0026-895X/96/040923-08$3.00/0

MOLECULAR PHARMACOLOGY, 50:923–930 (1996).

Coupling of Metabotropic Glutamate Receptors 2 and 4 to $G_{\alpha 15}$, $G_{\alpha 16}$, and Chimeric $G_{\alpha q/i}$ Proteins: Characterization of New Antagonists

JESÚS GOMEZA,[1] SOPHIE MARY, ISABELLE BRABET, MARIE-LAURE PARMENTIER, SOPHIE RESTITUITO, JOËL BOCKAERT, and JEAN-PHILIPPE PIN

UPR Centre Nationale de Recherche Scientifique 9023, Mécanismes Moléculaires des Communications Cellulaires, CCIPE, 34094 Montpellier, Cedex 5, France

Received April 22, 1996; Accepted June 5, 1996

SUMMARY

Together with the calcium-sensing receptor, the metabotropic glutamate receptors (mGluRs) share no sequence homology with the other G protein-coupled receptors (GPCRs) and therefore constitute a new family of receptors. Recently, it was reported that $G_{\alpha 15}$ and $G_{\alpha 16}$ subunits allow many GPCRs to activate phospholipase C (PLC). Furthermore, the exchange of a few carboxyl-terminal residues of $G_{\alpha q}$ by those of $G_{\alpha i2}$ or $G_{\alpha o}$ allows the resulting chimeric G_{α} subunits ($G_{\alpha qi}$ and $G_{\alpha qo}$, respectively) to couple G_i-coupled receptors to PLC. We report that mGluR2 and mGluR4, two receptors negatively coupled to adenylyl cyclase, activate PLC when coexpressed with $G_{\alpha 15}$, $G_{\alpha qi}$, or $G_{\alpha qo}$. This indicates that the carboxyl-terminal end of the G_{α} subunit also plays an important role in the specific interaction between mGluRs and the G proteins. In addition, the measurement of PLC activation by G_i-coupled mGluRs coexpressed with these G_{α} subunits constitutes an easy functional assay for the pharmacological characterization of these receptors. The rank order of potency of antagonists was found to be (2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine ~ (*R*,*S*)-α-methyl-4-phosphonophenylglycine > (*R*,*S*)-α-methyl-4-sulfonophenylglycine > (*R*,*S*)-α-methyl-4-tetrazolylphenylglycine = (*S*)-2-amino-2-methyl-4-phosphonobutyrate for mGluR2 and to be (*R*,*S*)-α-methyl-4-phosphonophenylglycine ≥ (*S*)-2-amino-2-methyl-4-phosphonobutyrate ≫ (*R*,*S*)-α-methyl-4-sulfonophenylglycine [(*R*,*S*)-α-methyl-4-tetrazolylphenylglycine and (2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine being inactive at 1 mM] for mGluR4. Using this functional assay, (*R*,*S*)-α-methyl-4-phosphonophenylglycine was found to have a similar K_B value for mGluR2 and mGluR4.

The major excitatory neurotransmitter in the brain, glutamate, activates ionotropic and metabotropic (G protein-coupled) receptors (1). These latter receptors modulate the activity of many synapses and play important roles in glutamate-mediated effects, including neurotoxicity, brain development, and memory formation (2–4). Eight genes encoding mGluRs have been identified. Based on their sequence homology, transduction mechanism, and pharmacology, they can be classified into three groups (2–4). The first one is composed of mGluR1 and mGluR5, which are coupled to PLC. The second group is composed of mGluR2 and mGluR3, both of which negatively coupled to AC. The other mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8) constitute the third group. They are negatively coupled to AC in heterologous expression systems and are selectively activated by L-AP4 (5–8). Although the pharmacology of PLC-coupled mGluRs has been studied extensively, the pharmacology of many AC-coupled mGluRs is not well characterized, probably because of the difficulty in developing simple functional assays for these receptors.

MGluRs possess seven TMDs but share no sequence homology with other GPCRs except the recently identified cal-

This work was supported by grants from the Centre National de la Recherche Scientifique, the European Community (Biomed 2 program, BMH4-CT96-0228), the French ministry of Education, Research and Professional Insertion (ACC-SV5, Grant 9505077), and Bayer Company (Wupertal, Germany) Direction des Recherches et Études Techniques (DRET 91/161). J.G. was supported by a fellowship from the Spanish Ministry of Education.

[1] Current affiliation: Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; AC, adenylyl cyclase; ACPD, 1-amino-1,3-cyclopentane dicarboxylate; 4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; 4CPG, (*S*)-4-carboxyphenylglycine; 3,5-DHPG, (*R*,*S*)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; IP, inositol phosphates; HEK, human embryonic kidney; L-AP4, L-2-amino-4-phosphonobutyrate; L-CCGI, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine; L-SOP, L-serine-*O*-phosphate; MAP4, (*S*)-2-amino-2-methyl-4-phosphonobutyrate; MCCGI, (2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine; MCPG, (*R*,*S*)-α-methyl-4-carboxyphenylglycine; MPPG, (*R*,*S*)-α-methyl-4-phosphonophenylglycine; MSPG, (*R*,*S*)-α-methyl-4-sulfonophenylglycine; MTPG, (*R*,*S*)-α-methyl-4-tetrazolylphenylglycine; PLC, phospholipase C; PTX, pertussis toxin; t-ADA, *trans*-azetidine diacid; TMD, transmembrane domain; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cium-sensing receptor (9). This absence of homology prevents the construction of a three-dimensional model for the seven-TMD region of mGluRs based on the known structure of bacteriorhodopsin or on other GPCR models (10). In contrast to many GPCRs, the agonist binding site of mGluRs is located in a large extracellular domain homologous to bacterial periplasmic binding proteins (11, 12). Finally, most of the structural and sequence requirements for G protein coupling in GPCRs are not fulfilled by mGluR intracellular sequences (4). We recently proposed that the second intracellular loop of mGluRs plays a role equivalent to that of the third intracellular loop of the other GPCRs (13, 14).

Recently, $G_{\alpha 15}$ and $G_{\alpha 16}$, which activate PLC, have been reported to be activated by many different GPCRs, including those recognized not to stimulate PLC (15). It has also been reported that the carboxyl-terminal end of the α subunit of G proteins plays an important role in the specific interaction between the G protein and the receptor. Accordingly, the exchange of the five carboxyl-terminal residues of the PLC activating G_{α} subunit, $G_{\alpha q}$, by those of either $G_{\alpha i2}$ or $G_{\alpha o}$ is sufficient to allow these chimeric $G_{\alpha qi}$ and $G_{\alpha qo}$ subunits to be activated by receptors coupled to $G_{\alpha i}$ or $G_{\alpha o}$ (16).

In the current study, we show that despite the absence of sequence homology between mGluRs and the other GPCRs, the representative members of group II and III mGluRs (i.e., mGluR2 and mGluR4, respectively) activate $G_{\alpha 15}$ and the chimeric $G_{\alpha qi}$ and $G_{\alpha qo}$ subunits. We also show that these wild-type and chimeric α subunits can be used to develop simple functional assays for the pharmacological characterization of group II and group III mGluRs. This is of particular interest because of the difficulty of maintaining stable cell lines expressing these receptors and because such an assay will prove to be useful for the functional characterization of a large number of chimeric or mutated mGluRs negatively coupled to AC. Such an assay allowed us to further characterize the pharmacological profiles of mGluR2 and mGluR4.

