

# CPCCOEt, a Noncompetitive Metabotropic Glutamate Receptor 1 Antagonist, Inhibits Receptor Signaling Without Affecting Glutamate Binding

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## ABSTRACT

Metabotropic glutamate receptors (mGluRs) are a family of G protein-coupled receptors characterized by a large, extracellular N-terminal domain comprising the glutamate-binding site. In the current study, we examined the pharmacological profile and site of action of the non-amino-acid antagonist 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt). CPCCOEt selectively inhibited glutamate-induced increases in intracellular calcium at human mGluR1b (hmGluR1b) with an apparent  $IC_{50}$  of 6.5  $\mu$ M while having no agonist or antagonist activity at hmGluR2, -4a, -5a, -7b, and -8a up to 100  $\mu$ M. Schild analysis indicated that CPCCOEt acts in a noncompetitive manner by decreasing the efficacy of glutamate-stimulated phosphoinositide hydrolysis without affecting the  $EC_{50}$  value or Hill coefficient of glutamate. Similarly, CPCCOEt did not displace [ $^3$ H]glutamate binding to membranes prepared from mGluR1a-expressing cells. To elucidate

the site of action, we systematically exchanged segments and single amino acids between hmGluR1b and the related subtype, hmGluR5a. Substitution of Thr815 and Ala818, located at the extracellular surface of transmembrane segment VII, with the homologous amino acids of hmGluR5a eliminated CPCCOEt inhibition of hmGluR1b. In contrast, introduction of Thr815 and Ala818 at the homologous positions of hmGluR5a conferred complete inhibition by CPCCOEt ( $IC_{50}$  = 6.6  $\mu$ M), i.e., a gain of function. These data suggest that CPCCOEt represents a novel class of G protein-coupled receptor antagonists inhibiting receptor signaling without affecting ligand binding. We propose that the interaction of CPCCOEt with Thr815 and Ala818 of mGluR1 disrupts receptor activation by inhibiting an intramolecular interaction between the agonist-bound extracellular domain and the transmembrane domain.

Metabotropic glutamate receptors (mGluRs) are coupled to heterotrimeric G proteins, and through this interaction they modulate intracellular concentrations of second messengers and ion channel functions (see reviews by Knoepfel et al., 1995; Pin and Duvoisin, 1995; Conn and Pin, 1997). Molecular cloning has revealed the existence of eight distinct mGluR subtypes subdivided into three groups based on sequence similarities, agonist profiles, and main signal transduction pathways activated in heterologous systems. Further multiplicity in this receptor family is generated by splice variants in the cytoplasmic C-terminal domain. Group I receptors (mGluR1 and -5) mobilize intracellular calcium ( $[Ca^{2+}]_i$ ) by stimulating phospholipase C and are activated selectively by dihydroxyphenylglycine (DHPG). Group II receptors (mGluR2 and -3) and group III receptors (mGluR4, -6, -7, and -8) inhibit adenylate cyclase. Group II receptors are activated

selectively by (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) and (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate [(2R,4R)-APDC], whereas L-2-amino-4-phosphonobutyrate (L-AP4) and L-serine-O-phosphate are selective agonists of group III mGluRs.

mGluRs, together with the  $\gamma$ -aminobutyric acid type B receptor (Kaupmann et al., 1997), the parathyroid calcium-sensing receptors (Brown et al., 1993), and the vomeronasal receptors (Bargmann, 1997) form a separate family within the G protein-coupled receptor (GPCR) superfamily and show no significant sequence homology to other cloned receptors. A particular feature of the mGluR family is the remarkable large, extracellular N-terminal domain that comprises the ligand-binding site (O'Hara et al., 1993; Takahashi et al., 1993; Tones et al., 1995; Wroblewska et al., 1997; Okamoto et al., 1998; Parmentier et al., 1998). This contrasts with most

**ABBREVIATIONS:** (1S,3R)-ACPD, (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylate; AIDA, (RS)-1-aminoindan-1,5-dicarboxylate; AP4, L-2-amino-4-phosphonobutyrate; CPCCOEt, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester; mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; TM, transmembrane; fura-2/AM, 1-[2-(5-carboxyoxal-2-yl)-6-aminobenzofuran-5-Oxyl]-2-(2'-amino-5'-methylphenoxyethane-N,N,N',N'-tetraacetic acid); PBP, periplasmic-binding protein.

other GPCRs, where ligand binding occurs in the transmembrane (TM) domain (Savarese and Fraser, 1992; Trumpp-Kallmeyer et al., 1992), and thus raises great interest to understand how extracellular signals are transmitted into the cells.

In addition, there is a need to identify novel selective ligands that are specific for each subtype to characterize unambiguously the physiological role of individual mGluRs. Thus far, group I mGluR ligands are rigidified analogs of glutamate, such as the phenylglycine derivatives (see review by Watkins and Collingridge, 1994), competitive mGluR ligands exhibiting agonist, antagonist, and/or partial agonist activity depending on the mGluR subtypes. However, most of these compounds are neither subtype-selective nor potent. Only recently synthesized antagonists such as (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (S)-(+)-2-(3'-carboxybicyclo[1.1.1]pentylglycine [(S)-CBPG], and (+)-2-methyl-4-carboxy-phenylglycine (LY-367385) exhibit good selectivity for group I mGluR subtypes (Pellicciari et al., 1995; Clark et al., 1997; Moroni et al. 1997).

A recently discovered member of a novel structural class of mGluR ligands is 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) (Annoura et al., 1996). This compound is structurally different from phenylglycines or glutamate and exhibited selective antagonist activity at cloned rat mGluR1a (Annoura et al., 1996) and human mGluR1b (Casabona et al., 1997). In this study, we have characterized the pharmacological profile of CPCCOEt at cloned rat and human receptors (rmGluR1a, hmGluR1b, -2, -4a, -5a, -7b, -8a) expressed in stable cell lines. The mode of inhibition by CPCCOEt was examined and the amino acids mediating the selective inhibition at hmGluR1b were identified using a series of chimeric receptors and point mutations in which segments or single amino acids of the hmGluR1b receptor were exchanged with the corresponding amino acids of hmGluR5a. Our results indicate that CPCCOEt is a selective noncompetitive mGluR1 antagonist interacting with Thr815 and Ala818 in TM segment VII. We propose that CPCCOEt specifically inhibits receptor signaling without affecting glutamate binding by disrupting an intramolecular interaction between the glutamate-bound extracellular domain and the TM region.

## Materials and Methods

**Compounds.** CPCCOEt was synthesized according to the procedure described by Annoura et al. (1996). Glutamate, DHPG, quisqualate, (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylate [(1S,3R)-ACPD], and L-AP4 were obtained from Tocris (Bristol, UK). Other chemicals were purchased from Sigma (Buchs, Switzerland).

