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Pharmacological Characterization and Identification of Amino Acids Involved in the Positive Modulation of Metabotropic Glutamate Receptor Subtype 2

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ABSTRACT

In the present study, we describe the characterization of a positive allosteric modulator at metabotropic glutamate subtype 2 receptors (mGluR2). *N*-(4-(2-Methoxyphenoxy)-phenyl-*N*-(2,2,2-trifluoroethylsulfonyl)-pyrid-3-ylmethylamine (LY487379) is a selective positive allosteric modulator at human mGluR2 and is without activity at human mGluR3. Furthermore, LY487379 has no intrinsic agonist or antagonist activity at hmGluR2, as determined by functional guanosine 5′(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding, single-cell Ca^{2+} imaging, and electrophysiological studies. However, LY487379 markedly potentiated glutamate-stimulated [^{35}S]GTPγS binding in a concentration-dependent manner at hmGluR2, shifting the glutamate dose-response curve leftward by 3-fold and increasing the maximum levels of [^{35}S]GTPγS stimulation. This effect of LY487479 was also observed to a greater extent on the concentration-response curves to selective hmGluR2/3 agonists. In radioligand binding studies to rat cortical membranes, LY487379 increased the affinity of the radiolabeled agonist, [^{3}H]DCG-IV, without affecting the binding affinity of the radiolabeled antagonist, [^{3}H]LY341495. In rat hippocampal slices, coapplication of LY487379 potentiated synaptically evoked mGluR2 responses. Finally, to elucidate the site of action, we systematically exchanged segments and single amino acids between hmGluR2 and hmGluR3. Substitution of Ser688 and/or Gly689 in transmembrane IV along with Asn735 located in transmembrane segment V, with the homologous amino acids of hmGluR3, completely eliminated LY487379 allosteric modulation of hmGluR2. We propose that this allosteric binding site defines a pocket that is different from the orthosteric site located in the amino terminal domain.

G-protein-coupled receptors (GPCRs) are a family of membrane bound proteins that play a central role in the recognition and signal transduction of neurotransmitters. It is generally believed that binding of the agonist induces a conformational change in the intracellular domain responsible for G-protein activation, thereby initiating a cascade of signaling events in the cell. In contrast, competitive antagonist binding will stabilize the inactive conformation of the receptor and block agonist-induced conformational changes and signal transduction (Gether and Kobilka, 1998). Although the exact mechanism of receptor/G-protein interaction is still unclear, GPCRs share a common motif of seven transmembrane helices connected by intra- and extracellular loops, an extracellular amino terminus, and a cytoplasmic carboxyl terminus. Based on their sequence homology, GPCRs have been subdivided into five families (Pin et al., 1994). Family 3 comprises the calcium-sensing receptors, pheromones, $GABA_B$, and metabotropic glutamate (mGlu) receptors. To date, eight mGlu receptor subtypes have been cloned and classified into three groups based on their primary sequence, second messenger coupling, and pharmacology. Signal transduction from group I receptor subtypes

B.A.R. and S.M. participated equally in this work.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; mGlu, metabotropic glutamate receptor; LY487379, *N*-(3-(2-methoxyphenoxy)-phenyl-*N*-(2,2,2-trifluoroethylsulfonyl)-pyrid-3-ylmethylamine; DCG-IV, (2*S*,2′*R*,3′*R*)-2-′,3′)-dicarboxycyclopropyl)glycine; LCCG-I, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycyclopro-1-yl)]-3-(xanth-9-yl) propanoic acid; LY379268, (−)-2-oxa-4-aminobicyclo-[3.1.0]-hexane-4,6-dicarboxylate; WT, wild type; PCR, polymerase chain reaction; Nt, nucleotides; HEK, human embryonic kidney; HBS, HEPES-buffered saline; IP_1, inositol monophosphate; MPP, medial perforant path; FEPSP, field excitatory postsynaptic potential; ANOVA, analysis of variance; CHO, Chinese hamster ovary; PI, phosphoinositide; DG, dentate gyrus; TM, transmembrane; MGS0028, (+)-2-amino-6-fluoro-4-oxobicyclo[3.1.0]-hexane-2,6-dicarboxylic acid monohydrate; Ro 67-7476, (*S*)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine; Ro 01-6128, diphenylacetyl-carbamic acid ethyl ester; CGP7930, 2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol; CGP62349, [3-[1-*R*-[[2-(*S*)-hydroxy-3-[hydroxy-4-methoxyphenyl]-methyl]-phosphinyl]-propyl]-aminoethyl]-benzoic acid; LY 508869, *N*-(4-(2-methoxyphenoxy)phenyl)-*N*-(2,2,2-trifluoroethylsulfonyl)pyrid-3-ylmethylamine.

(mGlu1 and mGlu5) occurs preferentially via the G-protein $G\alpha_q$, whereas group II (mGlu2, -3) and group III receptors (mGlu4, -6, -7, and -8) couple with proteins from the $G\alpha_i/G\alpha_o$ family (Conn and Pin, 1997). X-ray crystallography, ligand binding studies, and mutagenesis reports have shown that the large N-terminal extracellular domain of mGlu receptors contains the glutamate binding or orthosteric site (Kunishima et al., 2000).

The allosteric modulation of ligand-gated ion channels is a well-known phenomenon and has been exploited therapeutically by development of the benzodiazepines (e.g., diazepam), which act to enhance the activity of GABA at $GABA_A$ receptors (Sigel and Buhr, 1997). Positive allosteric modulators at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ionotropic glutamate receptors, with activities similar to cyclothiazide and aniracetam, have also been developed (Shepherd et al., 2002). Galanthamine, which is an acetylcholinesterase inhibitor, has also been reported to be an allosteric modulator at neuronal nicotinic acetylcholine receptors (Erkinjuntti et al., 2002). In a similar manner, small molecules have recently been reported that positively modulate multiple GPCRs by binding to a site distinct from the orthosteric site (Christopoulos and Kenakin, 2002). Several lines of evidence suggest that allosteric modulators may offer advantages over GPCR agonists (Pin et al., 2001) because of receptor desensitization after repeated dosing of the agonist. The most thoroughly studied allosteric binding site on a GPCR is on muscarinic receptors (Birdsall et al., 1995). Recently, many reports of new small molecules that positively modulate family 3 GPCRs at Ca^{2+}-sensing receptors (Hammerland et al., 1998), $GABA_B$ (Urwyler et al., 2001), or mGlu1 receptors (Knoflach et al., 2001) have been reported.

Group II mGlu receptors are expressed in several brain and spinal cord regions. In situ hybridization (Ohishi et al., 1993a,b), immunohistochemistry (Carlton et al., 2001), and autoradiography (Schaffhauser et al., 1998) have confirmed expression of mGlu2/3 receptors in the hippocampus, olfactory bulb, neocortical regions, and cerebellar Golgi neurons, with lower levels of expression in thalamic nuclei and striatum. The activation of these receptors has been shown to decrease synaptic transmission and glutamate release in the hippocampus (Macek et al., 1996). The distribution and prominent functions of group II mGlu receptors in neuronal excitability and synaptic transmission suggests that modulation of these GPCRs is a promising strategy for the treatment of neurological and neuropsychiatric disorders [e.g., anxiety, schizophrenia, and pain (Conn and Pin, 1997; Chavez-Noriega et al., 2002; Varney and Gereau, 2002)].

The synthesis of pyridine-sulfonamide derivatives, a novel class of compounds that behave as selective positive allosteric modulators of mGlu2 receptors, has been disclosed in a international patent application (Colemna et al., 2001) and recently reported at the IV International Meeting on Metabotropic Glutamate Receptors (Johnson et al., 2002). In the present study, we characterized the activity of *N*-(4-(2-methoxyphenoxy)-phenyl-*N*-(2,2,2-trifluoroethylsulfonyl)-pyrid-3-ylmethylamine (LY487379; Fig. 1A) at mGlu2 and mGlu3 receptors, employing both biochemical and electrophysiological techniques in native and recombinant systems. This compound is structurally different from glutamate and exhibits selective positive modulation at cloned hmGlu2 receptors. In addition, the amino acids mediating the selective modulation of mGlu2 receptor were identified using a series of chimeric receptors and point mutations in which segments or amino acid residues of hmGlu2 receptor were exchanged with the corresponding amino acids of the hmGlu3 receptor. We found that LY487379, a selective mGlu2 receptor positive modulator, binds to a pocket defined by 3 amino acids located in transmembrane IV and V of the mGlu2 receptor.

