A Cluster of Basic Residues in the Carboxyl-terminal Tail of the Short Metabotropic Glutamate Receptor 1 Variants Impairs Their Coupling to Phospholipase C*

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Among phospholipase C-coupled metabotropic glutamate receptors (mGluRs), some have a surprisingly long carboxyl-terminal intracellular domain (mGluR1a, -5a, and -5b), and others have a short one (mGluR1b, -1c, and -1d). All mGluR1 sequences are identical up to 46 residues following the 7th transmembrane domain, followed by 313, 20, 11, and 26 specific residues in mGluR1a, mGluR1b, mGluR1c, and mGluR1d, respectively. Several functional differences have been described between the long isoforms (mGluR1a, -5a, and -5b) and the short ones (mGluR1b, -1c, and -1d). Compared with the long receptors, the short ones induce slower increases in intracellular Ca\(^{2+}\), are activated by higher concentrations of agonists, and do not exhibit constitutive, agonist-independent activity. To identify the residues responsible for these functional properties, a series of truncated, chimeric, and mutated receptors were constructed. We found that the deletion of the last 19 carboxyl-terminal residues in mGluR1c changed its properties into those of mGluR1a. Moreover, the exchange of the long carboxyl-terminal domain of mGluR5a with that of mGluR1c generated a chimeric receptor that possessed functional properties similar to those of mGluR1c. Mutagenesis of specific residues within the 19 carboxyl-terminal residues of mGluR1c revealed the importance of a cluster of 4 basic residues in defining the specific properties of this receptor. Since this cluster is part of the sequence common to all mGluR1 variants, we conclude that the long carboxyl-terminal domain of mGluR1a suppresses the inhibitory action of this sequence element.

Although they possess 7 transmembrane domains, the G-protein-coupled metabotropic glutamate receptors (mGluRs)\(^1\) (1–3), Ca\(^{2+}\)-sensing (4), and γ-aminobutyric acid, type B (GABA\(_B\)) receptors (5) share no sequence homology with any other G-protein-coupled receptors (GPCRs) and constitute therefore a distinct family of receptor proteins. Whereas the agonist binding site is located within a hydrophobic cleft formed by the 7 transmembrane domains in most GPCRs, it is located within the large extracellular domain homologous to bacterial periplasmic binding proteins in this receptor family (6, 7). Moreover, the second intracellular loop of mGluRs likely plays a role equivalent to that of the third intracellular loop of the other GPCRs for G-protein coupling and activation (8–10).

Among the mGluR subtypes cloned so far, three that are coupled to phospholipase C (PLC), mGluR1a and the two splice variants mGluR5a and mGluR5b, possess a surprisingly long (350 residues) carboxyl-terminal intracellular domain (11–15). The role of this domain is not yet fully characterized, but it may be involved in specific regulation of the receptor function, possibly by interacting with specific proteins (16, 17). Several splice variants have been isolated for mGluR1 that differ in the length of their intracellular tail (18–21). In the rat mGluR1b, mGluR1c, and mGluR1d, the 313 carboxyl-terminal residues of mGluR1a are replaced by 20, 11, and 26 residues, respectively. All these splice variants do couple to PLC indicating that the long carboxyl-terminal domain of mGluR1a is not critical for this function of the protein. Differences in PLC coupling have been reported between the long forms, mGluR1a, -5a, and -5b, and the short forms, mGluR1b, mGluR1c, and mGluR1d. In contrast to the long forms, which generate fast chloride current responses upon agonist application when expressed in Xenopus oocytes, mGluR1c induces slowly developing responses (14, 18), and in baby hamster kidney (BHK) cells stably expressing mGluR1b, glutamate (Glu) induced slower Ca\(^{2+}\) responses than in cells expressing mGluR1a (22). Moreover, only the long forms display a significant constitutive, agonist-independent activity when expressed in human embryonic kidney (HEK) 293 cells (14, 23, 24), and mGluR1a possesses a higher apparent affinity (EC\(_{50}\)) for agonists than the short mGluR1 isoforms (25, 26). Accordingly, it has been proposed that the long carboxyl-terminal domain enables better PLC coupling efficacy (25, 27).