Experimental Procedures

Materials. MCPG, 4CPG, 4C3HPG, 3,5-DHPG, L-AP4, L-SOP, L-CCGI, MAP4, MCCGI, MTPG, MSPG, and MPPG were obtained from Tocris Cookson (Essex, UK). The four different stereoisomers of ACPD were a generous gift of Dr. K. Curry (University of British Columbia, Vancouver, Canada). (±)-t-ADA was a gift from A. P. Kozikowski (Trophic Pharmaceuticals, Inc., South Plainfield, NJ), (+)-t-ADA (CGP-61919) and (−)-t-ADA (CGP-61920) were gifts from Dr. T. Knöpfel (Ciba, Basle, Switzerland).

For the transient expression of mGluRs, the cDNA of mGluR1a (17), mGluR2 (isolated like mGluR3; 13), and mGluR4a (a generous gift from Prof. S. Nakanishi, University of Kyoto, Kyoto, Japan) were subcloned into the eukaryotic expression vector pRK5 containing a cytomegalovirus immediate-early promoter. The plasmids pcDNA-G_{qwt}, pcDNA-G_{qi5}, pcDNA-G_{qi9}, and pcDNA-G_{qo5} were gifts from Dr. B. Conklin and H. Bourne (University of California, San Francisco, San Francisco, CA). The plasmids pCIS-$G_{\alpha 15}$ and pCIS-$G_{\alpha 16}$ were kindly provided by Dr. M. Simon (California Institute of Technology, Los Angeles, CA).

Culture and transfection of HEK 293 cells. HEK 293 cells were cultured in DMEM (GIBCO BRL, Baltimore, MD) supplemented with 10% fetal calf serum and antibiotics (final concentration of penicillin and streptomycin, 100 units/ml). Electroporation was performed in a total volume of 300 μl with 10 μg of carrier DNA, 4 μg of mGluR2 plasmid DNA, 10 μg of mGluR4 plasmid DNA, 4 μg of wild-type or mutated G_{α} subunit plasmid DNA when indicated, and 10 million cells in electroporation buffer (50 mM K_2HPO_4, 20 mM CH_3COOK, 20 mM KOH). After electroporation [260 V, 960 μF; BioRad gene pulser electroporator (Hercules, CA)], cells were resuspended in DMEM supplemented with 10% fetal calf serum and antibiotics and split into 12-well clusters (Falcon Plastics, Oxnard, CA) (10 million cells/cluster) previously coated with poly-L-ornithine (15 μg/ml; molecular weight, 40,000; Sigma Chemical, St. Louis, MO) to favor adhesion of the cells.

Determination of IP accumulation. Determination of IP accumulation in transfected cells was performed as described previously (18). At 3–4 hr after electroporation, cells were washed and incubated for 12 hr in DMEM/Glutamax-I (GIBCO) containing 1 μCi/ml *myo*-[^{3}H]inositol (23.4 Ci/mol, Dupont-New England Nuclear, Les Ulis, France). Cells were then washed three times and incubated for 1–2 hr at 37° in 1 ml of HEPES buffer saline (146 mMNaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.4) supplemented with 1 unit/ml glutamate pyruvate transaminase (Boehringer-Mannheim, Meylan, France) and 2 mM pyruvate (Sigma, Lisle d'Abeau, France). Cells were then washed again twice with the same medium and preincubated for 5 min in medium containing 10 mM LiCl. The agonist was then added for a period of 30 min. The reaction was stopped by replacing the incubation medium with 1 ml of perchloric acid (5%) on ice. The total IP were then extracted and purified on Dowex columns as described previously (18). Total radioactivity remaining in the membrane fraction was counted after solubilization in 10% Triton X-100 and 0.1 N NaOH and used as standard. Results are expressed as the amount of IP produced over the radioactivity present in the membranes. The dose-response curves were fitted using the equation $y = [(y_{max} - y_{min})/1 + (x/EC_{50})^n] + y_{min}$ and the Kaleidagraph program.

Results

To examine whether group II and III mGluRs that are negatively coupled to AC could activate the promiscuous G protein α subunits $G_{\alpha 15}$ and $G_{\alpha 16}$, these α subunits were expressed alone or in combination with either mGluR2 or mGluR4 in HEK-293 cells. As shown in Fig. 1, glutamate stimulated IP formation in cells coexpressing mGluR2 and either $G_{\alpha 15}$ or $G_{\alpha 16}$. The glutamate-stimulated IP formation was higher in cells expressing mGluR2 and $G_{\alpha 15}$ than in cells expressing mGluR2 and $G_{\alpha 16}$. When coexpressed with

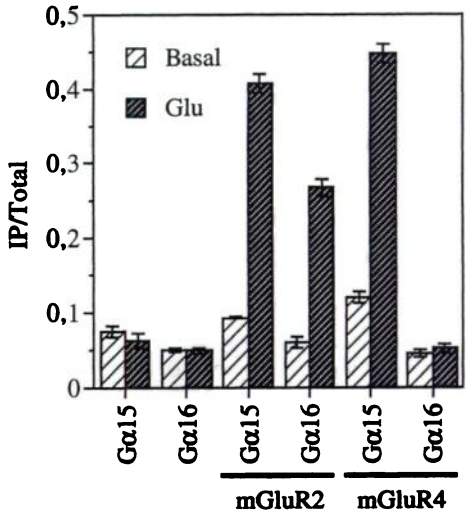

Fig. 1. Basal and 1 mM glutamate (*Glu*)-stimulated IP formation in cells expressing $G_{\alpha 15}$ or $G_{\alpha 16}$ alone or in combination with mGluR2 or mGluR4. Values are mean ± standard error of triplicate determinations from a typical experiment. Similar data were obtained in at least five other experiments.

mGluR4, $G_{\alpha 15}$ allowed glutamate to stimulate PLC, whereas $G_{\alpha 16}$ did not. No glutamate stimulation of IP formation was observed in cells expressing $G_{\alpha 15}$, $G_{\alpha 16}$ (Fig. 1), mGluR2, or mGluR4 (Fig. 2) alone.

We next examined whether the carboxyl-terminal domain of the G_α subunit plays a critical role in the specific interaction with mGluRs, as already observed with the other GPCRs. MGluR2 and mGluR4 were therefore coexpressed with the chimeric $G_{\alpha q}$ subunits in which the five or nine carboxyl-terminal residues of $G_{\alpha q}$ have been replaced by those of $G_{\alpha i2}$ ($G_{\alpha qi5}$ and $G_{\alpha qi9}$, respectively) or $G_{\alpha o}$ ($G_{\alpha qu5}$). As shown in Fig. 2, glutamate stimulated IP formation in cells coexpressing mGluR2 or mGluR4 with any of these three chimeric G_α subunits. The larger glutamate stimulation of IP formation was observed when mGluR2 or mGluR4 was coexpressed with either $G_{\alpha qi9}$ or $G_{\alpha qu5}$. No stimulation of IP formation was observed in cells expressing mGluR2 or mGluR4 alone or in combination with the wild-type $G_{\alpha q}$, indicating that the activation of PLC did not result from an overexpression of the $G_{\alpha q}$-like subunit but that the exchange of the carboxyl-terminal end was indeed necessary for this glutamate-induced response.

