The Non-competitive Antagonists 2-Methyl-6-(phenylethynyl)pyridine and 7-Hydroxyiminocyclopropan[b]chromen-1a-carboxylic Acid Ethyl Ester Interact with Overlapping Binding Pockets in the Transmembrane Region of Group I Metabotropic Glutamate Receptors*

We have investigated the mechanism of inhibition and site of action of the novel human metabotropic glutamate receptor 5 (hmGluR5) antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP), which is structurally unrelated to classical metabotropic glutamate receptor (mGluR) ligands. Schild analysis indicated that MPEP acts in a non-competitive manner. MPEP also inhibited to a large extent constitutive receptor activity in cells transiently overexpressing rat mGluR5, suggesting that MPEP acts as an inverse agonist. To investigate the molecular determinants that govern selective ligand binding, a mutagenesis study was performed using chimeras and single amino acid substitutions of hmGluR1 and hmGluR5. The mutants were tested for binding of the novel mGluR5 radioligand [3H]2-methyl-6-(3-methoxyphenyl)ethynyl pyridine (M-MPEP), a close analog of MPEP. Replacement of Ala-810 in transmembrane (TM) VII or Pro-655 and Ser-658 in TMIII with the homologous residues of hmGluR1 abolished radioligand binding. In contrast, the reciprocal hmGluR1 mutant bearing these three residues of hmGluR5 showed high affinity for [3H]M-MPEP. Radioligand binding to these mutants was also inhibited by 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), a structurally unrelated non-competitive mGluR1 antagonist previously shown to interact with residues Thr-815 and Ala-818 in TMVII of hmGluR1. These results indicate that MPEP and CPCCOEt bind to overlapping binding pockets in the TM region of group I mGluRs but interact with different non-conserved residues.

Metabotropic glutamate receptors are G protein-coupled receptors that play important roles in regulating the activity of many synapses in the central nervous system. At present, eight mGluR subtypes (mGluR1 through mGluR8) have been cloned and functionally expressed (1, 2). Based on their amino acid sequence homologies, pharmacology, and functional profiles, these subtypes are classified further into three groups. Members of group I (mGluR1 and -5) stimulate the activity of phospholipase C and mobilize intracellular Ca2+. Members of group II (mGluR2 and -3) and group III (mGluR4, -6, -7, -8) inhibit adenyl cyclase. Despite the differences in primary structures and functional roles, all mGluRs feature a large conserved N-terminal extracellular domain, which is involved in the recognition of agonists and competitive antagonists (3–8).

Most ligands for mGluRs were derived from amino acids and act at the conserved glutamate binding site (9). Recently, novel subtype-selective group I mGluR antagonists emerged that are structurally unrelated to amino acids and to each other. The first non-amino acid-like antagonist described was CPCCOEt (Fig. 1), a selective mGluR1 antagonist (10, 11). CPCCOEt inhibits receptor activity by a non-competitive mechanism which does not affect the binding affinity of glutamate (12, 13). Molecular characterization of the site of inhibition in mGluR1 revealed that CPCCOEt interacts with two non-conserved residues at the top of transmembrane (TM) helix VII (13). The first described selective mGluR5 antagonists, SIB-1757 and SIB-1893 (Fig. 1), are also unrelated to amino acids and were shown to act via a non-competitive mechanism (14).

To address the question whether these structurally unrelated mGluR1 and mGluR5 antagonists interact with different sites of the mGluR subtypes or share a common binding site in the 7TM domain, we have studied the binding site and mode of action of the selective mGluR5 antagonist MPEP (15). MPEP is a novel derivative of SIB-1893 with nanomolar potency (Fig. 1); it is an effective antihyperalgesic in animal models of chronic inflammatory pain (16), a neuroprotectant in excitotoxin-induced striatal lesions (17) and an anticonvulsant in several epilepsy models (18). We generated a number of chimeric receptors and point mutations in which segments or single residues of hmGluR5 were exchanged with the corresponding residues of hmGluR1 and vice versa. These mutants were tested for inhibition of glutamate-induced calcium signals by MPEP.
and for binding affinity of [3H]M-MPEP (Fig. 1), a closely related radioligand with a Kᵦ of 3.5 nM. We found that the key amino acids for selective and high affinity binding of [3H]M-MPEP to hmGluR5 are Pro-655 and Ser-658 in TMIII and furthermore, [3H]M-MPEP binding to these mutants was inhibited by CPCCOEt, suggesting that the subtype-selective antagonists MPEP and CPCCOEt bind to overlapping binding pockets in the TM region of group I mGluRs. These experimental findings are plausibly explained by a three-dimensional model of the mGluR5-MPEP complex that we constructed detailing findings are plausibly explained by a three-dimensional model of the mGluR5-MPEP complex that we constructed departing from the bovine rhodopsin structure.