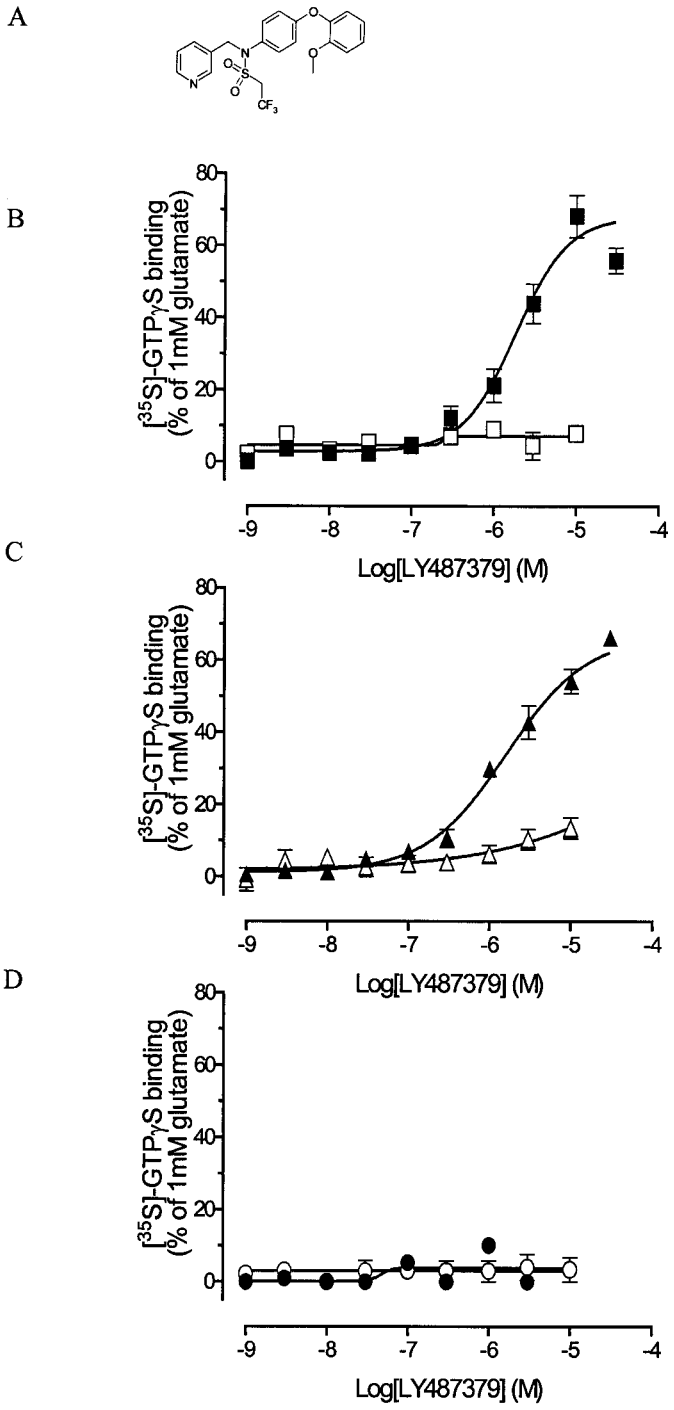

Fig. 1. A, chemical structure of LY487379. B to D, concentration-response curves for LY487379 in the absence (open symbol) or presence (closed symbol) of glutamate (EC_{10}) generated from stimulation of [^{35}S]GTPγS binding to membrane from rat brain (B), or cell lines expressing hmGlu2 (C) and hmGlu3 receptors (D). Results are expressed as a percentage of the response to glutamate (1 mM) and are the mean of three individual experiments performed in triplicate.

Materials and Methods

Materials. Glutamate, GDP, probenecid and GTPγS, were obtained from Sigma Chemical (St Louis, MO). [^{35}S]GTPγS (1,250 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA). (2*S*,2′*R*,3′*R*)-2-(2′,3′)-dicarboxycyclopropyl)glycine (DCG-IV), (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (LCCG-I), LY341495, [^{3}H]DCG-IV (18.3 Ci/mmol), and [^{3}H]LY341495 (36.5 Ci/mmol) were all obtained from Tocris Cockson (Ellisville, MO). LY487379 and LY379268 were synthesized in-house (Merck Research Laboratories, San Diego, CA). Male Sprague-Dawley rats (250–300 g) or Wistar rats (2–4 weeks old) were purchased from Harlan (Indianapolis, IN); fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). Unifilter GF/B plates and Microscint 20 were obtained from PerkinElmer Life Science. pcDNA3.0, pcDNA3.1-based mammalian expression vectors, WesternBreeze chemiluminescent immunodetection kit, Nitrocellulose (0.45-μm membrane), and NuPage 4 to 12% Bis-Tris Gels were purchased from Invitrogen (Carlsbad, CA). QuikChange site-directed mutagenesis kit was obtained from Stratagene (San Diego, CA). Anti-mGluR2/3 was purchased from Upstate Biotechnology (Lake Placid, NY). Actin (β) antibody was purchased from Abcam (Hartford, CT). Effectene kit was purchased from QIAGEN (Valencia, CA). BioCoat Fibronectin 24-well tissue culture plates were obtained from BD Biosciences Discovery Labware (Bedford, MA). Dowex-1-X8 (200–300 mesh in the formate form) was obtained from Bio-Rad (Hercules, CA). [*myo*-2-^{3}H]inositol (specific activity, 18 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Protease inhibitor cocktail was obtained from Roche Diagnostics (Indianapolis, IN).

Cloning of Wild-Type Human mGlu2 and mGlu3 Receptor cDNAs. The cloning of wild-type (WT) human mGlu2 receptor cDNA was performed as described by Flor et al. (1995). The hmGlu2 receptor cDNA was subsequently cloned into the pcDNA3.1-based mammalian expression vector. The full-length WT human mGlu3 receptor cDNA was cloned from two partial cDNAs isolated from human hippocampus. All of the 5′-untranslated sequence was removed from the resulting cDNA,and a consensus ribosomal sequence was added to the very 5′-end of the cDNA by PCR techniques. The hmGlu3 receptor cDNA was cloned into the mammalian expression vector pcDNA3.0.

Construction of Chimeric And Point Mutations of hmGlu2/3 and hmGlu3/2 Receptor cDNAs. Chimeric receptor cDNAs were generated by initially performing site-directed mutagenesis on the WT cDNAs to introduce multiple restriction sites into conserved regions of the WT cDNAs that did not alter the resulting amino acid coding sequence. *Pin*AI (nt 1651–1656), *Eco*47III (nt 1944–1949), and *Mlu*I (nt 2245–2250) restriction sites were introduced into the WT mGlu2 receptor cDNA. *Pin*AI (nt 1678–1683), *Eco*47III (nt 1971–1976) and *Mlu*I (nt 2272–2277) restriction sites were introduced into the mGlu3 receptor cDNA. Once the restriction mutations were introduced into the WT clones, the resulting constructs were digested with *Pin*AI and *Eco*47III to produce the hmGlu2/3 chimeric construct, hmGlu2/3 receptor TM1–3 or with *Eco*47III and *Mlu*I to produce the chimeric construct, hmGlu2/3 TMIII-V. All of the single- and multiple-point mutations of hmGlu2/3 receptor cDNAs were synthesized using the QuikChange site-directed mutagenesis kit following the manufacturer's instructions. The chimeric and point mutant cDNAs were cloned into the pcDNA3.1-based mammalian expression vectors. The samples were completely sequenced from the CMV promoter to the termination sequence by automated DNA sequence protocols to determine whether the correct mutation had been incorporated and no other mutations had been misincorporated (Applied Biosystems, Foster City, CA).

Transient Transfection of Chimeric hmGlu2/3 cDNAs into HEK293 Cells. All the generated cDNA constructs, including the wild type, were cotransfected with Gα16 cDNA (kindly provided by Aurora Pharmaceuticals, San Diego, CA) into HEK293 cells with the lipid-based transfection reagent Effectene using a modified version of the reverse transfection protocol (Ziauddin and Sabatini, 2001). Briefly, hmGlu2 or hmGlu3 receptor cDNA (0.35 μg/well), Gα16 cDNA (0.35 μg/well), and enhanced green fluorescent protein cDNA (0.1 μg/well) were mixed with 45 μl/well of DNA-condensation buffer (buffer EC, Effectene kit) in which sucrose had been dissolved to a concentration of 0.3 M. Enhancer solution was added to the mixture (4.5 μl/well), remixed by gentle vortexing, and allowed to incubate for 5 min at room temperature. Effectene reagent (11 μl/well) was added to the mixture, remixed by gentle vortexing, and allowed to incubate for 10 min at room temperature. Finally, 90 μl/well of 0.25% glycogen was added to the mixture and remixed by gentle vortexing. The resulting DNA-lipid-polymer transfection reagent (150 μl/well) was added to the bottom of a fibronectin-coated, 24-well tissue culture plate; gently tapped to insure complete coating of the bottom of the well; and then allowed to incubate overnight at 4°C. The plate was then dried under vacuum and seeded with HEK293 cells at 0.7×10^6 cells/well. The cells were incubated in a 37°C, CO_2 incubator for 36 h. The transient expression of enhanced green fluorescent protein was used as an indicator of transfection efficiency.

Membrane Preparation and [^{35}S]GTPγS Binding. hmGlu2 and hmGlu3 receptor-expressing stable cell lines (Varney et al., 1999) were grown to confluence in a T-225-cm^2 flask and washed twice with ice-cold PBS. The cells were then scraped with a cell scraper in phosphate-buffered saline and harvested by centrifugation (200*g*) using a tabletop centrifuge. The cell pellet was homogenized in hypotonic buffer A (20 mM HEPES and 10 mM EDTA, pH 7.4) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 40,000*g* for 20 min. The resulting pellet was washed once in the same buffer and once with buffer B (20 mM HEPES and 0.1 mM EDTA, pH 7.4). At the last centrifugation, the pellet was resuspended in buffer B and the homogenate was aliquoted and stored at −80°C at a protein concentration of approximately 1 mg/ml. Protein measurement was determined with the Bio-Rad detergent-compatible protein assay kit using bovine serum albumin as standard.

Rats (250–300 g) were decapitated; the whole brain was removed, placed on ice, and homogenized in 6 volumes (w/v) of 10% sucrose at 4°C using a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000*g* for 10 min, and the supernatant was centrifuged at 40,000*g* for 20 min. The pellet was resuspended in 25 ml of water using a Polytron homogenizer and centrifuged at 8,000*g* for 10 min. The supernatant was further centrifuged at 40,000*g* for 20 min at 4°C. The supernatant was removed and the pellet was resuspended in buffer C (5 mM HEPES-KOH, pH 7.4). The homogenate was freeze-thawed twice before being centrifuged at 40,000*g* for 20 min. Finally the resulting pellet was resuspended in buffer C, aliquoted and stored at −70°C until used.

[^{35}S]GTPγS Binding Assay. Membranes were thawed and homogenized in 25 ml of 20 mM HEPES containing 0.1 mM EDTA, pH 7.4, and centrifuged at 40,000*g* for 20 min. The pellet was resuspended in assay buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, and 3 mM $MgCl_2$ at a final protein concentration of 0.5 mg/ml (hmGlu2 and hmGlu3 receptors) or 0.1 mg/ml (rat brain). In a 96-well plate (Beckman Coulter, Fullerton, CA), test compounds were added along with 5 μM GDP, membrane (10 μg/well for rat brain and 50 μg for recombinant mGlu receptors), and 0.05 nM [^{35}S]GTPγS to achieve a total volume of 0.5 ml in assay buffer. The plate was incubated at 30°C for 1 h, and the assay was terminated by rapid filtration over Unifilter GF/B plate using a 96-well cell harvester (Brandel, Gaithersburg, MD). The plate was rinsed three times with ice-cold assay buffer, dried, and 50 μl of Microscint 20 was added to each well. The plate was counted in a Topcount scintillation counter (PerkinElmer Life Science).

