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Changes in the Carboxyl-Terminal Domain of Metabotropic Glutamate Receptor 1 by Alternative Splicing Generate Receptors with Differing Agonist-Independent Activity

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SUMMARY

The metabotropic glutamate receptors (mGluRs) share no sequence homology and show different structural features compared with most other G protein-coupled receptors (GPCRs). In particular, some isoforms of the phospholipase C (PLC)-coupled mGluRs (mGluR1a, mGluR5a, and mGluR5b) have a surprisingly long carboxyl-terminal intracellular domain of more than 350 residues, whereas the splice variants mGluR1b and mGluR1c have a much shorter carboxyl terminus. In the current study, the different splice variants of mGluR1 were expressed in porcine kidney epithelial (LLC-PK1) or the human embryonic kidney (HEK 293) cells, and their levels of expression were examined with the use of Western blot analysis. Expression of the short isoforms mGluR1b and mGluR1c did not modify the basal inositol phosphate production. In contrast, expression to similar levels of mGluR1a resulted in a 2-fold increase in the basal inositol phosphate formation. This increase in basal PLC activity was due to neither the presence of a low concentration of glutamate in the incubation medium nor a modification of the PLC pathway, resulting, for example, from the constant activation of mGluR1a by glutamate during the culture. Surprisingly, none of the known competitive antagonists of mGluR1 inhibited the basal PLC activity, indicating that none of these molecules act as inverse agonists. Taken together, these results indicate that the long carboxyl-terminal domain confers a small agonist-independent activity to mGluR1. This indicates that, as already observed for other GPCRs, little constitutive activity of wild-type mGluRs can be detected. Our results also add to the functional differences already observed among the mGluR1 splice variants and further suggest that the long carboxyl-terminal domain of mGluR1a confers better coupling efficiency to the G proteins.

The recent cloning of both ionotropic glutamate receptors and mGluRs revealed that these proteins constitute different receptor families compared with the other ligand-gated channels and GPCRs, respectively (1–3). Although they are coupled to G proteins, mGluRs share no sequence homology with any other GPCRs. Their hydrophobicity profiles suggest that they possess seven TMDs, like the other GPCRs. However, in contrast to TMDs of GPCRs, in which charged residues play critical roles in ligand binding (4), signal transduction (5), and TMD interactions (6), there are no charged residues in any mGluR TMDs. The absence of primary sequence 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 (7). In agreement with an original topological organization of the seven TMDs of mGluRs, the second intracellular loop of mGluRs plays a role similar to that of the third intracellular loop of the other GPCRs (8, 9). Finally, the most striking difference between most GPCRs and mGluRs resides in their ligand binding domain. Although it is located in a cavity formed by the seven TMDs in most GPCRs (4, 7), the glutamate binding site in mGluRs is located in a large (500 amino acid residues) extracellular domain homologous to bacterial periplasmic binding proteins (10, 11). This domain consists of

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; TMD, transmembrane domain; PLC, phospholipase C; AT1, angiotensin II type 1; V1a, arginine vasopressin type 1a; PACAP, pituitary adenylyl cyclase-activating polypeptide; LLC-PK1, porcine kidney epithelial; HEK 293, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MCPG, (*RS*)-α-methyl-4-carboxyphenylglycine; 4CPG, (*S*)-4-carboxyphenylglycine; 4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

two lobes with a hinge region where glutamate binds (11). By analogy with periplasmic binding proteins, it has been proposed that these two lobes close like a clamshell on binding of the agonist, inducing the conformational changes required for the activation of the receptor.

Site-directed mutagenesis of GPCRs revealed that point mutations can generate receptors that are active in the absence of agonists (12–15). Such mutation-generating receptors with constitutive activity are likely to be responsible for a number of diseases (13, 16–19). A more careful analysis of the transduction mechanism of wild-type receptors revealed that some are also constitutively active (15, 17, 20–24). Interestingly, some but not all antagonists can inhibit the constitutive activity and are therefore called inverse agonists (21–27). With the use of such molecules, it has been reported that muscarinic acetylcholine (28, 29), β-adrenergic (30–32), 5-hydroxytryptamine$_{2c}$ (22), and B_2 bradykinin (33) receptors are constitutively active in normal tissue, indicating that these observations are not simply the consequence of the heterologous expression of cloned receptors in cell lines. These observations lead to an extension of the ternary complex model of GPCR action (15, 25, 27, 34, 35). According to this model, receptors may exist in two conformational states (an active and an inactive one), and the equilibrium between these two states is displaced toward the active state when an agonist binds on the receptor. In contrast, the binding of an inverse agonist may displace the equilibrium toward the inactive state of the receptor. Neutral antagonists, i.e., antagonists with no inverse agonist action, may have the same affinity for the two states and therefore do not modify the equilibrium.

