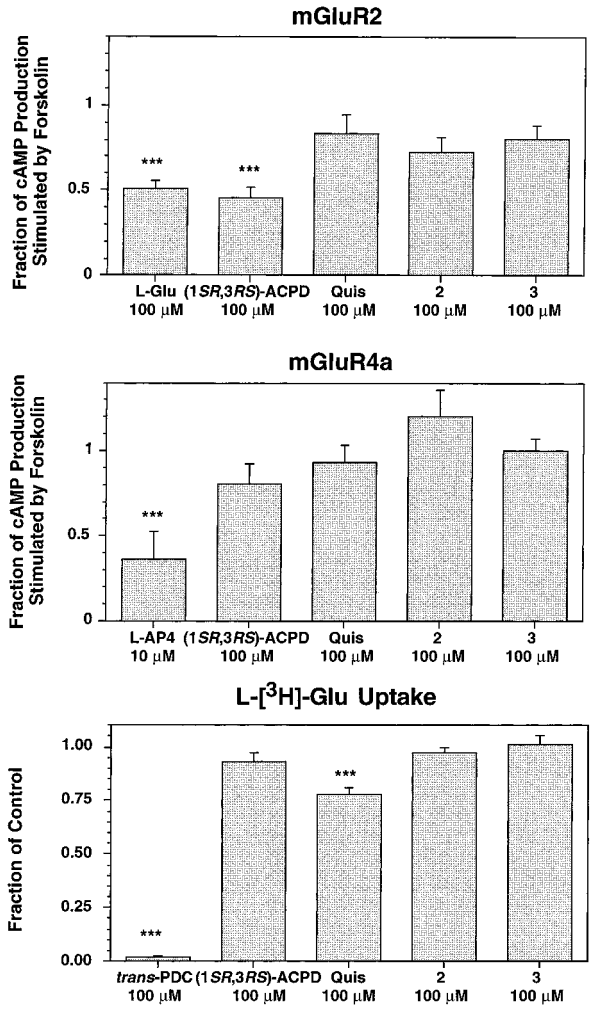


Figure 7. Effect of quisqualic acid analogues on cAMP accumulation stimulated by forskolin and L-[^{3}H]glutamic acid uptake. The effects of L-glutamic acid, (1*SR*,3*RS*)-ACPD, quisqualic acid, **2**, **3**, and L-2-amino-4-phosphonobutanoic acid (L-AP4) on cAMP production stimulated by forskolin at the concentrations indicated were examined in BHK cells which expressed either mGluR2 (top panel) or mGluR4 (middle panel). None of the quisqualic acid analogues stimulated cAMP accumulation in the absence of forskolin (data not shown). The effects of L-*trans*-pyrolidine-2,4-dicarboxylic acid (*trans*-PDC), (1*SR*,3*RS*)-ACPD, quisqualic acid, **2**, and **3** on L-[^{3}H]glutamic acid uptake in synaptosomes (bottom panel) were also examined at the concentrations indicated. These data represent the mean ± SEM of three to four experiments. ***$p < 0.001$ for difference from control.

compared to mGluR1a are at least 270-fold for **2** and 60-fold for **3**. Compounds **2** and **3**, therefore, show greater selectivity than quisqualic acid in these systems. In addition, both **2** and **3** were substantially more potent than (*RS*)-2-chloro-5-hydroxyphenylglycine, a compound which had previously been identified as a specific agonist for mGluR5a metabotropic receptors.

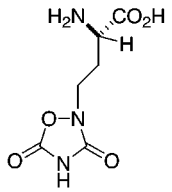
The selectivity of **2** and **3** for the group 1 mGluR5a metabotropic receptor was also demonstrated by the fact that neither compound showed significant activity at the group 2 mGluR2 metabotropic receptor and the group 3 mGluR4a metabotropic receptor. Although quisqualic acid shows some effect in blocking L-[^{3}H]glutamic acid uptake into synaptosomal membranes and produces the QUIS effect, **2** and **3** did not show any significant activity in either of these systems.

