Role of the Second and Third Intracellular Loops of Metabotropic Glutamate Receptors in Mediating Dual Signal Transduction Activation*

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(Received for publication, May 15, 1997, and in revised form, November 7, 1997)

On the basis of sequence homology and structural similarities, metabotropic glutamate receptors (mGluRs), extracellular Ca2+-sensing receptor, γ-aminobutyric acid type B receptor, and pheromone receptors are enlisted in a distinct family within the larger G protein-coupled receptor superfamily. When expressed in heterologous systems, group I mGluRs can activate dual signal transduction pathways, phosphoinositides turnover and cAMP production. To investigate the structural basis of these coupling properties, we introduced single amino acid substitutions within the second and third intracellular loops (i2 and i3) of mGluR1α. Wild-type and mutant receptors were expressed in human embryonic kidney 293 cells and analyzed for their capacity to stimulate both signaling cascades. Each domain appeared to be critical for the coupling to phospholipase C and adenyl cyclase. Within i2, Thr695, Lys697, and Ser702 were found to be selectively involved in the interaction with Gq class α subunit(s), whereas mutation of Pro698 and the deletion Cys694-Thr695 affected only Gs coupling. Furthermore, the mutation K690A profoundly altered mGluR1α signaling properties and imparted to the receptor the ability to couple to the inhibitory cAMP pathway. Within i3, we uncovered two residues, Arg777 and Phe781, that are crucial for coupling to both pathways, since their substitution leads to receptor inactivation.

Metabotropic glutamate receptors (mGluRs)1 are coupled to heterotrimeric G proteins, and through this interaction they regulate the intracellular level of second messenger molecules such as inositol trisphosphate and cyclic AMP (cAMP). Furthermore, they have been shown to modulate the activity of voltage-sensitive Ca2+ and K+ channels, G protein-regulated inward rectifier K+ channels, as well as GABA_A, α-aminoadipic acid-3-hydroxy-5-methylisoxazole-4-propionate, and N-methyl-D-aspartate receptors (1–2). Thus far, eight mGluRs (mGluR1 through mGluR8; see Refs. 3–10) have been cloned. They have been categorized into three groups according to their sequence homology, agonist selectivity, and main signal transduction pathway activated in heterologous systems (1, 11–12). Group I mGluRs (mGluR1 and -5) mobilize intracellular Ca2+ by stimulating phosphoinositide (PI) turnover and promote cAMP accumulation; group II (mGluR2 and 3) and group III (mGluR4, -6–8) receptors inhibit adenyl cyclase.

mGluRs, together with the parathyroid Ca2+-sensing receptors (PCaR1 (13)) and the GABA_B receptor (14), form a separate family within the seven transmembrane domain G protein-coupled receptor (GPCR) superfamily (15–17). mGluRs are characterized by a large amino-terminal extracellular domain that comprises the glutamate binding site (18–19). The intracellular loops connecting the putative membrane-spanning helices are also distinctive. In mGluRs, they are relatively small compared with those of seven transmembrane domain receptors belonging to the rhodopsin/β-adrenergic family. Most importantly, there is no significant sequence homology between members of the mGluR family and other cloned GPCRs.

The structure-function relationships supporting the coupling to G proteins have been well investigated for members of the rhodopsin/β-adrenergic family. Although all the cytoplasmic domains of these receptors take part in G protein activation to some degree, i3 appears to harbor the structural elements that impart specificity to the interaction (20–21).

However, much less is known about the structural determinants of the coupling of mGluRs to G proteins. Recently, the analysis of chimeric receptors derived from the Gi-coupled mGluR3 bearing different portions of the cytoplasmic domains of the Gi-coupled mGluR1 has shown that i2 of group I receptors is necessary, but not sufficient, for the specific activation of phospholipase C (PLC) and that both i2 and the cytoplasmic tail of the receptor appear to be also necessary for efficient coupling to this pathway (22–23). The finding that a point mutation in i3 of the human PCaR1 is responsible for loss of receptor function (24) also suggests that this domain could be important for mGluR-G protein interaction.

In heterologous systems, group I mGluRs have been shown to stimulate cAMP accumulation (25–26). However, the domains of the receptor and the signaling partners involved in the activation of this pathway have not been characterized.