**Construction of Chimeric Receptors and Site-Directed Mutagenesis.** cDNAs encoding wild-type hmGluR1b, -5a, and the chimera hmGluR4/1b were described previously (Daggett et al., 1995; Tones et al., 1995; Lin et al., 1997). cDNAs encoding chimeric hmGluR5/1b and hmGluR1/5a receptor proteins were constructed in the mammalian expression vector pCMV-T7-3 (Daggett et al., 1995) using standard cloning techniques (Sambrook et al., 1989) based on unique restriction sites in hmGluR1b and -5a, novel restriction sites introduced by site-directed mutagenesis, or the polymerase chain reaction (PCR)-based overlap extension technique (Horton et al., 1989). The authenticity of the chimeric cDNAs (Table 1) was confirmed by restriction enzyme analysis and sequencing of all amplified DNA fragments. Site-directed mutagenesis of sequences encoding amino acids of TMVII in hmGluR1b was performed using a 679-bp *AccI*-*NotI* fragment cloned into pBluescript SK(-) vector and

the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The authenticity of each point mutation was confirmed by DNA sequencing of the entire fragment before recloning into pCMV-T7-3. Point mutations were introduced into TMVII of hmGluR5a using a 469-bp *AccI*-*ApaI* fragment cloned into pBluescript KS(-) (Stratagene, La Jolla, CA).

**Cell Lines, Cell Culture, and Transfections.** Culturing of baby hamster kidney cells stably expressing rmGluR1a and Chinese Hamster Ovary (CHO) and L cell lines stably expressing hmGluR1b, -2, -4a, -5a, and -7b was performed as described previously (Thomsen et al., 1993; Daggett et al., 1995; Flor et al., 1995a,b, 1997). The generation of a human embryonic kidney (HEK)-hmGluR8a cell line is described by Gasparini et al. (1999). Mammalian expression constructs for wild-type and mutant mGluR cDNAs were transiently transfected into COS1 cells (American Type Culture Collection, CRL1650) by the DEAE dextran method as described by Al-Moslih and Dubes (1973). Transient expression of rmGluR1a in HEK 293 cells was performed as described previously (Parmentier et al., 1998).

**[<sup>3</sup>H]Glutamate Binding.** [<sup>3</sup>H]glutamate binding to membranes prepared from baby hamster kidney cells expressing rmGluR1a was performed essentially as described by Thomsen et al. (1993). In brief, [<sup>3</sup>H]glutamate with a specific activity of 56 Ci/mmol (Amersham, Buckinghamshire, UK) was mixed with test compounds and membranes (1 mg protein/sample) suspended in assay buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM CaCl<sub>2</sub>). After 60-min incubation at 0°C samples were centrifuged (4000g, 3 min, 0°C) and the pellets were rinsed twice with 1 ml of cold assay buffer, solubilized in 2 N NaOH, and transferred to scintillation vials. Nonspecific binding was defined as the binding in the presence of 10 μM quisqualate.

**Measurements of cAMP Formation.** Mammalian cells stably expressing hmGluR2, -4a, -7b, and -8a were seeded in 24-well plates and cultured for 20 to 40 h. Treatment of cells with drugs and cytoplasmic cAMP determinations were performed as described previously (Flor et al., 1995a,b).

**Measurement of [<sup>3</sup>H]Inositol Phosphate Formation.** Clonal cell lines expressing hmGluR1b or hmGluR5a receptors were seeded in 24-well tissue culture plates. Cells were labeled to equilibrium with 2 μCi/ml myo-[<sup>3</sup>H]inositol (American Radiolabeled Chemicals, St. Louis, MO) for 20 h in Dulbecco's modified Eagle's medium, washed twice in Krebs-Henseleit buffer (Sigma), and incubated for 30 min at room temperature. Subsequently, cells were washed in buffer containing 10 mM LiCl and incubated in the same medium for 20 min at 37°C. After aspiration of medium, compounds were added to triplicate wells. For test of antagonist activity, a submaximal concentration of quisqualate (hmGluR1b: 20 μM; hmGluR5a: 0.3 μM) was added immediately after application of the test compound.

Inositol phosphate formation was measured essentially as described by Seuwen et al. (1988). In brief, the reaction was stopped

TABLE 1  
Chimeric receptor constructs

Name	Chimeric mGluR constructs
p252	1-562 <sub>R1</sub> /550-1180 <sub>R5a</sub>
p253	1-549 <sub>R5</sub> /563-906 <sub>R1b</sub>
p254	1-617 <sub>R5</sub> /631-906 <sub>R1b</sub>
p255	1-679 <sub>R5</sub> /693-906 <sub>R1b</sub>
p257	1-839 <sub>R5</sub> /853-906 <sub>R1b</sub>
p305	1-630 <sub>R1</sub> /618-1180 <sub>R5a</sub>
p306	1-692 <sub>R1</sub> /680-1180 <sub>R5a</sub>
p308	1-852 <sub>R1</sub> /840-1180 <sub>R5a</sub>
p309	1-617 <sub>R5</sub> /631-852 <sub>R1</sub> /840-1180 <sub>R5a</sub>
p310	1-630 <sub>R1</sub> /618-839 <sub>R5</sub> /853-906 <sub>R1b</sub>
p317	1-784 <sub>R5</sub> /798-906 <sub>R1b</sub>
p318	1-797 <sub>R1</sub> /785-1180 <sub>R5a</sub>
p321	1-797 <sub>R1</sub> /785-839 <sub>R5</sub> /853-906 <sub>R1b</sub>
p322	1-784 <sub>R5</sub> /798-852 <sub>R1</sub> /840-1180 <sub>R5a</sub>

Numbering indicates amino acid fragments of wild-type hmGluR1b (R1b) and hmGluR5a (R5a) used to generate chimeric receptors.

after an incubation of 20 min at 37°C by aspiration of the medium and lysis of the cells with 0.75 ml of ice-cold 10 mM formic acid (pH 3). After 30 min the extract was diluted into 2 ml of 5 mM NH<sub>3</sub> solution (yielding a final pH of 8–9) and applied to a column containing DOWEX-1 × 8 (Fluka, Buchs, Switzerland). After flow-through of the extract, columns were washed with 10 ml of H<sub>2</sub>O and 6 ml of 5 mM sodium tetraborate, 60 mM sodium formate, respectively. Inositol monophosphates were eluted with 6 ml of 100 mM formic acid, 200 mM ammonium formate. The eluted samples were counted 7 h after addition of 15 ml Irgasave Plus scintillation cocktail (Packard, Zurich, Switzerland) in a Tricarb 2700tr counter (Packard, Zurich, Switzerland). Each data point represents triplicate measurements expressed as mean ± S.E.M.

Measurement of IP production in HEK293 cells transiently expressing the rat mGluR1a has been described previously (Parnetier et al., 1998).