To identify the sequence responsible for the specific functional properties of mGluR1 splice variants, a series of truncated and chimeric receptors were constructed and analyzed both in Xenopus oocytes and HEK 293 cells. This approach allowed us to identify a basic tetrapeptide in the carboxyl-terminal end of these receptors that confers to the short variants mGluR1b, -c, and -d their specific PLC-coupling properties, i.e. slow responses in oocytes, lower potency of agonists, and absence of constitutive activity. Since this sequence is

kidney; PCR, polymerase chain reaction; IP, inositol phosphate; LLC-PK1, porcine kidney epithelial; PBS, phosphate-buffered saline.
conserved in mGluR1a, we propose that the apparent inhibitory action of this basic tetrapeptide is prevented by the presence of the long carboxy-terminal tail of this receptor.

**MATERIALS AND METHODS**

**Construction of Truncated Receptors**—The obtention of mGluR cDNAs and the construction of the eucaryotic expression plasmids has been described previously (14, 18, 21). To generate mGluR1Δ1139 and mGluR1Δ11093, polymerase chain reactions (PCR) were performed using mGluR1a cDNAs as template (20 ng), dNTP (200 μM), 2 units of Vent DNA polymerase (New England Biolabs), and 100 pmol of sense and antisense primers in a 100-μl reaction made with the 10 × buffer supplied by the manufacturer. The PCR amplification was performed for 30 cycles of 1 min at 95 °C, 1 min at 50 °C, and 2 min at 72 °C. The sense primer was 5′-CCG TGG CCC TGG GGT GC-3′, and the antisense primers containing the additional stop codon and an XhoI site located downstream of the second

The basal IP formation was determined after a 30-min incubation in the presence of 10 mM LiCl. The Glu degrading enzyme glutamate pyruvate transaminase (1 unit/ml) and 2 mM pyruvate were also added to avoid the possible action of Glu released from the cells. Results are expressed as the amount of IP produced over the radioactivity present in the membranes. The dose-response curves were fitted according to the equation

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\frac{y_m}{y_max} (EC_{50}) + y_2\). The IP was expressed as the concentration of agonist giving a response equal to 50% of the maximum, \(y_m\), and \(y_{max}\) correspond to the maximal and minimal values, and \(y_{H}\) is the Hill coefficient, using the Kaleidagraph program.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—HEK 293 cell membranes were prepared as described previously (23).

**Immunofluorescence of Transfected Cells**—The generation and characterization of the mGluR1 antibody (generous gift of Drs. V. Matarrese and F. Ferraguti, Glaxo, Verona, Italy) raised against a chimeric protein containing part of the extracellular domain has been described previously (29). Eighteen hours after transfection, HEK 293 cells grown on coverslips were washed three times with PBS, fixed for 20 min at room temperature in 4% paraformaldehyde in PBS, and washed three times in PBS. The cells were then incubated for 1 h at room temperature in PBS containing 3% bovine serum albumin and rabbit anti-mGluR1 (1:250). Cells were washed in PBS containing 3% bovine serum albumin, and bound primary antibodies were detected with a fluorescein isothiocyanate mouse anti-rabbit secondary antibody (1:50; Sigma, L’Ile d’Abeau, France) for 45 min at room temperature. Cells were washed, and the coverslips were mounted with Mowiol 4.88 and visualized with a Zeiss (Axiopt) microscope.

**Statistical Analysis**—Statistical differences were examined using the Stat-View Student program (Abacus Concept, Berkeley, CA) using t test or analysis of variance (Fisher’s LSD test).

