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The G Protein-Coupling Profile of Metabotropic Glutamate Receptors, as Determined with Exogenous G Proteins, Is Independent of Their Ligand Recognition Domain

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ABSTRACT

Metabotropic glutamate (mGlu), Ca^{2+}-sensing, γ-aminobutyric $acid_B$, and a large number of pheromone receptors constitute a peculiar family of G protein-coupled receptors. They possess a large extracellular domain that has been proposed to constitute their ligand binding domain. The aim of the current study was to examine whether this large ligand binding domain had any influence on the G protein-coupling selectivity of the receptor, and vice versa. We chose mGlu receptors, which are classified into three groups according to their sequence homology and pharmacology, as representatives of this receptor family. To define a G protein-coupling profile for these receptors, we used a set of exogenous phospholipase C-activating G proteins in the same way that synthetic ligands are used to define agonist and antagonist pharmacological profiles. This set includes $G_{\alpha 15}$, $G_{\alpha 16}$, $G_{\alpha q}$, and chimeric $G_{\alpha q}$ proteins with the last few amino acids of either $G_{\alpha i2}$ ($G_{\alpha qi}$), $G_{\alpha o}$ ($G_{\alpha qo}$), or $G_{\alpha z}$ ($G_{\alpha qz}$). Cotransfection of mGlu receptors with these G proteins and examination of their coupling to phospholipase C revealed that group I, II, and III receptors have distinct G protein-coupling profiles. By swapping the extracellular domains of the most distantly related mGlu receptors (the rat group I mGlu1a and the *Drosophila melanogaster* group II DmGluA receptors), we show that the extracellular domain determines the agonist pharmacological profile and that this domain does not modify the G protein-coupling profile determined by the seven-transmembrane-domain region of mGlu receptors.

Several families of GPCRs can be defined based on their sequence homologies. Although they all possess seven transmembrane domains, members from different families share no sequence homology, even within these transmembrane domains. Family 1 is constituted by the large number of receptors homologous to rhodopsin and to adrenergic and glycoprotein receptors (Savarese and Fraser, 1992). Family 2 is composed of the receptors homologous to the glucagon and pituitary adenylyl cyclase-activating polypeptide receptors (Spengler *et al.*, 1993), whereas family 4 corresponds to a subset of pheromone receptors (Dulac and Axel, 1995). The eight mGlu receptors (Conn and Pin, 1997; Nakanishi, 1992), the Ca^{2+}-sensing receptor (Brown *et al.*, 1993), the $GABA_B$ receptor (Kaupmann *et al.*, 1997), and a second subset of ≈100 pheromone receptors define the family three GPCRs (Herrada and Dulac, 1997; Matsunami and Buck, 1997).

In contrast to the other GPCRs, family 3 receptors possess a long amino-terminal domain (550–600 residues) that shares some sequence homology with bacterial PBPs (O'Hara *et al.*, 1993). Based on the known three-dimensional structure of some PBPs and subsequent site-directed mutagenesis, it has been proposed that the amino terminus of mGlu receptors is the glutamate recognition domain (O'Hara *et al.*, 1993). In agreement with this proposal, swapping the extracellular domain of the PLC-coupled mGlu1 receptor with that of either the mGlu2 or mGlu4 receptor that inhibits adenylyl cyclase generates chimeric receptors that activate PLC and possess agonist pharmacological characteristics that resemble those of mGlu2 and mGlu4 receptors, respectively (Takahashi *et al.*, 1993; Tones *et al.*, 1995).

As in the other GPCRs, most intracellular loops and the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; ACPD, 1-amino-1,3-cyclopentane-dicarboxylate; ACPT-I, (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-tricarboxylic acid; DL-AP4, DL-2-amino-4-phosphonobutanoate; APDC, 4-aminopyrrolidine-2,4-dicarboxylate; L-CCGI, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine; DMEM, Dulbecco's modified Eagle's medium; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DCG IV, (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine; IP, inositol phosphate; HEK, human embryonic kidney; LH, luteinizing hormone; mGlu, metabotropic glutamate; PBP, periplasmic binding protein; PLC, phospholipase C; L-SOP, L-serine-*O*-phosphate; 7TM, seven transmembrane domain.

carboxyl-terminal tail of family 3 receptors are responsible for the G protein coupling (Pin *et al*., 1994; Gomeza *et al*., 1996a; Mary *et al*., 1998). Although some short segments in the intracellular loops of 7TM receptors play an important role in G protein-coupling selectivity, the conformational state of the 7TM region also may play a role. Indeed, the current hypothesis regarding 7TM receptors is that they can adopt various conformations stabilized by different ligands. These different conformations of a receptor can have distinct G protein-coupling selectivities or desensitization properties (Spengler *et al*., 1993; Eason *et al*., 1994; Robb *et al*., 1994; Gether *et al*., 1995; Perez *et al*., 1996).

The aim of the current study was to examine whether the ligand binding extracellular domain of these receptors has any influence on the G protein-coupling selectivity of family 3 receptors. For that purpose, we used mGlu receptors as representatives of this receptor family. Three groups of mGlu receptors can be distinguished based on their sequence homology and pharmacology. The group I includes mGlu1 and mGlu5 receptors, which activate PLC. The second group is composed of mGlu2 and mGlu3 receptors. The recently cloned *Drosophila melanogaster* DmGluA receptor belongs to this group of mammalian receptors (Parmentier *et al*., 1996). The third group includes mGlu4, mGlu6, mGlu7, and mGlu8 receptors. Both group II and III mGlu receptors inhibit cAMP formation (for a review, see Conn and Pin, 1997).

In the current study, we first show that a G protein-coupling profile of receptors can be defined using exogenous and chimeric G proteins (Conklin *et al*., 1993; Offermanns and Simon, 1995) in the same way that synthetic ligands are commonly used to define an agonist or antagonist pharmacological profile. To analyze the influence of the extracellular domain on the G protein-coupling profiles of mGlu receptors, we first examined whether this profile may be agonist dependent. Then, the extracellular domains of the most distantly related mGlu receptors, the rat mGlu1 and the *D. melanogaster* DmGluA receptors, were swapped. Our data demonstrate that the G protein-coupling and ligand-recognition selectivities are exclusively determined by the 7TM and extracellular regions of mGlu receptors, respectively.

Experimental Procedures

Materials. Ibotenate, L-quisqualate, L-CCGI, DHPG, DCG IV, DL-AP4, and L-SOP were obtained from Tocris Cookson (Essex, UK). The two isomers (1*S*,3*S*)-ACPD and (1*S*,3*R*)-ACPD were a generous gift of Dr. K. Curry (Vancouver, Canada). Glutamate was from Sigma-Aldrich (L'isle d'Abeau, France). LY354740 and (2*R*,4*R*)-APDC were a generous gift of Dr. D. D. Schoepp (Eli Lilly, Indianapolis, IN). ACPT-I was a gift from Dr. F. Acher.