The concentration of [^{35}S]GTPγS in saturation studies using whole-brain membrane was varied from 0.04 to 10 nM. Glutamate (10 μM) was used as an agonist, and the experiment was performed in the absence or presence of LY487379 (3 μM). Nonspecific binding was determined by addition of 10 μM unlabeled GTPγS. Each exper-

iment was performed using triplicate samples per data point and then repeated on separate occasions to obtain a total of three determinations. Data were normalized to the response obtained with 1 mM glutamate. The curves were fitted to a four-parameter logistic equation giving EC_{50} values, Hill coefficient, and maximal effect using Prism (GraphPad Software, San Diego, CA).

Membrane Preparation and Radioligand Binding Study. Cortices were dissected on ice and homogenized in 25 volumes of 50 mM Tris-HCl, pH 7.1, with a Polytron homogenizer. The homogenate was centrifuged at 40,000*g* for 10 min. The pellet was resuspended in the same buffer, incubated at 37°C for 10 min, and centrifuged as described above. The final pellet was resuspended, aliquoted, and stored at −80°C.

[^{3}H]DCG-IV binding assay was adopted from the method described previously by Mutel et al. (1998). Isotherm saturation studies were performed by incubating 0.5 mg of protein with various concentrations of [^{3}H]DCG-IV (0.1–1000) for 1 h at room temperature in the absence or presence of LY487379 (3 μM). Nonspecific binding was defined in the presence of 100 μM DCG-IV. Isotherm saturations were analyzed using GraphPad Prism.

The effect of the potentiator on the binding of 4 nM [^{3}H]LY341495 binding to cortical membrane (12 μg of protein) was assayed as described previously by Wright et al. (2001). Nonspecific binding was defined by 1 mM glutamate.

Measurement of Phosphoinositide Hydrolysis. HEK293 cells that were previously transiently transfected were labeled overnight with 1 μCi/well [*myo*-2-^{3}H]inositol in a glutamine-free Dulbecco's modified Eagle's medium. The following day, the medium was removed and the cells were washed two times with HBS containing 125 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 6 mM glucose, and 20 mM HEPES, pH 7.4, for 45 min at 37°C. After the wash, cells were incubated with HBS containing 10 mM LiCl. After a 20-min incubation in the LiCl-containing buffer, glutamate alone [5 μM (EC_{10}) or 1 mM (EC_{100})] or glutamate (5 μM, EC_{10}) in combination with varying concentrations of LY487379 was added, and the mixture incubated for an additional hour at 37°C. The reactions were terminated by aspiration of the media and the accumulated [^{3}H]IP_1 was extracted by adding 1 ml of ice-cold chloroform/methanol/HCl (4 N; 200:100:2). The mixtures were transferred to glass tubes containing 300 μl of chloroform and 400 μl of H_2O and were vortexed briefly. The aqueous phase was separated from the organic phase by settling for 15 min. The [^{3}H]IP_1 fraction was separated using Dowex-1-X8 (200–300 mesh in the formate form) and quantified by liquid scintillation counting. Data were normalized to the response obtained with 1 mM glutamate.

Immunoblot Analysis. Selected mGlu receptor chimeric or mutant cDNAs were transiently transfected as described above and total membranes were harvested by solubilization of the cells in 1× radioimmunoprecipitation assay buffer containing 1× Complete protease inhibitor cocktail (Roche Diagnostics). The resulting membranes were electrophoresed in a NuPage 4–12% Bis-Tris gel then transferred to a nylon membrane (0.45 μm). The detection of mGluR2/3 and actin proteins was performed with anti-mGluR2/3 polyclonal antibody and anti-actin polyclonal antibody. The chemoluminescence detection of proteins was performed using a WesternBreeze chemiluminescence immunodetection kit.

Single-Cell Ca^{2+} Imaging. In single-cell Ca^{2+} imaging measurements, stable CHO cells expressing hmGlu2 were assayed on 22 mm poly-D-lysine–coated glass coverslips at a density of approximately 500,000 cells per coverslip. The cells were washed in HBS and loaded with 3 μM fura-2 acetoxymethyl ester in 1 mM probenecid for 90 min at room temperature. The coverslips were washed of unloaded dye and transferred to a coverslip chamber (Warner Instruments, Hamden, CT). The chamber was placed on the stage of a microscope equipped for fluorescence microscopy (TE300; Nikon, Tokyo, Japan). A xenon-arc lamp combined with a DeltaRAM photometer (Photon Technology International, Lawrenceville, NJ) was used for excitation of the loaded fura-2 at 345 and 380 nm. Excitation light was deflected by a dichroic mirror into the 40×-oil immersion microscope objective (Nikon CFI Super Fluor 40×) and onto the sample. Emitted light was collected from the sample through a dichroic mirror, and a 510 nm interference filter and directed into a Quantix 57 charge-coupled device camera (Photometrics, Tucson, AZ). Pairs of images were captured using software and hardware from Photon Technology International (Lawrenceville, NJ). Ratio images were generated after background subtraction. Agonists and antagonists were added to the cells using a perfusion system controlled by a valve bank (Cell Microcontrols, Virginia Beach, VA) with a flow rate of between 2 and 4 ml/min.

Hippocampal Slice Electrophysiology. Experiments were carried out in accordance with procedures approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories, San Diego, in accordance with National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Transverse hippocampal slices, 400 μm thick, were prepared from young (2–5 week old) Wistar rats. Animals were anesthetized with isoflurane; the brain was then extracted and sliced using a vibratome (Pelco 100; Pelco International, Redding, CA). A modified Krebs' solution containing sucrose (240 mM) substituting for NaCl was used for slice sectioning (Chavez-Noriega and Stevens, 1994). Slices were kept in a submerged incubation chamber at room temperature for at least 1 h before recording. In the recording chamber, slices were fully submerged and continuously perfused (2 ml/min) with Krebs' solution saturated with 95% O_2/5% CO_2, pH 7.4, at a temperature of 32°C. The Krebs' solution consisted of 120 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 11 mM D-glucose, 23 mM $NaHCO_3$, and 2 mM $CaCl_2$ (osmolarity, 290 mOsM). Stimulation and recording were performed in the middle third of the molecular layer, upper blade of the dentate gyrus. A bipolar tungsten microelectrode (FHC, Bowdoinham, ME) was used for stimulation of medial perforant path (MPP) fibers; stimuli (150–700 μA, 100 μs) were delivered at 0.1 Hz using an Isolator-11 stimulator (Axon Instruments, Union City, CA). Recordings were performed with an Axoclamp 2A amplifier (Axon Instruments) or an IE-210 amplifier (Warner Instruments Corp., Hamden, CT); glass micropipettes were filled with 3 M NaCl (1–3 MΩ resistance). Data were digitized and filtered at 10 and 2 kHz, respectively. Pulse and PulseFit (HEKA Elektronik, Lambrecht, Germany) were used for data acquisition and analysis. The field excitatory postsynaptic potential (fEPSP) slope was measured near the end of the deflection of the presynaptic fiber volley. An input-output curve was performed before each experiment, and the stimulus intensity was adjusted to produce a fEPSP of ≈50 to 70% of maximal amplitude. GraphPad Prism software was used to calculate EC_{50} values using nonlinear regression analysis. Statistical significance was determined using SigmaStat (SPSS Science, Chicago, IL) with a Student's *t* test; paired *t* tests were performed where indicated in the text when effects were measured in the same slice; the one-way repeated measures ANOVA with Bonferroni multiple comparisons test was also used where indicated.

Effect of LY487379 on mGlu5a/L38–20 Cells and hmGlu7 + Gα16 Receptors. The activity of LY487379 was examined against the hmGlu5a and hmGlu7 + Gα16 receptor stably expressed in mouse fibroblast Ltk cells (the hmGluR5a/L38–20 cell line) and CHO cells, respectively. Receptor activity was detected by changes in $[Ca^{2+}]_i$, measured using the fluorescent, Ca^{2+}-sensitive dye fura-2 as described previously (Varney et al., 1999). For antagonist testing, submaximal glutamate concentrations 10 μM and 2 mM were used for hmGlu5 and hmGlu7 receptor, respectively.

Results

Effect of LY487379 on Rat and Recombinant Human Group II mGlu Receptors. Stimulation of [^{35}S]GTPγS binding was used to provide a functional measure of $G\alpha_i$-coupled receptors in native and recombinant receptor mem-

brane preparations (Lazareno and Birdsall, 1993). Concentration-response curves for a number of selective group II mGlu receptor agonists were generated using membranes from rat brain or in cells stably expressing either human mGlu2 or mGlu3 receptors. Glutamate-induced [^{35}S]GTPγS binding to rat brain membranes or recombinant cell lines was mediated via group II mGlu receptors because the response was blocked by 1 μM LY341495 (data not shown), a competitive antagonist for group II mGlu receptors. LY487379 alone did not stimulate [^{35}S]GTPγS binding in membranes prepared from rat brain or from cells expressing hmGlu2 receptors or hmGlu3 receptors (Fig. 1, B to D). However, in the presence of the low concentration of glutamate that evoked a small response (an approximate EC_{10} of 1 μM for rat brain and hmGlu2 and 10 nM for hmGlu3 receptors), LY487379 increased the magnitude of glutamate-induced [^{35}S]GTPγS binding. The magnitude of the potentiation was similar between rat brain and hmGlu2 membranes and occurred in a concentration-dependent manner (Fig. 1, B and C). The EC_{50} values for this modulation are summarized in Table 1. Interestingly, LY487379 was without effect on glutamate-induced [^{35}S]GTPγS binding in membranes prepared from cells expressing hmGlu3 receptors (Fig. 1D).

To further characterize the effect of this positive modulator on mGlu2 receptors, concentration-response curves to the agonists DCG-IV, LCCG-I, and glutamate were performed using [^{35}S]GTPγS binding in the presence of a fixed concentration of LY487379 (3 μM). These agonists exhibited different potencies and efficacies at mGlu2 receptors (Fig. 2; Table 2). At 1 mM, the highest concentration tested, the responses to DCG-IV and LCCG-I were similar to but statistically different from the maximal stimulation induced by glutamate (50–70%) (Fig. 2, A-C; Table 2). The effect of LY487379 on agonist-induced [^{35}S]GTPγS binding showed a dual effect, with a 2- to 5-fold increase in the apparent potency and a 2-fold increase in the maximal response magnitude of DCG-IV and LCCG-I (Fig. 2, B and C). However, LY487379 had very little effect on the maximum response induced by glutamate or LY379268 in hmGlu2 receptor membranes (Fig. 2, A and D). Furthermore, LY487379 was devoid of any effects on glutamate-induced [^{35}S]GTPγS binding in hmGlu3 receptor membranes (Fig. 2E). In addition, LY487379 was tested for its ability to agonize, antagonize, or potentiate a glutamate-induced response in cell lines expressing a representative human group I (hmGlu5a) or group III (hmGlu7a) receptor. LY487379 was devoid of any agonist, antagonist, or allosteric modulator activities on these mGlu receptor subtypes (data not shown). These results suggest that LY487379

TABLE 1

Activity of LY487379 on glutamate-induced [^{35}S]GTPγS binding.