**RESULTS**

As shown in Fig. 1, several functional differences were observed between the long mGluR1 isoform mGluR1a and the short variant mGluR1c in agreement with our previous studies (18, 23, 25). When expressed in Xenopus oocytes, mGluR1a induced faster Chloride currents than mGluR1c upon activation with Glu (Fig. 1a). When the time needed to reach the maximal amplitude of the current after the beginning of the response was measured (time to peak), it was found to be 5 s in oocytes expressing mGluR1a and 15 s in oocytes expressing mGluR1c (p < 0.001) (Fig. 2a), even though the amount of cRNA injected was adjusted to obtain responses similar in amplitude (414 ± 28 nA (n = 111) for mGluR1a and 385 ± 35 nA (n = 140) for mGluR1c). In mGluR1a-expressing HEK 293 cells, a 2-fold higher basal Glu-independent PLC activity was measured compared with mock-transfected cells or cells expressing mGluR1c. Glu stimulated IP formation to similar extents in cells expressing mGluR1a or mGluR1c, but the EC50 value for Glu was smaller than determined in mGluR1a-expressing cells than in cells expressing mGluR1c (1.08 ± 0.12 μM (n = 10; 1.03 ± 0.14; n = 8) and 2.8 ± 0.89 (n = 11.3 ± 0.26; n = 8) (p < 0.001), respectively) (Fig. 1c). These functional differences did not result from a lower level of expression of the short variant as shown by Western blotting of membrane proteins prepared from both cell types (Fig. 1d).

To identify the sequence element within the mGluR1 carboxy-terminal tail responsible for the different functional properties of the long versus the short variant, a series of truncated receptors was constructed (Fig. 3). In mGluR1Δ1139, a stop codon was introduced at position 1139 so that the last 60 amino acids including a large number of serine and threonine
residues and a PDZ interacting sequence (17) were removed. In mGluR1Δ1093, an additional segment rich in acidic residues was removed. Finally, an additional truncated receptor mGluR1Δ879 with a carboxyl-terminal intracellular tail shorter than that of mGluR1c was also constructed (Fig. 3).

The coupling to PLC of these truncated receptors was first analyzed after expression in Xenopus oocytes. In oocytes expressing mGluR1Δ1139, mGluR1Δ1093 as well as in oocytes expressing mGluR1Δ879, Glu induced fast responses similar in shape to those measured in oocytes expressing mGluR1a (Figs. 2b and 4a). These truncated receptors were also expressed in HEK 293 cells. In these cells all truncated and wild type receptors stimulated IP formation to a similar extent when activated with Glu (data not shown). All truncated receptors also exhibited constitutive activity like mGluR1a (Figs. 2b and 4b).

Taken together, these results indicate that the truncated receptor mGluR1Δ879, which possesses a carboxyl-terminal intracellular domain shorter than that of mGluR1c, displays the same functional properties as the long variant mGluR1a both in Xenopus oocytes and HEK 293 cells (Fig. 4). This suggests that sequence elements within the 19 carboxyl-terminal residues of mGluR1c are responsible for the specific functional properties of this short mGluR1 variant.

To examine whether the carboxyl-terminal end of mGluR1c was sufficient to explain its functional properties, we exchanged the carboxyl-terminal domain of the other PLC-coupled mGluR (mGluR5a) with that of mGluR1a or mGluR1c taking advantage of a conserved Sphl site in the mGluR1 and mGluR5 sequences (Figs. 2c and 5). Like the wild type mGluR5a that also possesses a large carboxyl-terminal domain (Fig. 2c), mGluR5/1a induced fast responses in oocytes (Figs. 2c and 6a) and possessed high constitutive activity (Figs. 2c and 6b). In contrast, the chimeric mGluR5/1c receptor with the carboxyl-terminal tail of mGluR1c induced slowly developing responses in oocytes (Figs. 2c and 6a) and had reduced constitutive activity (Figs. 2c and 6b). Moreover, when various concentrations of Glu were used to stimulate IP formation in cells expressing these chimeric receptors, a lower EC50 value was measured with mGluR5/1a-expressing cells than with cells expressing mGluR5/1c (0.74 ± 0.25 μM (n1 = 0.62 ± 0.12; n = 4) and 4.39 ± 1.13 (n1 = 1.05 ± 0.20; n = 4) (p < 0.02), respectively) (Fig. 6c). Finally, a truncated mGluR5 receptor with a carboxyl-terminal intracellular tail similar in length to that of mGluR1c (see Fig. 5) has the same functional properties as the wild-type mGluR5a (Fig. 2c). The presence of the carboxyl-terminal half of the mGluR1c intracellular tail is therefore sufficient to confer to mGluR5 the specific PLC coupling properties of mGluR1c.