In this work, we introduced single amino acid substitutions within i2 and i3 of mGluR1 to identify specific residues that contribute to the interaction with G proteins. To gain insights...
on the structural elements involved in selective interaction with either pathway, we tested the mutant receptors for their ability to induce PI turnover and cAMP accumulation. Here we show that both i2 and i3 play a role in determining selective coupling to Gα and Gβγ class α subunits and that mutations of single residues differentially affect the activation of the two transduction pathways.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by a modification of the polymerase chain reaction (PCR) technique as described by Nelson and Long (27). The oligonucleotides required for the mutagenesis (Ransom Hill Bioscience, Ramona, CA; Table I) consisted of the following: (a) forward mutagenic primers containing single/multiple base mismatches; (b) reverse hybrid primer serving as 3′-anchor, harboring at the 5′-end 20–30 nucleotides randomly selected and unique and not complementary to the mGluR1α sequence and, at the 3′-end, 21 complementary nucleotides; (c) forward 5′-anchor primer; (d) reverse complementary hybrid primer (c-primer) identical to the random 20–30 nucleotides. All polymerase chain reaction reactions were carried out in a 50-μl reaction volume containing 200 μM deoxynucleotides and 2.5 mM MgCl₂; 1× PCR buffer, 0.25 μM each primer, 0.025 μM each deoxynucleoside triphosphate, 2.5 U Taq polymerase (Boehringer Mannheim). In the Step 1 polymerase chain reaction reaction, 50 pmol each of mutagenic and hybrid primer were used with the template pBluescriptSK+ (pRC-CMV vector; Invitrogen, San Diego, CA) to yield an amplified product comprising the mutation and the unique sequence. Step 1 was 20 cycles of 1 min at 94 °C denaturation, 3 min at 37 °C annealing, and 3 min at 74 °C elongation. The amplified DNA was fractionated on agarose gel, purified, and used together with pBluescriptSK+ in the Step 2 reaction. Step 2 was a single cycle of 5 min at 94 °C, 2 min at 37 °C, and 10 min at 74 °C. At Step 2 completion, 50 pmol each of the 5′-anchor and c-hybrid primer was added, and 30 additional cycles were completed (Step 3). Step 3 products were digested with BglII and SphI for all mutants but i2* (Table I), which were digested with NcoI and SphI and cloned into pBS-mGluR1α (pBlueScriptSK+ vector; Stratagene, San Diego, CA). The sequence of the amplified DNA was determined by the Sanger method (Sequenase, U. S. Biochemical Corp./Amersham Life Science, Inc.). Mutant receptors were subcloned from the pBlueScript construct into pRC-CMV by digestion with SacII and ApaI (i2 and i2* mutants) or SacII and BspEI (i3 mutants).

Expression in HEK 293 Cells—HEK 293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected by electroporation with an Electro Cell Manipulator (BTX, San Diego, CA) using 4-mm gap cuvettes. The DNA used in transfection experiments was purified with Qiagen columns (Qiagen, Chatsworth, CA) according to the procedure recommended by the manufacturer. In a standard experiment, cells were harvested by trypsinization and washed 3 times with PBS; for each electroporation 5 or 10 million cells were used with either 10 or 20 μg of DNA (according to the purpose of the experiment) in 0.3-ml final volume. Electroporations were conducted at 230 V, 950 microfarads, 72 Ω, yielding 20 ms as average time constant. The transfection efficiency, about 30%, was determined by co-transfection (1:1 ratio) with the pRc-lacZ vector and subsequent chromogenic reaction. All the tissue culture reagents were purchased from Life Technologies Inc. HEK 293 were generously provided by Dr. M. Chao at Cornell University Medical College.