Plasmids. The cDNAs encoding mGlu1a, mGlu2, mGlu4a, and DmGluA receptors were in the pRK vector under the control of a CMV promoter, as described previously (Joly *et al*., 1995; Gomeza *et al*., 1996b; Parmentier *et al*., 1996). The cDNAs of mGlu7a and mGlu8a originally in the pBluescript vector (gifts from Dr. J. Saugstad) were subcloned into the pRK vector. The cDNAs of the wild-type $G_{\alpha q}$ and the chimeric $G_{\alpha qi}$ ($G_{\alpha q}$ with the carboxyl-terminal nine amino acids of $G_{\alpha i2}$), $G_{\alpha qo}$, and $G_{\alpha qz}$ ($G_{\alpha q}$ with the carboxyl-terminal five amino acids of $G_{\alpha o}$ and $G_{\alpha z}$, respectively) were in the pcDNA-I expression vector (gift from Drs. B. Conklin and H. Bourne) (Conklin *et al*., 1993). The cDNAs of the mouse $G_{\alpha 15}$ and the human $G_{\alpha 16}$ were in the pCIS vector (gift from Drs. S. Offermanns and M. Simon) (Offermanns and Simon, 1995).

Culture and transfection of HEK 293 cells. HEK 293 cells were cultured in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum and transfected by electroporation as described previously (Gomeza *et al*., 1996b). Electroporation of 5×10^6 cells was performed in a total volume of 150 μl with 6 μg of carrier DNA (500 μF, 260 V). Plasmid DNA expressing mGlu receptors (100 ng of mGlu1a, 1 μg of mGlu2, 2.5 μg of DmGluA (Parmentier *et al*., 1996); 5 μg of mGlu4, mGlu7a, or mGlu8a; or the chimeric mGlu1a/D receptors) was transfected alone or with 3 μg of plasmid DNA expressing $G_{\alpha qi}$, $G_{\alpha qo}$, $G_{\alpha qz}$, $G_{\alpha q}$ (Conklin *et al*., 1993), $G_{\alpha 15}$, or $G_{\alpha 16}$ (Offermanns and Simon, 1995). Cells were resuspended and split into half of a 12-well cluster. For the cyclase experiments, 10^7 cells were electroporated in a total volume of 300 μl with 12.5 μg of carrier DNA, 5 μg of plasmid DNA expressing DmGluA receptor, and 600 ng of LH receptor-expressing plasmid (950 μF, 280 V). Cells were resuspended and split into 12-well clusters.

Determination of IP accumulation. Determination of IP accumulation in transfected cells was performed as described previously after labeling the cells overnight with *myo*-[^{3}H]inositol (23.4 Ci/mol; New England Nuclear, Le Blanc Mesnil, France) in DMEM Glutamax I (GIBCO-BRL, Life Technologies, Cergy Pontoise, France) (Gomeza *et al*., 1996b). The stimulation was conducted for 30 min in a medium containing 10 mM LiCl and the agonists. The basal IP formation was determined after a 30-min incubation in the presence of 10 mM LiCl and of the glutamate-degrading enzyme glutamate pyruvate transaminase (1 unit/ml plus 2 mM pyruvate). Results are expressed as the amount of IP produced divided by the radioactivity present in the membranes. The concentration-response curves were fitted using the equation $y = [(y_{max} - y_{min})/1 + (x/EC_{50})^n] + y_{min}$ and the Kaleidagraph program, where n is the Hill coefficient, x is the concentration of agonist, and y_{max} and y_{min} are the maximal and minimal responses, respectively.

Determination of cAMP accumulation. The cellular cAMP production was measured using the prelabeling technique as described previously (Parmentier *et al*., 1996). Four hours after being electroporated, cells were washed and incubated for 14 hr in DMEM Glutamax I containing 1 μCi/ml [^{3}H]adenine (27 Ci/mmol). Stimulation of the cells was performed in HEPES buffer saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.4). cAMP formation is expressed as percentage conversion of [^{3}H]ATP to [^{3}H]cAMP: ([^{3}H]cAMP $\times$ 100)/([^{3}H]cAMP + [^{3}H]ATP).

***Xenopus laevis* oocytes assay.** The preparation of oocytes and the *in vitro* synthesis of RNA transcripts from the cloned cDNA were performed as described previously (Pin *et al*., 1994). Recordings were performed in Barth's medium using the two-electrode voltage-clamp technique (Axoclamp-2A; Axon Instruments, Burlingame, CA) 3–4 days after injection. Data were recorded on a PC and analyzed using the pCLAMP software.

List and sequence of the oligonucleotides used to generate chimeras. The sequence of the oligonucleotide primers used to generate the chimeric receptors are presented in the 5′-to-3′ direction. The following nucleotides have their 5′-end sequences corresponding to mGlu1a receptor, with the junction between the mGlu1 and DmGluA receptor sequences indicated by a hyphen: 1aD/1S, ACA AAA GCG GAA TGG TAC GA-T CGG CCT GTT CAC TAC CAT G; 1aD/2S, GGA AAG GAG AAG TGA GCT GC-T GCT GGA TAT GCG ACA GCT G; 1aD/3S, AGT TCA CCT GCA GAG CCT GT-G GTC CTG GAC TTT GGC CCT A; 1aD/4S, TCC GTT ATC TTG AGT GGA GT-T CGT TGT TTG CCC TTA TTC C; D1a/1AS, CAA GGC TCA CTGCAC ACA GA-A GTA GGT TGT TCG GTT TCC T; D1a/2AS, CAG GCC GTG CAG ATC CAG CA-A CAG GTA TCC CCC TGT TGT T; D1a/3AS, TTG GGC CAC CAC CCC AGG TC-G CAG TCT TTA CAC GTA AAC T; and D1a/4AS, GCT ATG ATA GAT TCT ATG TC-G TTC CAT TTC ATA TAC TGG A. The following oligonucleotides have their 5′ sequence corresponding to DmGluA, with the junction between the DmGluA and mGlu1a receptor sequences indicated by a hyphen: D1a/1S, AGG AAA CCG AAC AAC CTA CT-T CTG TGT GCA GTG