**MATERIALS AND METHODS**

**Compounds**

MPEP and CPCCOEt were synthesized as described previously (15, 10). [3H]M-MPEP was synthesized using 2-methyl-6-(3-hydroxy-phenylethynyl)pyridine as starting material by methylation with tritiated methyl iodide. Further details of the synthesis and characteristics of the radioligand will be published elsewhere.2 Glutamate was obtained from Tocris (Bristol, United Kingdom). Other chemicals were purchased from Sigma (Buchs, Switzerland).

**Plasmids**

CDNAs encoding wild-type hmGluR1b and hmGluR5a and the chimeric hmGluR15a and hmGluR51b receptors termed p254, p255, p317, p257, p205, p306, p310, and p322 were described previously (13). Additional chimeras were constructed in pCMV-T7–3 (19) using standard cloning techniques based on unique restriction sites in hmGluR1b and -5a, novel restriction sites introduced by site-directed mutagenesis or the polymerase chain reaction-based overlap extension technique. The authenticity of the chimeric CDNAs (Table I) was confirmed by sequencing of all amplified DNA fragments. Point mutations in TMIII and TMVII of hmGluR1b and hmGluR5a cDNAs were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The authenticity of each point mutation was confirmed by DNA sequencing.

The cDNA encoding Go₅ (q4WT-pcDNA-I) was described previously by Conklin et al. (20), rat mGluR5a (pRK5a) by Joly et al. (21), and EAAC1 by Brabet et al. (22), respectively.

**Cell Culture and Transfections**

Chinese hamster ovary and L cell lines stably expressing human mGluR1b and -5a were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal calf serum as described previously (13). For radioligand binding experiments, wild-type and mutant cDNAs were transfected into COS1 cells using the DEAE-dextran method (23). For measurements of intracellular [Ca²⁺], mutant cDNAs were transiently expressed in HEK293 cells by electroporation using a Gene Pulser apparatus (Bio-Rad). Briefly, 5 µg of plasmid DNA were used to transfect 1.5 × 10⁶ cells in a total volume of 150 µl of electroporation buffer (K₂HPO₄, 50 mM; CH₃COOK, 20 mM; KOH, 20 mM; pH 7.4). After electroporation (250 V, 300 microfarad), cells were resuspended in Dubelco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and antibiotics. 5 × 10⁶ cells were plated on glass coverslips (9 × 18 mm) coated with 100 mg/ml poly-D-lysine (Sigma).

**IP and [Ca²⁺]^2**

Cells were seeded in 24-well tissue culture plates and were labeled to equilibrium with nycos-[3H]inositol at 2 × 10⁻⁶ Ci/ml in Dulbecco’s modified Eagle’s medium for 20 h. Preincubation with LiCl, stimulation with agonist and/or antagonist, and extraction of total inositol phosphates (IP) were performed as described previously (13). For the experiments addressing constitutive receptor activity, the cells were incubated in the presence of a glutamate-degrading enzyme (1 unit/ml glutamate pyruvate transaminase plus 2 mM pyruvate), 1 h before and during the incubation period with LiCl, except when cells were stimulated by added glutamate. Concentration-response curves were obtained by fitting the four-parametric logistic equation to the data using Prism2.0 (GraphPad Software, San Diego, CA).

Microfluorimetric measurements of [Ca²⁺], were performed as described previously (13).