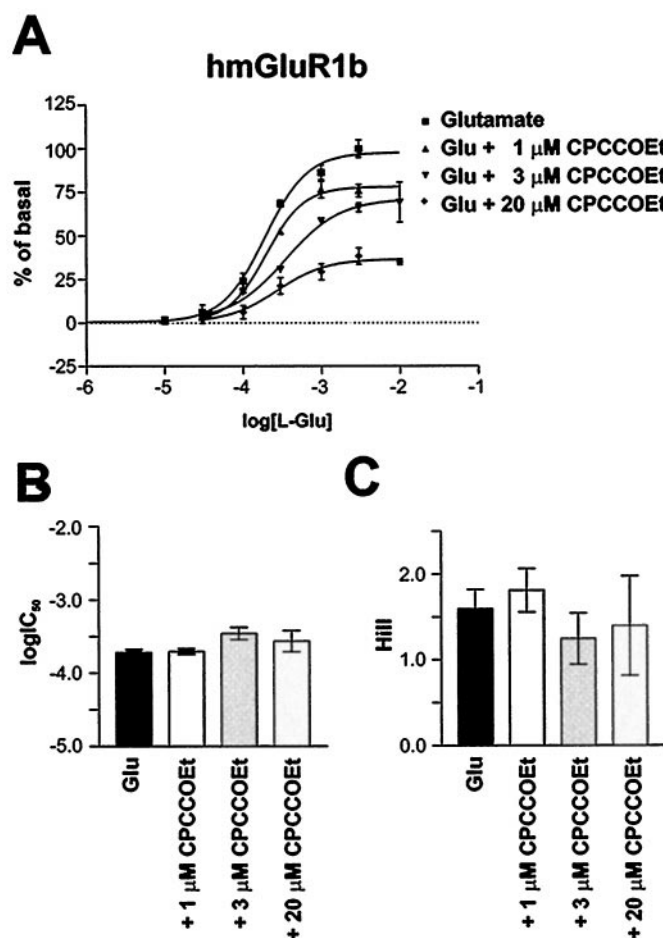
**Measurement of [Ca<sup>2+</sup>]<sub>i</sub>.** Cells were cultured and grown until confluency on glass coverslips (9 × 18 mm; Vitromed, Basel, Switzerland). Cells were loaded with the fluorescent indicator for 30 min at room temperature in HEPES-buffered saline solution supplemented with 1.8 mM CaCl<sub>2</sub> (Life Technologies, Basel, Switzerland) containing 10 μg/ml 1-[2-(5-carboxyoxal-2-yl)-6-aminobenzofuran-5-Oxyl]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid (fura-2/AM; Molecular Probes, Eugene, OR) and 0.5% Pluronic F-127 (Molecular Probes). After dye loading, cells were washed in buffer and kept at room temperature to recover for at least 60 min. For recordings of [Ca<sup>2+</sup>]<sub>i</sub>, glass coverslips were mounted into a continuously perfused cuvette (flow rate, 1 ml/min) in a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan) to measure fura-2 fluorescence intensity at 510 nm (bandpass filter, 20-nm bandwidth) while alternating excitation wavelengths between 340 and 380 nm (excitation filters, 20-nm bandwidth) at a switching frequency of 1.6 Hz. In a typical recording, the resting fluorescence intensity was measured during the first minute. The perfusate then was switched to one containing the test drugs at the desired concentrations for 1 min, after which the perfusion was switched back to buffer to wash. When a second drug application was performed during a recording, a 3- to 5-min wash-out period was allowed between applications. Recordings lasted 5 or 10 min (one or two applications, respectively). Before actual experiments, proper dye loading and positioning of the coverslips was ascertained by directly monitoring the resting fura-2 fluorescence intensities excited alternately at the two excitation wavelengths.

To quantitatively assess changes in [Ca<sup>2+</sup>]<sub>i</sub> in response to receptor stimulation, two-wavelength ratiometry was used. The fluorescence intensity ratio (FIR), as calculated from the fluorescence intensity measurements at 340 nm (F<sub>340</sub>) and 380 nm (F<sub>380</sub>),  $r = F_{380}/F_{340}$ , is a direct, but nonlinear measure for [Ca<sup>2+</sup>]<sub>i</sub>. Therefore, response amplitudes were expressed in terms of FIR rather than absolute calcium concentrations. Because the actually measured FIR values depend on the optical characteristics of the recording device, they cannot be directly compared with FIR values obtained with another optical apparatus. Concentration-response curves were obtained by fitting the four-parametric logistic equation to the data using GraphPad Prism 2.0 (GraphPad, San Diego, CA). Maximum and minimum parameters were fixed to 1 and 0, respectively.

## Results

**CPCCOEt Is a Selective Noncompetitive mGluR1 Antagonist.** We previously showed that CPCCOEt potently reduces stimulation of phosphoinositide (PI) hydrolysis by quisqualate in a concentration-dependent manner in CHO cells stably expressing hmGluR1b with an IC<sub>50</sub> value of 9.9 μM, whereas in L cells stably expressing hmGluR5a CPCCOEt had no significant effect up to a concentration of 100 μM (Casabona et al., 1997). To analyze the mode of inhibition

of CPCCOEt on hmGluR1b, concentration-response curves for stimulation of PI hydrolysis in response to glutamate were compared in the absence and presence of 1 μM, 3 μM, and 20 μM CPCCOEt. With increasing concentrations of CPCCOEt a profound reduction in the amplitude of PI hydrolysis was observed as compared with the stimulation evoked by glutamate alone (Fig. 1A). However, the reduction in the amplitude affected neither the EC<sub>50</sub> value nor the Hill coefficient of glutamate (Fig. 1, B and C). Similarly, CPCCOEt (100 μM) was equally effective in inhibiting PI hydrolysis to basal levels in hmGluR1b expressing cells maximally stimulated with quisqualate (200 μM), glutamate (1 mM), DHPG (1 mM), and (1*S*,3*R*)-ACPD (3 mM) (Fig. 2A). As shown previously, (1*S*,3*R*)-ACPD displayed only a 2.5-fold stimulation of PI hydrolysis over basal levels, corresponding to approximately 45% of the effect of 1 mM glutamate (Lin et al., 1997). No CPCCOEt inhibition was noted when PI hydrolysis was stimulated with ATP (10 μM), which activates an endogenously expressed purinoreceptor in CHO-K1 cells (EC<sub>50</sub> of 1.9 μM; R. Kuhn, unpublished observation). When tested as an agonist, CPCCOEt did not significantly enhance



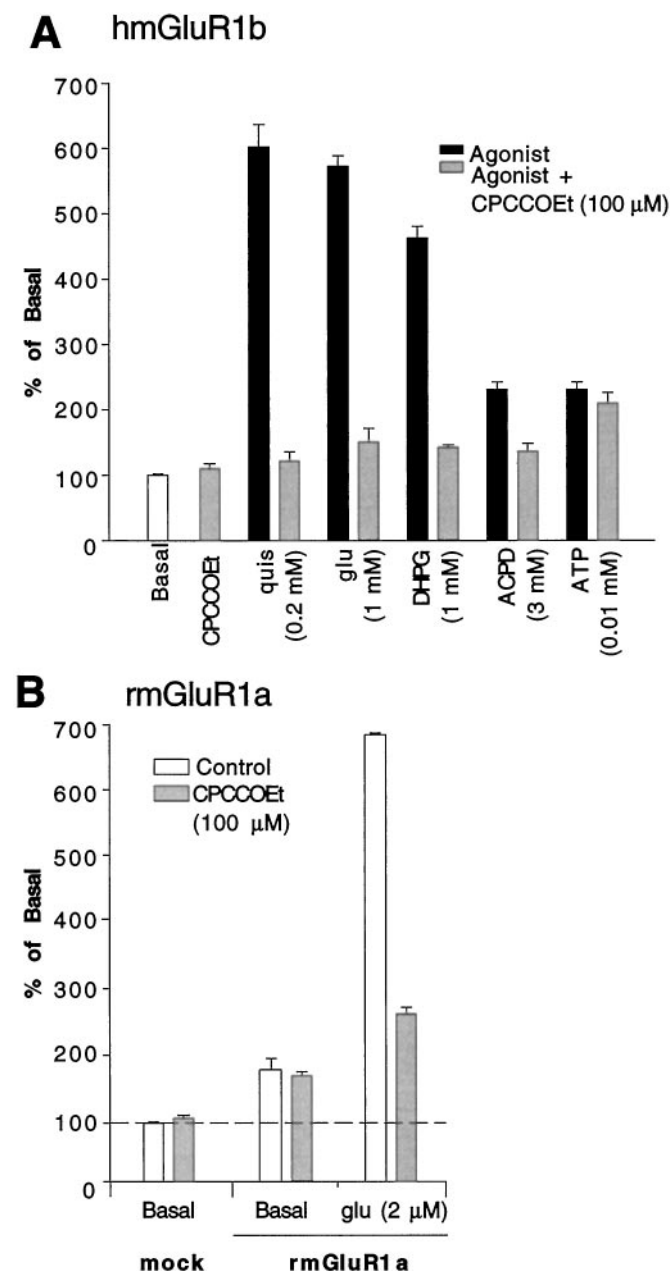
**Fig. 1.** Noncompetitive inhibition of glutamate-stimulated PI hydrolysis by CPCCOEt in CHO cells expressing hmGluR1b. A, concentration-response curves of glutamate alone or together with 1 μM (▲), 3 μM (▼), and 20 μM (◆) CPCCOEt. Data are expressed as a percentage of PI hydrolysis over basal level and are mean ± S.E.M. of two to three experiments performed in triplicate. B, Log IC<sub>50</sub> values (means ± S.E.M.) of indicated concentration-response curves. C, Hill coefficients (means ± S.E.M.) of indicated concentration-response curves.