Table 1. AMPA Receptor-Mediated Depolarization by Quisqualic Acid (**1**) and Analogues **2** and **3** and Sensitization of CA1 Pyramidal Neurons to Depolarization by L-AP6[a] after Exposure to **1**−**3**

compd	depolarization IC_{50} (μM)	QUIS effect conditions of exposure		IC_{50} for L-AP6 (mM) ± SD		
		concn (μM)	time (min)	before exposure	after exposure	after reversal with L-α-AA[b]
1	7.0 ± 0.8	16	4	>10	0.04 ± 0.008	1.9 ± 0.1
2	69 ± 3	200	20	>10	7.0 ± 0.8	>10
3	390 ± 140	400	20	>10	>10	nd

[a] L-AP6, L-2-amino-6-phosphonohexanoic acid. [b] Treatment with 2 mM L-α-aminoadipic acid (L-α-AA) for 10 min.

Structurally, **2** and **3** can be viewed as analogues of (*S*)-homoquisqualic acid (**9**) in which a methylene bridge has been placed between the α- and γ-carbon atoms. Previously, **9** was found to be a full agonist at group 1 metabotropic receptors with an EC_{50} of 50.2 ± 1.6 μM in rat pup cerebrocortical slices.[37] In comparison, in our system **2** and **3** exhibited IC_{50} values of 18 ± 6 and 53 ± 19 μM, respectively. It is also interesting to compare



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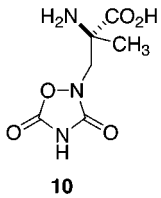

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the activity seen with **2** and **3** to that reported recently with L-α-methylquisqualic acid (**10**).[38] In contrast to **2** and **3**, **10** was found to be a weak antagonist at the group 1 metabotropic receptors. The IC_{50} values of **10** for mGluR1a and mGluR5a receptors were 500 and 1000 μM, respectively. Although **2** and **3**, like **10**, are substituted at the α-carbon, the nature of this substituent clearly has a major impact on whether the compound will be an agonist or antagonist.

Although both **2** and **3** possessed the ability to act as agonists at the mGluR5a receptor, the isomer with the amino and oxadiazolidinedione ring system in a syn relationship was found to be the most potent, suggesting that this conformationally constrained analogue comes closest to mimicking the bioactive conformation needed for the selective stimulation of the mGluR5a metabotropic receptor.

Experimental Section

General. Melting points were determined on a Thomas-Hoover Unimelt melting point apparatus model 6406-K and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Unless otherwise indicated, all analytical results were within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded on either a Bruker 200-MHz or a GE 300-MHz spectrometer. The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in either $CDCl_3$, MeOH-d_4, or acetone-d_6 and to TSP in D_2O. ^{13}C NMR spectroscopy was performed on either a Bruker 200-MHz or a GE 300-MHz spectrometer at 50 and 75 MHz, respectively. FAB mass spectra were obtained on a Finnigan 4000 spectrometer. Column chromatography was performed on silica gel (Merck, grade 60, 240−400 mesh, 60 Å) from Aldrich Chemical Co. Thin-layer chromatography (TLC) was carried out on Analtech 250-mm silica gel GHLF Uniplates. Visualization was achieved with either UV, I_2, or ninhydrin spray. THF was freshly distilled from sodium and benzophenone ketyl. CH_2Cl_2 was distilled from sodium hydride.

[^{3}H]-*myo*-Inositol (15.4 Ci/mmol) was obtained from DuPont Co. (Boston, MA). (1*SR*,3*RS*)-1-Amino-1,3-cyclopentanedicarboxylic acid, quisqualic acid, and L-2-amino-4-phosphonobutanoic acid were obtained from Tocris Cookson (Bristol, England). L-Glutamic acid was obtained from Sigma Chemical Co. (St. Louis, MO).

***(Z)*- and *(E)*-Ethyl 1-[(*tert*-Butoxycarbonyl)amino]-3-[(benzyloxy)amino]cyclobutane-1-carboxylate (5a and 5b).** *O*-Benzylhydroxylamine (1.5 g, 10.0 mmol), ethyl 1-[(*tert*-butoxycarbonyl)amino]-3-oxocyclobutane-1-carboxylate[21] (**4**; 1.0 g, 3.89 mmol), and sodium cyanoborohydride (0.326 g, 5.18 mmol) were dissolved in 25 mL of anhydrous methanol. The pH of the solution was brought to 5 by addition of HCl in dioxane, and the reaction was stirred for 48 h at room temperature. The pH of the solution then was brought to 8 by addition of 1 M $NaHCO_3$. The solvents were removed under aspirator pressure. The crude product was partitioned between EtOAc (50 mL) and H_2O (25 mL). The organic layer was washed with water and brine and dried. The product was purified using flash column chromatography with hexanes/EtOAc (3:1) as an eluant. The product was obtained as an oil in a yield of 0.80 g (50%). Anal. ($C_{19}H_{28}N_2O_5$) C, H, N. HPLC analysis (Waters Novapak silica cartridge column, 8 × 100 mm, hexanes/EtOAc (2:1), 4 mL/min) indicated the product was a mixture of *Z* and *E* isomers in the ratio of 1:1 with retention times of 18 and 20 min, respectively. The two isomers were separated by HPLC under the condition described above.