Concentration-response curves were determined in the presence of 1 μM glutamate in membranes prepared from rat brain or recombinant cells expressing hmGlu2 receptors, or 10 nM glutamate for the hmGlu3 receptor preparation. Results are expressed as mean EC_{50} values (lower-upper S.D.) and mean efficacy (E_{max}) ± S.E.M., expressed relative to the maximal stimulation achieved by glutamate alone. Summary data are calculated from three individual experiments performed in triplicate.

Membrane Preparation	EC_{50}	E_{max}
	μM	
Rat brain (cortex)	2.8 (2.1–3.7)	68 ± 4
hmGluR2	1.7 (0.7–4.0)	52 ± 4
hmGluR3	N.E.	N.E.

N.E., no effect at concentrations up to 10 μM.

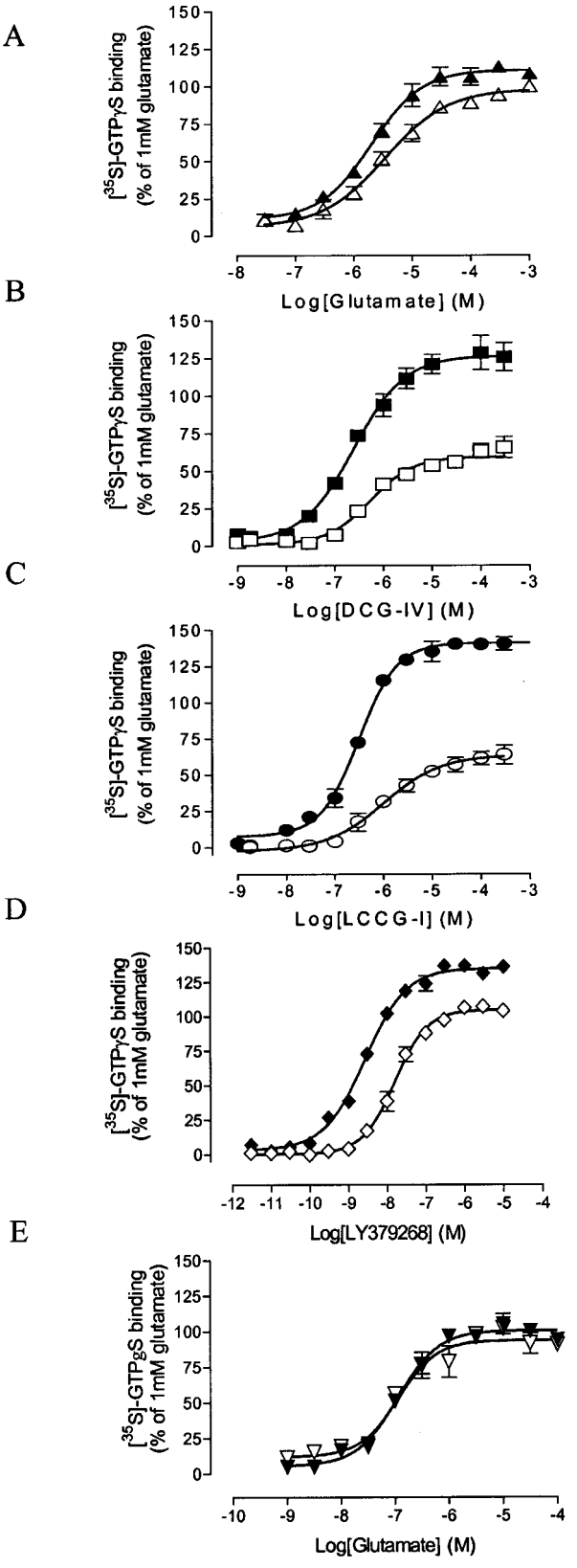

Fig. 2. Concentration response curves for glutamate (A), DCG-IV (B), LCCG-I (C), and LY379268 (D)-induced [^{35}S]GTPγS binding to membrane prepared from recombinant hmGlu2 or (E) hmGlu3 receptor cell lines in the absence (open symbols) or presence (filled symbols) of 1 μM LY487379. Results are expressed as a percentage of the response to glutamate (1 mM) and are the means ± S.E.M. from three individual experiments performed in triplicate.

is a selective mGlu2 receptor positive modulator that increases both the affinity and the efficacy of an agonist ligand.

One possible mechanism that may explain this dual action of LY487379 is that there is an increase in the coupling to G-proteins. To test this hypothesis, we performed saturation binding experiments to determine the effect of LY487379 on glutamate (10 μM)-stimulated [^{35}S]GTPγS binding. Under these conditions, LY487379 (3 μM) significantly increased the number of binding sites (B_{max}) without affecting the affinity (K_d) of the [^{35}S]GTPγS for G-proteins (Table 3), suggesting that there is an increased coupling to G-proteins.

Potentiator Properties of LY487379 Using Single-Cell Ca^{2+} Imaging Assay. We characterized the effect of LY487379 on a stable (CHO) cell line coexpressing the hmGlu2 receptor with the promiscuous G-protein Gα_{16}. Previous studies have revealed that mGlu2 receptors can couple to Gα_{16} to activate phospholipase C, increasing phosphoinositide (PI) hydrolysis and the subsequent release of Ca^{2+} from intracellular stores, which can be monitored by single cell Ca^{2+} fura-2 imaging (Gomeza et al., 1996). In these cells, application of LY487379 alone did not elicit a Ca^{2+} response; however, coapplication with glutamate (10 μM), a concentration that evoked a submaximal response when applied alone, resulted in a 2-fold enhancement of the response to glutamate (Fig. 3A) that recovered after washout. The potentiation with LY487379 was blocked by the selective group II antagonist LY341495 (1 μM) (Fig. 3B).

Effect of LY487379 on [^{3}H]DCG-IV and [^{3}H]LY341495 Binding in Rat Cortical Membranes. In rat cortical membranes, LY487379 increased the amount of specific binding of 50 nM [^{3}H]DCG-IV with an EC_{50} of 56 nM (46–68) (geometric mean; values in parentheses are the lower and upper S.D.) and with an efficacy (E_{max}) of 177 ± 4% (Fig. 3C). Saturation isotherms with [^{3}H]DCG-IV in the presence of 3 μM LY487379 indicated a 2-fold increase in affinity (K_d) without a change in the number of binding sites (B_{max}) (Table 3). In contrast to the radiolabeled agonist, the binding of the radiolabeled mGlu receptor antagonist [^{3}H]LY341495 was unaffected by the presence of the allosteric modulator LY487379 (Fig. 3C).

Effect of LY487379 on Medial Perforant Path-Dentate Gyrus Synapses of Rat Hippocampus. The hippocampal slice preparation was used to evaluate the activity of LY487379 on excitatory glutamatergic transmission in medial perforant path-dentate gyrus (MPP-DG) synapses, in which transmission is inhibited by activation of group II mGlu receptors (Kew et al., 2001). Application of LY487379 (3–30 μM) produced minimal effects on fEPSPs when applied on its own, but it significantly potentiated the inhibition produced by DCG-IV alone (Fig. 4A). LY487379 produced similar effects on both the fEPSP peak and slope. The potentiation produced by LY487379 was observed when LY487379 (3 μM) was applied either before (+40.3%, $n = 7$) or after (+50.9%, $n = 3$) DCG-IV. The group II mGlu receptor antagonist LY341495 (0.5–1 μM) blocked the effects of DCG-IV alone (93% block) and DCG-IV + LY487379 (60% block; data not shown). The small and variable effect of LY487379 (3 μM) applied on its own (7%) was reduced by LY341495 on average by 50%, although this effect did not reach statistical significance.

DCG-IV inhibited MPP fEPSPs with an IC_{50} of 92 nM with a maximal inhibition of 63%. Coapplication with LY487379 (3 μM) shifted the concentration-response curve for DCG-IV to the left by approximately 3-fold, to 32 nM, with a maximal inhibition of 63% (Fig. 4B). Coapplication of LY487379 potentiated the inhibition of MPP fEPSPs produced by an $\approx IC_{20}$ concentration of DCG-IV (50 nM) by up to 148% (maximal inhibition of baseline, predrug fEPSPs, 53%). The enhancement produced by coapplication with LY487379 at concentrations 3 to 40 μM was statistically significant compared with slices treated with DCG-IV alone ($P < 0.001$, ANOVA). LY487379 potentiated DCG-IV responses with an EC_{50} of 3.9 μM (Fig. 4C).

The Transmembrane Domain of hmGlu2 Receptor Mediates Positive Allosteric Modulation of LY487379. The lack of displacement of [^{3}H]LY341495 prompted us to

TABLE 2

Effect of LY487379 on agonist-evoked [^{35}S]GTPγ S stimulation at human mGlu2 receptors

Potency (EC_{50}), and efficacy (E_{max}) for the stimulation of [^{35}S]GTPγS binding to recombinant human mGlu2 receptors preparation in the absence or presence (+ LY) of 3 μM LY487379. Summary data represent the geometric mean EC_{50} values (lower-upper S.D.), and mean efficacy ± S.E.M. (compared with glutamate alone), calculated from three individual experiments performed in triplicate. Ratio values are calculated from the EC_{50} or E_{max} values obtained by the agonist alone, divided by those obtained in the presence of LY487379.