In the current study, coupling to PLC of the different mGluR1 splice variants was examined in either LLC-PK1 or HEK 293 cells. Our results indicate that despite the differences between mGluRs and the other GPCRs, agonist-independent activity of these glutamate receptors can be detected. Interestingly, only the mGluR1a isoform with the long carboxyl-terminal domain possesses this property. These results further suggest that the long carboxyl-terminal domain of mGluR1a confers a better coupling efficiency of this receptor protein to G proteins.

Materials and Methods

Plasmids. The cDNAs encoding mGluR1a, mGluR1b, mGluR1c, mGluR5a, and mGluR5b (36, 37) were subcloned into an eucaryotic vector that possesses a cytomegalovirus immediate-early promoter (38). The plasmids carrying the cDNA encoding the rat AT1 receptor, the rat V1a receptor, and the rat PACAP receptor were gifts from Drs. J. Marie, C. Barberis, and L. Journot (C.C.I.P.E., Montpellier, France) respectively.

Culture and transfection of LLC-PK1 and HEK 293 cells. Culture and electroporation of the LLC-PK1 and HEK 293 cells were conducted as previously described (37). Briefly, cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 units/ml final). Electroporation was performed in a total volume of 300 μl with 10 μg of carrier DNA, 200–600 ng of plasmid DNA, 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, Bio-Rad gene pulser electroporator), cells were resuspended in DMEM supplemented with 10% fetal calf serum and antibiotics and split into 12-well clusters (Falcon) (10 million cells/cluster). For HEK 293 cells, culture wells were previously coated with poly-L-ornithine (15 μg/ml; molecular mass, 40,000 Da; Sigma Chemical Co.) to facilitate adhesion of the cells.

Determination of IP accumulation. Determination of IP accumulation in transfected cells was performed as previously described (37). 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; NEN). Cells were then washed three times and incubated for 1–2 hr at 37° in 1 ml of HEPES buffer saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.4). The cells were washed again twice with the same medium and preincubated for 10 min in medium containing 10 mM LiCl. Then the agonist was added for a period of 30 min. The reaction was stopped by replacement of the incubation medium with 1 ml of perchloric acid (5%) on ice. The total IP was then extracted and purified on Dowex columns as previously described (37). 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 current in the membranes.

Labeled inositol lipids were determined after chloroform/methanol/HCl (100:100:1) extraction, as previously described (39). To discriminate among PI, PIP, and PIP_2, the extracted inositol lipids were deacylated with NaOH (1 M) in the presence of MeOH. The resulting products (glycerophosphoinositol, glycerophosphoinositol-4-phosphate, and glycerophosphoinositol-4,5-bisphosphate) were separated by the use of chromatography on Dowex columns. They were consecutively eluted with 20-ml fractions of 0.18 M ammonium formate/5 mM disodium tetraborate, 0.3 M ammonium formate/0.1 formic acid, and 0.75 M ammonium formate/0.1 M formic acid.

Determination of glutamate concentration. glutamate concentration in the incubation medium was determined as previously described using precolumn derivatization with orthophtalaldehyde and separation on a reversed-phase C-18 high performance liquid chromatography column (40).

SDS-PAGE and immunoblotting. To prepare membranes, cells were washed three times with ice-cold PBS without calcium and magnesium and suspended in a homogenization buffer composed of 10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted at 4° with a Dounce homogenizer equipped with a loose pestle (15 strokes). Intact or partially disrupted cells were eliminated by low-speed centrifugation (100 × *g* for 5 min at 4°). The supernatant was centrifuged at 30,000 × *g* for 15 min at 4°. The final pellet (crude membrane preparation) was resuspended in the homogenization buffer and used immediately. The protein content of each preparation was determined by the Bradford method using bovine serum albumin as standard.

Samples (40 μg of protein) were solubilized in Laemmli sample buffer (2.5% w/v SDS, 25 mM Tris·HCl, pH 6.8, 5% v/v β-mercaptoethanol, and 6.25% glycerol), resolved with the use of SDS-PAGE (7.5% acrylamide), and transferred by electroblotting onto a nitrocellulose membrane. Blots were blocked for 60 min at room temperature with 5% (w/v) milk powder dissolved in TBS (10 mM Tris·HCl, pH 7.4, and 500 mM NaCl) complemented with 0.1% Tween-20 and further incubated overnight at 4° with the primary antibodies (anti-mGluR1, 1/250 v/v, or anti-actin, 1/2000) in TBS complemented with 0.1% Tween-20 with 5% (w/v) milk powder. After being washed, the blots were probed with peroxidase-linked anti-rabbit IgG, and bands were visualized with the enhanced chemiluminescence method (Amersham). The generation and characterization of the mGluR1 antibody (generous gift of Drs. V. Matarese and Ferraguti, Glaxo, Verona, Italy) raised against a chimeric protein containing part of the extracellular domain have been previously described (41). The densitometric intensity of each band was determined with the National Institutes of Health Image program (version 1.55).