**Ligand Binding**

Membranes from transfected COS1 cells were collected 2 days after transfection. Cells were washed with phosphate-buffered saline and mechanically detached in ice-cold phosphate-buffered saline containing 10 mM EDTA. Cells were centrifuged at 4000 rpm for 20 min at 4 °C and resuspended in binding buffer (30 mM NaHepes, 110 mM NaCl, 1.2 mM MgCl₂, 5 mM KCl, 2.5 mM CaCl₂·2H₂O, pH 8.0). Cells were then disrupted on ice with a Polytron homogenizer for 20 s, and membranes were collected by centrifugation at 18,000 rpm for 20 min at 4 °C. The pellet was resuspended in binding buffer, homogenized with a Teflon homogenizer, and used immediately for binding. Ligand binding assays were performed using [3H]M-MPEP and cold M-MPEP (10⁻⁶ M) to determine nonspecific binding.2 Briefly, samples consisted of 200 µl of membrane suspension (50 µg), 25 µl of radioligand (2–50 nM), and 25 µl of binding buffer. The reaction was terminated after a 30-min incubation at 25 °C by dilution and rapid filtration through Whatman GF/B glass fiber filters. The filters were washed three times with cold binding buffer, and the bound radioactivity was counted using a β-counter in 5 ml of Ultima Gold NV Packard (Canberra Packard, Zurich, Switzerland). Specific [3H]M-MPEP binding was defined as total binding minus nonspecific binding in the presence of 1 × 10⁻⁶ M cold M-MPEP. In one experiment all measurements were performed in duplicate. Saturation analysis and competition curves were analyzed using Prism2.0 (GraphPad Software).

**Molecular Modeling**

Construction of a hmGluR5 Transmembrane Domain Model—To suggest a plausible binding mode of MPEP to hmGluR5, a seven-transmembrane model was built and optimized using the programs SYBYL (SYBYL 6.4 software; Tripos Inc., St. Louis, MO) and X-PLOR.

**FIG. 1. Structures of CPCCOEt, SIB-1757, MPEP, and [3H]M-MPEP.**

2 I. Vranesic, personal communication.
TABLE II
Assignment of α-helical transmembrane segments of the hmGluR5 receptor based on the rhodopsin receptor family

| TM | SN | Amino acid sequence | EN | Baldwin numbering |
|----|----|---------------------|----|-------------------|
| I  | 582 | (PEP1) AAVAFACLGLLALTLPFVTTVVFITYRDTP | 608 | 101–127 |
| II | 617 | LCVIILLAGCGLYLCFTFLAIKFKQYI | 643 | 200–226 |
| III| 649 | IGGILSPAMSYSLATKTNRIARILAGGSKKCTK | 683 | 298–332 |
| IV | 695 | VIAFLICMGIGIVALF1MEPFDI | 719 | 401–425 |
| V  | 738 | L5V5PgLYNGILLISCTFAYRTNVPAN | 767 | 505–532 |
| VI | 770 | EAKYIAFTMYTTCIIWLAFVPIYFGSNYK | 799 | 596–625 |
| VII| 802 | MCFVSLSATVSVGCFVFPMYI1 (LAK) | 825 | 703–726 |

(X-PLOR 3.1 software; Molecular Simulations, Inc., San Diego, CA) based on the α-carbon template of the transmembrane helices of the rhodopsin receptor family (24, 25). In brief, seven individual polyalanine standard α helices of lengths 27, 27, 35, 25, 30, 30, and 24 were built, and each helix was superimposed on the corresponding helix of the α-carbon template derived from the rhodopsin family of GPCRs (25). The root-mean-square distances of these superimposed Cα atoms of TM helices I–VII were 0.14, 0.79, 0.19, 0.13, 0.84, 1.35, and 0.12, respectively.

All 198 alanines were mutated according to the putative assignment of transmembrane segments given in Table II by making the corresponding side chain changes of alanines. All prolines were fixed using the SYBYL Biopolymer FIX_PROLINE command, hydrogen atoms were added with the ADDH command, and Gasteiger partial charges were computed with the CHARGE GST_HUC command. Owing to the presence of charged residues (arginines (4), lysines (11), aspartic acid (2), and glutamic acids (2)), the net charge was +1.1e. Groups forming termini of helices where kept neutral as NH₂ and COOH.