Agonists	EC_{50}	EC_{50}(+ LY)	Ratio (EC_{50})	E_{max}	E_{max}(+LY)	Ratio(E_{max})
	nM	*nM*				
Glutamate	8,000(7,000–9,000)	3,300(2,000–6,000)	2.4	101 ± 2	108 ± 5	1.1
DCG-IV	400(270–630)	270(200–360)	1.5	56 ± 5*	127 ± 8	2.3
LCCG-I	1,600(600–4,000)	300(260–400)	5.3	68 ± 10*	136 ± 7	2.0
LY379268	17(18–40)	4(2–9)	4	103 ± 4	130 ± 7	1.26

* Significantly different from glutamate ($p < 0.01$ paired t test).

TABLE 3

Effect of LY487379 (3 μM) on the saturation binding of ([^{35}S]GTPγS) or [^{3}H]DCG-IV to native group II mGlu receptors in rat cortical membranes

The K_d values are expressed as the geometric mean (lower-upper S.E.) and the B_{max} as mean ± S.E.M. from data obtained from three independent experiments.

	LY487379	K_d	B_{max}
	μM	*nM*	*fmol/mg of protein*
[^{35}S]GTPγS	0(control)	0.4(0.2–0.9)	2360 ± 330
	3	0.6(0.34–0.9)	4,110 ± 150*
[^{3}H]DCG-IV	0(control)	214 (188–242)	1326 ± 90
	3	115 (87–150)*	1304 ± 95

* Significantly different from control ($p < 0.05$, Student t test).

speculate that LY487379 does not act at the glutamate-binding site located in the large N-terminal extracellular domain. To elucidate the precise location of the binding site of LY487379 on the hmGlu2 receptor, we initially constructed three chimeras in which we exchanged segments or a single amino acid between hmGlu3 and hmGlu2 receptors spanning the transmembrane domains (Fig. 5A). These different chimeric constructs, in addition to the wild-type receptor, were transiently transfected into HEK293 cells with Gα16. In the wild-type receptor, glutamate induced the production of [^{3}H]IP$_1$ in a concentration-dependent manner with an EC$_{50}$ of 40 μM (30–53) [geometric mean (lower, upper S.D.); data not shown]. Addition of LY487379 alone (10 μM) slightly increased production of [^{3}H]IP$_1$. However, this concentration of LY487379, in the presence of a low concentration of glutamate (EC$_{10}$ of 5 μM of glutamate), significantly increased the magnitude of the glutamate-induced phosphoinositide hydrolysis (Fig. 5A). The exchange of the transmembrane region TMI-III (Thr550 to Leu656) or a single amino acid residue in TMVII (Ser817) from hmGlu2 receptor residues with hmGlu3 receptor sequences did not significantly change the enhancing property of LY487379 on glutamate-induced [^{3}H]IP$_1$ accumulation (Fig. 5, B and D). However, exchanging the region containing the amino acids between TMIII and V (Leu656 to Arg750) from the hmGlu2 receptor with hmGlu3 receptor sequences completely abolished the potentiator effect of LY487379 (Fig. 5C). Western blot analysis of the hmGlu2/3 receptor chimeras revealed no significant difference in the level of protein expression compared with wild-type mGlu2 receptor, and the magnitude of glutamate-stimulated [^{3}H]IP$_1$ was similar to that of wild-type mGlu2 receptor, suggesting that this enhancing effect of LY487379 is mediated by amino residue(s) present between Leu656 and Arg750 (Fig. 5E).

Sequence alignments of hmGlu2 and hmGlu3 receptors in this TMIII-V segment revealed multiple amino acid differences in residues contained within these transmembrane domains and extracellular loop. To precisely identify the residues governing the positive allosteric modulation of the hmGlu2 receptor by LY487379, we constructed a series of mutations in which multiple or single amino acid residues of the hmGlu2 receptor were substituted with the homologous amino acids of hmGlu3 receptor (Fig. 6A). In TMIV, substitutions of single residues A681F or double residues V695S-A696V had no significant effect on the functional effect of LY487379 (Table 4). A partial (60%) and significant reduction of the potentiation by LY487379 of glutamate-induced [^{3}H]IP$_1$ accumulation was observed in the hmGlu2 S688L-G689V receptor mutant (Fig. 6B; Table 4). Interestingly, however, the single point mutations S688L and G689V had no effect on their own, suggesting that these residues are important in the binding of LY487379 only when present together (Fig. 7A, Table 4). The analysis of the extracellular segment between TMIV and V mutants by single mutation (H723V) or double mutations (A710L-P711A; V716T-T718I) (Table 4) revealed no significant reduction in the potentiator effect of LY487379 on glutamate-induced [^{3}H]IP$_1$ production. In TMV, the single point mutations G730I and A740I also failed to show a significant loss of LY487379 activity (Table 4). In contrast, the double mutant A733T-N735D significantly reduced (70%; Fig. 6C, Table 4) the positive modulation by LY487379 on glutamate-induced [^{3}H]IP$_1$ accumulation. However, only the single mutation N735D was able to significantly reduce the effect of LY487379, suggesting that Ala733 is not involved in the binding of the compound (Fig. 7A, Table 4). The combined double mutants S688L-N735D and G689V-N735D or the triple mutant S688L-G689V-N735D completely prevented the effects of LY487379 binding

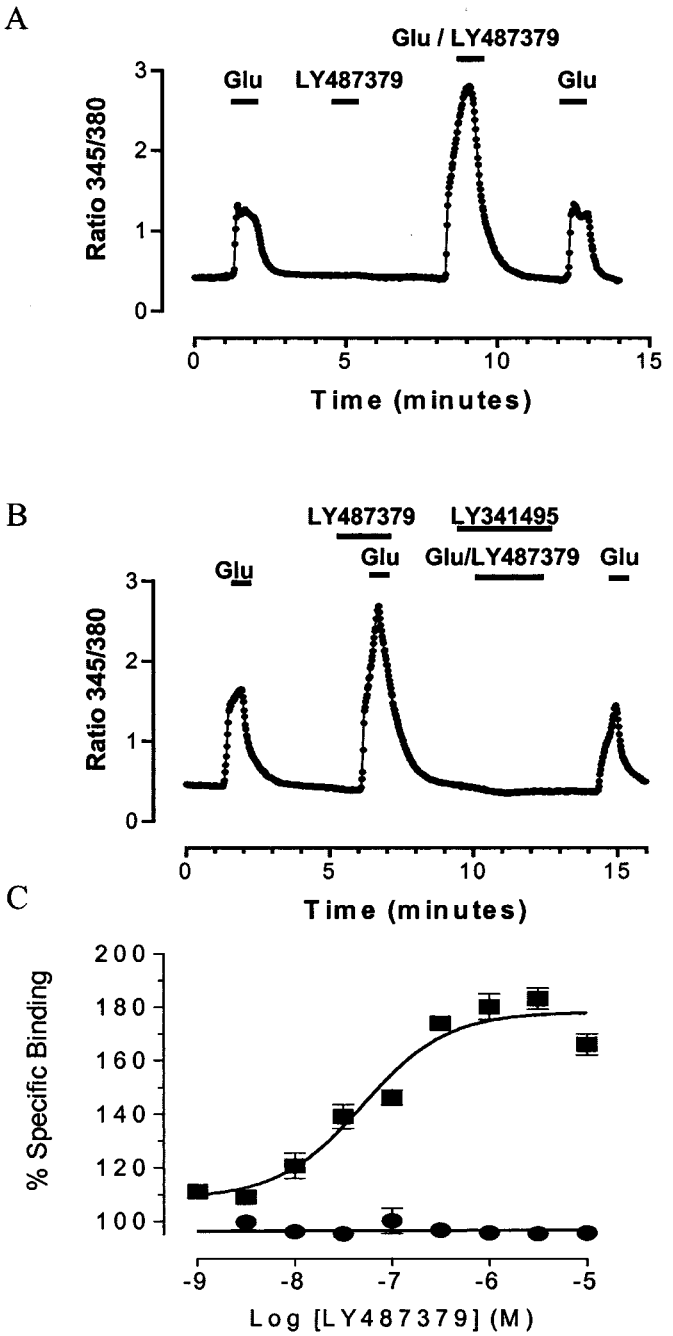

Fig. 3. A and B, effect of LY487379 alone and on 10 μM glutamate-stimulated Ca^{2+} signals in CHO cells expressing hmGlu2 receptors and Gα_{16}. Cells were challenged with a submaximal concentration of glutamate (10 μM) in the absence or presence of 3 μM LY487379. The antagonist LY341495 was used at 1 μM. The numbers of cells analyzed in these studies were 15 (A) and 14 (B). Results are from a single experiment, representative of at least two additional experiments. C, effect of LY487379 on the binding of the agonist [^{3}H]DCG-IV (50 nM) (■) or the antagonist [^{3}H]LY341495 (4 nM) (●) in rat cortical membranes. Results are expressed as percentage of specific binding in the absence of LY487379 and are the mean ± S.E.M. from triplicate determination.