Inositol Phosphates Accumulation—Following electroporation, cells were left to recover in DMEM supplemented with 10% dialyzed FBS and 2 mM GlutaMAX™ (Life Technologies Inc.) for 3–5 h and metabolically labeled for approximately 16 h with 1 μCi/ml [3H]myo-inositol (NNL Life Science Products) in isositol-free DMEM. Cells were washed with PBS and incubated for 2 h with 2 mM pyruvate and 2 units/ml glutamic-pyruvic transaminase (Sigma) (28). After washing with PBS, cells were incubated for 10 min in Hank's balanced saline solution containing 10 mM LiCl; 1 mM Glu (Sigma) was then added for 20 min. Agonist stimulation was stopped by placing the cells on ice and adding 5% ice-cold trichloroacetic acid. Separation of total [3H]inositol phosphates was carried out as described (29) by chromatography on AG 1-X8 anion-exchange resin (Bio-Rad); total inositol phosphates were left to recover in DMEM supplemented with 10% dialyzed FBS and 2 mM GlutaMAX™, and preincubated for 30 min with Hanks' balanced saline solution containing 1 mM 3-isobutyl-1-methylxanthine (Sigma). 1 mM Glu in the presence of 3-isobutyl-1-methylxanthine was then added for 20 min. Cells were lysed with ice-cold 5% trichloroacetic acid, and metabolically labeled for approximately 16 h with 1 μCi/ml [3H]myo-inositol (NNL Life Science Products) in isositol-free DMEM. Cells were washed with PBS and incubated for 2 h with 2 mM pyruvate and 2 units/ml glutamic-pyruvic transaminase (Sigma) (28). After washing with PBS, cells were incubated for 10 min in Hank's balanced saline solution containing 10 mM LiCl; 1 mM Glu (Sigma) was then added for 20 min. Agonist stimulation was stopped by placing the cells on ice and adding 5% ice-cold trichloroacetic acid. Separation of total [3H]inositol phosphates was carried out as described (29) by chromatography on AG 1-X8 anion-exchange resin (Bio-Rad); total inositol phosphates were left to recover in DMEM supplemented with 10% dialyzed FBS and 2 mM GlutaMAX™. Cells were then washed with PBS, treated with glutamic-pyruvic transaminase for 2 h (see above), and preincubated for 30 min with Hanks' balanced saline solution containing 1 mM 3-isobutyl-1-methylxanthine (Sigma). 1 mM Glu in the presence of 3-isobutyl-1-methylxanthine was then added for 20 min. Cells were lysed with ice-cold 5% trichloroacetic acid, and adenine nucleotides were fractionated according to the method of Salomon (30). Alternatively, after stimulation with Glu, unlabeled cells were lysed with 65% ethanol, 4 mM EDTA, and cAMP concentration was determined by the cyclic AMP [3H] assay system (Amersham Life Science Inc.)

### Table I

| Primer | Orientation | Position | Sequence |
|--------|-------------|----------|----------|
| R696A | Forward | 2062-2081 | 5′-GGCCAGCCGAAGAGAGTCTG-3′ |
| Cys694-Thr695 | Forward | 2062-2100 | 5′-GGCCAGAGAAAGAGATCCGG-3′ |
| Cys694-Thr695/K692G | Forward | 2059-2100 | 5′-GGCTGGCAGCAAGAGGGGATCG-3′ |
| T695A | Forward | 2077-2097 | 5′-ATCTCGCAGCCGGAGGAGCAAGA-3′ |
| T695E | Forward | 2077-2097 | 5′-ATCTCGCAGCCGGAGGAGCAAGA-3′ |
| R696G | Forward | 2072-2097 | 5′-AGAAGATGCTGACCGGGAGAAAAGC-3′ |
| K697M | Forward | 2086-2105 | 5′-CCAGATCGCAGCCGGAGGAGCAACCGCTGAC-3′ |
| P689R | Forward | 2072-2102 | 5′-GAATGCTGACCGGGAGGAGCAACCGCTGAC-3′ |
| S702R | Forward | 2101-2120 | 5′-ATGAGATCGTGGCCCAAGTA-3′ |
| S702T | Forward | 2100-2120 | 5′-CATGAGATCGTGGCCCAAGTA-3′ |
| A705S | Forward | 2111-2131 | 5′-GGCCAGATGATCAATGCTGATC-3′ |
| R775W | Forward | 2341-2364 | 5′-CCTGAGAATCGTCCGGCCAACAATTCA-3′ |
| R775K | Forward | 2317-2341 | 5′-AGAAGACCAAGTGCTGGCCGACACT-3′ |
| P778Q | Forward | 2327-2349 | 5′-ACGTCAGGCGCAAGTTCAATGAG-3′ |
| A779E | Forward | 2333-2352 | 5′-GGCCAGATCTTCATGAGGGAGCT-3′ |
| F781P | Forward | 2337-2357 | 5′-ACGTCAGGCGCAAGTTCAATGAG-3′ |
| N782I | Forward | 2337-2360 | 5′-ACGTCAGGCGCAAGTTCAATGAG-3′ |
| E783Q | Forward | 2341-2360 | 5′-TTCAATATGGCTAAATTCAT-3′ |
| Hybrid | Reverse | 3072-3092 | 5′-GAGATGATGATATGATGATGATGATG-3′ |
| 5′-Anchor I | Forward | 1807-1826 | 5′-GGCCATCTGCTGCAGGCTTGT-3′ |
| 5′-Anchor II | Forward | 1123-1142 | 5′-GGCCATCTGCTGCAGGCTTGT-3′ |
| c-Hybrid | Reverse | 1123-1142 | 5′-GGCCATCTGCTGCAGGCTTGT-3′ |
Mutational Analysis of mGluR-G Protein Coupling