AGC CTT G; D1a/2S, AAC AAC AGG GGG ATA CCT GT-T GCT GGA TCT GCA CGG CCT G; D1a/3S, AGT TTA CGT GTA AAG ACT GC-G ACC TGG GGT GGT GGC CCA A; D1a/4S, TCC AGT ATA TGA AAT GGA AC-G ACA TAG AAT CTA TCA TAG C; 1aD/1AS, GTA CCA TCA CTT GTC CGG CT-T CGT ACC ATT CCG CTT TTG T; 1aD/2AS, CAG CTG TCG CAT ATC CAG CA-G CAG CTC ACT TCT CCT TTC C; 1aD/3AS, TAG GGC CAA AGT CCA GGA CC-A CAG GCT CTG CAG GTG AAC T; and 1aD/4AS, GGA ATA AGG GCA AAC AAC GA-A CTC CAC TCA AGA TAA CGG A. The following oligonucleotides also were used: MGR15, Kos3, Dchim1, MGR42: MGR 15 (CGC TTC CAG TGT CGC CTA CC), Kos3 (TCT AGA ACT TAT TTA CTA TAT GAC AGA T), and Dchim1 (GGC CGG ATC CTT GGT TTG CTG AAT AC), MGR 42 (AGT GTA CAT GGT GAA GGC G).

Construction of chimeric receptors. Chimeras were constructed using the PCR overlap extension method as described previously (Pin *et al.*, 1994). To construct 1a/D-1 chimera that contains the extracellular domain of the mGlu1a receptor and the 7TM and carboxyl-terminal regions of DmGluA receptor, a PCR was conducted with pKosDMGRAs as template and Kos3 and 1a/D-1S as primers to amplify the DmGluA receptor part of the chimera. A parallel PCR was conducted using pRKG1aS (Prézeau *et al.*, 1996) as template DNA and MGR15 and 1a/D-1AS as primers to amplify the mGlu1a receptor part of the chimera. The products of both reactions were mixed and used as template in a third PCR using MGR15 and Kos3 as primers. The resulting chimeric PCR product was digested with *Pml*I and *Xba*I and subcloned into pmGR1a [mGlu1a receptor cDNA inserted into pBluescript-SK$^{(-)}$ (Pin *et al.*, 1994)] cut with the same enzymes. After sequence verification, the resulting plasmid was digested with *Bam*HI and *Esp*I, and the smaller fragment was inserted into pKosDMGRAs previously cut with the same enzymes. The same strategy was used for the construction of 1a/D-2, 1a/D-3, and 1a/D-4 with the corresponding chimeric sense and antisense oligonucleotides.

The chimeric receptor cDNA D/1a-1 containing the extracellular domain of the DmGluA receptor and the 7TM and intracellular domains of the mGlu1a receptor was constructed using a similar strategy with D/1a-1S, D/1a-1AS, Dchim1, and MGR42 as primers. The final chimeric PCR product was cut with *Bam*HI and *Eco47*III and subcloned into pRKG1 as cut previously with the same enzymes. The *Bam*HI/*Eco*RV fragment of the resulting plasmid then was replaced by the corresponding fragment of pKosDMGRAs. The same strategy was used for the construction of D/1a-2, D/1a-3, and D/1a-4 using the corresponding chimeric sense and antisense oligonucleotides.

For each construct, the amplified region was sequenced on both strands using 17–25-mer primers with the dideoxynucleotide method using Sequenase (United States Biochemical, Cleveland, OH).

Results

Exogenous G proteins can be used to define a G protein-coupling profile of mGlu receptors. To study the G protein-coupling selectivity of mGlu receptors, we needed tools that define a G protein-coupling profile of receptors. For that purpose, we used a series of exogenous wild-type or modified G protein α subunits in the same way that synthetic ligands are commonly used to define the agonist and antagonist pharmacological profiles.

The promiscuous G protein α subunits $G_{\alpha 15}$ and $G_{\alpha 16}$ or the chimeric $G_{\alpha q}$ proteins bearing the nine carboxyl-terminal residues of $G_{\alpha i2}$ ($G_{\alpha qi}$) or the five carboxyl-terminal residues of $G_{\alpha o}$ or $G_{\alpha z}$ ($G_{\alpha qo}$ or $G_{\alpha qz}$) are known to activate PLC (Conklin *et al.*, 1993; Offermanns and Simon, 1995). When expressed alone, $G_{\alpha 16}$, $G_{\alpha qi}$, or $G_{\alpha qo}$ had no effect on the basal IP formation, whereas $G_{\alpha 15}$ and $G_{\alpha qz}$ induced a 2-fold increase (Fig. 1a). To analyze whether these G proteins can be activated by group II or III mGlu receptors, we measured the agonist-induced IP formation in cells expressing any of these receptors alone or with one of the G proteins.

When expressed alone or in combination with $G_{\alpha q}$, group II (mGlu2 and DmGluA receptors) and group III (mGlu4, mGlu7, and mGlu8 receptors) mGlu receptors did not stimulate PLC (Fig. 1). In contrast, when coexpressed with $G_{\alpha 15}$, $G_{\alpha qi}$, or $G_{\alpha qo}$, they all induced an increase in IP production on agonist application (Fig. 1). We previously reported that in the case of mGlu2 and mGlu4a receptors, the PLC activation resulted from the coupling of these receptors to the cotransfected G proteins (Gomeza *et al.*, 1996b). In cells expressing mGlu2 or mGlu4a receptors together with $G_{\alpha qi}$ or $G_{\alpha qo}$, the