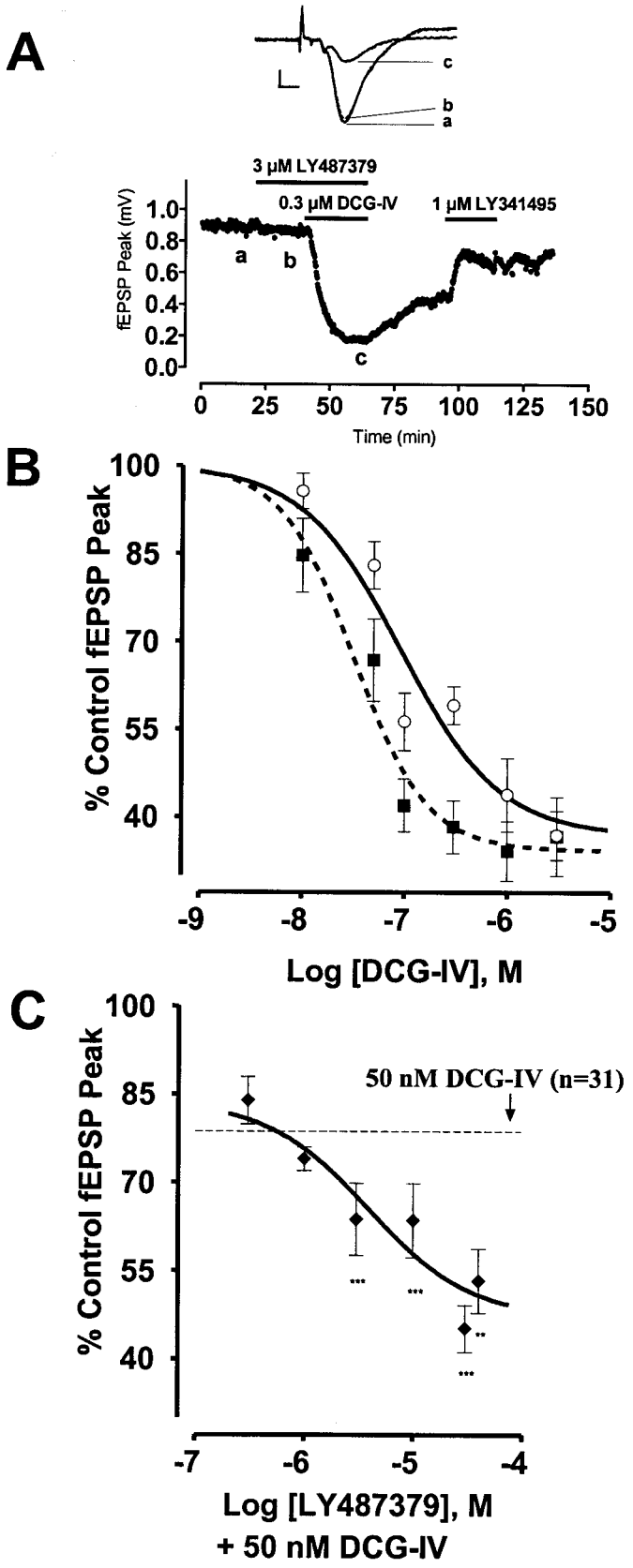

Fig. 4. LY487379 potentiates the effect of DCG-IV on MPP-DG synapses in rat hippocampal slices. A, time course of the effect produced by LY487379 (3 μM) on the inhibition of fEPSPs produced by DCG-IV (0.3 μM). Partial reversal of this inhibition is observed upon washout and application of LY341495 (1 μM). Inset shows fEPSPs recorded at the times indicated in B and C. Calibration bars, 0.2 mV and 2 ms. B, concentration-response curves for the effect of DCG-IV (○) and DCG-IV + 3 μM LY487379 (■) on MPP-DG synapses. Data points indicate the mean ± S.E.M. inhibition of fEPSP peak amplitude expressed as a percentage of the predrug control baseline (n = 3–6 slices per data point). IC_{50} estimates obtained from the fitted curves were 92 and 32 nM for DCG-IV and DCG-IV + LY487379, respectively. C, concentration-response curve for LY487379 in the presence of an ≈IC_{20} concentration of DCG-IV (50 nM) on MPP-DG synapses. Data points indicate the mean ± S.E.M. inhibition of control fEPSP peak amplitude (n = 4–8 slices per data point) (predrug). The mean inhibition produced by DCG-IV (50 nM) alone is indicated by the dotted line (79 ± 12%, mean ± S.D.; n = 31). *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$, ANOVA/Bonferroni multiple comparisons test for effect of DCG-IV + LY487379 versus DCG-IV alone. The EC_{50} estimate obtained from the fitted curve (see text) is 3.9 μM.

(Fig. 7, B and C, Table 4). The lack of potentiation in these mutants is not the result of a different level of protein expression (data not shown) or sensitivity to glutamate (Table 4).

Discussion

To date, the described group II mGlu receptor-selective agonists, including the heterobicyclic amino acids LY354740, LY379268, and MGS0028, which are believed to act at the glutamate binding site, activate both mGlu2 and mGlu3 receptors with similar potencies (Monn et al., 1999; Pin et al., 1999; Nakazato et al., 2000). In this study, we characterized a mGluR2-specific allosteric positive modulator and identified the novel site of action of this modulator on the receptor. The presence of a novel allosteric site on the mGlu2 receptor has allowed for the development of compounds that selectively potentiate mGlu2 but not mGlu3 receptors. LY487379 is a novel, low molecular weight compound that acts as a selective mGlu2 receptor allosteric modulator in preparations expressing native (rat brain membranes and rat hippocampal brain slices) and recombinant (stably or transiently transfected cells) mGlu2 receptors.

Previous reports have established that group II mGlu receptors stimulate [^{35}S]GTPγS binding in recombinant membrane preparations (Kowal et al., 1998). Using this functional binding assay, we demonstrated that LY487379 alone does not enhance [^{35}S]GTPγS binding in membranes prepared from rat brain or from cells stably expressing human mGlu2 receptors; rather, it produces a robust enhancement of glutamate-induced responses. These effects are similar to those reported for the positive allosteric modulators Ro 67-7476 (Knoflach et al., 2001) and CGP7930 (Urwyler et al., 2001) acting on recombinant mGlu1 and $GABA_{B(1b/2A)}$ receptors, respectively. The modulatory effect we observed was highly selective for mGlu2 receptors, because there was an absence of modulatory activity in cells stably expressing human mGlu3, mGlu5, and mGlu7 receptors.

Dose-response curves to the selective group II agonists revealed a rank order of potency of LY379268 > DCG-IV > LCCG-I > glutamate at human mGlu2 receptors, consistent with previous reports (Kowal et al., 1998). Interestingly, DCG-IV and LCCG-I were not fully efficacious agonists compared with LY379268 or glutamate, which is in contrast with the previous pharmacological characterization of these compounds on mGlu2 receptors (Kowal et al., 1998). This is most probably explained by a difference in the level of expression of the receptor and/or G-protein. In the presence of LY487379 (1 μM), the potencies and the maximum responses of the

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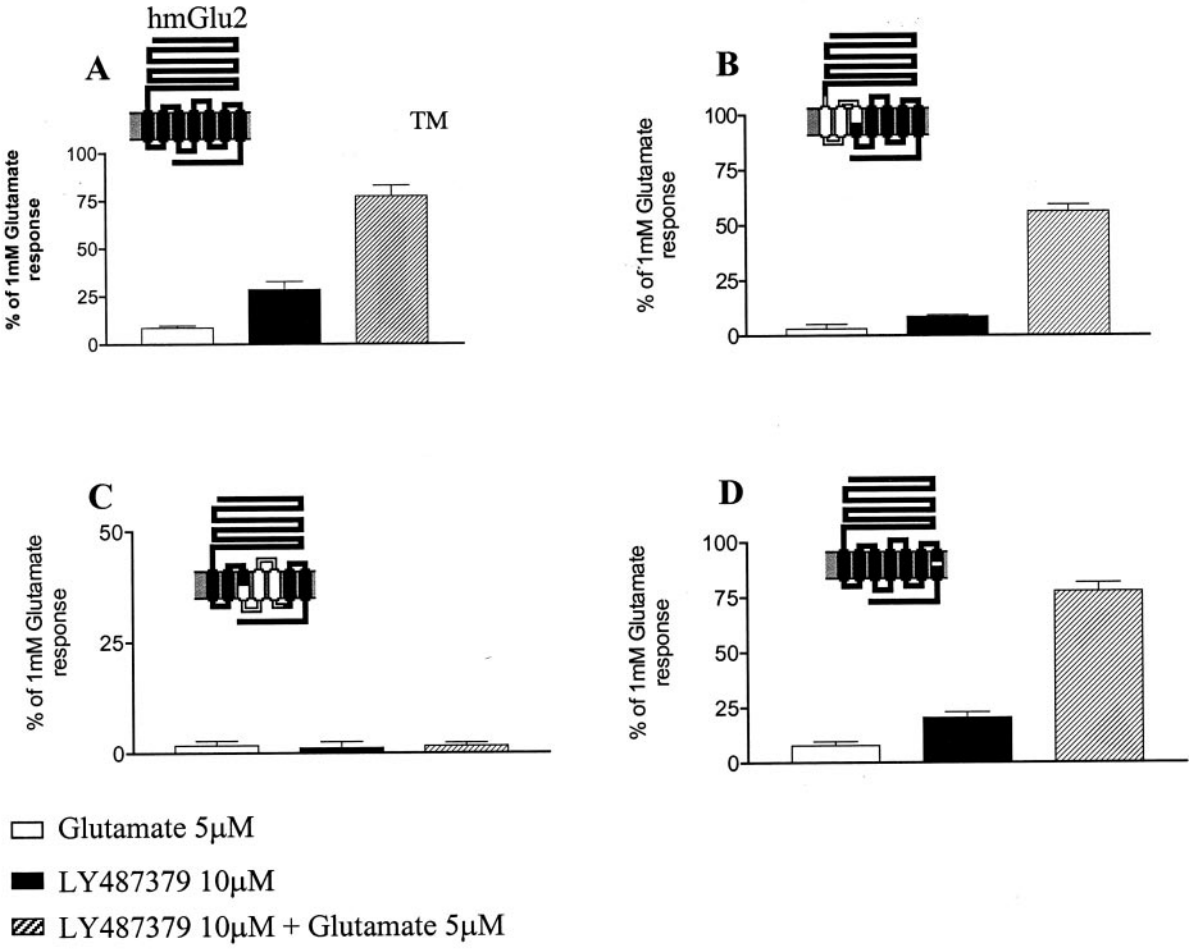