***Z* Isomer 5a**: ^{1}H NMR ($CDCl_3$) δ 7.35 (br s, 5 H, Ar−H), 5.77 (br s, 1 H, HNOBn), 5.29 (br s, 1 H, HNBoc), 4.71 (s, 2 H, CH_2Ph), 4.24 (q, J = 6 Hz, 2 H, OCH_2), 3.78 (dd, J = 6 and 9 Hz, 1 H, 3-CH), 2.71−2.78 (m, 2 H, 2- and 4-CH_2), 2.30−2.39 (m, 2 H, 2- and 4-CH_2), 1.42 (s, 9 H, $C(CH_3)_3$) 1.26 (t, J = 6 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.8 (CH_3), 28.9 ($C(CH_3)_3$), 37.9 (2- and 4-C), 49.8 (CNOBn), 53.6 (1-C), 62.2 (OCH_2), 77.0 (OCPh), 80.5 ($OC(CH_3)_3$), 128.5, 129.1, 138.5 (Ar−C), 155.3 (NC=O), 174.7 (CO_2Et); FAB MS *m*/*z* 365 (100%) $[M + H]^+$; FAB HRMS *m*/*z* 365.2081 ($C_{19}H_{28}N_2O_5 + H^+$ requires 365.1998).

***E* Isomer 5b**: ^{1}H NMR ($CDCl_3$) δ 7.34 (br s, 5 H, Ar−H), 5.77 (br s, 1 H, HNOBn), 5.22 (br s, 1 H, HNBoc), 4.69 (s, 2 H, CH_2Ph), 4.17 (q, J = 6 Hz, 2 H, OCH_2), 3.83 (m, 1 H, 3-CH), 2.50−2.58 (m, 2 H, 2-and 4- CH_2), 2.30−2.45 (m, 2 H, 2-and 4-CH_2), 1.42 (s, 9 H, $C(CH_3)_3$) 1.21 (t, J = 6 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.8 (CH_3), 28.9 ($C(CH_3)_3$), 37.1 (2- and 4-C), 50.8 (CNOBn), 54.6 (1-C), 62.2 (OCH_2), 77.0 (CH_2Ph), 80.7 ($C(CH_3)_3$), 128.5, 129.0, 138.5 (Ar−C), 155.8 (NC=O), 174.1 (CO_2Et); FAB MS *m*/*z* 365 $[M + H]^+$.

***(Z)*- and *(E)*-Ethyl 1-[(*tert*-Butoxycarbonyl)amino]-3-[*N*-(benzyloxy)-*N*-(ethoxycarbonyl)ureido]cyclobutane-1-carboxylate (6a and 6b)**. Each isomer of **5** (0.10 g, 0.28 mmol) was dissolved in 5 mL of dry THF under N_2, and ethoxycarbonyl isocyanate (0.03 g, 0.28 mmol) was added after which the reaction was stirred for 5 min. An additional 1 equiv of ethoxycarbonyl isocyanate was added, and the reaction was stirred for an additional 10 min. The solvent was removed on a rotatory evaporator under aspirator pressure. The crude product was purified using flash chromatography with hexanes/EtOAc (1:1) as the eluant.