RESULTS

Receptors belonging to the rhodopsin/β-adrenergic family are characterized by the presence of short amphipathic α-helices at the amino and carboxyl termini of their third intracellular loop. Amino acids within these microdomains play a key role in determining selective coupling to heterotrimeric G proteins (20–21). Unlike these GPCRs, mGluR1 i3 is small, non-homologous, and well conserved among all members of the family, despite their different coupling properties (Fig. 1a).

To evaluate the contribution of single amino acid residues in i3 of mGluR1 to G protein coupling, we mutagenized Arg775 (R775W and R775K), corresponding to Arg796 in PCaR1; Pro778 (P778Q); Phe781 (F781P and F781S); Asn782 (N782I); and Glu783 (E783Q). These residues are conserved among all metabotropic and calcium-sensing receptors and were substituted with amino acids designed to change the charge distribution of the loop (Fig. 1b).

The only residue in i3 that distinguishes receptors coupled to different signal transduction pathways is Ala779, which is conserved in group I mGluRs, whereas Glu is present in groups II and III receptors. Since this seems to suggest that the amino acid at this position could participate in coupling selectivity, we replaced Ala779 with Glu (A779E).

Within i2 (the largest intracellular loop in mGluRs) only few amino acids are in common between groups I and II/III receptors (Fig. 1c). The regions of homology are concentrated at the amino and carboxyl termini of the domain, which are predicted to adopt α-amphipathic conformation. The intervening stretch

2 Developed at the National Institutes of Health and available by anonymous ftp from zippy.nimh.nih.gov.

Fig. 1. a, schematic representation of mGluR1α (adapted from Ref. 1). The first 20 amino acid residues corresponding to the signal peptide are underlined. The putative extracellular glycosylation sites are indicated by Y; the putative intracellular phosphorylation sites are indicated by *.

The filled circles correspond to second (i2) and third (i3) intracellular loops, b and c, sequence alignment of i3 and i2 of mGluRs and PCaR1. Residues that are shared by all mGluRs are boxed in black or in white if the conservation among members of the family is not perfect. Residues that are shared only by group I receptors (mGluR1 and mGluR5) are in bold letters. The positions where amino acid substitutions were introduced are indicated above the aligned sequences.

For immunofluorescence and Immunoblot Analysis—For immunofluorescence studies, electroporated cells were collected by centrifugation, and lysed in alkaline sample buffer (0.12 M Tris base, pH 9.5, 3.3% sodium dodecyl sulfate, 10% glycerol, 3.1% dithiothreitol, and 0.004% bromphenol blue). Total proteins were quantified with the Bio-Rad Protein Assay System (Bio-Rad) according to the protocol indicated by the manufacturer, and the membranes were washed, mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA), and analyzed using an Arcus II scanner (AGFA), and densitometric analysis was performed with the software package NIH-Image 1.60.