To confirm that the chimeric G_α subunits were responsible for the coupling of mGluR2 and mGluR4 to PLC, we examined the sensitivity of the glutamate-stimulated IP formation to PTX. This toxin is known to block the activation of $G_{\alpha i}$ and $G_{\alpha o}$ subunits by ADP-ribosylating a cysteine residue located three residues from their carboxyl terminus (19). This cysteine residue is therefore in the carboxyl-terminal end of the chimeric $G_{\alpha qi5}$, $G_{\alpha qi9}$, and $G_{\alpha qu5}$ subunits, making these subunits possible targets for PTX. Accordingly, the glutamate-stimulated IP formation in cells coexpressing mGluR2 and $G_{\alpha qi9}$ was inhibited dose-dependently by PTX (Fig. 3). Similar data were obtained in cells expressing mGluR4 (data not shown). Although high concentrations of PTX are necessary for the complete inhibition of the glutamate-induced IP formation, no effect of such high PTX concentrations were observed on the activation of PLC by mGluR1a (Fig. 3).

Full dose-response curves for glutamate were constructed by measuring IP formation in cells coexpressing mGluR2 or mGluR4 with the promiscuous and chimeric α subunits (Fig. 4). For mGluR2, the EC_{50} values (mean ± standard error from more than two experiments) were 3.7 ± 1.2, 11.5 ± 1.7, 8.4 ± 0.9, 7.6 ± 1.5, and 11.1 ± 0.6 μM with $G_{\alpha 15}$, $G_{\alpha 16}$, $G_{\alpha qi5}$, $G_{\alpha qu5}$, and $G_{\alpha qi9}$ respectively. For mGluR4, these values were 9.9 ± 2.3, 20.7 ± 3.7, 16.1 ± 1.3, and 19.1 ± 2.7 μM when determined with $G_{\alpha 15}$, $G_{\alpha qi5}$, $G_{\alpha qu5}$, and $G_{\alpha qi9}$ respectively.

The effects of well-characterized agonists of mGluR2 and mGluR4 were examined in cells coexpressing $G_{\alpha qi9}$ and $G_{\alpha qu5}$, respectively. Glutamate, L-CCGI, 1*S*,3*R*-ACPD, and 4C3HPG stimulated IP formation in mGluR2 expressing cells with EC_{50} values very similar to those reported by measuring the inhibition of forskolin-stimulated cAMP formation in Chinese hamster ovary cells stably expressing mGluR2 (Fig. 5a and Table 1) (20, 21). Furthermore, quisqualate and L-AP4, the potent agonists of group I and group III mGluRs, respectively, had no effects (Table 1). Similarly, the specific and potent agonists of group III mGluRs, L-AP4 and L-SOP, potently stimulated IP formation in cells coexpressing mGluR4 and $G_{\alpha qu5}$ (Fig. 5b and Table 1). The EC_{50} values were very similar to those reported using cell lines stably expressing mGluR4 (22–25). Taken together, these results indicate that mGluR2 and mGluR4 couple to chimeric $G_{\alpha qi/o}$ subunits and activate PLC and indicate that the few last carboxyl-terminal residues of the G_α subunits play an important role in the specific recognition of mGluRs.

As shown in Fig. 6, the pharmacological profiles of mGluR2 and mGluR4 determined by measuring PLC activation after coexpression with the chimeric $G_{\alpha qi9}$ or $G_{\alpha qu5}$ subunits per-

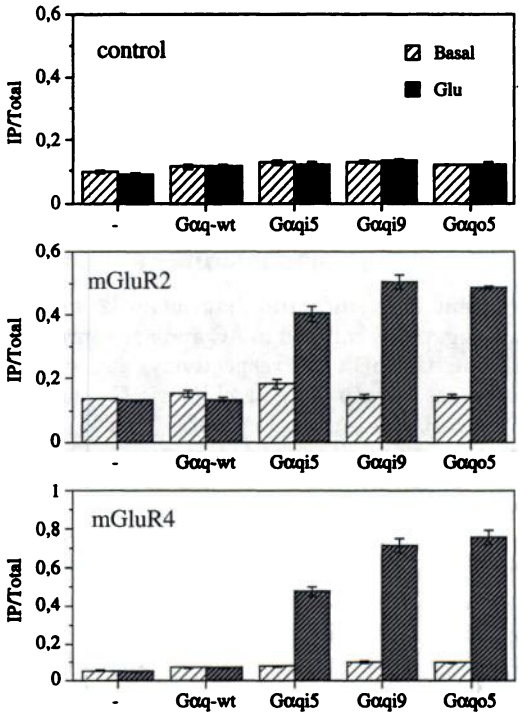

Fig. 2. PLC activation by mGluR2 or mGluR4 coexpressed with chimeric $G_{\alpha q}$ subunits. *Top*, basal and 1 mM glutamate (*Glu*)-stimulated IP formation in mock-transfected cells or cells expressing the wild-type $G_{\alpha q}$ ($G_{\alpha qwt}$) or the chimeric $G_{\alpha q}$ subunits in which the carboxyl-terminal five amino acid residues have been replaced by those of $G_{\alpha i2}$ ($G_{\alpha qi5}$) or $G_{\alpha o}$ ($G_{\alpha qu5}$) or in which the nine carboxyl-terminal residues have been replaced by those of $G_{\alpha i2}$ ($G_{\alpha qi9}$). *Middle*, same as *top* except that cells also expressed mGluR2. *Bottom*, same as *top* except that cells also expressed mGluR4. Values are mean ± standard error of triplicate determinations from typical experiments. Similar data were obtained in at least five other experiments.

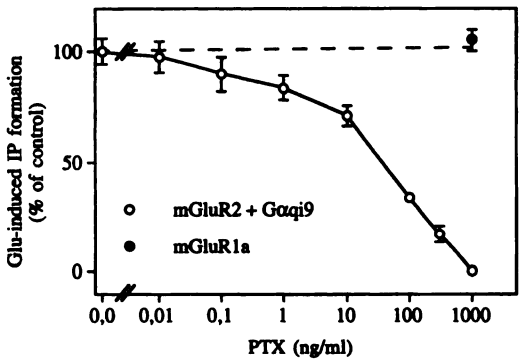

Fig. 3. Effect of PTX treatment on IP formation stimulated by 1 mM glutamate (*Glu*) in cells expressing mGluR1a or mGluR2 plus $G_{\alpha qi9}$. After electroporation, cells were incubated for 16 hr in the presence of the indicated concentration of PTX. Basal and 1 mM glutamate-induced IP formations were determined as described in the text. Values are mean ± standard error of triplicate determinations from a typical experiment and are expressed as a percentage of the effect of glutamate in cells not treated with PTX.

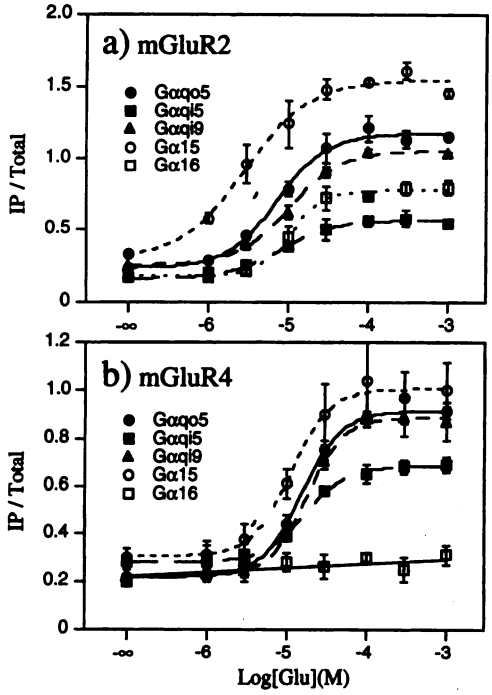

Fig. 4. Dose-response curves for glutamate (*Glu*) on the IP production in cells coexpressing mGluR2 (a) or mGluR4 (b) with $G_{\alpha qu5}$, $G_{\alpha qi5}$, $G_{\alpha qi9}$, $G_{\alpha 15}$, or $G_{\alpha 16}$. Results are mean ± standard error of at least three independent experiments performed in triplicate.