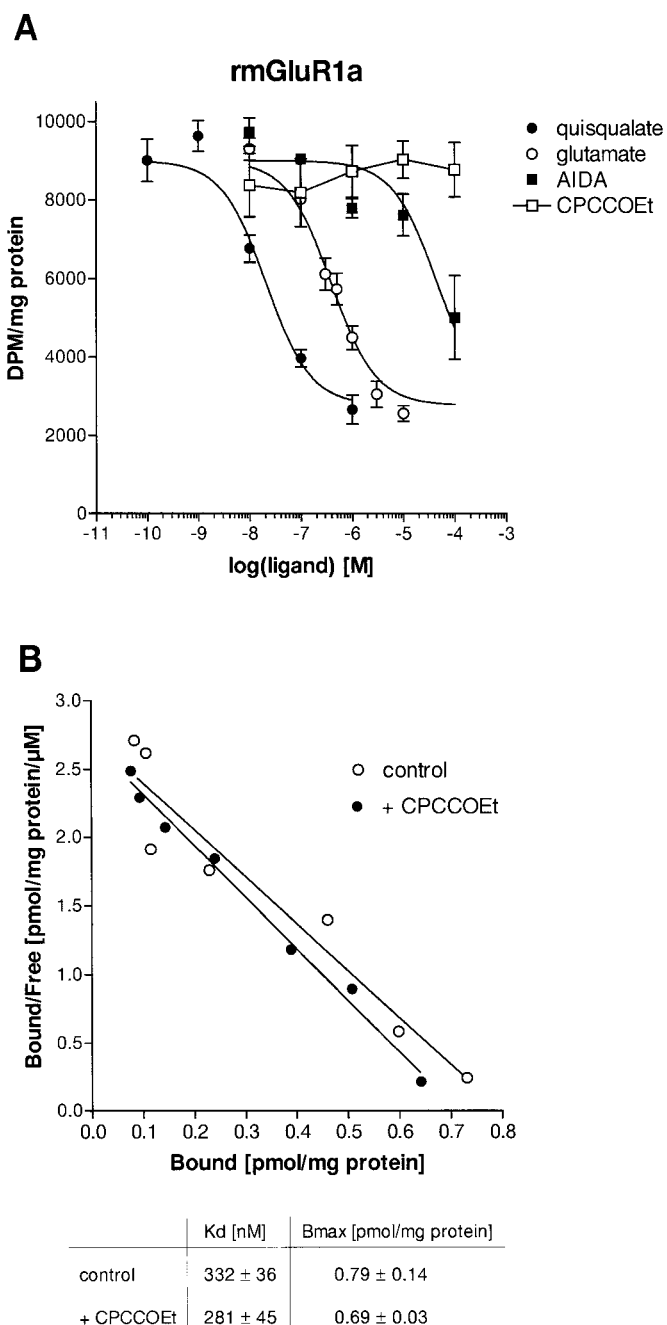
basal PI hydrolysis in hmGluR1b expressing cells up to a concentration of 100  $\mu$ M.

We reported previously that the mGluR1a splice variant displayed detectable constitutive activity when transiently overexpressed in HEK 293 cells (Prézeau et al., 1996). Surprisingly, none of the characterized mGluR1 competitive antagonists were able to inhibit this constitutive activity. Therefore, we wondered whether or not CPCCOEt could act as an inverse agonist, inhibiting the mGluR1a constitutive activity. In HEK 293 cells transiently expressing rmGluR1a, CPCCOEt also inhibited in a noncompetitive manner the effect of glutamate (Fig.

2B and data not shown). However, CPCCOEt was unable to inhibit the basal IP production measured in mGluR1a-expressing cells (Fig. 2B). The basal IP production in mGluR1a-expressing cells can be potentiated by coexpressing this receptor with the  $\alpha$  subunit of the Gq protein (Parmentier et al., 1998).



**Fig. 2.** Effect of CPCCOEt on PI hydrolysis stimulated by quisqualate (Quis), glutamate (Glu), DHPG, (1S,3R)-ACPD (ACPD), and ATP at hmGluR1b-expressing cells (A) and on constitutive activity of rmGluR1a (B). Results are expressed as a percentage of PI hydrolysis over basal level measured in hmGluR1b-expressing CHO cells (A) or mock-transfected HEK293 cells (B) (mean  $\pm$  S.E.M.). Data shown are representative examples of three separate experiments performed in triplicate.



**Fig. 3.** Effect of CPCCOEt on [ $^3$ H]glutamate binding to rmGluR1a. A, displacement of [ $^3$ H]glutamate binding to membranes prepared from rmGluR1a cells by mGluR1 agonists glutamate ( $\circ$ ) and quisqualate ( $\bullet$ ) and competitive antagonist AIDA ( $\blacksquare$ ). CPCCOEt ( $\square$ ) did not displace binding at concentrations of up to 100  $\mu$ M. Data are expressed as total [ $^3$ H]glutamate binding and are mean  $\pm$  S.E.M. of two to three experiments, which were performed in triplicate. B, saturation-binding experiments with [ $^3$ H]glutamate in absence ( $\circ$ ) or presence ( $\bullet$ ) of 100  $\mu$ M CPCCOEt. [ $^3$ H]glutamate (40 nM) was diluted with nonlabeled L-glutamate at seven concentrations ranging from 0.01  $\mu$ M to 10  $\mu$ M, and experiments ( $n = 3$ ) were performed in triplicate as described in *Materials and Methods*. Nonspecific binding was defined as binding in presence of 10  $\mu$ M quisqualate.

Even under this condition, CPCCOEt did not inhibit the basal IP formation (data not shown).