***Z* Isomer 6a**: obtained as an oil in a yield of 0.215 g (100%); ^{1}H NMR ($CDCl_3$) δ 7.79 (br s, 1 H, CONHCO), 7.41 (br s, 5 H, Ar−H), 5.29 (br s, 1 H, HNBoc), 4.89 (s, 2 H, $PhCH_2O$), 4.81 (t, 1 H, 3-CH), 4.07−4.26 (m, 4 H, OCH_2), 2.78−2.84 (m, 4 H, 2- and 4-CH_2), 1.42 (s, 9 H, $C(CH_3)_3$), 1.26 (t, 6 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.8 and 15.2 (CH_3), 28.9 ($C(CH_3)_3$), 37.6 (2-

and 4-C), 49.0 (CNOBn), 53.3 (1-C), 61.7 and 62.4 (OCH_2), 62.8 (OCH_2Ph), 80.7 ($OC(CH_3)_3$), 129.8, 130.3, 134.5, 145.5 (Ar−C), 155.3, 153.8, 151.4 (NCOO, NCON, and NCO_2Et), 174.1 (CO_2Et); FAB MS *m*/*z* 478 $[M - H]^-$. Anal. ($C_{23}H_{33}N_3O_8$) C, H, N.

***E* Isomer 6b**: obtained as an oil in a yield of 0.115 g (100%); ^{1}H NMR ($CDCl_3$) δ 7.80 (br s, 1 H, CONHCO), 7.41 (br s, 5 H, Ar−H), 5.05 (br s, 1 H, HNBoc), 5.01 (t, J = 8.4 Hz, 1 H, 3-H), 4.89 (s, 2 H, $PhCH_2O$), 4.13−4.26 (m, 4 H, OCH_2), 2.94−3.01 (m, 2 H, 2- and 4-CH_2), 2.31−2.44 (m, 2 H, 2- and 4-CH_2), 1.43 (s, 9 H, $C(CH_3)_3$), 1.26 (t, 6 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.8 (CH_3), 28.9 ($C(CH_3)_3$), 36.0 (2- and 4-C), 49.6 (CNOBn), 54.1 (1-C), 62.3 and 62.8 (OCH_2), 63.5 (OCH_2Ph), 81.0 ($OC(CH_3)_3$), 129.8, 130.2, 134.4 (Ar−C), 151.3, 153.9, 156.0 (NCOO, NCON, and NCO_2Et), 173.0 (CO_2Et); FAB MS *m*/*z* 478 $[M - H]^-$. Anal. ($C_{23}H_{33}N_3O_8$) C, H, N.

(*Z*)- and (*E*)-Ethyl 1-[(*tert*-Butoxycarbonyl)amino]-3-[*N*-(hydroxy)-*N*-(ethoxycarbonyl)ureido]cyclobutane-1-carboxylate (7a and 7b). Each isomer of **6** (0.10 g, 0.22 mmol) was dissolved in 20 mL of EtOH, and this solution was mixed with 0.05 g of 10% Pd/C. The solution was hydrogenated at 30 psi for 3 h. The solution was then filtered through Celite, and the solvent was removed on a rotatory evaporator under aspirator pressure. The crude compound was purified by flash chromatography with CH_2Cl_2/MeOH (20:1) as the eluant.

***Z* Isomer 7a**: obtained as a solid in a yield of 0.17 g (90%); ^{1}H NMR ($CDCl_3$) δ 8.65 (s, 1 H, NOH), 8.13 (s, 1 H, CONHCO), 5.82 (s, 1 H, BocNH) 4.96−5.01 (m, 1 H, 3-CH), 4.19−4.31 (m, 4 H, OCH_2), 3.02−3.16 (br m, 2 H, 2-and 4-CH_2), 2.82−2.92 (m, 2 H, 2-and 4-CH_2), 1.44 (s, 9 H, $C(CH_3)_3$), 1.27−1.35 (m, 6 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.1 and 14.2 (CH_3), 28.2 ($C(CH_3)_3$), 36.2 (2-C and 4-C), 45.7 (CNOBn), 52.3 (1-C), 61.6 and 61.8 (OCH_2), 79.9 ($OC(CH_3)_3$), 151.8, 153.1, 155.0 (NCOO, NCON, and NCO_2Et), 173.7 (CO_2Et); FAB MS *m*/*z* 342 $[M - EtOH]^+$. Anal. ($C_{16}H_{27}N_3O_8$) C, H; N: calcd, 10.79; found, 10.31.