The carboxyl-terminal sequences of mGluR1b, mGluR1c, and mGluR1d display no sequence homology after the splice junction site (Fig. 5). They share however similar functional properties (18, 21, 25, 30) suggesting that their specific few carboxyl-terminal residues may not play a critical role in these properties. In agreement with this proposal, a truncated mGluR1a receptor (mGluR1Δ939) with a carboxyl-terminal tail slightly longer than that of mGluR1d shares functional properties with these short receptor variants: it induces slow responses in oocytes and displays no constitutive activity (data not shown). Therefore, the short sequence located between Arg-878 and the splice junction site (KKGPGAGNA, see Fig. 5) may be responsible for the specific functional properties of these mGluR1 variants. Interestingly, residue Arg-878 is the second of a cluster of 4 basic residues, RRKK (Fig. 5). To examine if this cluster of basic residues could be responsible for the specific properties of the short mGluR1 splice variants, we constructed mutated mGluR1a and mGluR1c receptors in

![Image](https://example.com/image.png)
which Arg-878, Lys-879, and Lys-880 were replaced by Met, Ala, and Ala, respectively. These mutated receptors were named mGluR1a<sub>D</sub>b and mGluR1c<sub>D</sub>b, respectively. Mutation of these 3 basic residues in mGluR1a did not modify its functional properties when examined either in <i>Xenopus</i> oocytes or in HEK 293 cells (Fig. 2d). However, mGluR1c<sub>D</sub>b induced fast current responses when expressed in <i>Xenopus</i> oocytes (Figs. 2d and 7a) and displayed agonist-independent constitutive activity (Figs. 2d and 7b) and an increased potency of glutamate (6.39 ± 1.76 μM (<i>n</i> = 103 ± 0.21; <i>n</i> = 4) and 0.97 ± 0.53 (μM = 96 ± 0.21; <i>n</i> = 4) (<i>p</i> < 0.01) for mGluR1c and mGluR1c<sub>D</sub>b, respectively) (Fig. 7c) even though it appeared to be expressed at a lower level than the wild type mGluR1c (Fig. 7d).

To verify that all wild type and mutated mGluR1 receptors were correctly targeted to the plasma membrane, immunostaining of HEK 293 cells expressing these receptors was performed using an antibody directed against their conserved extracellular domain. As shown in Fig. 8, all receptors were found at the plasma membrane level. Interestingly, the labeling was found as large patches along the plasma membrane in many cells expressing mGluR1c. Such large patches were never observed in cells expressing mGluR1a, mGluR1Δ879, or mGluR1cΔβ.

**DISCUSSION**

Our data indicate that a cluster of 4 basic residues located 36 amino acid residues after the 7th transmembrane domain is responsible for the specific PLC coupling properties of the short mGluR1 variants. Since this sequence is conserved in the long isoform mGluR1a, the long extra carboxyl-terminal domain of this receptor may simply prevent the action of this cluster of basic residues (Fig. 9).

The functional properties due to this cluster of basic residues in the short mGluR1 variants include a slow activation of the Cl<sup>-</sup> current in <i>Xenopus</i> oocytes, a very low or no constitutive activity, and a low potency of Glu in stimulating IP<sub>formation</sub>.