Immunohistochemistry and confocal microscopy. Immunostaining experiments were performed essentially as previously described (37). Briefly, cells grown on coverslips were fixed with 4%

paraformaldehyde in 120 mM glucose for 20 min at room temperature. Coverslips were rinsed in 0.1 M glycine, pH 7.4, for 30 min and then permeabilized with 0.05% Triton X-100 in PBS for 10 min. After being washed with PBS containing 10% gelatin (PBS/gelatin), cells were incubated overnight at 8° with the anti-mGluR1 antibody (1:200) in PBS/gelatin buffer. Coverslips were washed three times for 30 min with PBS/gelatin buffer and exposed to goat anti-rabbit IgG second antibody conjugated to fluoresceine (1:100 in PBS/gelatin buffer; Sigma, Lisle d'Abeau, France) for 1–2 hr at room temperature. After being washed with PBS/gelatin, coverslips were mounted on glass slides and visualized with a Bio-Rad 600 confocal microscope.

Materials. MCPG, 4CPG, and 4C3HPG were obtained from Tocris Cookson (Essex, UK). PACAP38 was obtained from Sigma, and arginine vasopressin and Sar-AII were obtained from Bachem (Bubendorf, Switzerland). Glutamate pyruvate transaminase was from Boehringer Mannheim (Meylan, France).

Results

The functional coupling to PLC of the three mGluR1 splice variants [mGluR1a, mGluR1b, and mGluR1c (Fig. 1)] was examined after transient expression in LLC-PK1 cells. These variants differ only in their carboxyl-terminal intracellular domain, with the last 318 carboxyl-terminal residues of mGluR1a being replaced by 20 and 11 residues in mGluR1b and mGluR1c, respectively (Fig. 1). Application of 1 mM glutamate resulted in an increase in IP formation in cells expressing these mGluR1 variants, with this effect being greater in mGluR1a-expressing cells than in cells expressing the short variants mGluR1b or mGluR1c (Fig. 2a). No effect of glutamate was observed in control or mock-transfected cells (Fig. 2a). Surprisingly, the basal production of IP in cells expressing mGluR1a was higher [mean ± standard error, 192.4 ± 12.0% of control (21 experiments); $p < 0.001$, t test] than that measured on mock-transfected cells or cells expressing mGluR1b or mGluR1c (Fig. 2a). This increased basal PLC activity was not due to a difference in the labeling or size of the pool of PIP_2, due to, for example, a change in PI kinase activity, because it was still observed when the amount of [^{3}H]IP produced was normalized to the amount of [^{3}H]PIP_2 present in the membranes (Fig. 2b). Because the glutamate-induced IP production was less in cells expressing mGluR1b or mGluR1c than in those expressing mGluR1a, it was possible that the increase in basal IP production induced by mGluR1b or mGluR1c was too small to be detected. However, in the HEK 293 cells, in which all splice variants stimulate IP production to the same extent when activated by 1 mM glutamate (Fig. 2c), only the expression of the long mGluR1a isoform resulted in an increase in basal IP production.

A similar increase in basal PLC activity is also observed after expression of the other PLC-coupled mGluRs, mGluR5a and mGluR5b, which also possess a long intracellular domain (Fig. 1) (37). This increase in basal PLC activity was, however, not observed after expression of other PLC-activating

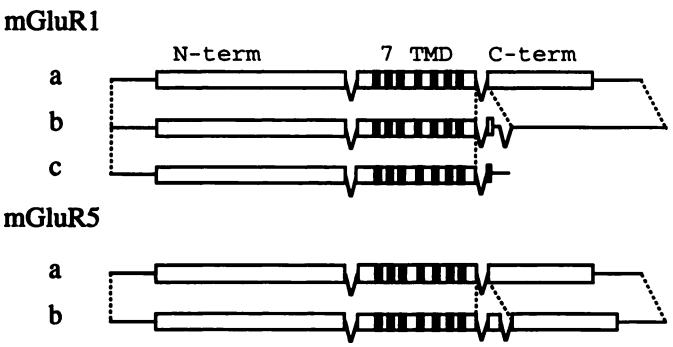

Fig. 1. Schematic representation of the PLC-coupled mGluR splice variants. *Open boxes*, coding sequences. *Horizontal lines*, noncoding sequences. *Black bars*, seven TMDs. Identical sequences found in the different variants are joined by *dashed lines*. Only the introns that have been identified are presented (*V*). *C-term*, carboxyl terminus; *N-term*, amino terminus.