***E* Isomer 7b**: obtained as a solid in a yield of 0.08 g (100%); ^{1}H NMR ($CDCl_3$) δ 9.04 (br s, 1 H, NOH), 8.63 (s, 1 H, CONHCO), 5.62 (s, 1 H, BocNH), 4.96−5.10 (m, 1 H, 3-CH), 4.11−4.27 (m, 4 H, OCH_2), 2.93−3.07 (m, 2 H, 2- and 4-CH_2), 2.25−2.38 (m, 2 H, 2- and 4-CH_2), 1.41 (s, 9 H, $C(CH_3)_3$), 1.21−1.29 (m, 6 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 14.1 and 14.2 (CH_3), 28.2 ($C(CH_3)_3$), 34.8 (2- and 4-C), 46.8 (3-C), 53.2 (1-C), 61.5 and 61.9 (OCH_2), 80.1 ($OC(CH_3)_3$), 151.8, 153.5, 155.6 (NCOO, NCON, and NCO_2Et), 172.9 (CO_2Et); FAB MS *m*/*z* 390 $[M + H]^+$. Anal. ($C_{16}H_{27}N_3O_8$) C, H, N.

(*Z*)- and (*E*)-1-[(*tert*-Butoxycarbonyl)amino]-3-[2′-(3′,5′-dioxo-1′,2′,4′-oxadiazolidinyl)]cyclobutane-1-carboxylic Acid (8a and 8b). Each isomer of **7** (0.05 g, 0.12 mmol) was dissolved in a mixture of 5 mL of THF and 5 mL of water. Sodium hydroxide (0.014 g, 0.25 mmol) was added, and the reaction was stirred at room temperature for 3 h. It then was quenched by the addition of 3 mL of 10% citric acid solution. The product was extracted with 3 × 25 mL of EtOAc. The organic layer was dried over $MgSO_4$ and the solvent then removed under aspirator pressure to give product.

***Z* Isomer 8a**: obtained in a 62% yield; ^{1}H NMR (MeOH-d_4) δ 4.67−4.72 (m, 1 H, 3-H), 2.79−2.91 (m, 2 H, 2- and 4-CH_2), 2.56−2.63 (m, 2 H, 2- and 4-CH_2), 1.46 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (MeOH-d_4) δ 27.8 ($C(CH_3)_3$), 37.1 (2- and 4-C), 43.1 (CNOBn), 52.5 (1-C), 79.9 ($OC(CH_3)_3$), 152.9 (NCO), 157.2 (NCON), 172.9 (CO_2H); FAB MS *m*/*z* 480 [M + MNBA matrix]$^+$. Anal. ($C_{12}H_{17}N_3O_7$) C, H, N.

***E* Isomer 8b**: obtained in a yield of 0.03 g (85%); ^{1}H NMR (acetone-d_6) δ 4.67−4.72 (m, 1 H, 3-CH), 2.86−3.00 (m, 2 H, 2- and 4-CH_2), 2.45−2.52 (m, 2 H, 2- and 4-CH_2), 1.42 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (acetone-d_6) δ 28.3 ($C(CH_3)_3$), 35.8 (2- and 4-C), 43.0 (3-C), 49.6 (1-C), 79.9 ($OC(CH_3)_3$), 152.1 (NCO), 156.2, 156.6 (NCON, NCOO), 171.6 (CO_2H); FAB MS *m*/*z* 314 $[M - H]^-$. Anal. ($C_{12}H_{17}N_3O_7$) C, H, N.

(*Z*)- and (*E*)-1-Amino-3-[2′-(3′,5′-dioxo-1′,2′,4′-oxadiazolidinyl)]cyclo-butane-1-carboxylic Acid Hydrochloride (2•HCl and 3•HCl). Each isomer of **8** (0.075 g, 0.24 mmol) was dissolved in 2 mL of THF under Ar, and 0.1 mL of 4 N HCl in dioxane was added. The solution was stirred for 1 h. The solvent and HCl were removed under vaccum. Water (5 mL) was added to the residue, and the solution was lyophilized.

***Z* Isomer 2·HCl**: obtained in an 80% yield; ^{1}H NMR (D_2O) δ 4.94 (dd, J = 7.5 and 9 Hz, 1 H, 3-CH), 3.60−3.79 (m, 2 H, 2- and 4-CH_2), 2.87−3.03 (m, 2 H, 2- and 4-CH_2); ^{13}C NMR (D_2O) δ 43.1 (2-C and 4-C), 46.3 (3-C), 53.1 (1-C), 145.9, 157.2 (NCON, NCOO), 174.2 (CO_2H); FAB MS *m*/*z* 216 $[M + H]^+$. Anal. ($C_7H_{10}N_3O_5Cl$) C, H, N.