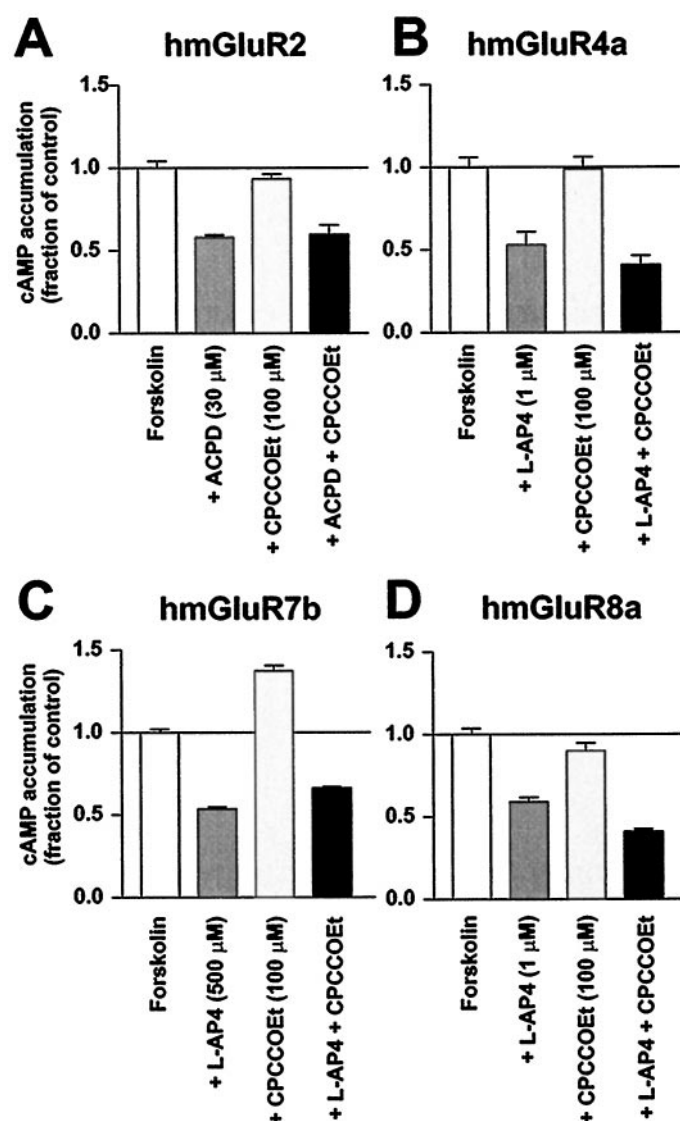
Because CPCCOEt concentration-dependently antagonized glutamate-stimulated PI hydrolysis in a noncompetitive manner, it was suggestive that CPCCOEt does not influence the binding of glutamate to the extracellular glutamate-binding domain. To confirm this hypothesis, displacement of [ $^3\text{H}$ ]glutamate from membranes prepared from baby hamster kidney cells stably expressing rmGluR1a was examined (Thomsen et al., 1993). As shown in Fig. 3A, [ $^3\text{H}$ ]glutamate binding was displaced in a concentration-dependent manner by the mGluR1 agonists glutamate and quisqualate and the competitive mGluR1 antagonist AIDA (Pellicciari et al., 1995; Moroni et al., 1997). In contrast, CPCCOEt did not displace [ $^3\text{H}$ ]glutamate binding at concentrations of up to 100  $\mu\text{M}$ . Saturation-binding experiments

with [ $^3\text{H}$ ]glutamate in the presence and absence of 100  $\mu\text{M}$  CPCCOEt also revealed no significant change in the  $K_d$  and  $B_{\text{max}}$  values (Fig. 3B). Thus, glutamate binding is not influenced in the presence of the noncompetitive mGluR1 antagonist CPCCOEt.

To determine the activity at group II and III metabotropic glutamate receptors, CPCCOEt was tested for agonist and antagonist activity in cell lines stably expressing the human subtypes mGluR2, -4a, -7b, and -8a (Fig. 4). Maximal inhibition of forskolin-stimulated cAMP formation was 80% for (1S,3R)-ACPD at hmGluR2 and between 45 and 80% for L-AP4 at hmGluR4a, -7b, and -8a, respectively (data not shown). When examined as an agonist, CPCCOEt (tested at 100  $\mu\text{M}$ ) did not reveal any inhibitory effect on forskolin-stimulated cAMP formation in all four cell lines. However, CPCCOEt (100  $\mu\text{M}$ ) slightly potentiated the effect of forskolin in cells expressing hmGluR7b by approximately 35% (Fig. 4C). When tested as an antagonist, CPCCOEt (100  $\mu\text{M}$ ) did not suppress the inhibition of forskolin-stimulated cAMP formation by an  $\text{EC}_{50}$  concentration of (1S,3R)-ACPD in hmGluR2 cells or by L-AP4 in hmGluR4a, -7b, and -8a cells. These results indicate that CPCCOEt (100  $\mu\text{M}$ ) is neither an agonist nor antagonist at the human mGluR subtypes 2, -4a, -7b, and -8a.

**Chimeric Receptors and Point Mutants Indicate that the Inhibition by CPCCOEt Is Mediated by Thr815 and Ala818 of hmGluR1b.** Because CPCCOEt selectively inhibits hmGluR1b activity in a noncompetitive manner and does not influence glutamate binding, it can be concluded that CPCCOEt acts at a site different from the glutamate-binding site. To localize the structural determinants mediating this inhibition, we generated a set of chimeric hmGluR1/5a, -5/1b, and -4/1b (Tones et al., 1995) receptors fused at the border between the large N-terminal extracellular domain and the first TM segment (Fig. 5). All chimeric receptors described are coupled to PI turnover and a subsequent release of  $[\text{Ca}^{2+}]_i$  from internal stores. As shown previously for rat mGluR2/1 and mGluR3/1, human mGluR4/1b, *Drosophila* mGluRA/rmGluR1, and rmGluR1/DmGluRA receptors (Takahashi et al., 1993; Tones et al., 1995; Wroblewska et al., 1997; Parmentier et al., 1998), the rank order of agonist potency of chimeric human mGluR1/5 and -5/1 receptors is determined by the N-terminal extracellular domain of the receptor. When COS1 cells were transfected with the different chimeric cDNAs, all transfected cells responded with a transient increase in  $[\text{Ca}^{2+}]_i$  after stimulation with 300  $\mu\text{M}$  glutamate (Fig. 5A). Coapplication of glutamate together with CPCCOEt (40  $\mu\text{M}$ ) completely inhibited the  $[\text{Ca}^{2+}]_i$  response in cells transfected with hmGluR1b and the chimeras hmGluR5/1b and -4/1b, respectively. In contrast, the glutamate-stimulated  $[\text{Ca}^{2+}]_i$  transient was not affected by CPCCOEt in hmGluR5a- or hmGluR1/5a-expressing cells. Comparison of the hmGluR5/1b chimera with wild-type hmGluR1b (Fig. 5B) revealed no significant difference in the  $\text{IC}_{50}$  values ( $7.7 \pm 2.0 \mu\text{M}$  versus  $6.5 \pm 1.4 \mu\text{M}$ , respectively). This indicates that the inhibitory effect of CPCCOEt is mediated by the TM and/or intracellular regions of hmGluR1b and not by the large N-terminal extracellular domain.