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of residues is highly hydrophilic and therefore constitutes a potential protein interaction site. Previous studies conducted with chimeric receptors showed that the 16-amino acid region from Lys691 to Gln706 is required to impart the ability to activate PLC to Gi-coupled mGluRs (22–23). We mutagenized residues within this region that, according to sequence alignment, are conserved among all mGluRs (Lys690, Pro698, and Ser702) and residues that are shared only by PLC-coupled receptors (Lys692, Thr695, Arg696, Lys697, and Ala705) (Fig. 1c). Among them, the positively charged amino acids that are part of the central hydrophilic region of i2 were substituted with hydrophobic residues to perturb the charge distribution. Group I mGluRs also differ from group II and III in the size of i2 (26 versus 24 amino acids). To test whether such a difference is structurally relevant for G protein coupling, we generated two deletion mutants. In the Cys694-Thr695 mutant, Cys694 and Thr695, which are shared only by group I receptors and are located in the center of i2, were deleted. In the K692G/Cys694-Thr695 mutant, the same deletion was accompanied by the substitution of Lys692 to Gly; this substitution reduces the number of positively charged residues located in the amino terminus of i2, thus increasing the structural similarity of the mutant mGluR1 with group II and III receptors.

Receptor-mediated Stimulation of PI Turnover—To assess the ability of the mutant receptors to activate the PLC signaling cascade, we measured the accumulation of inositol phosphates (IPs) upon stimulation with Glu in HEK 293 cells. Wild-type receptor activation induces a 3–4-fold increase of IP production compared with basal level (not-stimulated cells); the specificity of this response is confirmed by the absence of effect of Glu in cells transfected with the reporter construct pRe-lacZ alone (Fig. 2a). Consistent with recent reports, mGluR1α expression generates a high basal level of inositol phosphates; this effect, distinctive to the long splice variant of the receptor, has been attributed by other investigators to intrinsic (agonist-independent) activity (28).

In HEK 293 cells, mGluR1α-mediated stimulation of the PLC pathway is insensitive to pertussis toxin (PTx) (data not shown). In contrast, both in Xenopus oocytes and Chinese hamster ovary (CHO) cells, the receptor activates the same signaling pathway through PTx-sensitive and -insensitive G proteins, Gα11, respectively (25, 33–34). Since HEK 293 cells do not express Gα11, PLC stimulation is likely to depend solely on the interaction of the receptor with Gα subunits of the Gq family.

Upon analysis of receptors mutated in i3, we observed that the Glu-stimulated IP accumulation is suppressed in the R775W, F781S, and F781P mutants (Fig. 2a) and significantly reduced in the P778Q. The R775W substitution introduced in rat mGluR1 corresponds to the R796W mutation in the human PCaR1 linked to familial hypocalciuric hypercalcemia (24); such mutation causes loss of function of the human receptor. In an analogous fashion, the same substitution in mGluR1α leads to loss of the Glu-dependent PLC stimulation but does not affect the high basal activity of the receptor. Notably, substitution of Arg775 with Lys (R775K) produces the opposite effect; this mutation generates a receptor that retains wild-type-like Glu-dependent activity but lacks intrinsic activity.

Substitution of Phe781 with either a polar (F781S) or a hydrophobic residue (F781P) completely blocks receptor-medi-
ated activation of the PLC pathway, both Glu-dependent and constitutive. In contrast, the mutations A779E, N782I, and E783Q do not alter significantly the Glu-dependent activity of the receptor. However, both the N782I and the E783Q mutants do not possess agonist-independent basal activity.

i2 mutant receptors carrying the substitutions T695A, R696G, P698R, S702T, and A705S do not differ from wild type in stimulating PI turnover (Fig. 2b), although a significantly higher level of basal activity is observed in the P698R mutant. On the other hand, the mutations K690A, T695E, K697M, and S702R cause a significant decrease in Glu-stimulated IP production. In addition, with the exception of K690A, these receptors show a significantly reduced intrinsic activity. None of the mutations introduced in i2 results in a nonfunctional receptor.