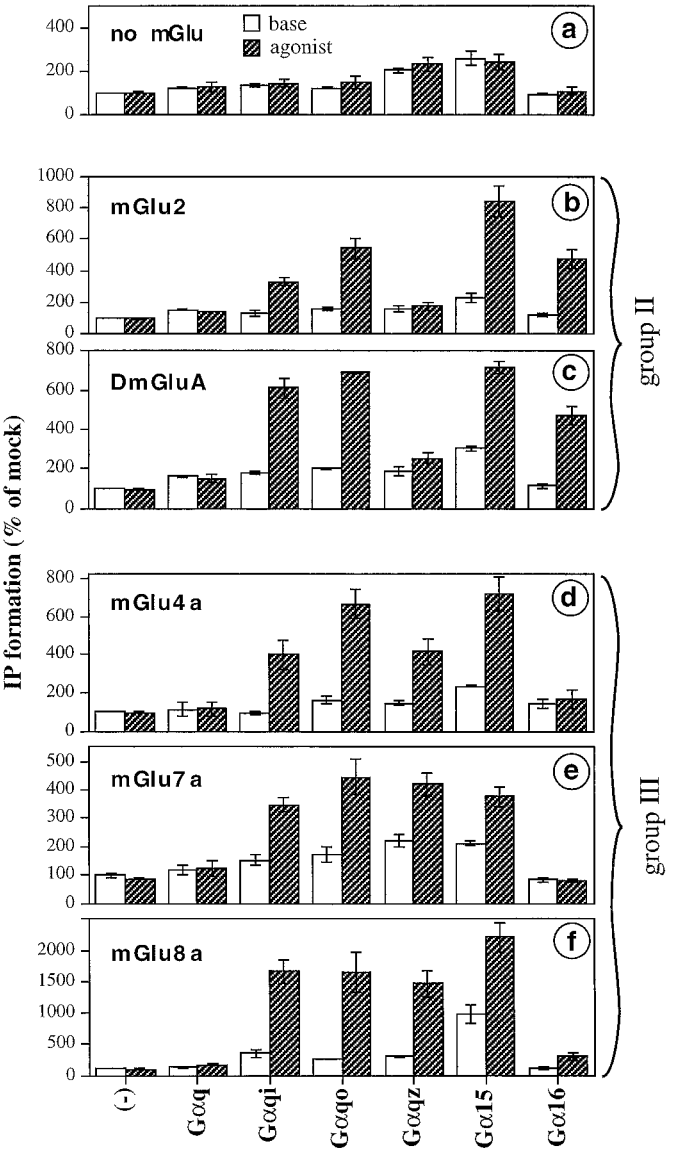

Fig. 1. PLC activation by mGlu2, DmGluA, mGlu4a, mGlu7a, or mGlu8a receptors coexpressed with the $G_{\alpha q}$ subunit, the promiscuous $G_{\alpha 16}$ and $G_{\alpha 15}$ subunits, or the chimeric $G_{\alpha q}$ subunits. Basal and agonist-stimulated IP formation in cells expressing (a) the G proteins only, (b) the G proteins plus mGlu2 or (c) DmGluA, (d) mGlu4a, (e) mGlu7a, or (f) mGlu8a receptors. The agonist used is glutamate (1 mM) except for the mGlu7a receptor, for which the more potent DL-AP4 agonist was used at 3 mM. Values are mean ± standard error of at least two experiments performed in triplicate.

agonist-induced IP formation was dose dependent, and the pharmacological profiles obtained were identical to those determined measuring the inhibition of cAMP formation (Gomeza *et al*., 1996b). Taken together, these results indicate that these receptors couple to $G_{\alpha 15}$, $G_{\alpha qi}$, and $G_{\alpha qo}$.

In cells expressing group II receptors (mGlu2 or DmGluA receptors), an agonist-induced increase in IP formation also was observed with $G_{\alpha 16}$ but not with $G_{\alpha qz}$ (Fig. 1, b and c). In contrast, in cells expressing group III receptors (mGlu4, mGlu7, and mGlu8 receptors), an increase in IP formation on agonist stimulation was observed with $G_{\alpha qz}$ but not with $G_{\alpha 16}$ (or to a much lower extent than with $G_{\alpha 15}$, $G_{\alpha qi}$, or $G_{\alpha qo}$ in the case of mGlu8 receptors) (Fig. 1, d–f). These observations cannot be explained by a different level of expression of the chimeric G proteins because Western blot analysis of these proteins tagged with the hemagglutinin epitope revealed similar level of expression (data not shown) as also reported by others (Liu *et al*., 1995; Conklin *et al*., 1996). Even though we cannot compare the level of expression of these receptors, the opposite coupling of group II and III mGlu receptors toward $G_{\alpha 16}$ and $G_{\alpha qz}$ (Table 1) indicates that the G protein-coupling profiles of group II and III receptors are different.

We also used this assay to compare the G protein-coupling profiles of the group I mGlu1a receptor with that of group II and III mGlu receptors. Unlike group II and III receptors, the mGlu1a receptor is able to activate PLC when expressed alone in HEK 293 cells (Fig. 2). Its possible coupling to $G_{\alpha 15}$, $G_{\alpha 16}$, or chimeric G_{α} proteins will be detected by an increase in the agonist-induced IP formation (Conklin *et al*., 1993; Offermanns and Simon, 1995). We also expect an increase in the basal PLC activity due to the constitutive activity of this receptor (Burstein *et al*., 1996; Prézeau *et al*., 1996). As expected for a $G_{\alpha q}$-coupled receptor, we observed an increased IP formation both in the presence and in the absence of glutamate when $G_{\alpha q}$ was cotransfected with the mGlu1a receptor (Fig. 2). This also was observed when the mGlu1a receptor was coexpressed with $G_{\alpha qi}$, $G_{\alpha qo}$, or $G_{\alpha qz}$ but not with $G_{\alpha 16}$ (Fig. 2). In cells expressing mGlu1a receptors and $G_{\alpha 15}$, we observed a significant increase in the basal IP formation but no modification of the IP formation measured in the presence of glutamate (Fig. 2). Taken together, these results indicate that the mGlu1a receptor couples to $G_{\alpha q}$, $G_{\alpha qi}$, $G_{\alpha qo}$, and $G_{\alpha qz}$ but not to $G_{\alpha 16}$ (Table 1). More experiments are required to test the possible coupling between the mGlu1a receptor and $G_{\alpha 15}$. These data further establish that the G protein-coupling profile of the group I mGlu1a receptor is distinct from those of group II and III mGlu receptors (Table 1).

Promiscuous and chimeric α subunits therefore seem to be good tools with which to define a G protein-coupling profile of mGlu receptors.

The G protein-coupling profile of mGlu receptors seems to not depend on the agonist used. Previous reports indicated that the G protein-coupling specificity of a receptor can vary depending on the agonist used (see, for example, Spengler *et al*., 1993). To test whether this also may be the case for mGlu receptors, the coupling of mGlu2 and mGlu4 receptors to the exogenous G proteins described above was examined after stimulation with various agonists. As shown on Fig. 3a, the three specific group II agonists LY354740 (5 μM), (2*R*,4*R*)-APDC (500 μM), and DCG IV (50 μM) (Conn and Pin, 1997) stimulated IP production in cells expressing mGlu2 receptor and $G_{\alpha qi}$, $G_{\alpha qo}$, $G_{\alpha 15}$, or $G_{\alpha 16}$ but not in cells coexpressing mGlu2 receptors with $G_{\alpha q}$ or $G_{\alpha qz}$, as observed with glutamate. Similarly, the three specific group III agonists DL-AP4 (50 μM), L-SOP (500 μM), and ACPT-I (500 μM) (Acher *et al*., 1997; Conn and Pin, 1997) stimulated IP formation in cells expressing the mGlu4a receptor and $G_{\alpha qi}$, $G_{\alpha qo}$, $G_{\alpha qz}$, or $G_{\alpha 15}$ but not in cells coexpressing mGlu4a receptors with $G_{\alpha q}$ or $G_{\alpha 16}$ (Fig. 3b). These results revealed no difference in the G protein-coupling specificity of mGlu2 and mGlu4a receptors on stimulation with various agonists.