This raw model was optimized with X-PLOR using the conjugate gradient method and the Tripos force field (X-PLOR/TAFF). Harmonic restraints with a force constant of 1.0 kcal/mol Å were applied to the Cα atoms. Minimization was carried out until the gradient (E) and the energy E were 0.4 kcal/mol Å and ~1814 kcal/mol Å, respectively.

The extracellular domain between TMVI and TMVII is, according to our sequence assignment to helical transmembrane segments, formed by a short sequence of about four residues, which may be part of the binding pocket. A plausible third extracellular loop between helix VI and VII was searched with SYBYL/BIOPOLYMER/LOOP assigning binding pocket. A plausible third extracellular loop between helix VI and VII was searched with SYBYL/BIOPOLYMER/LOOP assigning binding pocket. A plausible third extracellular loop between helix VI and VII was searched with SYBYL/BIOPOLYMER/LOOP assigning binding pocket.
**Binding Sites of Non-competitive Group I mGluR Antagonists**

**Fig. 3.** MPEP inhibits basal constitutive activity of rat mGlur5a. A, IP production was measured in HEK293 cells (control (Ctrl)) and cells transiently expressing rat mGlur5a. The constitutive activity is evident when comparing columns labeled Ctrl Basal and mGlur5a Basal. MPEP was applied at 1 μM and glutamate (Glu) at 3 μM. B, same as in A, except that the G protein αi subunit was overexpressed alone (Ctrl-Basal) or together with mGlur5a in order to increase the constitutive activity. MPEP was applied at 1 μM, MCPG at 3 mM, and glutamate (Glu) at 3 μM. C, concentration-response curve of the inhibitory effect of MPEP on the basal IP production measured in cells expressing both mGlur5a and the Goi protein. In all experiments, the glutamate transporter EAAC1 was coexpressed to deplete the extracellular medium of glutamate and the glutamate degrading enzyme glutamate-pyruvate transaminase (plus 2 mM pyruvate) was added to avoid a putative activation of the mGlur5a by residual extracellular glutamate produced by the cells. Results are expressed as the percentage of IP production over the total radioactivity remaining in the membrane fraction of the cells. The data presented in A and B are the means ± S.E. of data from four different experiments performed in triplicate, and data shown in C are a representative example of three separate experiments performed in triplicate.

Inhibition by MPEP Is Mediated by the Transmembrane Domain of mGlur5—The non-competitive mode of inhibition of MPEP prompted us to speculate that the compound does not act at the glutamate-binding site located in the large N-terminal extracellular domain. In order to localize the structural determinants mediating this inhibition, we first used two chimeras of hmGlur1 and hmGlur5 (hmGlur1/5 and -5/1) with a fusion site after the first TM segment (Fig. 4). When HEK293 cells were transfected with wild type hmGlur1 and hmGlur5 as well as two chimeras hmGlur1/5 and -5/1 (p305, p254), all transfected cells responded with a transient increase in [Ca²⁺], upon application of 50 μM glutamate (Fig. 4). Coapplication of glutamate and MPEP (1 μM) caused a complete inhibition of [Ca²⁺] responses in cells transfected with wild type hmGlur5 and the chimera hmGlur1/5 (p305). In contrast, the glutamate-stimulated change in [Ca²⁺], was not affected by MPEP in cells expressing hmGlur1 and hmGlur5/1 (p254). This indicated that the inhibitory effect of MPEP is mediated by the C-terminal part of the receptor including the TM segments II–VII and/or intracellular and extracellular loops of hmGlur5. Reversibility of the inhibition by MPEP was demonstrated by reapplying glutamate after a 10-min washout period.