**FIG. 2.** Summary table of the wild type and mutant receptors constructed and analyzed in this study. a, wild type receptors mGluR1a and mGluR1c; b, truncated mGluR1a receptors; c, wild type, truncated, and chimeric mGluR5 receptors; d, mGluR1a and -1c mutants. In the first column are the names of the receptors with the scheme of their carboxyl-terminal intracellular tail. The end of the 7th transmembrane domain is indicated. The mGluR1a and mGluR5 sequences are in black and white, respectively. The specific sequence of mGluR1c is indicated with a hatched rectangle. The position of the 3 mutated basic residues in mGluR1aΔβ and mGluR1cΔβ are indicated by a thin white line. In the second column are the means ± S.E. of the time-to-peak values in seconds for responses smaller than 1000 nA obtained upon stimulation with 300 μM Glu of oocytes injected with 0.5–10 ng of cRNA. In a, b, and d, asterisks indicate that the values are statistically different (**, <i>p</i> < 0.01) from that measured in mGluR1a-expressing cells. In c, asterisks indicate that the values are statistically different (**, <i>p</i> < 0.01; *, <i>p</i> < 0.05) from that measured in mGluR5a-expressing cells.
These properties may be explained if this sequence element impairs the expression of the receptor in the plasma membrane. This hypothesis cannot be tested directly using binding experiments because of the absence of high affinity radioligand for this receptor. However, our previous and present data indicate that this is unlikely to be the case (18, 23). Slowly developing currents are rarely observed in oocytes expressing mGluR1a even when very low amounts of mRNA are injected (18). By changing the amount of plasmid DNA transfected into HEK 293 cells, we previously reported that the ratio of basal over Glu-stimulated PLC activity was independent of the amount of receptor protein and was higher with mGluR1a than with mGluR1c (23). Finally, Western blots suggest a higher level of expression of mGluR1 than mGluR1a or mGluR1cΔβ, and immunostaining experiments revealed the presence of all wild type and mutated receptors at the plasma membrane level. Accordingly, it can be proposed that the cluster of basic residues decreases the PLC coupling efficacy of the short mGluR1 variants. In agreement with this hypothesis, several authors reported slowly developing currents induced in oocytes by receptors that have a low PLC coupling efficacy (8, 31–35). Moreover, GPCR constitutive activity is often associated with a higher G-protein coupling efficacy (for example, see Refs. 36 and 37) and higher potency of agonists (36, 38–40). A higher PLC coupling efficacy of mGluR1a may be associated with a higher Glu-induced IP formation in cells expressing this receptor compared with cells expressing mGluR1c. However, under our experimental conditions, Glu stimulated IP formation to a similar extent in cells expressing any of the wild type or mutated receptors. This may be explained if a high level of expression of these receptors is reached so that the PLC pathway is saturated upon activation with Glu. In agreement with this hypothesis, the maximal Glu-induced IP formation is lower in mGluR1c-expressing cells than in cells expressing mGluR1a when lower amounts of plasmid are transfected or when porcine kidney epithelial (LLC-PK1) cells, which expressed fewer receptors than the HEK 293 cells are used (18).

Finding that the carboxyl-terminal end of a GPCR decreases G-protein coupling is not unique to mGluRs. Truncation of the last 59 amino acid residues of the thyrotropin-releasing hormone receptor causes constitutive activity (41). The last 12 residues of bovine rhodopsin have also been proposed to operate as a negative regulator of guanine nucleotide exchange (42–44). In that case, the inhibitory action of the carboxyl terminus is abolished when the receptor is depalmitoylated (45). Similarly, removal of the extended carboxyl-terminal domain of the avian β-adrenergic receptor increases its activity (46). Finally, removal of the carboxyl-terminal tail of the human parathyroid hormone receptor suppresses its G-protein coupling selectivity, suggesting that this region inhibits coupling to some G-proteins (47). However, there are no primary sequence similarities between these domains.

Several hypotheses can be proposed to explain the lower PLC coupling efficacy due to this basic tetrapeptide in the carboxyl terminus of the short mGluR1 variants. One possibility is that this basic tetrapeptide directly interacts with the G-protein