Construction of chimeric mGlu1a/DmGluA receptors. Because the ligand PBP-like recognition domain of mGlu receptors seems to be distant from the 7TM region, it may be proposed that when occupied by an agonist, the domain of the family 3 receptors acts as the activator (as an agonist) of the 7TM region. The PBP-like domain of family 3 receptors may therefore, by stabilizing a specific conformation of the 7TM region, have some influence on the G protein-coupling specificity of the receptor. To test this possibility, the extracellular domains of the most distantly related mGlu receptors (i.e., the rat mGlu1a and the *D. melanogaster* DmGluA receptors) were swapped. These two receptors were chosen because they have distinct pharmacological profiles (Parmentier *et al*., 1996) and G protein-coupling specificities (Table 1). Four chimeras containing the PBP-like domain of

TABLE 1

G protein coupling specificity of mGlu receptors from the different groups

Group	$G_{\alpha q}$	$G_{\alpha qi}$	$G_{\alpha qo}$	$G_{\alpha qz}$	$G_{\alpha 15}$	$G_{\alpha 16}$
I	+	+	+	+	?	−
II	−	+	+	−	+	+
III	−	+	+	+	+	−

+, Coupling (i.e., interaction and activation) to the G protein; −, lack of coupling to the G protein studied.

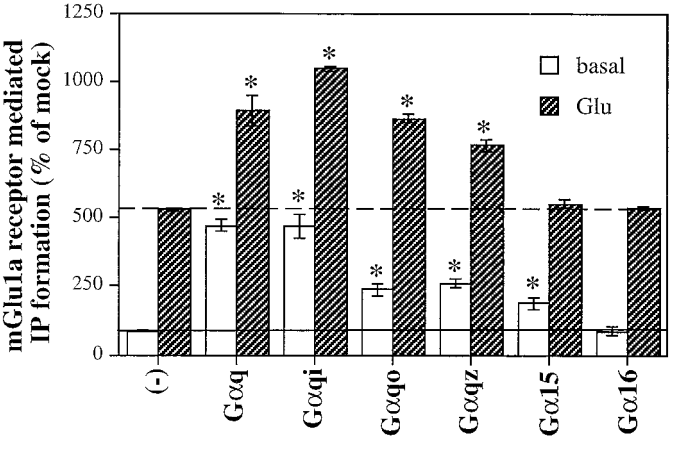

Fig. 2. PLC activation by the mGlu1a receptor expressed alone or with the wild-type or chimeric $G_{\alpha q}$ subunits or the promiscuous $G_{\alpha 15}$ or $G_{\alpha 16}$ receptor. Basal and 1 μM glutamate-stimulated IP formation was determined in cells expressing the mGlu1a receptor alone (−) or with the indicated G proteins. Basal IP formation also was determined in cells expressing the indicated G proteins alone. Values presented are obtained by subtracting this latter value corresponding to the effect of the G protein alone from the basal and glutamate-induced IP formation measured in cells coexpressing the mGlu1a receptor and the indicated G protein. *, Significantly different ($p < 0.05$ by analysis of variance) from the basal (*unbroken line*) or glutamate-induced (*dotted line*) activities in cells expressing the mGlu1a receptor alone. Values are mean ± standard error of one typical experiment performed in triplicate.

the mGlu1a receptor and the transmembrane and intracellular domains of the DmGluA receptor (1a/D-1–1a/D-4 chimeras) were constructed. They differ by the position of the junction within the two sequences (Fig. 4). The converse chimeras also were constructed and called D/1a-1–D/1a-4.

G protein-coupling profiles of chimeric mGlu receptors. The ability of the four D/1a chimeric mGlu receptors to activate PLC was examined by injecting their corresponding *in vitro* synthesized transcripts into *X. laevis* oocytes. It is established that among G protein-coupled receptors, only those coupled to PLC activate Ca^{2+}-dependent Cl^- channels by releasing Ca^{2+} from internal stores. Each of the D/1a chimeric mGlu receptors elicited an inward current when activated with 300 μM glutamate, whereas DmGluA receptors did not (data not shown). The D/1a-1 chimeric protein elicited the highest inward current, which was similar to that obtained with the wild-type mGlu1a receptor (data not shown). These results are in agreement with previous studies showing that mGlu1a chimeric receptors with the extracellular domain of the group II mGlu2 or the group III mGlu4 receptors activate PLC (Takahashi *et al.*, 1993; Tones *et al.*, 1995).

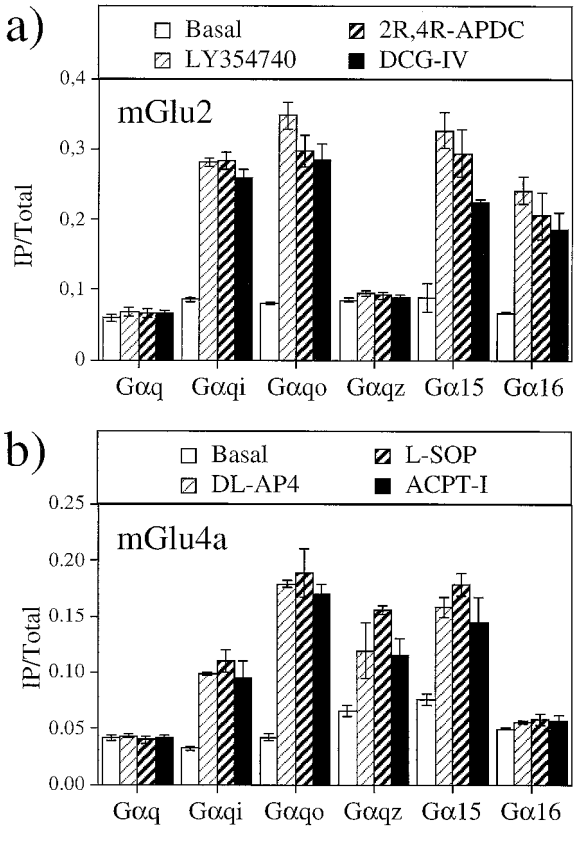

Fig. 3. The G protein-coupling profile of mGlu2 and mGlu4a receptors is the same whatever the agonist used. a, HEK293 cells coexpressing mGlu2 receptors with the wild-type $G_{\alpha q}$; the chimeric $G_{\alpha qi}$, $G_{\alpha qo}$, or $G_{\alpha qz}$; or the promiscuous $G_{\alpha 15}$ and $G_{\alpha 16}$ were incubated for 30 min with control medium (basal) or medium containing LY354740 (5 μM), (2*R*,4*R*)-APDC (500 μM), or DCG IV (50 μM), and the IP formation measured. b, Same as in a with cells expressing the mGlu4a receptor and stimulated with DL-AP4 (50 μM), L-SOP (500 μM), or ACPT-I (500 μM). Values are mean ± standard error of triplicate determinations. The experiment were repeated twice with the same results.