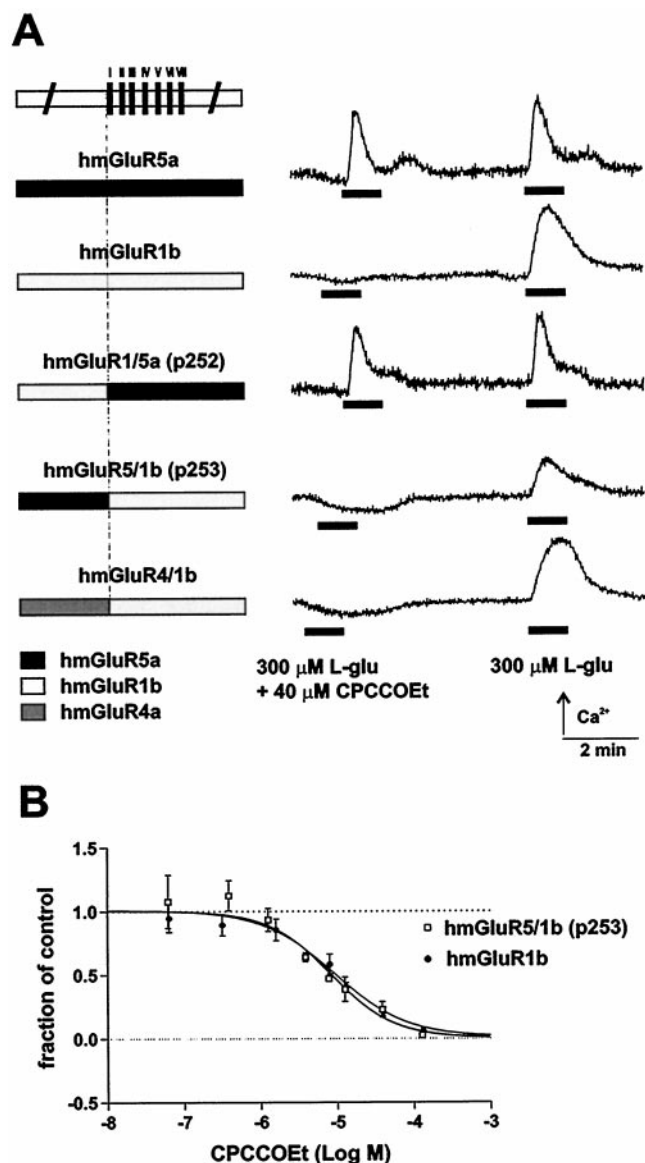
To determine more precisely the region(s) mediating the inhibition by CPCCOEt, we created a second series of chimeric hmGluR1/5a and -R5/1b receptors fused at different positions in the TM domains. Each cDNA construct was



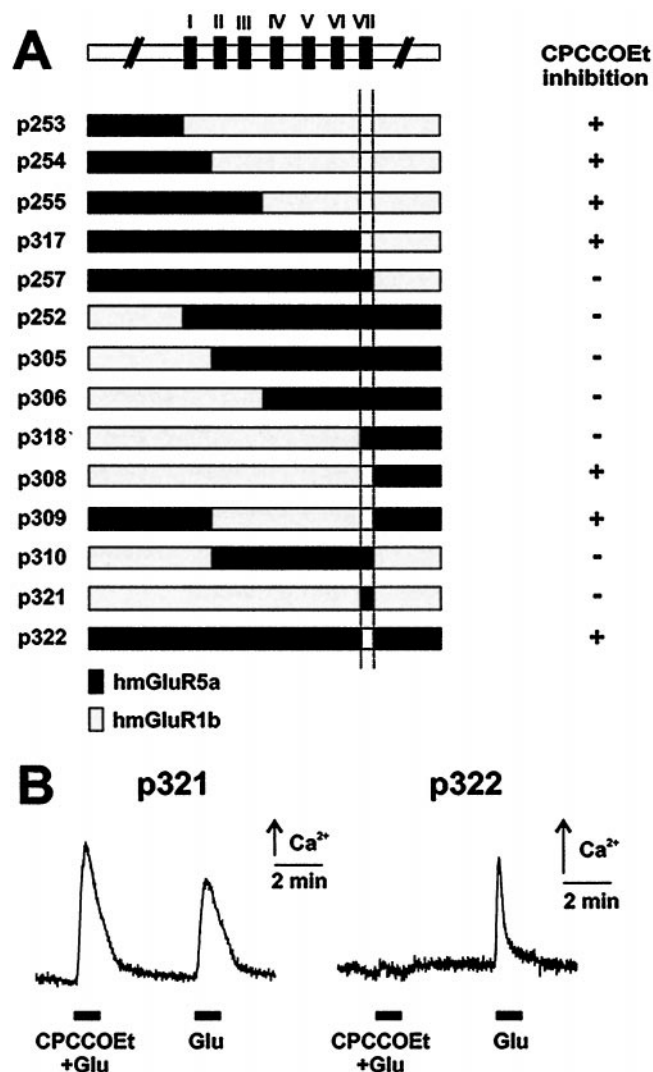
**Fig. 4.** Lack of agonist and antagonist effect of CPCCOEt in cell lines stably expressing human mGluR2, -4a, -7b, and -8a. Forskolin (10  $\mu\text{M}$ ) stimulated cAMP levels about 40-fold (taken as control). Results are expressed as fraction of control. Antagonist activity was measured against approximately  $\text{EC}_{50}$  concentrations of agonists. A, hmGluR2: 30  $\mu\text{M}$  (1S,3R)-ACPD. B, hmGluR4a: 1  $\mu\text{M}$  L-AP4. C, hmGluR7b: 500  $\mu\text{M}$  L-AP4. D, hmGluR8a: 1  $\mu\text{M}$  L-AP4. Columns represent mean values  $\pm$  S.E.M. from at least two experiments ( $n \geq 5$ ).

transiently transfected into COS1 cells, and the activity of the chimeric receptor was measured in the calcium assay (Fig. 6). Whereas all chimeras responded to a test application of glutamate (300  $\mu$ M), inhibition of glutamate-induced

$[Ca^{2+}]_i$  responses by CPCCOEt was observed only in chimeras containing the TMVII segment of hmGluR1b. All chimeric receptors containing the TMVII segment derived from hmGluR5a were not inhibited by CPCCOEt. These findings were substantiated further with chimeras in which only parts of the TM domain were exchanged between hmGluR1b and -5a (Fig. 6). For instance, CPCCOEt selectively inhibited glutamate-induced  $[Ca^{2+}]_i$  responses in p322, a chimeric hmGluR5a receptor with the TMVII segment derived from hmGluR1b. In contrast, CPCCOEt was ineffective at the reciprocal hmGluR1b chimera p321 with TMVII derived from hmGluR5a.



**Fig. 5.** Effect of CPCCOEt at wild-type hmGluR1b and -5a and chimeric receptors hmGluR1/5a, -5/1b, and -4/1b. **A**, CPCCOEt selectively inhibits glutamate-induced changes in  $[Ca^{2+}]_i$  in cells transiently expressing receptor constructs hmGluR1b, -5/1b, and -4/1b, but not hmGluR5a and -5a/1b. Left, schematic diagram of receptor constructs indicating location of fusion sites in front of TMI. Right, traces show representative time courses of  $[Ca^{2+}]_i$  transients as measured with fura-2 microfluorimetry. Black column indicate application of drugs below each trace. Glutamate (Glu) was applied at a concentration of 300  $\mu$ M and CPCCOEt at 40  $\mu$ M, respectively. Between drug applications a 3- to 5-min period was allowed for washout and recovery. Measurements were repeated at least five times in separate transfection experiments. **B**, concentration-response curves for inhibition of glutamate-induced  $[Ca^{2+}]_i$  transients by CPCCOEt in cells expressing wild-type hmGluR1b and hmGluR5/1b chimera p253. Results are normalized as a percentage of stimulation of  $[Ca^{2+}]_i$  with 300  $\mu$ M (hmGluR1b) and 100  $\mu$ M glutamate (p253), respectively, monitored every 30 min during recording of a concentration-response curve. Between successive recordings with different concentration of CPCCOEt, an interval of at least 10 min was allowed for washout and recovery. Measurements were performed six times in separate transfection experiments.