FIG. 4. The truncated receptor mGlur1Δ879 with a carboxyl-terminal tail shorter than that of mGlur1c has functional properties different from those of mGlur1c, but similar to those of mGlur1b. a, mGlur1Δ879 induces fast responses in Xenopus oocytes. Schematic representation of the mutant and typical current trace obtained upon application of 300 μM Glu on oocytes expressing mGlur1Δ879 and voltage-clamped at −70 mV. Scale bars: vertical, 200 nA; horizontal, 20 s (left). On the right, the time-to-peak values of individual responses are plotted against the maximal current amplitude measured upon Glu (300 μM) application (I max). b, truncated mGlur1Δ879 mutant stimulates IP production in the absence of agonist. Basal IP formation occurs in mock-transfected cells and in cells expressing mGlur1c and mGlur1Δ879. In these cells, the maximal IP formation induced by 1 mM Glu was 100 ± 2, 1075 ± 110, and 1326 ± 483 in mock-transfected cells, in cells expressing mGlur1c, and in cells expressing mGlur1Δ879, respectively. Values correspond to the [3H]IP level. c, truncated mGlur1Δ879 mutant is expressed at a level similar to mGlur1c. Membranes prepared from mock-transfected cells or cells transfected with 0.5 μg of plasmid containing the mGlur1c or mGlur1Δ879 cDNAs and the mGlur1 proteins (top panel) and actin (bottom panel) were detected using selective antibodies after transfer on membrane. The upper band observed with the mGlur1 antibody likely corresponds to mGlur1 dimer, as already described (23, 49).

FIG. 5. Alignment of the sequence of the carboxyl-terminal domain of mGlur1 splice variants and that of mGlur5a. The position of the conserved Sph I site used for the generation of the mGlur5/1 chimeric receptor is indicated. The position where the amino acid sequences of mGlur1 splice variants diverge (splice site) and where the stop codon is inserted in mGlur1Δ879 are indicated. The specific sequence of the different mGlur1 variants is in lowercase. The cluster of basic residues in mGlur1 is highlighted in black. The position where the stop codon is inserted in mGlur5Δ is also indicated.
and inhibits GDP/GTP exchange as observed with the carboxyl terminus of bovine rhodopsin (44). Another possibility is that the presence of this cluster of basic residues decreases the affinity of the receptor for the G-protein. This could be due to an interaction of the cluster of basic residues with one of the intracellular loop of the receptor, masking the G-protein recognition site as proposed for rhodopsin, or to the interaction of these basic residues with the membrane phospholipids, preventing the positive action of the amino-terminal part of the intracellular tail on G-protein coupling (8, 10, 48). Alternatively, this sequence element may stabilize the receptor in the inactive state. Another possibility is that, like the avian β-adrenergic receptor (46), the carboxyl terminus of the short mGluR1 variants reduces their accessibility to G-proteins possibly by decreasing their plasma membrane mobility. This could result either from its interaction with a cytoskeletal protein or from a clustering of the receptor. Interestingly, our preliminary immunostaining experiments revealed that in many cells expressing mGluR1c, large clusters of receptors can be seen at the level of the plasma membrane. No such clusters are seen with mGluR1a, mGluR1Δ879, or in cells expressing the mutated mGluR1cΔβ, suggesting that the clustering of the receptors and their low PLC coupling efficacy are related.

Although mGluR1a also contains this cluster of basic residues, it has a higher PLC coupling activity than the short variants. The long carboxyl-terminal tail of mGluR1a may therefore prevent the inhibitory action of this sequence (Fig. 9). The functional analysis of our deletion mutants indicates that the carboxyl-terminal acidic residue-rich region or serine/threonine-rich domain do not impair PLC coupling indicating that these sequences are not necessary to prevent the action of the cluster of basic residues nor are the last few carboxyl-terminal residues interacting with PDZ domains (17). This further suggests that the long intracellular tail of mGluR1a has numerous other regulatory roles such as the control of receptor desensi-
no longer interact with another domain of the receptor or with another protein responsible for the impaired coupling.

In conclusion, the truncation of the long carboxyl-terminal domain of mGluR1a by alternative splicing unmask a short sequence that decreases the ability of the receptor to activate PLC. Such an inhibitory sequence is not found in mGluR5 for which no short carboxyl-terminal tail splice variant has been described yet. Additional experiments need to be performed to see whether this inhibitory sequence also affects other signal transduction pathways activated by mGluR1, such as Ca2+- and K+-channel modulation or phospholipase A2 and adenylyl cyclase activation which may be mediated by G-proteins different from the Gq type.

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