The G protein-coupling specificity of the converse chimeric mGlu receptors with the PBP-like domain of mGlu1 and the 7TM domain of the cyclase inhibiting DmGluA receptor was examined after expression in HEK293 cells. The four chimeric receptors were first coexpressed with $G_{\alpha qi}$, which can be activated by the wild-type DmGluA receptor (Parmentier *et al.*, 1996), and their ability to stimulate PLC on activation with glutamate was analyzed. The best response was obtained with the 1a/D-4 chimera (data not shown). The G protein-coupling specificity of this chimeric receptor therefore was studied in greater detail.

As observed with the DmGluA receptor, when the 1a/D-4 chimera was coexpressed with the cyclase-stimulating LH receptor, glutamate was able to decrease by 30% the LH-stimulated cAMP formation (Fig. 5a). This indicates that, like the wild-type DmGluA receptor, 1a/D-4 is able to inhibit adenylyl cyclase. Under similar conditions, the mGlu1a receptor activation by glutamate did not inhibit adenylyl cyclase but instead induced a 2-fold increase in cAMP formation (data not shown). To further compare the G protein-coupling selectivity of DmGluA and 1a/D-4 receptors, the chimeric receptor was coexpressed with the various promiscuous and chimeric G protein α subunits described above. Fig. 5b reveals that 1a/D-4 receptor displays the same G protein-coupling profile as the DmGluA receptor and not that of the mGlu1a receptor: it activates $G_{\alpha 15}$, $G_{\alpha 16}$, $G_{\alpha qi}$, and $G_{\alpha qo}$ but neither $G_{\alpha qz}$ nor $G_{\alpha q}$. This strongly suggests that the extracellular domain of mGlu receptors has no major influence on their G protein-coupling selectivity.

The chimeric mGlu1a/D-4 receptor displays the same agonist recognition profile as the mGlu1a receptor. To analyze whether the 7TM region of the DmGluA receptor influences the agonist selectivity of the receptor, we systematically compared the agonist pharmacological profiles of DmGluA, mGlu1a, and 1a/D-4 receptors using seven agonists (Table 2, Fig. 6, a and b). The rank order of potency of these agonists was found to be identical between the mGlu1a receptor and the 1a/D-4 chimeric receptor, with the PBP-like domain of the mGlu1a receptor (correlation coefficient r = 0.97, $p < 0.005$) whereas it was not between DmGluA and 1a/D-4 receptors (r = 0.115) (Fig. 6c). This indicates that the 7TM region of mGlu receptors does not have a major influence on the agonist pharmacological profile of these receptors.

Discussion

Our results indicate that exogenous G protein α subunits can be used to define the G protein-coupling selectivity pro-

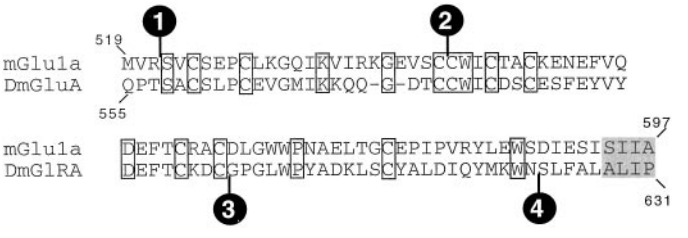

Fig. 4. Positions of the junction points between mGlu1a and DmGluA receptor sequences in the different chimeras. *Boxed*, residues conserved in all mGlu receptors. *Shaded*, beginning of the first transmembrane domain.

file of mGlu receptors, in the way synthetic ligands are commonly used to define their pharmacological profile. By using $G_{\alpha q}$; the chimeric $G_{\alpha qi}$, $G_{\alpha qo}$, and $G_{\alpha qz}$; and the promiscuous $G_{\alpha 15}$ and $G_{\alpha 16}$ and measuring the ability of most mGlu receptors to activate PLC, we show here that group I, II, and III mGlu receptors have distinct G protein-coupling profiles. This constitutes the first demonstration that group II and III mGlu receptors can be distinguished not only by their pharmacological profiles and sequence homology (Nakanishi, 1992; Conn and Pin, 1997) but also by their G protein-coupling selectivity.

Our experiments were not aimed at identifying the endogenous G proteins activated by mGlu receptors *in vivo*. However, because the carboxyl-terminal end of the G protein α subunits plays a critical role in their specific interaction with GPCRs (Hamm *et al*., 1988; Wong *et al*., 1992; Conklin *et al*., 1993, 1996; Conklin and Bourne, 1993), our data may help identify the endogenous G proteins that could be activated by these receptors. The pertussis toxin-sensitive $G_{\alpha o}$ and $G_{\alpha i}$ and the pertussis toxin-insensitive $G_{\alpha z}$ are expressed in many brain areas (Hinton *et al*., 1990; Wong *et al*., 1992), as are mGlu receptors. Our analysis of the coupling of group II and III mGlu receptors to $G_{\alpha qi}$, $G_{\alpha qo}$, and $G_{\alpha qz}$ (Table 1) suggests that group III receptors couple to $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha z}$, whereas group II receptors couple to $G_{\alpha i}$ and $G_{\alpha o}$ but may not couple to $G_{\alpha z}$. The possible endogenous coupling of group II and III mGlu receptors to $G_{\alpha i}$ and $G_{\alpha o}$ is supported by the pertussis toxin-sensitive inhibition of adenylyl cyclase and voltage-sensitive Ca^{2+} channels in neurons induced by selective group II and III agonists (for a review, see Conn and Pin, 1997). However, the endogenous coupling of group III mGlu receptors to $G_{\alpha z}$ remains to be demonstrated. Because $G_{\alpha 15}$ and $G_{\alpha 16}$ are found only in mouse and human hematopoietic cells, respectively (Amatruda *et al*., 1991; Wilkie *et al*., 1991), they cannot be involved in the natural transduction of mGlu receptors in the brain.