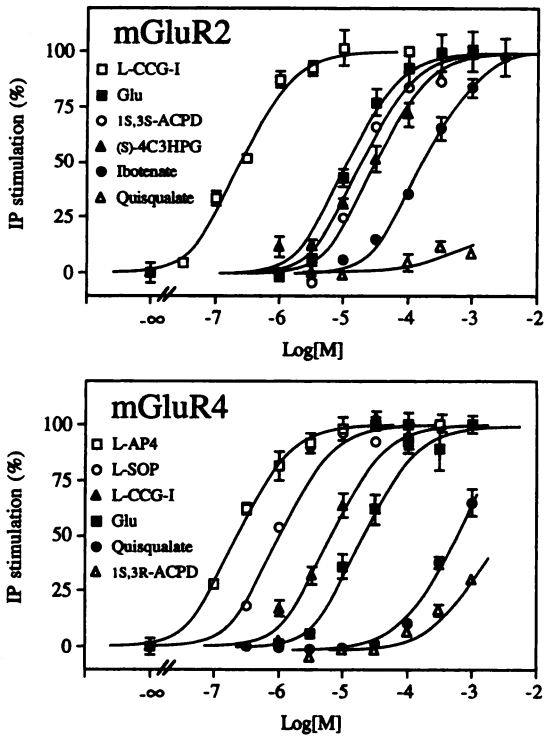

Fig. 5. Effect of various agonists on IP formation in cells expressing mGluR2 plus $G_{\alpha qi9}$ (a) or cells expressing mGluR4 plus $G_{\alpha qu5}$ (b). Transfected cells were washed, preincubated for 10 min with 10 mM LiCl in Krebs' buffer, and then stimulated for 30 min with the agonist at the indicated concentration. IP formation was then determined as described in the text. Values are mean ± standard error of triplicate determinations from typical experiments and are expressed as a percentage of the maximal effect of glutamate.

fectly correlate with those previously reported by others measuring inhibition of cAMP formation in cells stably expressing these receptors. The use of the chimeric G_α subunits therefore seems to be a simple functional assay for the pharmacological characterization of these receptors. We took advantage of this system to study further the effects of recently described drugs on these two cloned receptors. 3,5-DHPG and (−)-t-ADA, which have been described as potent agonists at mGluR1a and mGluR5a (26–29), were inactive at either mGluR2 or mGluR4 (Table 1). Among the four stereoisomers of ACPD, none were potent agonists at mGluR4, but both 1*S*,3*S*-ACPD and 1*S*,3*R*-ACPD were potent agonists at mGluR2 (Table 1).

Recently, the α-methyl derivatives of L-AP4 (MAP4) and of L-CCGI (MCCGI) (30) and a series of new derivatives of phenylglycine, MPPG, MSPG, and MTPG (31, 32), were described as putative specific antagonists of group II and group III mGluRs. None of these compounds tested at 300 μM inhibited the effect of 3 μM glutamate in cells expressing mGluR1a (data not shown). A significant inhibition was observed with MPPG and MTPG when used at 1 mM (46 ± 4% and 65 ± 5% inhibition, respectively). The effect of these drugs was therefore examined on mGluR2 and mGluR4 and compared with the other antagonist, MCPG. The rank order of potency of these antagonists was MCCGI ≈ MPPG > MSPG > MCPG = MTPG = MAP4 at mGluR2 and MPPG > MAP4 ≫ MSPG at mGluR4, with MCPG, MTPG, and MCCGI being inactive at this receptor (Fig. 7 and Table 1). The Schild plot analysis of MPPG and MCCGI on mGluR2 revealed K_B values of 54.4 ± 2.8 and 69.5 ± 8.1 μM with a slope of 0.98 ± 0.08 and 1.09 ± 0.06, respectively (n = 3), as expected for competitive antagonists (Fig. 8). The Schild plot analysis of MAP4 and MPPG also revealed that these compounds behave as competitive antagonists of mGluR4 (K_B = 69.6 ± 13.3 and 50.3 ± 16.0 μM, slope = 1.29 ± 0.10 and 1.12 ± 0.07, n = 3, respectively; Fig. 9).

Discussion

The current data indicate that mGluR2 and mGluR4, which are negatively coupled to AC and are representative of group II and III mGluRs, respectively, can activate PLC when coexpressed with $G_{\alpha 15}$ or chimeric $G_{\alpha q}$ subunits that possess the carboxyl-terminal end of $G_{\alpha i2}$ or $G_{\alpha o}$.

$G_{\alpha 15}$ and $G_{\alpha 16}$ subunits cloned from mice and human cDNA libraries, respectively, are specifically expressed in hematopoietic cells (33, 34). They have been recently shown to couple many receptor types to PLC, even receptors that usually stimulate or inhibit AC (15). These proteins share 85% amino acid sequence identity, so $G_{\alpha 16}$ is often considered the human homolog of $G_{\alpha 15}$. According to their high homology, these two G_α subunits are equally able to couple many different receptors to PLC (15). However, although $G_{\alpha 15}$ can couple both mGluR2 and mGluR4 to PLC, $G_{\alpha 16}$ was found to be activated only by mGluR2, suggesting that the region of low homology between $G_{\alpha 15}$ and $G_{\alpha 16}$ found 30–50 residues from the carboxyl terminus affects the interaction of these G_α subunits with the receptor. Accordingly, it has recently been reported that the 100 carboxyl-terminal residues of $G_{\alpha 16}$ play a role in the coupling specificity to the C5a receptor (35), and the equivalent region in transducin has been shown to contact rhodopsin (19, 36). Although mGluR2 and mGluR4 are unlikely to couple to $G_{\alpha 15}$ and $G_{\alpha 16}$ *in vivo* because these G_α

TABLE 1

Pharmacological profile of mGluR2 and mGluR4 as determined by stimulation of IP formation after coexpression of these receptors with $G_{\alpha qi9}$ and $G_{\alpha qo5}$, respectively

Each dose-response curve was fitted, and the EC_{50} and IC_{50} values were determined using the Kaleidagraph program as described in the text. The antagonists were tested using 20 and 30 μM Glu for mGluR2 and mGluR4, respectively. Values represent mean ± standard error of three or four independent experiments performed in triplicate.

	EC_{50}	
	mGluR2	mGluR4
	μM	
Agonist		
Glu	11.1 ± 0.6	16 ± 1
1*S*,3*S*-ACPD	12.9 ± 2.9	48 ± 3
1*S*,3*R*-ACPD	18.1 ± 0.3	>1000
1*R*,3*S*-ACPD	111 ± 11	>1000
1*R*,3*R*-ACPD	≥1000	NE
Ibotenate	272 ± 72	1000
L-CCGI	0.49 ± 0.10	8.9 ± 3.4
4C3HPG	51 ± 19	NE
L-Quisqualate	≥1000	≥1000
L-AP4	NE	0.4 ± 0.2
L-SOP	ND	2.3 ± 1.5
(*S*)3-hydroxyphenylglycine	NE	NE
3,5-DHPG	NE	NE
4CPG	24 ± 2	NE
(+)-t-ADA	>1000	NE
(−)-t-ADA	NE	NE
	IC_{50}	
Antagonist		
(±)-MCPG	671	NE
MCCG-I	84 ± 8	NE
MPPG	100 ± 9	54 ± 14
MSPG	250 ± 50	>1000
MTPG	453 ± 71	NE
MAP4	447 ± 72	88 ± 10

NE, no effect; ND, not determined.

subunits are expressed at very low levels in the brain, our results indicate that these two mGluRs do not couple with the same potency to different G proteins, reinforcing their classification into two different groups of mGluRs.