Receptor-mediated Stimulation of the cAMP Pathway—Wild-type and mutant receptors were tested for their ability to induce cAMP accumulation in HEK 293 cells. The endogenous adenylyl cyclase isoform in this cell line has not been identified yet; however, it is known to be Gs and forskolin-activated but not sensitive to Ca2+, protein kinase C, or βγ stimulation (36).
Mutational Analysis of mGluR-G Protein Coupling

Upon stimulation with Glu, mGluR1α induces a small but significant increase in cAMP (Fig. 3, a and b). The properties of the endogenous cyclase make it likely that such effect is generated through direct activation of Gs, although with low efficiency. This was ascertained by co-transfecting the wild-type mGluR1α together with increasing amount of the cDNA coding for Gs\(_{\alpha}\), which resulted in a proportional increase of Glu-induced mGluR1α-mediated cAMP accumulation (Fig. 3a).

Next, we tested for cAMP accumulation those i2 mutants that are affected in their ability to stimulate PI turnover. The parallel analysis of two separate signaling cascades should help determine whether the mutations affect selectively the coupling to the Gs\(_{\alpha}\) class \(\alpha\) subunits or whether they cause a general decrease in the affinity of the activated receptor for G proteins. All the mutants tested, except K690A, have activities comparable with wild type (Fig. 3b). Remarkably, the K690A mutant shows a much increased basal level and Glu-dependent stimulation of cAMP; this is likely to result from an increased affinity for Gs\(_{\alpha}\).

However, since the level of cAMP production is generally quite small, it is possible that some change in the efficiency of coupling could go undetected. To circumvent this possible problem we have increased the basal level of cyclase to amplify the coupling could go undetected. To circumvent this possible problem we have increased the basal level of cyclase to amplify the action of the mGluRs to be more easily detected. Expression of the K690A mutant to inhibitory G proteins. Properties of the K690A Mutant—From the characterization of the K690A mutant activity profile, it became clear that Lys690 is critical for coupling to both Gs\(_{\alpha}\) and Gq\(_{\alpha}\). In particular, in the K690A mutant the interaction with Gq\(_{\alpha}\) is weakened, whereas that with Gs\(_{\alpha}\) is strengthened compared with wild type.

It has been suggested that mGluR1α could interact with PTx-sensitive G protein(s) coupled to inhibition of adenylyl cyclase in CHO cells (25). Since the K690A substitution apparently changes the affinity of the receptor for at least two different G proteins, we examined the possibility that the same mutation could enable the receptor to interact with members of the Gi family. To this end, we co-transfected the β2-adrenergic receptor (20) with either mGluR1α, mGluR2, or K690A cDNAs in HEK 293 cells. β2-Adrenergic receptor potently stimulates adenylyl cyclase via coupling to Gi\(_{\alpha}\), allowing any inhibitory action of the mGluRs to be more easily detected. Expression of mGluR2, which is used as control for effective inhibition of adenylyl cyclase (6), induces in this experimental paradigm about 30% inhibition of cAMP accumulation (Fig. 5a). Although mGluR1α has no effect, the K690A mutant is able to inhibit cAMP accumulation with an efficiency comparable to mGluR2 (Fig. 5a). This effect is blocked by preincubating the transfected cells with PTx before stimulation with Glu (Fig. 5b), whereas no significant change is observed in the wild-type receptor (data not shown). This finding indicates a direct coupling of the K690A mutant to inhibitory G proteins.

Expression Level and Localization of mGluR1α and Mutant Receptors—To verify that the effects brought about by the amino acid substitutions on receptor effector-function are not due to changes in the synthesis and/or localization of the protein, we analyzed the expression level and distribution of wild-type and mutant receptors, transfected in HEK 293 cells, by

![Graph showing Receptor-mediated stimulation of cAMP accumulation in HEK 293 expressing type II adenylyl cyclase.](image)

**Fig. 4.** Receptor-mediated stimulation of cAMP accumulation in HEK 293 expressing type II adenylyl cyclase. cAMP levels upon induction with 1 mM Glu are expressed as fold increase over nonstimulated cells. Results are the mean ± S.E. of at least two independent experiments performed in duplicate (*p ≤ 0.05; **p ≤ 0.01; t test, two-tailed).
in the syntheses of wild-type and mutant receptors, we trans-
sected HEK 293 cells with an adequate amount of the corre-
sponding DNAs (2.5 μg) to obtain a level of protein expression
within the linear range of increase (Fig. 6b). This analysis does
not show any significant difference in the level of synthesis of
the mutant receptors tested compared with the wild type.