Our data reveal that the carboxyl-terminal end of the α subunits plays a critical role in their specific coupling not only to the large family of GPCRs (Conklin and Bourne, 1993; Dratz *et al*., 1993; Liu *et al*., 1995) but also to the mGlu receptor family, even though these two families of receptors do not share any sequence homology. Moreover, they show that a difference of only three residues (between $G_{\alpha qo}$ and $G_{\alpha qz}$; see Fig. 7) in this short domain can discriminate between cyclase-inhibiting receptors. However, the carboxyl-terminal end of the α subunit is not the only region involved in the specific recognition of mGlu receptors. Although $G_{\alpha 15}$ and $G_{\alpha 16}$ possess an identical carboxyl terminus (Amatruda *et al*., 1991; Wilkie *et al*., 1991), $G_{\alpha 15}$ couples to group II and III mGlu receptors, whereas $G_{\alpha 16}$ couples to group II mGlu receptors only. Other regions with low sequence homology between these α subunits therefore may be involved in the interaction with these receptors. Among these regions is the sequence around the loop L9 of the α subunit that has been proposed to contact the receptor (Hamm *et al*., 1988; Conklin and Bourne, 1993; Lee *et al*., 1995; Lichtarge *et al*., 1996).

The *D. melanogaster* mGlu receptor DmGluA belongs to group II mGlu receptors. Indeed, this G_i-coupled receptor share 47% sequence similarity with group II mammalian mGlu receptors, whereas it share 42% and 35% similarity with group I and III receptors, respectively. Moreover, it displays a pharmacological profile closer to that of group II

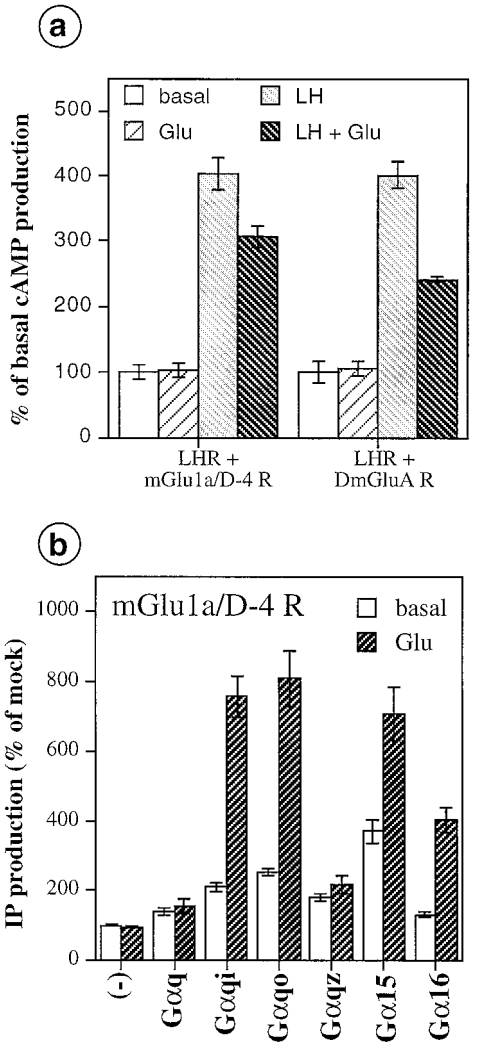

Fig. 5. Analysis of the G protein coupling of mGlu1a/D-4 receptor chimera. a, Cotransfection of DmGluA or mGlu1a/D4 receptors with the LH receptor. Percentage of conversion of ATP in cAMP is estimated without any agonist and in the presence of either 1 mM glutamate or 100 ng/ml LH or in the presence of both agonists. Values are mean ± standard error of triplicate determinations from one typical experiment. b, Basal and 1 mM glutamate-stimulated IP formation in cells expressing the mGlu1a/D-4 receptor alone or with the $G_{\alpha q}$, $G_{\alpha 16}$, $G_{\alpha 15}$, $G_{\alpha qi}$, $G_{\alpha qo}$, or $G_{\alpha qz}$ subunits. Values are mean ± standard error of three experiments performed in triplicate.

TABLE 2

Agonists EC_{50} values for DmGluA, mGlu1, and the chimeric mGlu1a/D-4 receptors

EC_{50} are determined as described in the text and are the mean ± standard error of at least two independent experiments performed in triplicate.

Agonist	EC_{50}		
	DmGluA	mGlu1	mGlu1a/D-4
		μM	
Glutamate	1.25 ± 0.07	0.9 ± 0.4	5.9 ± 3.0
L-Quisqualate	37 ± 7	0.03 ± 0.01	0.14 ± 0.03
Ibotenate	38 ± 29	1.9 ± 0.6	7.0 ± 1.9
1*S*, 3*S*-ACPD	29 ± 8	87 ± 49	236 ± 124
1*S*, 3*R*-ACPD	67 ± 1	4.7 ± 1.4	12.3 ± 2.3
L-CCG-I	5.8 ± 5	1.87 ± 0.06	5.7 ± 0.6
DHPG	>1000[a]	5.7 ± 2.5	7.5 ± 0.1

[a] The molecule at 1 mM induced a response <50% of the maximal effect of glutamate.