***E* Isomer 3·HCl**: obtained in an 88% yield; ^{1}H NMR (D_2O) δ 4.97 (t, J = 6 Hz, 1 H, 3-CH), 3.19−3.27 (m, 2 H, 2- and 4-CH_2), 2.65−2.74 (m, 2 H, 2- and 4-CH_2); ^{13}C NMR (D_2O) δ 44.0 (2- and 4-C); 47.1 (3-C), 54.0 (1-C), 145.5, 156.8 (NCON, NCOO), 174.1 (CO_2H); FAB MS *m*/*z* 216 $[M + H]^+$. Anal. ($C_7H_{10}N_3O_5Cl$) C, H, N.

X-ray Diffraction. Colorless platelike crystals of **7a** were grown by vapor diffusion from THF/pentane. All measurements were made on the Siemens SMART platform CCD diffractometer with graphite monochromated Mo Kα radiation (λ = 0.71073 Å) using the hemisphere collector technique at 193(2) K. The structure was solved by direct methods and refined by full matrix least-squares using SHELXTL-V5.0.[39] All non-hydrogen atoms were refined with anisotropically displacement parameters unless otherwise stated. All hydrogen atoms were placed in ideal positions and refined as riding atoms with individual (or group if appropriate) isotropic displacement parameters. The compound crystallized with two independent molecules plus one THF molecule per asymmetric unit. One showed normal crystallographic behavior, while the other possessed disorder with rotation about both carbonyl carbon to OEt bonds. Crystallographic data are provided as Supporting Information.[40]

Cell Cultures. BHK570 cell lines stably expressing mGluR1a and mGluR5a were generously provided by Novo Nordisk A/S (Malov, Denmark).[41] A BHK570 cell line stably expressing mGluR2 was prepared by the methods of Chen and Okayama[42] using a pZEM219b expression vector that was generously donated by Zymogenetics, Inc. (Seattle, WA). BHK570 cells stably expressing mGluR4a was generously provided by Zymogenetics, Inc. BHK570 cells were grown in DMEM supplemented with 5% heat-inactivated fetal bovine serum, 1 μM sodium pyruvate, 2 mM glutamine, 50 mg/mL penicillin, and 50 μg/mL streptomycin, in an atmosphere of 5% CO_2. Stable expression of metabotropic receptor subtypes was maintained by inclusion of appropriate selection media: 1 μM methotrexate and 0.5 mg/mL G418 (Geneticin) for mGluR1a or mGluR4a; 0.5 mg/mL G418 (Geneticin) for mGluR2; or 2 μM methotrexate for mGluR5.

Phosphoinositide Hydrolysis. Phosphoinositide (PI) hydrolysis was measured in neonatal (6−11-day-old) rat hippocampal or cerebellar slices as previously described.[43] Adult Sprague−Dawley rats obtained from Charles River (Wilmington, MA) were bred at Children's Hospital of Philadelphia. For measurement of PI hydrolysis in cell cultures, BHK cells were grown to 60−90% confluence in 12-well tissue culture plates as described above. Cells were treated for 16−20 h with DMEM containing 1 μCi/mL [^{3}H]-*myo*-inositol. PI hydrolysis in cell cultures was measured using the method of Thomsen et al.[44] Measurements of cAMP formation, l-[^{3}H]glutamic acid uptake, and electrophysiological assays were performed as previously described.[27,43,45]

The results from PI hydrolysis experiments were normalized to 100 000 DPM of the total amount of radiolabel incorporated (the amount of radiolabel in the lipid fraction plus the amount of radiolabel in the soluble IP fraction) under basal conditions. For adenylate cyclase experiments, data are presented as fractions of forskolin control. Concentration−response curves were analyzed using nonlinear regression analysis. Curves were fit to the data from each experiment, and the EC_{50} values and maximal stimulatory effects from each experiment were used to determine the average EC_{50} values and maximal stimulatory effect. All data presented are the mean ± SEM. Statistical significance was determined using either Student's *t*-test or ANOVA with a Fisher PLSD post-hoc test. A $p < 0.05$ was considered significant.

AMPA Receptor-Mediated Depolarization and QUIS Effect. The procedures for AMPA receptor-mediated depolarization and its QUIS effect assay were conducted on rat hippocampal slices in the same manner as reported previously.[46]

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Supporting Information Available: X-ray crystallographic data including tables of positional parameters, bond distances, and bond angles for **7a**. This information is available free of charge via the Internet at http://pubs.acs.org.

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