[¹H]M-PMEP Binding Requires Residues in TMIII and TM-VII of mGlur5—To identify the molecular determinants governing MPEP selectivity and inhibition, we constructed a series of chimeric hmGlur1/5 and -5/1 receptors fused at different position in the TM domains (Fig. 5). Each of these mutants was tested in a radioligand binding assay using the novel antagonist [¹H]M-PMEP (Fig. 1), a close analog of MPEP with a KD of 3.5 ± 0.7 nM on wild-type hmGlur5a. [¹H]M-PMEP was displaced by MPEP with an IC₅₀ of 16 nM (data not shown). Saturation binding studies with membranes prepared from cells transfected with the hmGlur1/5 chimera p305 yielded a KD value of 4.0 ± 0.5 nM (Fig. 5). Binding affinities comparable to wild type hmGlur5 (KD < 5 nM) were also obtained for all mutants containing the third and seventh TM segments of hmGlur5 (p257, p305, p310, p316, p323, p324). In contrast, no significant binding was observed in wild type hmGlur1 or chimeric receptors lacking TMIII and/or TMVII of hmGlur5 (p254, p265, p317, p306, p312, p361, p322). All chimeras were shown to be functional as revealed by a transient increase in [Ca²⁺], upon stimulation with 50 μM glutamate. Consistent with the ligand binding data, chimeras containing TMIII and TMVII of hmGlur5 were also functionally inhibited by MPEP (1 μM), whereas mutants lacking one or both of these TM segments of hmGlur5 were insensitive to application of MPEP (data not shown).

Sequence alignments of the hmGlur subtypes revealed a high degree of conservation of TMIII and TMVII. In fact, only
MPEP at 1 m to [3H]M-MPEP. All mutants demonstrated changes in [Ca\(^{2+}\)] with fura-2 microfluorimetry. Bars indicate application of drugs below each trace. Glutamate (glu) was applied at a concentration of 50 \(\mu\)M and MPEP at 1 \(\mu\)M, respectively. Between drug applications, a 3–5-min period was allowed for washout and recovery. Measurements were repeated at least five times in separate transfection experiments.

![Diagram](image)

**Fig. 4. Effect of MPEP at wild-type hmGluR5 and -1 and chimeric hmGlu1/5 and -5/1 receptors.** MPEP selectively inhibits glutamate-induced changes in [Ca\(^{2+}\)], in cells transiently expressing the constructs hmGluR5 and -1/5, but not hmGluR1 and -5/1. Left, schematic diagram of receptor constructs indicating the location of the fusion sites. Right, traces show representative time courses of [Ca\(^{2+}\)], transients as measured with fura-2 microfluorimetry. Bars indicate application of drugs below each trace. Glutamate (glu) was applied at a concentration of 50 \(\mu\)M and MPEP at 1 \(\mu\)M, respectively. Between drug applications, a 3–5-min period was allowed for washout and recovery. Measurements were repeated at least five times in separate transfection experiments.

six residues in TMIII and six residues in TMVII differ between hmGluR1 and hmGluR5 (Fig. 6). To precisely identify the residues governing the specific binding of \([\text{H}]\)M-MPEP, we constructed point mutants of hmGluR5, in which the non-conserved amino acids in TMIII and TMVII were substituted by the homologous amino acids of the closely related subtype hmGluR1. The analysis of the TMVII mutants R5-M802T, -S805A, -V819T, -V822M, and -L826I revealed no significant decrease in binding affinity of \([\text{H}]\)M-MPEP when compared with wild-type hmGluR5 (Table III). In contrast, \([\text{H}]\)M-MPEP binding could not be detected in the mutant R5-A810I. A loss of binding affinity was also observed by replacing Ala-810 with glycine (R5-A810G), the homologous residue present in the hmGluR2 and -3 subtypes. In TM III, replacements of the non-conserved residues Ile-649 (R5-I649L), Gly-650 (R5-G650L), and Ile-651 (R5-I651V) had no significant effect on the binding affinity of the radioligand. Replacement of Pro-655 (R5-P655S) and Ser-658 (R5-S658C) caused a 7.7- and 4.7-fold decrease in affinity of \([\text{H}]\)M-MPEP (KD = 27.1 \(\pm\) 3.2 nM and KD = 16.5 \(\pm\) 5.7 nM, respectively. The combined double mutant R5-P655S,S658C revealed a complete loss of binding affinity for \([\text{H}]\)M-MPEP.