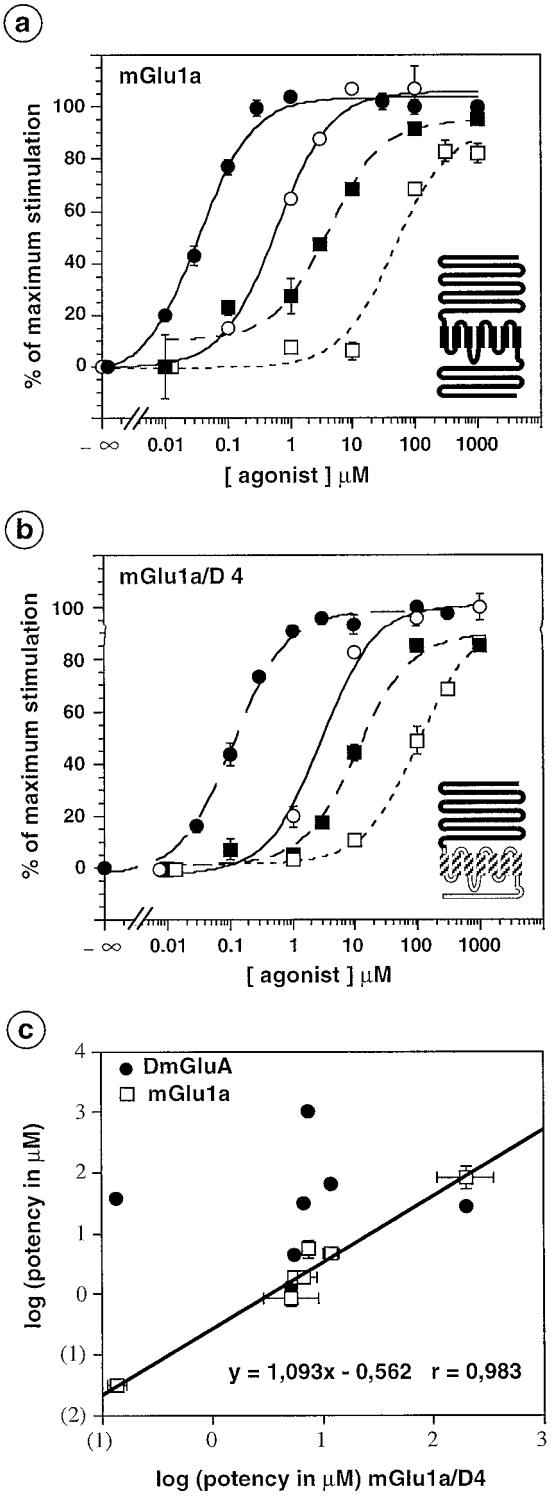

Fig. 6. Dose-response curves of agonist-induced IP stimulation (expressed in percentage of maximal glutamate stimulation) in mGlu1a receptor-expressing cells (a) and in cells expressing the mGlu1a/D-4 receptor and $G_{\alpha qi}$ (b). In a and b, dose-response curves were obtained with quisqualate (●), glutamate (○), (1*S*,3*R*)-ACPD (■), and (1*S*,3*S*)-ACPD (□). c, For each of these drugs [glutamate, L-quisqualate, ibotenate, L-CCGI, DHPG, (1*S*,3*R*)-ACPD, and (1*S*,3*S*)-ACPD], the logarithm of the EC_{50} value obtained with the mGlu1a (□) or DmGluA (●) receptors was plotted versus the logarithm of the EC_{50} value obtained with the chimeric mGlu1a/D4 receptor. Only the significative linear regression obtained between the mGlu1 and mGlu1a/D-4 receptor values is shown (as well as the corresponding equation). Value are mean ± standard error (□) or mean only (●) of at least two independent determinations.

than to that of the other groups of mGlu receptors (Parmentier *et al.*, 1996). The use of the chimeric and promiscuous G proteins also reveals that despite sequence differences between the 7TM region of the DmGluA receptor and those of mammalian group II mGlu receptors, the DmGluA receptor has the same G protein-coupling specificity as the mGlu2 receptor. This confirms that DmGluA receptor is a *D. melanogaster* homologue of group II mammalian mGlu receptors, indicating that group II mGlu receptors were already defined in the common ancestor of vertebrates and arthropods.

It has been proposed that the large amino-terminal extracellular domain of mGlu receptors constitutes their ligand recognition domain. Homology modeling and site-directed mutagenesis of the mGlu1a receptor allowed the identification of two residues within this domain involved in glutamate binding (O'Hara *et al.*, 1993). Moreover, the exchange of the extracellular domain of the group I mGlu1a receptor with that of either mGlu2 or mGlu4 receptors generates chimeric receptors able to activate PLC. These receptors also were shown to have pharmacological profiles similar to those of mGlu2 and mGlu4 receptors, respectively, using two or three different agonists (Takahashi *et al.*, 1993; Tones *et al.*, 1995). In the current study, we analyzed in greater detail the pharmacological profile of a converse chimeric receptor with the extracellular domain of mGlu1 and the 7TM region of the cyclase-inhibiting DmGluA receptor. Our results show that the agonist pharmacological profile of this chimeric receptor is identical to that of mGlu1 receptors. They further indicate that the extracellular domain of mGlu receptors is responsible for the ligand recognition in this receptor family. Moreover, they demonstrate that the 7TM region has no influence on the agonist pharmacological profile of these receptors. According to these data, the extracellular domain of mGlu receptors may be considered the activator of the 7TM region.

It recently was been reported that different agonists, probably by stabilizing different conformational states of the 7TM domains, can lead to the activation of different G proteins (Spengler *et al.*, 1993; Eason *et al.*, 1994; Robb *et al.*, 1994; Gether *et al.*, 1995; Perez *et al.*, 1996). This means that the G protein-coupling selectivity of a 7TM receptor depends not only on the sequence of its intracellular loops but also on its conformational state. Our results revealed, however, no difference in the G protein-coupling profiles of either mGlu2 or mGlu4a receptor on activation with four different agonists. However, because the extracellular domain of mGlu receptors, when occupied by glutamate, can be considered the activator (or agonist) of the 7TM region, we also tested whether it could influence the G protein-coupling specificity

relative position to the C-terminus	-5	-4	-3	-2	-1
Gα15	E	I	N	L	L
Gαi2	D	C	G	L	F
Gαo	G	C	G	L	Y
Gαz	Y	I	G	L	C
Gαq	E	Y	N	L	V

Fig. 7. Alignment of the five carboxyl-terminal amino acids of $G_{\alpha 15}$ (which are the same for $G_{\alpha 16}$), $G_{\alpha i2}$, $G_{\alpha o}$, $G_{\alpha z}$, and $G_{\alpha q}$. *Boxed*, amino acids conserved in the five sequences. *Shaded*, amino acids that differ between $G_{\alpha z}$ and $G_{\alpha i/o}$.

by stabilizing a specific conformation of the 7TM region. By using exogenous G proteins, we show here that the G protein-coupling profile of the 7TM region of the DmGluA receptor is the same regardless of whether the extracellular domain is that of DmGluA or mGlu1 receptors, although these two domains share only 35% of sequence homology. This supports the conclusion that the extracellular domain of mGlu receptors may have no influence on their G protein-coupling profiles.

Taken together, these results demonstrate that mGlu receptors are composed of two domains, with the PBP-like domain entirely responsible for the ligand recognition and the 7TM region responsible for the G protein-coupling specificity. Because the agonist pharmacological and G protein-coupling profiles are not influenced by the 7TM and PBP-like domains, respectively, one may expect these two domains to evolve independently. Surprisingly, the only example of a mGlu receptor from a distant species, the DmGluA receptor, does not fit this hypothesis. Indeed, although the DmGluA receptor shares only 45% homology with the mGlu2 receptor, both its agonist pharmacological (Parmentier *et al*., 1996) and G protein-coupling profiles have been conserved during evolution. The functional analysis of other distant mGlu receptors would help us to understand this discrepancy between the apparent independence of the agonists and G protein-coupling specificities of mGlu receptors and their parallel conservation during evolution.

Within the 7TM domain, it has been proposed that the sequence of the intracellular loops plays an important role in G protein-coupling selectivity. We previously reported that the second intracellular loop was critical in determining the coupling of the mGlu1 receptor to PLC (Pin *et al*., 1994; Gomeza *et al*., 1996a). Further experiments would reveal whether this loop is indeed responsible for the different G protein-coupling profiles observed here between group II and III mGlu receptors.

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