The carboxyl-terminal end of the α subunit of G proteins has been shown to play an important role in the interaction with GPCRs (19). A synthetic peptide corresponding to the carboxyl terminus of transducin stabilizes rhodopsin in its active state (36, 37), and chimeric $G_{\alpha q}$ subunits that possess the carboxyl-terminal end of $G_{\alpha i2}$ or $G_{\alpha o}$ can be activated by G_i- or G_o-coupled receptors (16). These latter results indicate that the carboxyl-terminal end of the G_α subunit is critical for the specific interaction between GPCRs and the G protein. Our results indicate that glutamate stimulates PLC in cells coexpressing mGluR2 or mGluR4 with the chimeric $G_{\alpha qi5}$, $G_{\alpha qi9}$, or $G_{\alpha qu5}$ subunits. This glutamate effect results from the activation of mGluR2 or mGluR4, as indicated by the use of specific mGluR2 and mGluR4 agonists. Furthermore, two observations indicate that the chimeric G_α subunits are responsible for the coupling of these mGluRs to PLC. First, coexpression of mGluR2 or mGluR4 with the wild-type $G_{\alpha q}$ did not allow glutamate to stimulate PLC. Second, as expected by the presence of the PTX ADP-ribosylating site in the chimeric G proteins, the coupling to PLC of mGluR2 and mGluR4 was inhibited after PTX treatment of the cells. Although a high concentration of PTX was required, such a concentration did not affect the stimulation of IP production by the PLC-coupled mGluR1a. That a high concentration of PTX was necessary for the inhibition of these chimeric G proteins in HEK 293 cells has been reported by others (16). This may be due either to the overexpression of this G_α subunit (so that more PTX is required for a complete ADP-ribosylation) or to a lower efficacy of PTX to ADP-ribosylate such a chimeric G_α subunit. Taken together, these results indicate that even though mGluRs and the other GPCRs constitute two distinct families of receptors, the carboxyl-terminal end of the G_α subunit directs its coupling to receptors of both families.

Determination of the potency of glutamate on stimulating IP formation in cells coexpressing mGluR2 or mGluR4 with the promiscuous or chimeric G protein α subunits revealed that the higher is the efficacy of glutamate in stimulating IP production, the lower is the EC_{50} value, as expected for a more efficient coupling to PLC. For both mGluR2 and mGluR4, the best coupling to PLC is obtained with $G_{\alpha 15}$. This may result either from better coupling of these receptors to this particular G protein or from a higher level of expression of this α subunit.

The pharmacological profiles of mGluR2 and mGluR4 determined using $G_{\alpha qi9}$ and $G_{\alpha qu5}$, respectively, correlates perfectly with those reported by measuring inhibition of cAMP formation in cells stably expressing these receptors (20–25). Measurement of PLC activation in cells transiently expressing G_i-coupled mGluRs and chimeric G proteins therefore seems to be a useful functional assay for the characterization of a large number of wild-types or mutated G_i-coupled mGluRs. Such an assay also seems to be useful for the pharmacological characterization of these receptors. Although the potencies measured using this assay may be slightly different from those on native receptors due to the difference in the transduction pathways analyzed, the rank order of potency of a series of agonists or antagonists may be maintained. Indeed, among the multiple GPCRs studied, only one has been reported to have a slightly different rank order of potency for agonists depending on the transduction mechanism examined (38).

With the limitations mentioned above, this assay was used to further analyze the pharmacological profiles of mGluR2 and mGluR4. Although t-ADA has been reported to be inactive on human mGluR1 and human mGluR5 (39), it has, together with the phenylglycine derivative 3,5-DHPG, a potent agonist action on rat mGluR1 and rat mGluR5 (26–29). Both t-ADA and 3,5-DHPG have therefore been proposed to be specific agonists of group I mGluRs (27, 40), although their action on rat group II and group III cloned mGluRs has not been examined. We show that 3,5-DHPG has no effect on mGluR2 and mGluR4 at 1 mM and that (−)-t-ADA is a very weak agonist of rat mGluR2, as reported for the human mGluR2 (39). The 1*S*,3*S* isomer of ACPD has been shown to activate G_i-coupled mGluRs in brain slices (41, 42) and to be a very weak agonist at group I mGluRs (18). Accordingly, it is often used as a selective agonist of group II mGluRs (30, 32, 43–46), but its effect on cloned group II and group III mGluRs has not been reported. A potent agonist action of 1*S*,3*S*-ACPD on mGluR3 can, however, be proposed based on its effect on chimeric mGluRs (13). We report here that 1*S*,3*S*-ACPD is a potent agonist of mGluR2, being even more potent than 1*S*,3*R*-ACPD. However, 1*S*,3*S*-ACPD is also an agonist at mGluR4, with a 4-fold lower potency only, suggest-

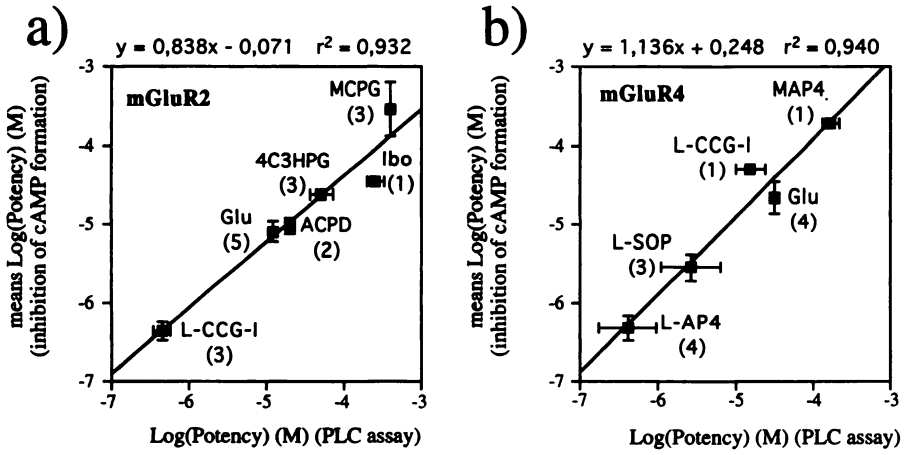

Fig. 6. Correlation between the potencies of several drugs on mGluR2 (a) and mGluR4 (b) determined via the PLC stimulation mediated by $G_{\alpha qi9}$ and $G_{\alpha qu5}$ respectively, and those reported in the literature by measuring inhibition of forskolin-stimulated cAMP formation. For the antagonists MCPG and MAP4, published K_B values or K_B values estimated using the Cheng and Prussof equation were used. *Numbers in parentheses*, number of values found in the literature and used for the analysis. *Glu*, glutamate. Values are mean ± standard error.