**Fig. 6.** Inhibition by CPCCOEt is mediated by TMVII of hmGluR1b. **A** left, schematic diagram of a series of chimeric receptor constructs indicating location of fusion sites between hmGluR1b and hmGluR5a. Right, receptor constructs were transiently expressed in COS1 cells and tested for  $[Ca^{2+}]_i$  responses to glutamate (Glu) and inhibition of glutamate-induced responses by CPCCOEt. +, Complete inhibition of glutamate-induced  $[Ca^{2+}]_i$  response by 40  $\mu$ M CPCCOEt. -, no inhibition of glutamate-induced calcium response by 40  $\mu$ M CPCCOEt. Data were reproduced at least three times in separate transfection experiments. **B**, traces show representative time courses of  $[Ca^{2+}]_i$  transients with constructs p321 and p322. Consecutive application of drugs is indicated below each trace. Glutamate was applied at a concentration of 300  $\mu$ M and CPCCOEt was applied at a concentration of 40  $\mu$ M, respectively. Data were repeated at least five times in separate transfection experiments.

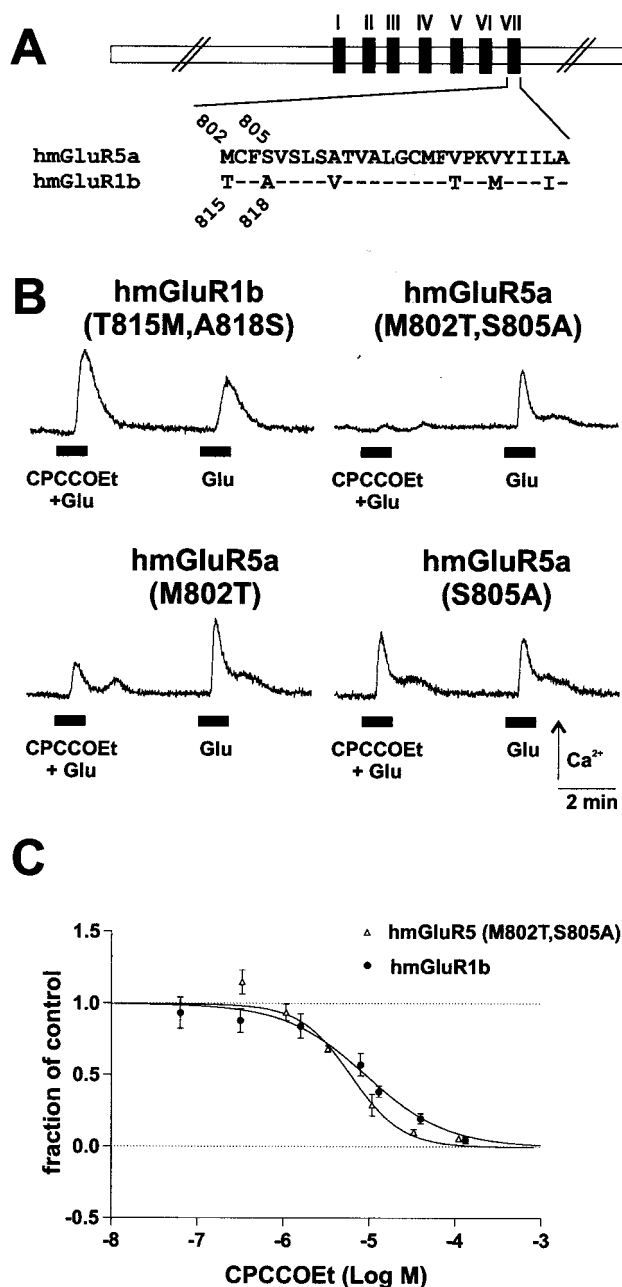


Sequence alignment of hmGluR1b and hmGluR5a revealed that the TMVII segment of hmGluR1 and hmGluR5 is highly conserved and differs in only 6 out of 26 amino acid residues (see Fig. 7A). To precisely identify the amino acids mediating

the inhibition by CPCCOEt, we constructed point mutations of hmGluR1b and -5a, in which the nonconserved amino acids were substituted by the homologous amino acids of the related receptor subtype. Substitution of Thr815 (T815) and Ala818 (A818) of hmGluR1b with the homologous amino acids Met802 (M802) and Ser805 (S805) of hmGluR5a eliminated the CPCCOEt inhibition at hmGluR1b but did not affect the induction of  $[Ca^{2+}]_i$  responses by glutamate (Fig. 7B). Substitution of Met802 of hmGluR5a with Thr815 of hmGluR1b (M802T) resulted in a partial inhibition by CPCCOEt (40  $\mu$ M), whereas substitution of Ser805 of hmGluR5a with Ala818 (S805A) had no effect. However, substitution of both amino acids in hmGluR5a (M802T, S805A) conferred complete CPCCOEt inhibition at 40  $\mu$ M. To quantitatively demonstrate that Thr815 and Ala818 of hmGluR1b are sufficient to mediate the antagonist effect of CPCCOEt, an inhibition curve was performed with the mutant hmGluR5a (M802T, S805A). As shown in Fig. 7C, the half-maximal inhibition of CPCCOEt was  $6.6 \pm 0.9$   $\mu$ M and, thus, very similar to the  $IC_{50}$  value of the wild-type hmGluR1b receptor ( $IC_{50} = 6.5 \pm 1.4$   $\mu$ M; see also Fig. 5). Taken together, these data indicate that the amino acids Thr815 and Ala818 of hmGluR1b are both necessary and sufficient to mediate the subtype-specific inhibition of CPCCOEt.

## Discussion

mGluRs together with the  $\gamma$ -aminobutyric acid type B receptor (Kaupmann et al., 1997), the parathyroid calcium-sensing receptors (Brown et al., 1993), and the vomeronasal receptors (Bargmann, 1997) form a separate family within the superfamily of GPCRs (Conn and Pin, 1997). The most striking difference between the mGluR family and most other GPCR resides in their ligand-binding domain. In the latter group numerous studies of structure-function relationships indicated that ligands either interact directly with amino acid residues in the TM domain or in addition with residues in the extracellular domains. For instance, monoamines and other small ligands are bound directly in a pocket formed within the TM segments, whereas the binding sites for neuropeptides and chemokines are defined by complex interactions with residues in the N-terminal extracellular domain, the extracellular loops, and TM segments (Betancur et al., 1997). In contrast, the ligand-binding site of mGluRs is thought to be located completely in the large (500-amino-acid-residue) extracellular domain homologous to bacterial periplasmic-binding proteins (PBPs) (O'Hara et al., 1993; Takahashi et al., 1993). Modeling based on the three-dimensional structures of several PBPs (Adams and Oxender, 1989; Sack et al., 1989; Quiocho, 1990) has predicted that the glutamate-binding domain of mGluR1 consists of two lobes with a hinge region where glutamate binds (O'Hara et al., 1993). In support of this model, mutation of two amino acids proposed to be critically involved in agonist binding completely abolished  $[^3H]$ glutamate binding (O'Hara et al., 1993). In addition, several studies using chimeric receptors demonstrated that the agonist selectivity is determined solely by the extracellular domain (Takahashi et al., 1993; Tones et al., 1995; Wroblewska et al., 1997; Parmentier et al., 1998). Furthermore, a soluble form of the extracellular domain of mGluR1 was shown recently to fully retain the ligand-binding characteristics of the full-length receptor (Oka-