The correct localization at the plasma membrane of
mGluR1α and mutant receptors was validated by immunoflu-
orescence combined with laser scanning confocal microscopy.
Representative mutants are shown in Fig. 6c. None of the
introduced substitutions or deletions appear to affect the sort-
ing and overall structure of the receptor protein, as can be
inferred by the correct targeting to the plasma membrane.

**DISCUSSION**

Physiological and pharmacological studies have shown that
mGluRs activate multiple second messenger cascades, inhibit
neurotransmitter release, and regulate K+ and Ca2+ channel
activity (1, 38, 39). These multi-signaling effects are thought to be mediated by multiple mGluRs. In addition, it is possible that
some receptors have the capacity to interact with several dif-
ferent subsets of G protein α subunits (3–4, 25). Furthermore,
the associated βγ subunits could also contribute to specific
signaling by regulating channel opening (40), different iso-
forms of adenyl cyclase (36, 41), and PLC (42, 43). Hence, the
final output of mGluR activation in the central nervous system
could also depend on the repertoire of signaling molecules
available in the vicinity of the receptor. This could be particu-
larly important in neurons, which can undergo activity-dependent
changes in dendritic and axonal compartments.

In neurons, group I mGluRs have been shown to stimulate
local intracellular calcium release via the PLC cascade and
modulate other biochemical pathways and channel activities.
This results from the stimulation of protein kinase C following
the release of diacylglycerol. Although there is, thus far, no
unequivocal demonstration for the direct involvement of group
I mGluRs in the activation of the cAMP stimulatory pathway
in vivo, evidence for the activation of the cAMP stimulatory
pathway in cortical neurons following both group I and group
II receptor activation has been presented (44–45). This discrep-
ancy with studies conducted on receptors expressed in hetero-
logous systems could be due to the paucity of pharmacological
tools capable of discriminating selectively group I receptors.

Here, we focused on the signaling properties of mGluR1α in
a heterologous system, HEK 293 cells. In these cells we have
demonstrated that the receptor activates at least two different
signaling cascades, the PLC pathway (via interaction with Gq)
and the stimulatory cAMP pathway (via interaction with Gs).
Recently several other GPCRs, such as α2-adrenergic receptors,
muscarinic receptors, and the parathyroid hormone (PTH)/
PTH-related peptide receptor, have been shown to possess dual
or multiple effector functions. There is now accumulating evi-
dence that this multi-signaling activity is due to coupling to
different subsets of G protein α subunits, sometimes leading to opposite
effects, for example Gs and Gi as in the case of the α2-adrener-
getic receptor (46). In several instances, the structural domains
involved in differential coupling have been partially uncovered;
however, it is not yet possible to draw general rules from these
studies. For example, in the case of the PTH/PTH-related pep-
tide receptor, some residues within the amino terminus of i3
appear to be independently involved either in PLC or adenyl-
cyclase stimulation, whereas mutation of other residues affects
both cascades (47). On the other hand, in the case of the
α2-adrenergic receptor, both i2 and the amino terminus of i3
are involved in stimulating cAMP production, and the deter-

Western blot and immunofluorescence. In immunoblots,
the polyclonal antibody M5-14 detects selectively the receptor pro-
tein, thus yielding a single band of 145 kDa estimated mole-
cular mass, as predicted for mGluR1α (Fig. 6a). The expression
level of the receptor increases in linear fashion with the
amount of DNA introduced into the cells. To detect variations

**FIG. 5.** a, Glu-induced inhibition of cAMP accumulation by mGluR2 and
K690A mutant receptor in HEK 293 cells. Cells were co-transfected
with the β2-adrenergic receptor (β2-AR) and mGluR1α, mGluR2, or the
K690A receptor. After 30 min incubation with 3-isobutyl-1-methylxan-
thine, the transfected cells were incubated for a further 20 min in the
same medium (basal level), with 10 μM (-)-isoproterenol, the transfected cells were incubated for a further 20 min in the

**cAMP/ATP**

**deviation from the mean.** cAMP accumulation is expressed as the
obtained in another independent experiment.

Shown is a typical assay performed in duplicate; similar results were

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minants of coupling to G_i are located in different regions of i3 (48, 49).