To exclude the possibility that the mutation of Pro-655, Ser-658, and Ala-810 disrupt the structure of the binding site, these residues were introduced singly or in combination at the corresponding position of hmGluR1. Single substitutions such as R1-V823A or double substitutions such as R1-S668P,C671S revealed no significant affinity for \([\text{H}]\)M-MPEP as observed with the wild type hmGluR1 receptor (Table III). However, introduction of all three residues (R1-S668P,C671S,V823A) resulted in high affinity binding with a KD of 21.8 \(\pm\) 0.3 nM. Further replacement of Val-664 by Ile-665 of hmGluR5 (R5-V664I) did not further affect the affinity for \([\text{H}]\)M-MPEP by a factor of 2 (KD = 12.5 \(\pm\) 1.6 nM). Replacements of all non-conserved residues in TMVII with the homologous residues of hmGluR1 (R1-L662I,L663G,V664I,S668P,C671S,V823A) did not further affect the affinity for \([\text{H}]\)M-MPEP (KD = 10.7 \(\pm\) 1.6 nM). Taken together, these data clearly indicate that the residues Pro-655 and Ser-658 of TMIII and Ala-810 of TMVII are critical molecular determinants for the selective binding of \([\text{H}]\)M-MPEP to hmGluR5.

To demonstrate that the mutants with reduced binding affinity or loss of binding affinity were properly expressed and targeted to the membrane, these mutants were tested in the calcium assay. All mutants tested showed transient elevations of [Ca\(^{2+}\)]\(\text{\textsuperscript{+}}\), upon application of 50 \(\mu\)M glutamate. Furthermore, the mutants that showed a complete loss of binding affinity also failed to show inhibition by MPEP (data not shown).

**Fig. 5. \([\text{H}]\)M-MPEP binding and functional inhibition by MPEP is mediated by TMIII and TMVII of hmGluR5.** Left, schematic diagram of a series of chimeric receptor constructs indicating the location of the fusion sites between hmGluR5 and hmGluR1. Right, receptor constructs were transiently expressed in COS1 cells and tested for binding to \([\text{H}]\)M-MPEP and [Ca\(^{2+}\)], responses to glutamate. + indicates KD values <5 nM; - indicates no measurable binding affinity to \([\text{H}]\)M-MPEP. All mutants demonstrated changes in [Ca\(^{2+}\)], by application of 50 \(\mu\)M glutamate (data not shown). Data were reproduced at least three times in separate transfection experiments.
the non-conserved amino acid residues Pro-655 and Ser-658 in TMIII and Ala-810 in TMVII. We wondered whether these antagonists bind to different or mutually exclusive binding sites in the transmembrane region of group I mGluRs. To address this question, CPCCOEt was tested for inhibition of radioligand binding in the hmGluR5 mutant R5-M802T,S805A (p330) and the hmGluR1 mutant R1-V664I,S668P, C671S,V823A (p381), which showed high affinity binding for [3H]M-MPEP (Table III) as well as functional inhibition by CPCCOEt (for p330, see Ref. 13; for p381, data not shown). [3H]M-MPEP binding to these mutants was fully inhibited by CPCCOEt in a concentration-dependent manner with IC50 values of 3.5 \pm 0.3 \mu M for R5-M802T,S805A and 15.9 \pm 3.4 \mu M for R1-V664I,S668P,C671S,V823A, respectively (Fig. 7). Hill coefficients in both cases were close to unity.

In parallel, we performed molecular modeling of the TM region of wild-type hmGluR5 and the mutant R5-M802T,S805A and manually docked MPEP and CPCCOEt to the key residues identified by site-directed mutagenesis (Fig. 8). After a series of 48 minimizations using a molecular dynamic protocol (for details, see “Materials and Methods”), the lowest energy structures were taken as a model for MPEP binding to hmGluR5 and CPCCOEt binding to the mutant R5-M802T,S805A, respectively. The MPEP/hmGluR5 model supports the experimental findings by predicting close interaction of MPEP with the side chains of the amino acid residues Val-806, Ser-809, and Ala-810 in TMVII and Pro-655, Ser-658, and Tyr-659 in TMIII. Further favorable contacts are suggested with Lys-638 in TMII. Contact predictions are consistent with the side chains of Pro-655, Ser-658, and Tyr-659 in TMIII. A comparison of both models suggests that the pyridine ring of MPEP occupies precisely the same space between TMVII and TMIII than the benzene ring of CPCCOEt.