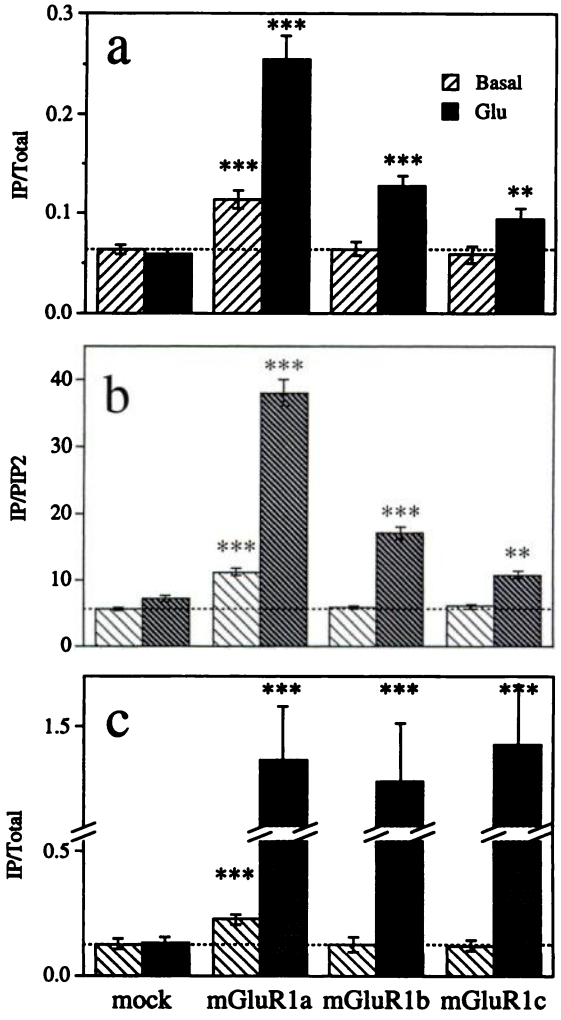

Fig. 2. Expression of mGluR1a but not mGluR1b or mGluR1c into mammalian cells leads to an increase in the basal PLC activity. a, LLC-PK1 cells transfected with the carrier DNA alone (mock) or expressing mGluR1a, mGluR1b, or mGluR1c were labeled with [^{3}H]inositol. After being washed with the culture medium, cells were incubated for 30 min in the absence (*basal*) or in the presence (*Glu*) of 1 mM Glu. Results are mean ± standard error of 20, 26, 13, and 10 triplicate determinations for mock-transfected cells and cells expressing mGluR1a, mGluR1b, and mGluR1c, respectively. Data are expressed as the total amount of radioactive IP divided by the amount of radioactivity in the membranes. b, Same as in a except that the values are expressed as the total amount of radioactive IP divided by the amount of radioactive PIP_2. Results are mean ± standard error of a triplicate determination from a typical experiment. c, Same as in a except that HEK 293 cells were used. Results are mean ± standard error of 4–9 independent experiments performed in triplicate. ***, $p < 0.001$; * $p < 0.05$); t test analysis revealed a significant difference with the base of control cells.

receptors, such as PACAP, V1a, and AT1 receptors (data not shown).

The high basal PLC activity in cells expressing mGluR1a was not due to the presence of a low concentration of glutamate in the incubation medium for several reasons. First, this was observed in cells expressing mGluR1a, but not in cells expressing mGluR1b and mGluR1c, and all of these receptors are similarly sensitive to Glu. The EC_{50} values for glutamate were 12.4 ± 2.7 μM (five experiments), 21.7 ± 6.7 μM (three experiments), and 19.3 ± 6.3 μM (four experiments) for mGluR1a, mGluR1b, and mGluR1c, respectively. Second, the glutamate concentration in the medium at the end of the incubation period was found to be 0.3 μM, a concentration that was not sufficient to activate these receptors expressed either in mammalian cells or in *Xenopus* oocytes (data not shown). Third, the enzyme degrading Glu, glutamate/pyruvate transaminase (2 units/ml plus 2 mM pyruvate), which was able to prevent the stimulatory effect of a 10 μM glutamate solution, was not able to suppress the basal activity (Fig. 3). Fourth, the three characterized competitive antagonists of mGluR1 [MCPG, 4CPG, and 4C3HPG], used at a concentration able to antagonize the effect of 10 μM Glu, did not inhibit the basal PLC activity (Fig. 4). The two other recently characterized mGluR1 antagonists [Br-homo-ibotenate (42) and a rigid analogue of 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylate, the amino-bicyclo[2.2.1]heptane dicarboxylate (43)] also failed to inhibit the basal PLC activity measured in mGluR1a-expressing cells (data not shown). These later results confirm that the basal PLC activity is not due to the presence of an agonist in the incubation medium and also revealed that none of the characterized competitive antagonists of mGluR1 have inverse agonist activity.