Since the mGluRs bear no sequence homology with the other GPCR families, their study, together with that of other GPCRs, will unravel conserved structural features involved in coupling to G proteins. By mutating amino acids that are shared by all mGluRs, we have identified two residues in i3, Arg^775 and Phe^781, that are required for productive coupling to both the PLC and stimulatory cAMP pathway. The finding that the R775W substitution can be “rescued” by replacing Arg^775 with another positively charged residue points to a requirement for a positive charge at this position. A more dramatic phenotype is obtained by substituting Phe^781 with either hydrophobic or polar residues; both mutations cause complete loss of function of the receptor. This finding suggests that Phe^781 is a key residue at the surface of interaction between receptor and G proteins. Together these observations indicate that i3 could play a pivotal role in providing a site for interactions with both G_q and G_s. On the other hand, two other mutants in i3 appear to operate independently in their ability to couple to G proteins.

**Fig. 6. Expression level and localization of mGluR1α, i2, and i3 mutant receptors in HEK 293 cells.**

(a) Western blot analysis of total protein lysate (approximately 20 μg per lane) from HEK 293 cells transfected with increasing amount of mGluR1α cDNA (the total DNA amount (20 μg) used in the transfection was held constant with carrier). Lane 1, untransfected cells; lanes 2-6, 0.5, 1, 2.5, 5, and 10 μg, respectively, of pRc-mGluR1α. The inset shows the quantification of mGluR1α expression level; arbitrary units represent the ratio between the optical density of mGluR1α-specific signal on autoradiographic film and that of the proteins transferred on the blot and visualized with india ink. 

(b) Relative expression level of wild-type, i2, and i3 mutant receptors as determined by Western blot analysis. 2.5 μg of each mutant and wild-type DNAs were transfected in HEK 293 cells; total protein lysate (20 μg) was used for immunoblot analysis. Quantification of the expression level was conducted as described above. Results represent mean ± S.E. of at least two independent experiments. 

(c) Transfected cells were immunolabeled with an antibody directed against mGluR1α and then analyzed by confocal microscopy. Untransfected cells have a weak background florescence.
as follows: Pro\textsuperscript{778} seems to be important for a full Glu-dependent \(G_s\) stimulation, and Asn\textsuperscript{782} is required for a full activation of the stimulatory cAMP pathway.

Our study has uncovered several residues within i2 that are selectively involved in either the PLC pathway or the stimulatory cAMP pathway, suggesting the existence of two independent G protein-specific interaction domains. One example is the deletion of Cys\textsuperscript{894} and Thr\textsuperscript{895}. These residues are shared only by group I receptors and are located in the center of i2, between two predicted α-helical regions at the amino and carboxyl termini of this loop. They appear to be necessary to support coupling to \(G_s\) but not to \(G_q\). Interestingly, the K690A substitution in i2 generates a receptor that is less effective than wild type in activating \(G_s\) but more effective in activating \(G_q\). This suggests that Lys\textsuperscript{869} could be located at a position critical for both \(G_s\) and \(G_q\) coupling. Searching for common motifs shared by mGluR1α i2 and \(G_s\)-coupled GPCRs, we found an interesting homology between the amino terminus of i2 and the equivalent region. Because inverse agonists are not yet available, the physiological relevance of the constitutive activity of mGluR1α in neurons cannot be assessed.

Taken together, these findings suggest that for mGluRs, as for other GPCRs, more than one domain is required to form the surface for the interaction with G protein α subunits. This improved understanding of mGluR-G protein interaction will provide a valuable paradigm to analyze the coupling properties of GABA\(_B\) receptors, the recently discovered pheromone receptors (52–53), and possibly other related receptors yet to be discovered.

Acknowledgments—We thank Drs. A. A. Hiran, L. R. Levin, J. A. Wagner, and J. G. Connolly for critical reading of the manuscript; Dr. J. P. Pin for having provided the mGluR1α clones and for sharing unpublished data. The mGluR2, \(G_\alpha\)-adrenergic receptor, and adenyl cyclase type II clones were kindly donated by Drs. S. Nakanishi and L. R. Levin. We also thank Dr. X.-Y. Huang for providing the \(G_s\) clone. The mGluR1α antibody was a generous gift from Dr. C. Romano.
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