**Fig. 7.** Inhibition by CPCCOEt is mediated by Thr815 and Ala818 of hmGluR1b. **A**, schematic diagram of hmGluR1b and -5a receptors indicating high degree of sequence conservation in TM segment VII. Conserved amino acid residues are indicated by (-). Numbers reveal positions of homologous amino acid residues in hmGluR1b and -5a, respectively. **B**, Traces show representative time courses of  $[Ca^{2+}]_i$  transients in mutant receptors. Application of drugs is indicated below each trace. Glutamate was applied at a concentration of 300  $\mu$ M and CPCCOEt was applied at a concentration of 40  $\mu$ M, respectively. Data were repeated at least five times in independent transfection experiments. **C**, Concentration-response inhibition of glutamate-induced  $[Ca^{2+}]_i$  transients by CPCCOEt in cells expressing mutant receptor hmGluR5a(M802T, S805A) and wild-type hmGluR1b. Results are expressed as a percentage of stimulation of  $[Ca^{2+}]_i$  with 100  $\mu$ M and 300  $\mu$ M glutamate, respectively. Measurements were performed six times in separate transfection experiments.

moto et al., 1998). This indicates that the ligand-binding event of mGluRs is dissociable from receptor signaling across the membrane and allows to predict that novel antagonist ligands may function either as competitive glutamate antagonists at the extracellular ligand-binding site or as inhibitors of G protein coupling by preventing receptor intramolecular signaling. Currently known mGluR antagonists such as the widely studied carboxyphenylglycines are glutamate analogs and, as such, are likely to interact with the extracellular glutamate-binding site in a competitive manner as demonstrated for (*R,S*)- $\alpha$ -methyl-4-carboxyphenylglycine, (*S*)-4-carboxyphenylglycine, and AIDA by a parallel right shift of concentration-response curves of agonist-stimulated PI hydrolysis (Birse et al., 1993; Eaton et al., 1993; Hayashi et al., 1994; Thomsen et al., 1994; Brabet et al., 1995; Moroni et al., 1997).

In this report we provide evidence for the existence of compounds with a different mode of inhibition. We show that CPCCOEt (Annoura et al., 1996), a selective mGluR1 antagonist structurally unrelated to glutamate-derived mGluR ligands, inhibits receptor activity without affecting glutamate binding. Coapplication of glutamate and increasing concentrations of CPCCOEt decreased the efficacy of hmGluR1b activity in the PI hydrolysis assay but did not influence the EC<sub>50</sub> or Hill coefficient of glutamate. In addition, [<sup>3</sup>H]glutamate binding to membranes prepared from rat mGluR1a-expressing cells was not inhibited in the presence of CPCCOEt. Very recently, CPCCOEt also was shown not to displace [<sup>3</sup>H]quisqualate binding to the glutamate-binding domain of rat mGluR1 expressed in a soluble form (Okamoto et al., 1998). Taken together, these data indicate a noncompetitive mode of inhibition via interaction of CPCCOEt with a novel receptor site independent from the glutamate-binding domain.

Different binding domains for agonists and antagonists previously have been identified for a number of GPCRs such as tachykinins, cholecystokinin, angiotensin, opioids, neurotensin, and vasopressin (for review see Betancur et al., 1997). However, in contrast to CPCCOEt, agonists and antagonists were shown to act as competitive ligands, which compete for binding to the receptor in a mutually exclusive fashion. To elucidate the site of action of the noncompetitive antagonist CPCCOEt, a detailed molecular investigation using chimeric receptors and point mutations of hmGluR1b and hmGluR5a was performed. We show that CPCCOEt specifically interacts with Thr815 and Ala818 located at the extracellular surface of TMVII of hmGluR1b. Substitution of these amino acids with the homologous amino acids of hmGluR5a (hmGluR1b-T815M, A818S) suppressed the CPCCOEt inhibition of glutamate-induced [Ca<sup>2+</sup>]<sub>i</sub> responses, whereas introduction of Thr815 and Ala818 from hmGluR1b at the corresponding position of hmGluR5a generated a gain-of-function mutant as sensitive to the inhibition by CPCCOEt as the wild-type receptor hmGluR1b (IC<sub>50</sub> of 5  $\mu$ M versus 12  $\mu$ M, respectively). Thus, Thr815 and Ala818 of hmGluR1b both are sufficient and necessary to fully mediate the subtype-specific inhibition of CPCCOEt. Because both residues are found only in mGluR1 but not in the homologous position at the subtypes mGluR2 to -8, these data are consistent with the observed lack of interaction of CPCCOEt in functional assays of other cloned mGluR subtypes.

The existence of distinct binding sites for glutamate and

CPCCOEt at mGluR1 and their noncompetitive interaction raises questions about the intramolecular mechanism of receptor activation and its inhibition by CPCCOEt. As described above, mGluRs are constituted of two main domains: the glutamate-binding domain (B) that corresponds to the extracellular PBP-like domain and a TM region (E) constituted of seven TM helices. The current hypothesis on the functioning of GPCRs that are constituted of a seven-TM region (TMVII) only is that they naturally oscillate between at least two conformations (or states), an active (R\*) and an inactive (R) one (Lefkowitz et al., 1993). This proposal results from the observation that many mutated as well as wild-type receptors possess constitutive activity. A follow-up of this model is that the agonists stabilize the R\* state whereas the antagonists with inverse agonist activity stabilizes the R state. This hypothesis also may be valid for the TM domain of mGluRs, because it is likely structurally related to the other seven TM receptors. This mGluR domain therefore also may oscillate between at least two states, E and E\*. The observation that CPCCOEt does not act as an inverse agonist (it does not inhibit the constitutive activity of mGluR1a) although it interacts directly with the TM region indicates that it does not modify the natural equilibrium between E and E\*. As already mentioned, glutamate binds on the B domain, which is structurally related to PBPs. It also has been proposed that this domain, like the PBPs, undergoes a large conformational change upon agonist binding (a closure of the two lobes) (Quiocho, 1990; O'Hara et al., 1993). Accordingly, glutamate is unlikely, by itself, to directly stabilize the E\* state of the TM region of mGluRs. Instead, glutamate may stabilize a conformation of the extracellular B domain that will stabilize E\*. By interacting on top of TMVII of mGluR1, CPCCOEt may prevent the activation of the TM domain by the glutamate-occupied B domain. CPCCOEt therefore may be considered as an inhibitor of the intramolecular-signaling mechanism of mGluR1, disconnecting the cross-talk between the two domains of the receptor. This may occur in several ways. CPCCOEt and the glutamate-bound B domain might compete directly for the same site on the TM region. Alternatively, interaction of CPCCOEt with Thr815 and Ala818 in TMVII might create a steric obstacle, preventing the glutamate-bound B domain to interact with the TM region and, subsequently, to stabilize.

Taken together, our data demonstrate that CPCCOEt is a subtype-selective, noncompetitive mGluR1 antagonist interacting with Thr815 and Ala818 in TMVII. To our knowledge, this is the first demonstration of a compound acting at a GPCR by specifically inhibiting TM signaling without affecting binding of the endogenous agonist. The discovery of a novel pharmacological site separated from the extracellular glutamate-binding domain may allow the discovery of new structural classes of subtype-specific mGluR ligands unrelated to amino acids.

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