It can be argued that the difference observed between splice variants was due to a different level of expression of these proteins. Because [^{3}H]glutamate (the only tritiated ligand interacting with mGluR1) has a low affinity, the exact

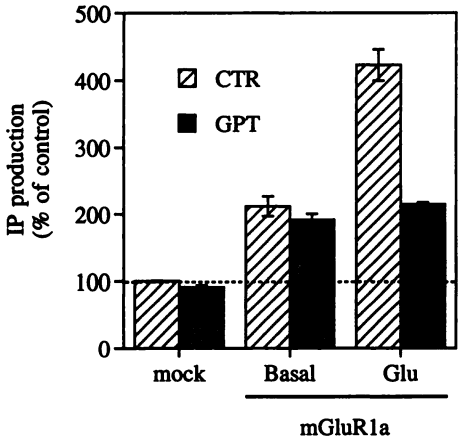

Fig. 3. The enzyme degrading Glu, *GPT* (glutamate-pyruvate transaminase), does not inhibit the basal PLC activity measured in mGluR1a-expressing cells. Mock-transfected LLC-PK1 cells (*mock*) or cells expressing mGluR1a were washed and incubated for 30 min in the absence (*CTR*) or in the presence (*GPT*) of 2 units/ml glutamate-pyruvate transaminase plus 2 mM pyruvate. The experiment with mGluR1a-expressing cells was conducted in the absence (*Basal*) or in the presence (*Glu*) of 10 μM Glu. Data are expressed as the total amount of radioactive IP divided by the amount of radioactivity in the membranes. Results are mean ± standard error of triplicate determinations from a representative experiment.

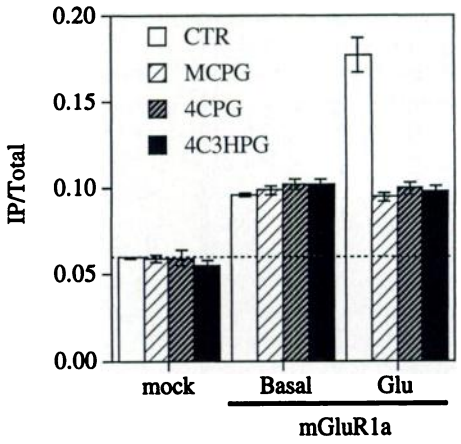

Fig. 4. The competitive mGluR1 antagonists do not inhibit the basal PLC activity measured in mGluR1a-expressing cells. Control LLC-PK1 cells (*mock*) or cells expressing mGluR1a were washed and incubated for 30 min in control buffer (*CTR*) or buffer containing 1 mM MCPG, 500 μM 4CPG, or 500 μM 4C3HPG. The experiment with mGluR1a-expressing cells was conducted in the absence (*Basal*) or in the presence (*Glu*) of 10 μM Glu. Data are expressed as the total amount of radioactive IP divided by the amount of radioactivity in the membranes. Results are mean ± standard error of triplicate determinations from two representative experiments.

density of receptors is difficult to determine with binding experiments. The level of expression of mGluR1a, mGluR1b, and mGluR1c was therefore examined with Western blot and immunofluorescence, using an antibody raised against the amino-terminal domain of the protein, a domain common to all of these variants (Fig. 1). In both HEK 293 cells (Fig. 5, a and b) and LLC-PK1 cells (data not shown), the level of expression of the three variants is comparable, with the amount of mGluR1a slightly more than that of mGluR1b, which is in agreement with previous reports for BHK cells (44), but less than that of mGluR1c. Moreover, the three splice variants were found at the plasma membrane level, as revealed by immunofluorescence staining (data not shown).

To check whether the increased basal PLC activity can be observed only after overexpression of mGluR1a, cells were transfected with varying amounts (0–3 μg/5 million cells) of

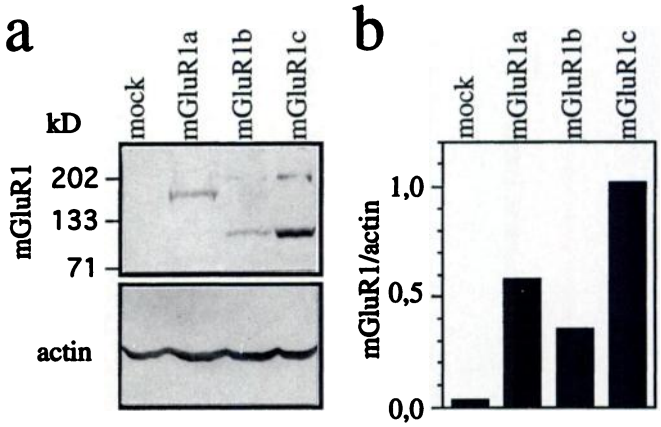

Fig. 5. Relative level of expression of mGluR1 variants. Membranes were prepared from mock-transfected HEK 293 cells or cells expressing mGluR1a, mGluR1b, or mGluR1c; solubilized; and subjected to SDS-PAGE. a, After transfer onto nitrocellulose membrane, mGluR1 (*top*) and actin (*bottom*) were detected with selective antibodies. b, Histogram of the ratio of the densitometric scan of the bands corresponding to mGluR1 to that of actin.