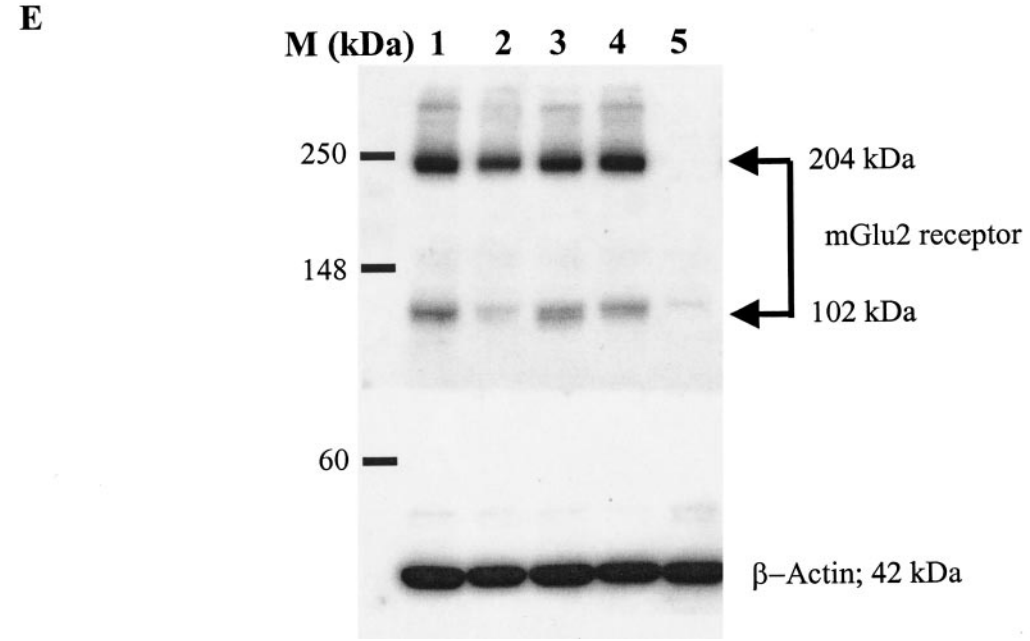

Fig. 5. Effect of LY487379 at wild-type hmGlu2 receptor (A), chimeric receptor TM I–III (B), TM III–V (C), and TM VII (D) on glutamate (5 μM)-induced PI hydrolysis. Results are expressed as percentage of glutamate (1 mM) and are the means ± S.E.M. from three individual experiments performed in triplicate. Inset, schematic diagram of receptor constructs indicating the location of mGlu3 receptor fusion sites (open areas). E, immunoblot analysis of wild-type and chimeric mGlu2/3 receptors transiently expressed in HEK293 cells; 5 μg of total membranes was run per lane. Lane M: molecular mass markers (in kilodaltons); Lane 1, membranes prepared from mGlu2 wild-type expressing cells; lane 2, membranes prepared from mGluR2/3 TMI–III expressing cells; lane 3, membranes prepared from mGluR2/3 TMIV–V expressing cells; lane 4, membranes prepared from mGluR2/3 TMVII expressing cells; lane 5, membranes prepared from untransfected HEK293 cells

agonists were increased approximately 2- to 5-fold, although the absolute magnitude of this effect was dependent upon the agonist. This change in the potency might reflect a change in the affinity of the receptor for the agonist, which is supported by radioligand binding studies. LY487379 significantly enhanced the binding of the agonist [^{3}H]DCG-IV, without affecting binding of the high-affinity group II mGlu receptor antagonist [^{3}H]LY341495. Further examination using saturation experiments revealed that LY487379 increased the affinity of the receptor for [^{3}H]DCG-IV, without significantly effecting the number of available binding sites. Similar to the effect we observed with LY487379, the mGlu1 receptor-selective allosteric potentiators Ro 67-7476 and Ro 01-6128 increased the affinity of [^{3}H]quisqualate for recombinant rat mGlu1 receptors (≈2-fold decrease in K_d) without a significant effect on B_{max} (Knoflach et al., 2001). Also, the $GABA_B$ positive allosteric modulator CGP7930 enhances the binding of the $GABA_B$ agonist [^{3}H]aminopropylphosphonous acid to native rat $GABA_B$ receptors via an increase in affinity (≈3-fold decrease in K_d) without a significant effect on B_{max} or on the binding of competitive $GABA_B$ antagonist [^{3}H]CGP62349 (Urwyler et al., 2001). Importantly, the lack of effect of LY487379 on [^{3}H]LY341495 binding indicates that LY487379 may preferentially bind to an active conformation of the receptor or that [^{3}H]LY341495 binds to different residues in the glutamate binding site that are relatively unaffected by binding of the potentiator. Saturation binding experiments suggest that in the presence of glutamate, the potentiator LY487379 increased the ability of the mGlu2 receptor to activate G-proteins. Mechanistically, there are

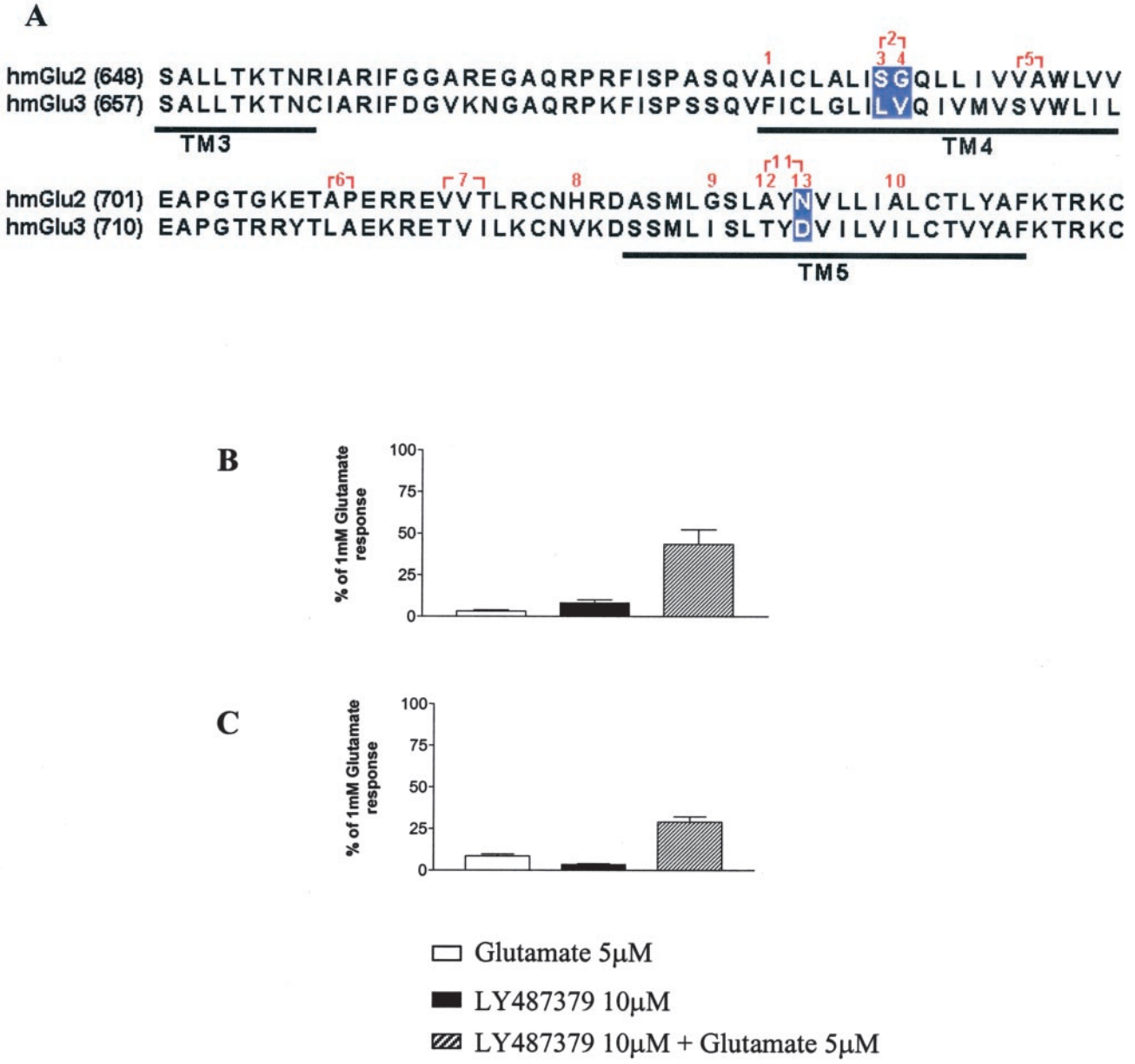

Fig. 6. A, sequence alignment of mGlu2 and mGlu3 receptors between TMIII-TMV. The numbers represent the different mutations (see Table 4). Highlighted residues represent the amino acids involved in the allosteric binding site. Effect of LY487379 at hmGlu2 receptor mutated at S688L-G689V (B) or at A733T-N735D (C) on glutamate-induced PI hydrolysis in HEK293 cells. Results are expressed as percentage of glutamate (1 mM) and are the means ± S.E.M. from three individual experiments performed in triplicate.

TABLE 4

Effect of glutamate (1 mM) and percentage of potentiation by LY48739 obtained from [^{3}H]IP$_1$ accumulation in wild-type and mutated receptors

Mutant and wild-type hmGlu2 receptors were transiently co-expressed in HEK293 cells with Gα16 and stimulated with or without glutamate (10 μM) and/or LY487379 (10 μM). Glutamate (1 mM) was determined to establish the maximum stimulation for wild-type and mutated hmGlu2 receptor. Summary data are calculated from three individual experiments performed in triplicate and represent the mean dpm (basal or glutamate 1 mM) or percentage of potentiation (LY487379 + glutamate at 10 μM, respectively) ± S.E.M. A two-way ANOVA revealed a significant effect of mutants [F (16.283) = 4.76; $p < 0.001$], a significant effect of condition [i.e., basal versus glutamate (1 mM) (F(1,283) = 882.4; $p < 0.001$)], and a significant effect mutant versus condition interaction [(F (16,283) = 3.6; $p < 0.001$)]. A Dunnett's post hoc analysis revealed that all the mutations respond to glutamate ($p < 0.05$) but only S688L-G689V gives a glutamate stimulation higher than wild-type ($p < 0.05$). No significant differences have been observed with the other mutations.