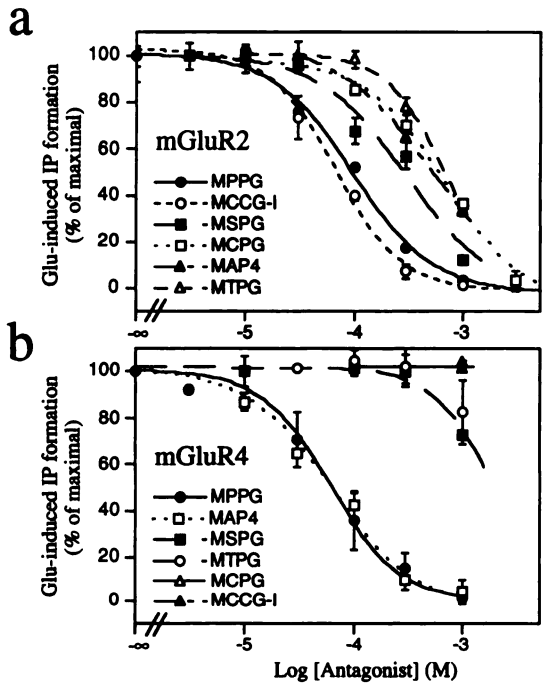

Fig. 7. Effect of various antagonists on IP formation in cells coexpressing mGluR2 and $G_{\alpha qi9}$ (a) or cells coexpressing mGluR4 and $G_{\alpha qu5}$ (b). Transfected cells were washed, preincubated for 10 min with 10 mM LiCl in Krebs' buffer, and then stimulated for 30 min with 20 μM glutamate (*Glu*) (a) or 30 μM glutamate (b) and the indicated concentration of antagonist. IP formation was then determined as described in the text. Values are mean ± standard error of triplicate determinations from typical experiments and are expressed as a percentage of the effect of glutamate alone.

ing that its action in the brain will not be exclusively mediated by group II mGluRs.

Although specific agonists will be useful for the characterization of the physiological roles of the different mGluR subtypes, much will be discovered as a result of the specific antagonists. Recently, several compounds have been synthesized and proposed to be specific antagonists of group II and group III mGluRs; these include MCCGI, MAP4, and the phenylglycine derivatives MSPG, MPPG, and MTPG (30–32). Among these, only the effect of MAP4 has been carefully examined on cloned rat receptors (47), and this was performed on mGluR4 only, so that the specificity of this drug is not known. Furthermore, MAP4 has been reported to have L-AP4-like actions in rat adult cortical slices (31). In agreement with Johansen *et al.* (47), we found MAP4 to be a potent and competitive antagonist of mGluR4, being inactive at mGluR1a. However, MAP4 is also an antagonist of mGluR2, with a 5-fold lower potency only, indicating that care should be taken before attributing a physiological role to a specific mGluR subtype based on the effect of MAP4. MCCGI was found to be inactive at mGluR1a and mGluR4 and to be a potent and competitive antagonist of mGluR2. Although its effect on other group I and III mGluRs remains to be determined, MCCGI seems to be a useful drug to characterize the effect of group II mGluRs. Recently, the new phenylglycine derivatives MSPG, MPPG, and MTPG have been proposed as antagonists of group II and group III mGluRs expressed in the spinal cord and responsible for the depressant action of 1*S*,3*S*-ACPD and L-AP4, respectively (31, 32). The rank order of potency of these antagonists is MTPG > MPPG > MSPG > MCCGI > MCPG for 1*S*,3*S*-ACPD-activated receptors and MPPG > MAP4> MSPG > MTPG for L-AP4 activated receptors. Here, we show that the rank order of potency of these antagonists is MCCGI ≈ MPPG > MSPG > MCPG = MTPG = MAP4 at mGluR2 and MPPG ≥ MAP4 ≫ MSPG at mGluR4 receptors, suggesting that mGluR2 is not responsible for the 1*S*,3*S*-ACPD presynaptic action in the spinal cord. It is, however, possible that differences in the diffusion properties of these molecules affect their apparent potencies determined in the spinal cord preparation.

These results indicate that despite the sequence and structural differences between mGluRs and the other GPCRs, the carboxyl-terminal end of the α subunit of G proteins plays an important role in the specific interaction with mGluRs. The total absence of homology between the mGluR seven-TMD region and the other GPCRs and the observation that the second intracellular loop of mGluRs plays a role equivalent to that of the third intracellular loop of the other GPCRs suggest a convergent evolution of these two receptor families. Interestingly, during evolution, different domains of these two families of proteins have been selected for the interaction with the same domains of the G_α subunits. This further suggests that the interaction of these regions of the α subunit with the receptor is important for the G protein activation (19, 37, 48). In addition, our results indicate that the promiscuous G protein α subunit $G_{\alpha 15}$ or the chimeric $G_{\alpha qi/o}$ proteins can be used to develop simple functional assays for the pharmacological characterization of group II and group III mGluRs.

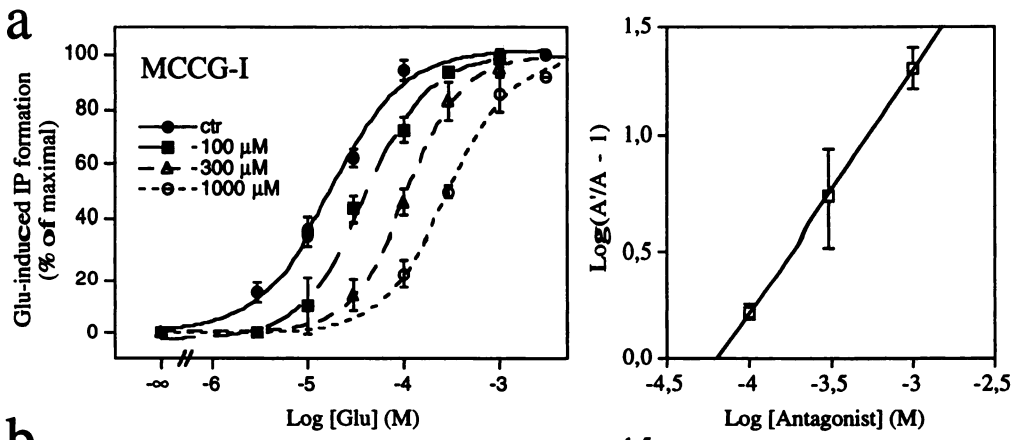

Fig. 8. Dose-response curves of glutamate for Schild plot determination of the antagonist potencies of MCCGI (a) and MPPG (b) on mGluR2. IP formation in cells expressing mGluR2 and $G_{\alpha qi9}$ was determined after 30-min stimulation with the indicated concentrations of glutamate alone (*ctr*) or in the presence of 100 µM, 300 µM, or 1000 µM antagonist. *Right*, Schild plots obtained from the EC_{50} values. Values are mean ± standard error of three independent experiments performed in triplicates. The K_B values and slopes were determined in individual experiments by linear regression analysis.