**DISCUSSION**

The major findings of this study concern the involvement of the hmGluR5 7TM domain in the high affinity binding of MPEP, which mediates non-competitive inhibition. This suggests that MPEP inhibits receptor activity without changing the affinity of glutamate to its binding site, and thus MPEP might act at a receptor site different from the glutamate binding domain. In agreement with this hypothesis is the inability of MPEP to displace [3H]quisqualate binding to rat mGluR5a and the lack of effect of mGluR5 agonists on [3H]M-MPEP binding.\(^3\)

In addition to its non-competitive antagonist action, MPEP also decreased basal IP production in the absence of an agonist

\(^3\)V. Mutel, personal communication.
Mutant and wild-type hmGluR1 and hmGluR5 receptors were transiently expressed in COS1 cells. Membranes prepared from these cells were equilibrated with increasing concentrations of [3H]M-MPEP (up to 40 nM). Nonspecific binding was determined by application of 1 μM M-MPEP. The amount of specifically bound [3H]M-MPEP after rapid filtration was plotted as bound/free versus bound, and the best fit linear regression lines for these saturation isotherms were used to calculate the corresponding [3H]M-MPEP equilibrium dissociation constant (K_D) for each receptor. The data presented are the means ± S.E. from at least three independent transfection experiments performed in duplicates.

| Receptor or mutant | [3H]M-MPEP K_D (nM) ± S.E. |
|-------------------|-----------------------------|
| R5                | 3.5 ± 0.7                   |
| R5-M802T          | 3.8 ± 0.5                   |
| R5-S805A          | 6.7 ± 1.1                   |
| R5-A810V          | 4.1 ± 2.0                   |
| R5-V819T          | 3.0 ± 1.2                   |
| R5-V822M          | 3.1 ± 0.2                   |
| R5-L826I          | 3.1 ± 0.9                   |
| R5-M802T,S805A    | 5.4 ± 0.7                   |
| R5-M802T,S805A,A810V | No binding                 |
| R5-I649L          | 3.6 ± 0.2                   |
| R5-G650L          | 2.6 ± 0.5                   |
| R5-I651V          | 5.1 ± 0.8                   |
| R5-I649L,G650L,I651V | 3.6 ± 0.2               |
| R5-P655S          | 27.1 ± 3.2                  |
| R5-S658C          | 16.5 ± 5.7                  |
| R5-P655S,S658C    | No binding                  |
| R1                | No binding                  |
| R1-V823A          | No binding                  |
| R1-S668P,C671S    | No binding                  |
| R1-S668P,C671S,V823A | 21.8 ± 0.3                |
| R1-V664L,S668P,C671S,V823A | 10.7 ± 1.6          |
| R1-L662I,L663G,V664I,S668P,C671S,V823A | 10.7 ± 1.4 |

Binding affinities for [3H]M-MPEP at wild-type and TMIII and TMVII mutant hmGluR1 and hmGluR5 receptors

| TABLE III |

A detailed molecular investigation using chimeric receptors and point mutants of hmGluR5 and hmGluR1 revealed that MPEP binds to and interacts with Ala-810 in TMVII and Ile-651, Pro-655, and Ser-658 in TMIII, respectively. Replacement of Ala-810 with valine, the homologous residue of hmGluR1, completely abolished [3H]M-MPEP binding and MPEP inhibition of glutamate-induced [Ca^{2+}]_i responses. Single replacement of Pro-655 and Ser-658 with the corresponding residues of hmGluR1 reduced the binding affinity of [3H]M-MPEP from 3.5 nM to 27.1 and 16.5 nM, respectively. However, a combination of both mutations lacked any significant binding affinity for [3H]M-MPEP, indicating the importance of both residues for radioligand binding. Likewise, introduction of Pro-655, Ser-658, and Ala-810 from hmGluR5 at the corresponding position of hmGluR1 generated a hmGluR1 mutant with a high affinity binding site for [3H]M-MPEP (K_D = 20 nM), indicating that these residues are critical molecular determinants for [3H]M-MPEP binding. A further 2-fold increase in binding affinity was achieved by introduction of Ile-651, mutation of which showed no change in binding affinity in wild type hmGluR5a. Because the combination of Pro-655, Ser-658, Ile-651, and Ala-810 is unique to mGluR5 and not found in the homologous position of other mGluR subtypes, it provides a molecular explanation for the high pharmacological selectivity of MPEP.