plasmid DNA encoding mGluR1a in the presence of carrier DNA (5 μg total DNA/5 million cells). Western blotting revealed that the amount of mGluR1a protein increased when greater amounts of plasmid DNA are used for transfection (Fig. 6a), as were basal and Glu-induced IP productions (Fig. 6b). Interestingly, the amount of protein corresponded to basal or Glu-induced IP production, and basal IP production increased linearly as a function of Glu-induced IP production (Fig. 7). In contrast, basal IP production remained constant regardless of Glu-induced response in mGluR1c- (Fig. 7) or mGluR1b- (data not shown) expressing cells. These results suggest that as has been observed with other GPCRs (15, 20, 24), the basal PLC activity in mGluR1a-expressing cells is proportional to the receptor density.

We then analyzed the possibility that the increase in basal PLC activity could be due to a change in the efficacy of the G protein PLC pathway (downstream from receptors) as a consequence of the presence of the mGluR1a protein. We therefore examined the PLC transduction cascade of cells expressing the AT1 receptor alone or in combination with mGluR1a. The IP production induced by a saturating concentration of Sar-Ang-II (a derivative of angiotensin II that is less susceptible to degradation) and by a concentration close to the reported EC_{50} value of Sar-Ang-II was identical in cells expressing the AT1 receptor alone or in combination with mGluR1a (Fig. 8). The basal PLC activity in cells expressing mGluR1a and the AT1 receptor was, however, higher than that of cells expressing the AT1 receptor alone (Fig. 8).

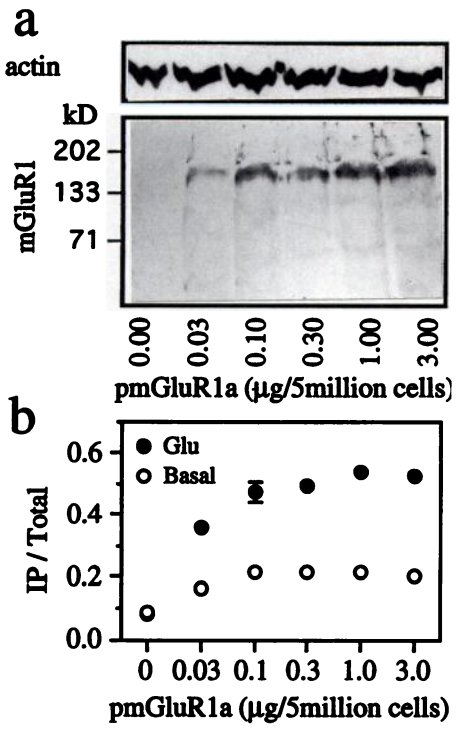

Fig. 6. Relationship between the level of expression of mGluR1a and the basal and Glu-induced IP productions. HEK 293 cells were transfected with various amounts of mGluR1a plasmid DNA. Membranes were prepared from half of the cells and subjected to SDS-PAGE. a, The mGluR1a (*bottom*) and actin (*top*) proteins were detected by specific antibodies after transfer onto nitrocellulose membrane. b, The other half of the cells were labeled with [^{3}H]inositol, and the basal and 1 mM Glu-induced IP productions were determined. Results are mean ± standard error of triplicate determinations.

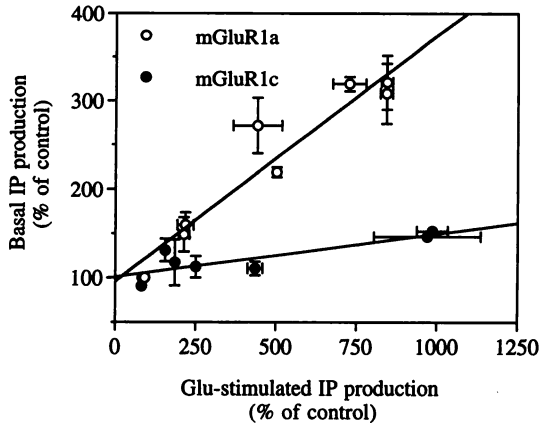

Fig. 7. Variation of the basal IP production as a function of the Glu-stimulated IP production in cells expressing different amounts of mGluR1a or mGluR1c. HEK 293 cells were transfected with various amounts of plasmid DNA containing the mGluR1a or mGluR1c cDNA (0, 1, 3, 10, 30, 100, 300, and 1000 ng/5 million cells). The IP production measured in the absence (*Basal IP production*) or in the presence (*Glu IP production*) of 1 mM glutamate were determined and expressed as the percentage of the basal IP production measured on mock-transfected cells. To avoid possible activation of the glutamate receptors by endogenous glutamate released from the cells, the basal IP production was determined in the presence of 2 units/ml glutamate-pyruvate transaminase plus 2 mM pyruvate. Results are mean ± standard error of triplicate determinations and are from a typical experiment.