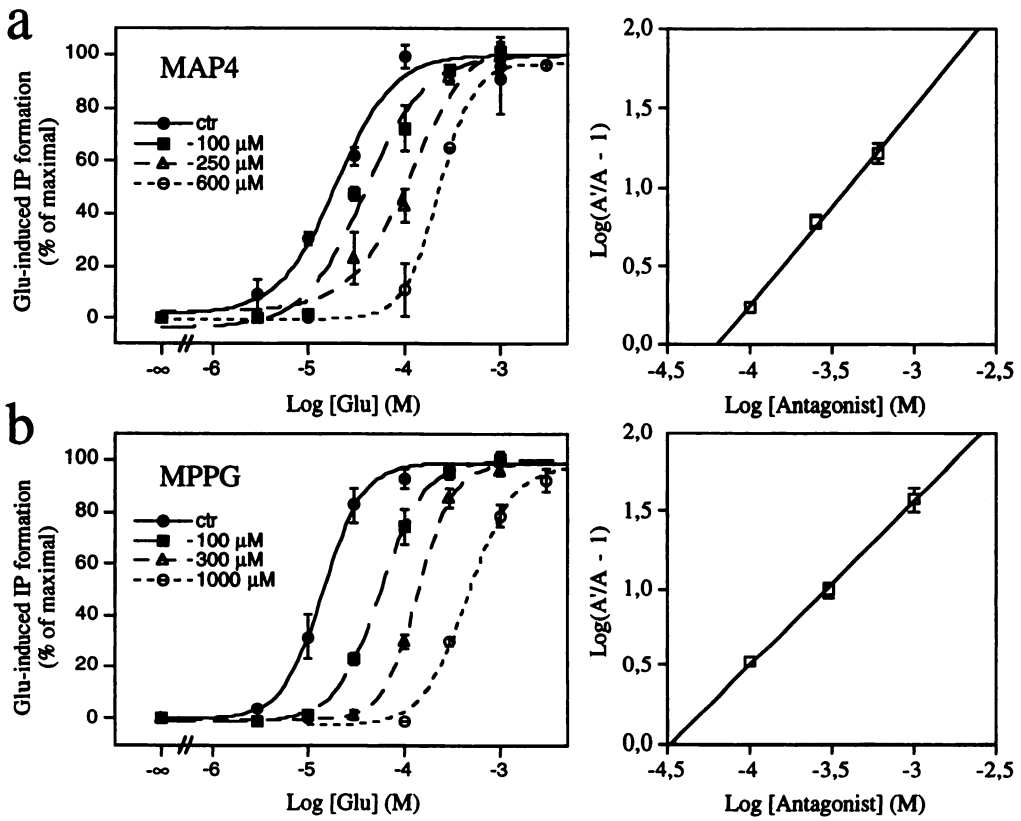

Fig. 9. Dose-response curves of glutamate for Schild plot determination of the antagonist potencies of MAP4 (a) and MPPG (b) on mGluR4. IP formation in cells expressing mGluR4 and $G_{\alpha qu5}$ was determined after 30-min stimulation with the indicated concentrations of glutamate alone (*ctr*) or in the presence of 100 µM, 250 µM, or 600 µM MAP4 (a) or in the presence of 100 µM, 300 µM, or 1000 µM MPPG (b). *Right*, Schild plots obtained from the EC_{50} values. Values are mean ± standard error of three independent experiments performed in triplicate. The K_B values and slopes were determined in individual experiments by linear regression analysis.

Acknowledgments

We thank Drs. Y. Grau and O. Manzoni for constructive discussion and critical reading of the manuscript. We thank Drs. B. Conklin and H. Bourne for the gift of the wild-type and chimeric $G_{\alpha q}$ subunit cDNAs, Drs. S. Offermanns and M. Simon for the gift of the $G_{\alpha 15}$ and $G_{\alpha 16}$ cDNAs, Drs. S. Nakanishi for the gift of the mGluR4 cDNA, and Drs. T. Knöpfel and A. P. Kozikowski for the generous gift of the t-ADA compounds and Dr. K. Curry for the ACPD molecules.