It has not yet been determined whether the structurally unrelated non-competitive antagonists MPEP and CPCCOEt interact with different sites of mGluR subtypes or share a common binding site in the TM domain. We showed in a previous study that two residues unique to hmGluR1, Thr-815 and Ala-818 on the extracellular surface of TMVII, were responsible for the selective action of the non-competitive mGluR1 antagonist CPCCOEt (13). The present study shows that binding of the mGluR5 antagonist MPEP requires an interaction with Ala-810 further down in the transmembrane helix VII as well as additional interactions with Pro-655 and Ser-658 in TMIII. Using hmGluR1 and -5 mutants (R1-V664L,S668P,C671S,V823A and R5-M802T,S805A), which show functional inhibition by MPEP and CPCCOEt, we unequivocally demonstrated complete inhibition of [3H]M-MPEP binding by CPCCOEt in a concentration dependent manner. This is further supported by docking studies of MPEP and CPCCOEt to 7TM domain models. These models suggest that the pyridine ring of MPEP precisely occupies the same space between TMVII and TMIII as the benzene ring of CPCCOEt, providing a molecular explanation for the displacement of MPEP by CPCCOEt. However, other parts of these antagonists do not overlap and suggest interactions with different TM helices. Thus, although MPEP and CPCCOEt are structurally unrelated, they recognize overlapping binding pockets in the 7TM region of group I mGluRs that are sufficiently diverse to allow subtype-specific interaction with different classes of compounds.

These findings may have important implications for the design of novel mGluR antagonists. Up to now, most compounds acting at the eight subtypes of mGluRs are phenylglycines or rigidified amino acid analogs such as LY354740 (9). These
FIG. 7. Inhibition of [3H]M-MPEP binding at the mutants R5-M802T,S805A and R1-V664I,S668P,C671S,V823A. hmGluR1 and hmGluR5 mutants were transiently expressed in COS1 cells. Membranes prepared from these cells were equilibrated with a fixed concentration of [3H]M-MPEP (~2 nM) and increasing concentrations of the competing drug CPCCOEt. The amount of specifically bound [3H]M-MPEP following rapid filtration is plotted as a function of the logarithm of the concentration of CPCCOEt. Nonspecific binding was determined by application of 1 μM M-MPEP. The values were determined from three independent transfection experiments performed in duplicate.

FIG. 8. Receptor models. A, model of MPEP (shown with green carbon atoms) docked to the model of hmGluR5 and minimized with X-PLOR. Closely interacting amino acid residues are shown in yellow and labeled with single-letter amino acid code (Val-806, Ser-809, Ala-810 (TMVII); Pro-655, Ser-658 (TMIII)). B, model of CPCCOEt (shown with green carbon atoms) docked to the model of the mutant R5-M802T,S805A (p330) receptor and minimized with X-PLOR. Closely interacting amino acid residues are shown in yellow (Thr-802, Ala-805 (TMVII); Pro-655, Tyr-659 (TMIII)).

compounds possess a wide spectrum of agonist, partial agonist, and antagonist activities and interact at the conserved glutamate binding site located in the large N-terminal extracellular domain. Although some of these compounds have reached nanomolar potencies and are able to discriminate among groups of mGluRs, as yet there are no known agonists or competitive antagonists that are able to sufficiently discriminate individual mGluR subtypes most likely due to structural constraints of the glutamate binding site. In contrast, the overlapping binding sites for the non-competitive antagonists CPCCOEt and MPEP in the 7TM region seem to be less conserved, and thus to tolerate binding of structurally diverse compounds. Therefore, it can be speculated that medicinal chemistry efforts toward non-competitive antagonists acting in the 7TM domain are more likely to generate subtype-selective compounds than efforts on competitive antagonists acting at the extracellular glutamate binding site.

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