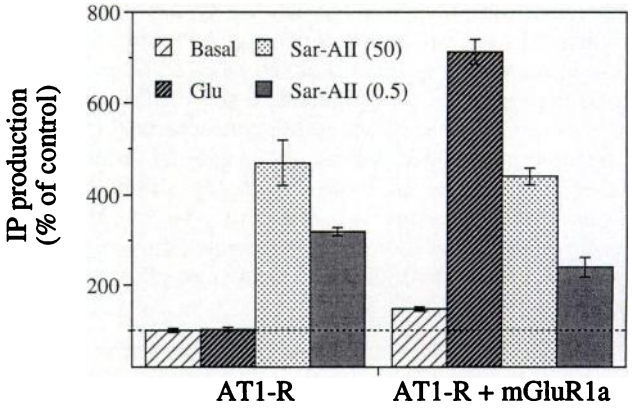

Fig. 8. Coupling of the AT1 receptor to PLC is not modified in the presence of mGluR1a. LLC-PK1 cells expressing the AT1-receptor (transfected with 50 ng plasmid DNA/5 million cells) alone (*AT1-R*) or in combination with mGluR1a (*AT1-R + mGluR1a*), were labeled overnight with [^{3}H]inositol. After the medium was washed and after a 10-min preincubation with LiCl, cells were stimulated for 30 min in the absence (*Basal*) or in the presence of 1 mM Glu, 50 nM Sar-AII, or 0.5 nM Sar-AII. The production of total IP was then determined. Results are expressed as percentage of the basal IP production measured in mock-transfected cells. Results are mean ± standard error of triplicate determinations from a typical experiment. a and b correspond to two distinct experiments.

Another possibility that we examined was that during the culture period after transfection, the glutamate concentration in the culture medium would be sufficient to activate the receptor and consequently modify cell physiology so that basal PLC activity is increased. This could be due, for example, to an increase in the amount of PLCγ or to a change in the subtype of PLCβ expressed in these cells. Indeed, we found that culture medium taken from cells 24 hr after transfection contained sufficient glutamate to activate mGluRs, an effect totally blocked by mGluR1a antagonists 4CPG or

4C3HPG (Fig. 9a). When cells were cultured in the continuous presence of these antagonists, the high basal PLC activity was still detected in cells expressing mGluR1a (Fig. 9b). This indicates that activation of mGluR1a by endogenous glutamate was not responsible for the increased basal PLC activity. However, the presence of the mGluR1a antagonists in the culture medium resulted in a significant increase in the Glu-induced IP production in cells expressing mGluR1a (Fig. 9b), suggesting that endogenous agonists were able to desensitize mGluR1a.

Discussion

Taken together, our results indicate that the long PLC-coupled mGluRs (mGluR1a, mGluR5a, and mGluR5b) but not the shorter variants (mGluR1b and mGluR1c) are slightly active even in the absence of agonist when expressed in LLC-PK1 or HEK-293 cells. This observation adds to the functional differences already reported between the short and the long PLC-coupled mGluR variants (36, 37, 44); these include faster electrophysiological responses in *Xenopus* oocytes (36, 37), and better activation of adenylyl cyclase in Chinese hamster ovary, baby hamster kidney, and LLC-PK1 cells (37, 44, 45) induced by the long compared with the short receptor isoforms. All of these functional differences could be explained if the short isoforms were expressed at a much lower density than the long isoforms. However, our previous experiments in *Xenopus* oocytes showed that mGluR1a did not generate slowly developing currents, even when expressed at low densities (36). Moreover, our current results indicate that the expression level of mGluR1c in HEK 293 cells is higher than that of the long isoform mGluR1a. Taken together, these results strongly suggest that the functional differences observed between the long and the short PLC-coupled mGluR isoforms do not result from a difference in their respective level of expression but rather from better coupling of the long isoforms to the G proteins. In agreement with this conclusion, mutations in the m3 muscarinic receptor that impair G protein coupling generate receptors that induce responses in *Xenopus* oocytes similar to those induced by the short mGluR1 isoforms (46). Moreover, a mutation in the β_2-adrenergic receptor that increases its agonist independent activity also increases its coupling efficiency (15).