References

1. Hollmann, M., and S. Heinemann. Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17**:31–108 (1994).
2. Nakanishi, S. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. *Neuron* **13**:1031–1037 (1994).
3. Pin, J.-P., and J. Bockaert. Get receptive to metabotropic glutamate receptors. *Curr. Opin. Neurol.* **5**:342–349 (1995).
4. Pin, J.-P., and R. Duvoisin. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34**:1–26 (1995).
5. Okamoto, N., S. Hori, C. Akazawa, Y. Hayashi, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular characterization of a new metabotropic glutamate receptor, mGluR7, coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.* **269**:1231–1236 (1994).
6. Saugstad, J. A., J. M. Kinzie, E. R. Mulvihill, T. P. Segerson, and G. L. Westbrook. Cloning and expression of a new member of the L-2-amino-4-phosphonobutyric acid class of metabotropic glutamate receptors. *Mol. Pharmacol.* **45**:367–372 (1994).
7. Nakajima, Y., H. Iwakabe, C. Akazawa, H. Nawa, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *J. Biol. Chem.* **268**:11868–11873 (1993).
8. Duvoisin, R. M., C. Zhang, and K. Ramonell. A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. *J. Neurosci.* **15**:3075–3083 (1995).
9. Brown, E. M., G. Gamba, D. Riccardi, M. Lombardi, R. Butters, O. Kifor, A. Sun, M. A. Hediger, J. Lytton, and S. C. Hebert. Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature (Lond.)* **366**:575–580 (1993).
10. Trumpp-Kallmeyer, S., J. Hoflack, A. Bruinvels, and M. Hibert. Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. *J. Med. Chem.* **35**: 3448–3462 (1992).
11. Takahashi, K., K. Tsuchida, Y. Tanabe, M. Masu, and S. Nakanishi. Role of the large extracellular domain of metabotropic glutamate receptors in agonist selectivity determination. *J. Biol. Chem.* **268**:19341–19345 (1993).
12. O'Hara, P. J., P. O. Sheppard, H. Thøgersen, D. Venezia, B. A. Haldeman, V. McGrane, K. M. Houamed, C. Thomsen, T. L. Gilbert, and E. R. Mulvihill. The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11**:41–52 (1993).
13. Pin, J.-P., C. Joly, S. F. Heinemann, and J. Bockaert. Domains involved in the specificity of G protein activation in phospholipase C coupled metabotropic glutamate receptor. *EMBO J.* **13**:342–348 (1994).
14. Gomeza, J., C. Joly, R. Kühn, T. Knöpfel, J. Bockaert, and J.-P. Pin. The second intracellular loop of mGluR1 cooperates with the other intracellular domains to control coupling to G-protein. *J. Biol. Chem.* **271**:2199–2205 (1996).
15. Offermanns, S., and M. I. Simon. Gα15 and Gα16 couple a wide variety of receptors to phospholipase C. *J. Biol. Chem.* **270**:15175–15180 (1995).
16. Conklin, B. R., Z. Farfel, K. D. Lustig, D. Julius, and H. R. Bourne. Substitution of three amino acids switches receptor specificity of Gqα to that of Giα. *Nature (Lond.)* **363**:274–276 (1993).
17. Pin, J.-P., C. Waeber, L. Prézeau, J. Bockaert, and S. F. Heinemann. Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **89**:10331–10335 (1992).
18. Joly, C., J. Gomeza, I. Brabet, K. Curry, J. Bockaert, and J.-P. Pin. Molecular, functional and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J. Neurosci.* **15**:3970–3981 (1995).
19. Conklin, B. R., and H. R. Bourne. Structural elements of Gα subunits that interact with Gβγ, receptors, and effectors. *Cell* **73**:631–641 (1993).
20. Tanabe, Y., M. Masu, T. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic glutamate receptors. *Neuron* **8**:169–179 (1992).
21. Hayashi, Y., Y. Tanabe, I. Aramori, M. Masu, K. Shimamoto, Y. Ohfune, and S. Nakanishi. Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. *Br. J. Pharmacol.* **107**:539–543 (1992).
22. Thomsen, C., P. Kristensen, E. Mulvihill, B. Haldeman, and P. D. Suzdak. L-2-Amino-4-phosphonobutyrate (L-AP4) is an agonist at the type IV metabotropic glutamate receptor which is negatively coupled to adenylate cyclase. *Eur. J. Pharmacol.* **227**:361–362 (1992).
23. Tanabe, Y., A. Nomura, M. Masu, R. Shigemoto, N. Mizuno, and S. Nakanishi. Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.* **13**:1372–1378 (1993).
24. Thomsen, C., and P. D. Suzdak. Serine-O-phosphate has affinity for type IV, but not type I, metabotropic glutamate receptor. *Neuroreport* **4**:1099–1101 (1993).
25. Johansen, P. A., L. A. Chase, A. D. Sinor, J. F. Koerner, R. L. Johnson, and M. B. Robinson. Type 4a metabotropic glutamate receptor: identification of new potent agonists and differentiation from the L-(+)-2-amino-4-phosphonobutanoic acid-sensitive receptor in the lateral perforant pathway in rats. *Mol. Pharmacol.* **48**:140–149 (1995).
26. Ito, I., A. Kohda, S. Tanabe, E. Hirose, M. Hayashi, S. Mitsunaga, and H. Sugiyama. 3,5-Dihydroxyphenylglycine: a potent agonist of metabotropic glutamate receptors. *Neuroreport* **3**:1013–1016 (1992).
27. Manahan-Vaughan, D., M. Reiser, J.-P. Pin, V. Wilsch, J. Bockaert, K. G. Reymann, and G. Riedel. Long-term potentiation in rat dentate gyrus in vivo: a role for class I metabotropic glutamate receptor subtypes. *Neuroscience* **72**:999–1008 (1996).
28. Brabet, I., S. Mary, J. Bockaert, and J.-P. Pin. Phenylglycine derivatives discriminate between mGluR1 and mGluR5 mediated responses. *Neuropharmacology* **34**:895–903 (1995).
29. Kozikowski, A. P., W. Tückmantel, Y. Liao, H. Manev, S. Ikonomovic, and J. T. Wroblewski. Synthesis and metabotropic receptor activity of the novel rigidified glutamate analogues (+)- and (−)-trans-azetidine-2,4-dicarboxylic acid and their N-methyl derivatives. *J. Med. Chem.* **36**:2706–2716 (1993).
30. Jane, D. E., P. L. S. J. Jones, P. C.-K. Pook, H.-W. Tse, and J. C. Watkins. Actions of two new antagonists showing selectivity for different subtypes of metabotropic glutamate receptor in the neonatal rat spinal cord. *Br. J. Pharmacol.* **112**:809–816 (1994).
31. Roberts, P. J. Pharmacological tools for the investigation of metabotropic glutamate receptors (mGluRs): phenylglycine derivatives and other selective antagonists: an update. *Neuropharmacology* **34**:813–819 (1995).
32. Jane, D. E., K. Pittaway, D. C. Sunter, N. K. Thomas, and J. C. Watkins. New phenylglycine derivatives with potent and selective antagonist activity at presynaptic glutamate receptors in neonatal rat spinal cord. *Neuropharmacology* **34**:851–856 (1995).
33. Wilkie, T. M., P. A. Scherle, M. P. Strathmann, V. Z. Slepak, and M. I. Simon. Characterization of G-protein α subunits in the Gq class: expression in murine tissue and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. USA* **88**:10049–10053 (1991).
34. Amatruda III, T. T., D. A. Steele, V. Z. Slepak, and M. I. Simon. Gα16, a G protein α subunit specifically expressed in hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **88**:5587–5591 (1991).
35. Lee, C. H., A. Katz, and M. I. Simon. Multiple regions of $G_{\alpha 16}$ contribute to the specificity of activation by the C5a receptor. *Mol. Pharmacol.* **47**:218–223 (1995).
36. Hamm, H. E., D. Deretic, A. Arendt, P. A. Hargrave, B. Koenig, and K. P. Hofmann. Site of G protein binding to rhodopsin mapped with synthetic peptides from the α subunit. *Science (Washington D. C.)* **241**:832–835 (1988).
37. Dratz, E. A., J. E. Furstenau, C. G. Lambert, D. L. Thireault, H. Rarick, T. Schepers, S. Pakhlevaniants, and H. E. Hamm. NMR structure of a receptor-bound G-protein peptide. *Nature (Lond.)* **363**:276–281 (1993).
38. Spengler, D., C. Waeber, C. Pantaloni, F. Holsboer, J. Bockaert, P. H. Seeburg, and L. Journot. Differential signal transduction by five splice variants of the PACAP receptor. *Nature (Lond.)* **365**:170–175 (1993).
39. Knöpfel, T., J. Sakaki, P. J. Flor, P. Baumann, A. I. Sacaan, G. Veliçelebi, R. Kuhn, and H. Allgeier. Profiling of trans-azetidine-2,4-dicarboxylic acid at the human metabotropic glutamate receptors mGlu1b, -2, -4a and -5a. *Eur. J. Pharmacol.* **288**:389–392 (1995).
40. Schoepp, D. D., J. Goldsworthy, B. G. Johnson, C. R. Salhoff, and S. R. Baker. 3,5-Dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. *J. Neurochem.* **63**:769–772 (1994).
41. Winder, D. G., and P. J. Conn. Activation of metabotropic glutamate receptors in the hippocampus increases cyclic AMP accumulation. *J. Neurochem.* **59**:375–378 (1992).
42. Cartmell, J., A. R. Curtis, J. A. Kemp, D. A. Kendall, and S. P. H. Alexander. Subtypes of metabotropic excitatory amino acid receptor distinguished by stereoisomers of the rigid glutamate analogue, 1-aminocyclopentane-1,3-dicarboxylate. *Neurosci. Lett.* **153**:107–110 (1993).
43. Gereau, R. W., and P. J. Conn. A cyclic AMP-dependent form of associative synaptic plasticity induced by coactivation of β-adrenergic receptors and metabotropic glutamate receptors in rat hippocampus. *J. Neurosci.* **14**: 3310–3318 (1994).
44. Gereau, R. W., and P. J. Conn. Potentiation of cAMP responses by metabotropic glutamate receptors depresses excitatory synaptic transmission by a kinase-independent mechanism. *Neuron* **12**:1121–1129 (1994).
45. Kemp, M., P. Roberts, P. Pook, D. Jane, A. Jones, P. Jones, D. Sunter, P. Udvarhelyi, and J. Watkins. Antagonism of presynaptically mediated depressant responses and cyclic AMP-coupled metabotropic glutamate receptors. *Eur. J. Pharmacol.* **266**:187–192 (1994).
46. Davies, C. H., V. R. J. Clarke, D. E. Jane, and G. L. Collingridge. Pharmacology of postsynaptic metabotropic glutamate receptors in rat hippocampal CA1 pyramidal neurones. *Br. J. Pharmacol.* **116**:1859–1869 (1995).
47. Johansen, P. A., and M. B. Robinson. Identification of 2-amino-2-methyl-4-phosphonobutanoic acid as an antagonist at the $mGlu_{4a}$ receptor. *Eur. J. Pharmacol.* **290**:R1–R3 (1995).
48. Desai, M. A., J. P. Burnett, N. G. Mayne, and D. D. Schoepp. Cloning and expression of a human metabotropic glutamate receptor 1α: enhanced coupling on cotransfection with a glutamate transporter. *Mol Pharmacol* **48**:648–657 (1995).

Send reprint requests to: Dr. Jean-Philippe Pin, UPR CNRS 9023, CCIPE, 141 rue de la Cardonille, 34094 Montpellier, Cedex 5, France. E-mail: pin@ccipe.montp.inserm.fr