Name	Mutation Constructs	Basal	Glutamate(1 mM)	Potentiation by LY487379 (10 μM)
		dpm	*dpm*	*%*
Wild-type	None	1,282 ± 51	8,950 ± 560	78 ± 5
PM1	A681F	1,510 ± 145	7,040 ± 1,290	90 ± 10
PM2	S688L-G689V	1,500 ± 150	13,791 ± 834	44 ± 9*
PM3	S688L	800 ± 75	6,611 ± 865	97 ± 10
PM4	G689V	934 ± 72	5,780 ± 895	90 ± 11
PM5	V695S-A696V	1,340 ± 113	10,840 ± 1,043	90 ± 5
PM6	A710L-P711A	1,226 ± 93	9,140 ± 735	80 ± 4
PM7	V716T-T718I	1,320 ± 120	10,550 ± 1,081	78 ± 3
PM8	H723V	1,570 ± 87	11,770 ± 1,020	62 ± 8
PM9	G730I	1,340 ± 127	7,800 ± 390	73 ± 6
PM10	A740I	1,660 ± 159	11,240 ± 1,292	70 ± 7
PM11	A733T-N735D	1,212 ± 116	9,160 ± 780	32 ± 4**
PM12	A733T	1,934 ± 110	10,540 ± 1,430	85 ± 7
PM13	N735D	1,775 ± 72	11,767 ± 1,250	14 ± 3**
PM14	S688L-N735D	940 ± 102	10,537 ± 1,421	3 ± 1**
PM15	G689V-N735D	1,070 ± 170	10,684 ± 2,000	4 ± 1**
PM16	S688L-G689V-N735D	1,045 ± 62	6,323 ± 730	5 ± 3**

* $p < 0.05$; significantly different from wild-type, paired Student t test.
** $p < 0.001$; significantly different from wild-type, paired Student t test.

several ways in which LY487379 could produce this effect. LY487379 might 1) increase the affinity of the mGlu2 receptor for the agonist; 2) enhance the ability of the active receptor to stimulate G-proteins through an increased coupling potency and/or coupling efficiency; 3) reduce the dissociation rate of the agonist from the receptor; or 4) increase the number of receptors in the active conformation or retain the receptors in an active conformation for a longer period. Our data do not allow us to discriminate among these potential mechanisms, although we determined that the potentiator increases the affinity of the agonist for the mGlu2 receptor. It seems unlikely that this effect alone can fully account for the potentiator activity, because it cannot explain how the apparent partial agonists LCCG-I and DCG-IV become full agonists at mGlu2 receptors in the presence of the potentiator.

LY487379 modulates not only recombinant human mGlu2 receptors but also native rat mGlu2 receptors. Previous reports have shown that activation of group II mGlu receptors modulates synaptic transmission at MPP-DG synapses in hippocampal slices (Macek et al., 1996). LY487379 significantly potentiated the inhibition of hippocampal MPP-DG synapses produced by DCG-IV but had no significant activity in the absence of DCG-IV, supporting a role for LY487379 as a positive allosteric modulator. The lack of effect of LY487379 when applied alone also supports the suggestion that mGlu2 receptors in the medial perforant pathway are not activated by synaptically released glutamate when these axons are stimulated at relatively low frequencies (Kew et al., 2001). Coapplication with LY487379 (3 μM) elicited a ≈3-fold leftward shift in the dose response for DCG-IV, without a significant enhancement in the efficacy of the agonist. The effect of LY487379 in this assay is similar to that produced by glutamate in the [^{35}S]GTPγS binding assay. Presumably, the absence or presence of 'spare receptors' at a synapse will determine whether the efficacy of the agonist response is increased.

We found that coapplication of low doses of DCG-IV with LY487379 can induce a maximal inhibition of MPP-DG synapses (53%) similar to that elicited by saturating concentrations of group II mGlu receptor agonists (63% for DCG-IV), suggesting that the inhibitory effect produced by nonselective group II mGlu receptor agonists on hippocampal MPP-DG synapses is predominantly mediated via the activation of mGlu2 receptors. The prominent role of mGlu2 receptors in MPP-DG synapses is supported by a report showing that in mGlu2 receptor knockout mice, there was a significant 68% loss in the inhibitory effect of the group II mGlu receptor agonist DCG-IV on MPP-DG synapses (Kew et al., 2002). This is consistent with a depletion of mGlu2 receptor immunoreactivity observed in the middle third of the dentate molecular layer in these mGlu2 receptor knockout mice (Yokoi et al., 1996).

The lack of effect of LY487379 on the binding of the competitive mGlu2/3 receptor antagonist [^{3}H]LY341495, suggests that LY487379 does not interact with the orthosteric binding site on the receptor. Therefore, to identify the site of action of LY487379, we performed a detailed molecular investigation using chimeric receptors and point mutations of hmGlu2 and hmGlu3 receptors. Under this transient expression, the cells are likely to express high levels of the mGlu2 receptor, LY487379 evoked a small response in the absence of added glutamate. This result suggests that, in this transient assay, mGlu2 receptors may have constitutive activity. Similar effects have also been reported for allosteric modulators at mGlu1 receptors in cell lines overexpressing the receptor (Knoflach et al., 2001). Alternatively, it is possible that LY487379 exhibits intrinsic efficacy because of contaminating glutamate levels. It should be noted that LY487379 had no intrinsic activity in assays using well-washed membranes or perfused cells in which contaminating glutamate levels were probably low. A lack of intrinsic activity was also observed in functional assays ([^{35}S]GTPγS binding and electrophysiology) using native tissue preparations. Mutagenic

analysis clearly demonstrated that LY487379 interacts with Ser688, Gly689, and Asn735 from hmGlu2 receptor located in TMIV and V, respectively. Baez et al. (2002) recently reported a partial lack of effect of another mGlu2 receptor positive modulator (LY508869) involving N735. Additionally, we identified the combination of either Ser688 or Gly689 with Asn735 or the combination of these residues together with Asn735 forms the binding pocket for LY487379 (Fig. 8). A novel negative allosteric binding site has been identified for the noncompetitive mGlu1 receptor antagonist 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[*d*]azepin-3-yl)-1,6-dihydropyrimidine-5-carbonitrile (Malherbe et al., 2003). The binding pocket for this modulator involves amino acids in multiple transmembrane domains (Val757 in TMV, Trp798, Phe801, and Tyr805 in TMVI and Thr815 in TMVII). These residues do not overlap with the residues we identified; however, the knowledge obtained from the mapping of

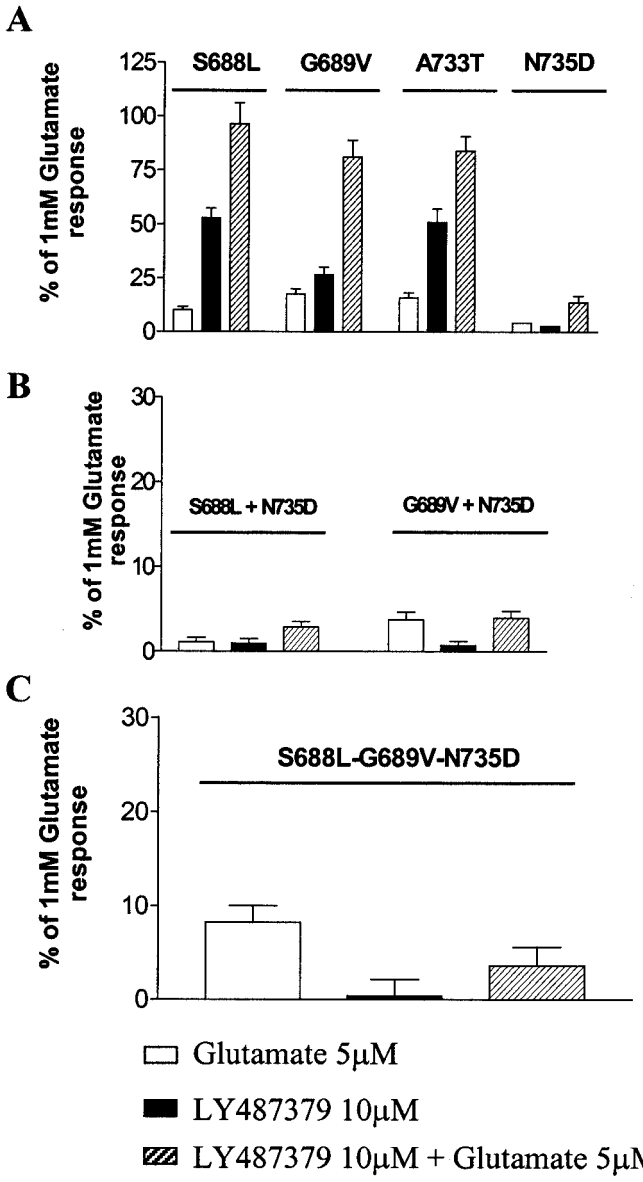

Fig. 7. Effect of LY487379 at single (A), double (B), or triple (C) hmGlu2 receptor mutants on glutamate-induced PI hydrolysis in HEK293 cells. Results are expressed as percentage of glutamate (1 mM) and are the means ± S.E.M. from three individual experiments performed in triplicate.

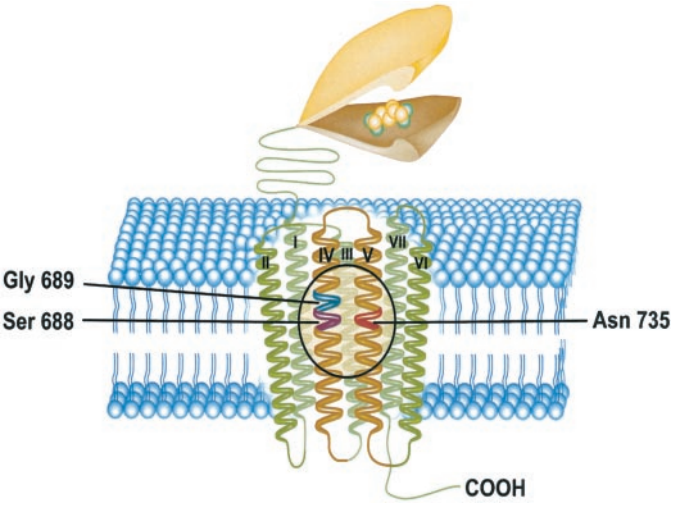

Fig. 8. Schematic diagram of the putative three-dimensional protein structure of the mGlu2 receptor based on the X-ray crystal structure of bovine rhodopsin receptor (Palczewski et al., 2000). The amino terminal, extracellular, glutamate-binding site is depicted as a clamshell-shaped object. The transmembrane domains are depicted as α-helical structures. The highlighted purple, blue, and red portions of TMIV and V α-helical structures mark the three amino acids that have been determined to be involved in the binding of LY487379.

new allosteric modulators is helping to map the regions of the mGlu receptors that are critical for modulation of agonist activity.

Positive allosteric modulators offer an attractive therapeutic approach for the activation of GPCRs because they would be efficacious only in the presence of endogenous agonist (i.e., use-dependent) and they might elicit less tachyphylaxis and/or receptor desensitization than competitive agonists. Furthermore, compounds that bind outside of the highly conserved agonist-binding site offer the potential for highly selective ligands, something that has been difficult to achieve. LY487379 and related compounds therefore constitute valuable tools to explore the role of mGlu2 receptors in neuronal function and the potential clinical utility of selective mGlu2 receptor allosteric potentiators.

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