In the large GPCR family, point mutations generating agonist-independent activity have been characterized. These mutations facilitate the understanding of the molecular basis for GPCR activation. Some of these mutations were localized within the transmembrane segments, as shown for rhodopsin (13) and the luteinizing hormone (16) and melanocyte-stimulating hormore receptors (18), and are thought to interfere with the conformational change induced by the agonists. Mutations of the domains involved in G protein activation, i.e., the third intracellular loop, can also result in a higher intrinsic activity of receptors (12, 14, 15, 17). Because small domains of GPCRs can alone directly activate G proteins (47–51), it has been proposed that the general structure of the receptor in the absence of agonists prevents this small domain from activating G proteins (12). In the case of PLC-coupled mGluRs, the G protein activation domain is probably located in the second intracellular loop (8, 9), which is therefore identical in both the long and the short variants of PLC-coupled mGluRs. Because inhibition of the intrinsic activity of mGluRs is stronger in the short receptors than in the long ones, the high intrinsic activity of the long receptors may be explained by partial removal of this inhibition by their long intracellular domain. This may explain why the long intracellular domain facilitates the coupling of the receptor to the G protein. Characterization of the segment within the long carboxyl-terminal domain of mGluR1a responsible for this property is under study.

It remains to be determined whether the agonist-independent activity of mGluR1a that we observed in transfected cells also occurs in neurons and whether it can be of physiological relevance. Recent data indicate that this may be the

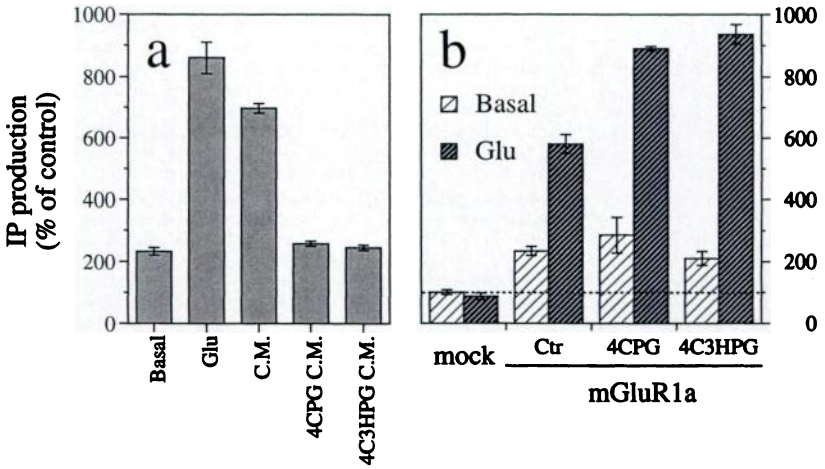

Fig. 9. Activation of mGluR1a during the culture by endogenous agonists is not responsible for the increase in basal PLC activity. a, LLC-PK1 cells expressing mGluR1a and labeled with [^{3}H]inositol were washed and then stimulated for 30 min in the absence (*Basal*) or in the presence (*Glu*) of 1 mM Glu, conditioned medium taken from cells expressing mGluR1a (*C.M.*), conditioned medium taken from cells cultured in the presence of 500 μM 4CPG (*4CPG C.M.*), or conditioned medium taken from cells cultured in the presence of 500 μM 4C3HPG (*4C3HPG C.M.*). Condition media were supplemented with LiCl (10 mM final concentration). b, Mock-transfected cells (*mock*) or cells expressing mGluR1a and cultured in control medium (*Ctr*), medium containing 500 μM 4CPG, or medium containing 500 μM 4C3HPG were washed several times and then stimulated for 30 min in the absence (*Basal*) or in the presence (*Glu*) of 1 mM Glu. The production of total IP was then determined. Data are expressed as percentage of the basal IP production measured in mock-transfected cells. Results are mean ± standard error of triplicate determinations from a typical experiment.

case. First, even though intrinsic activity of wild-type GPCRs in heterologous expression systems is often described at a high receptor density, it can also be detected at a low receptor density (50–300 fmol/mg) (23, 24). In the case of PLC-coupled mGluRs, the exact density in neurons is not known. However, because of their easy detection with specific antibodies compared with other GPCRs (52–55), they are likely expressed at a high density in some neurons, such as in Purkinje neurons for mGluR1a (52–54). This may be the case particularly in the postsynaptic element located in these small neuronal compartments that constitute the dendritic spines (52–55). Second, intrinsic activity of wild-type receptors has been described not only in heterologous expression systems but also in normal cells, by using inverse agonists (22, 28–33).

In conclusion, our current observations further suggest that the long carboxyl-terminal domain of the PLC-coupled mGluRs facilitates their coupling to the G proteins. However, the putative physiological relevance of the agonist-independent activity of mGluRs requires the characterization of inverse agonists.

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