Metabotropic glutamate receptors (mGluR) share no sequence homology with any other G-protein-coupled receptors (GPCRs). The characterization of their G-protein coupling domains will therefore help define the general rules for receptor-G-protein interaction. To this end, the intracellular domains of mGluR3 and mGluR1, receptors coupled negatively to adenylyl cyclase and positively to phospholipase C, respectively, were systematically exchanged. The ability of these chimeric receptors to induce Ca2+ signals was examined in Xenopus oocytes and HEK 293 cells. The chimeric receptors that still possessed the second intracellular loop (i2) of mGluR3 induced little or no Ca2+ signals, even though these proteins were targeted correctly to the plasma membrane. Consistent Ca2+ signals could be recorded only with chimeric mGluR3 receptors that contains i2 and at least one other intracellular domain of mGluR1. However, most intracellular domains of mGluR3 have to be replaced by their mGluR1 equivalent to produce optimal coupling to G-protein. These observations indicate that i2 of mGluR1 is a critical element in determining the transduction mechanism of this receptor. These results suggest that i2 of mGluRs may play a role similar to i3 of most other GPCRs in the specificity of coupling to the G-proteins. Moreover, as in many other GPCRs, our data revealed cooperation between the different mGluR intracellular domains to control efficient coupling to G-proteins.

Receptors coupled to G-proteins (GPCRs) constitute a large family of receptor proteins with seven transmembrane domains. Their agonist recognition site has been localized in a cavity formed by the seven transmembrane domains in receptors activated by small ligands like catecholamines (1) and peptides (2, 3). Their effector domains, the domains interacting with and activating the G-proteins, have been studied extensively (4, 5). It has been proposed that their third intracellular loop (i3) plays a critical role for the activation of G-proteins (6–12). However, the binding and selective recognition of the G-protein depends not only on the sequence of the i3 loop but is also influenced by other intracellular domains (13–19).

The cloning of 8 metabotropic glutamate receptors (mGluR1–8) and Ca2+-sensing receptors revealed the existence of a new GPCR family (20, 21). Whereas mGluR1, mGluR5 (22–24), and the Ca2+-sensing receptors (25, 26) activate phospholipase C, the other mGluRs inhibit adenylyl cyclase (27–32). Although mGluRs also possess 7 putative TMD, they do not show sequence homology with other GPCRs. Several lines of evidence suggest that mGluRs do not operate like the other GPCRs. Their large (about 600 residues) extracellular N-terminal domains are homologous to bacterial periplasmic binding proteins and constitute the agonist recognition domain (33, 34). Their intracellular domains also show striking differences with the corresponding domains of the other GPCRs. For example, i3 is very short and highly conserved among mGluRs and Ca2+-sensing receptors (21), whereas it is the longest and more variable loop in all other GPCRs. The DRY (or ERW) tripeptide at the N-terminal end of i2 that is conserved in most GPCRs and plays a critical role in G-protein coupling (13, 35) is absent in mGluRs. Nevertheless, and in spite of these differences, mGluRs and Ca2+-sensing receptors probably activate the same set of heterotrimeric G-proteins as all other GPCRs. The understanding of how mGluRs interact with and activate G-proteins will therefore help define the general rules for receptor-G-protein interaction.

In our preliminary analysis of rat mGluR domains involved in G-protein coupling selectivity, we only examined the role of i2 and the C-terminal tail of mGluR1 in the coupling to PLC (36). In contrast to what has been reported for many GPCRs in which only one short segment plays a critical role in G-protein coupling, we found that the simultaneous exchange of those two domains of the adenylyl cyclase-coupled mGluR3 with those of mGluR1 generated a chimeric receptor which coupled to PLC. However, the kinetics of the responses induced in Xenopus oocytes by this chimeric receptor differed from those of the wild type mGluR1, suggesting the involvement of other intracellular domains in G-protein coupling. The aim of the present work was therefore: 1) to examine whether only one, like in other GPCRs, of the two previously identified domains was critical for the PLC coupling and 2) to examine whether other intracellular domains (i1 and i3) could also influence the coupling of mGluR1 to PLC.
RESULTS

In our preliminary analysis of mGluR domains involved in G-protein coupling, we examined only the role of i2 and the C terminus of mGluR1c in determining the coupling to PLC (36). However, sequence alignment of the mGluR intracellular domains also revealed that several residues in i1 and i3 are conserved in adenylyl cyclase-coupled mGluRs, but different in PLC-coupled mGluRs. For example, Arg-618, Ser-625, and Ser-627 in mGluR1a (corresponding to the first intracellular loop of mGluR1 and mGluR5, are replaced by Asn, Ala, and Gly, respectively, in the adenylyl cyclase-coupled mGluRs (Fig. 1). Moreover, the neutral residue Ala-779 in the middle of i3 in PLC-coupled mGluRs is replaced by a glutamic acid residue in all adenylyl cyclase-coupled mGluRs (Fig. 1). Finally, the C-terminal intracellular domains show little homology between mGluRs (21). A role for the C terminus in G-protein coupling is suggested by the observation that mGluR1 splice variants, mGluR1a, mGluR1b, and mGluR1c, which have different C-terminal intracellular domains, are all coupled to PLC but have slightly different functional properties (37, 40) (see Fig. 2a). In mGluR1b and mGluR1c, the C-terminal 318 residues of mGluR1a are replaced by 20 and 11 residues, respectively (32, 37). These observations suggested that additional intracellular domains may somehow be involved in the control of the interaction between mGluRs and the G-protein.

In order to analyze the exact role of the different intracellular domains of mGluR1 in its coupling to PLC, we exchanged the equivalent domains of the adenylyl cyclase-coupled mGluR3 with those of mGluR1a or mGluR1c. The coupling to PLC of the resulting chimeric receptors was first analyzed after
transient expression in Xenopus oocytes. In these cells, it is well established that activation of PLC results in the activation of a Ca$^{2+}$-activated chloride current due to the IP$_3$-induced release of intracellular Ca$^{2+}$ (41). Not only the amplitude of the current was measured, but also the time needed to reach the maximal current after the response began (time-to-peak value). This later parameter has previously been shown to be a functional characteristic of a given receptor, not directly related to the level of expression of this receptor (7, 11, 37, 42). This parameter can therefore be considered as a more accurate measure of the efficiency of coupling of the receptor to this transduction pathway in oocytes (7, 11, 37, 42–44).

As shown in Fig. 2b, none of the chimeric receptors that still possess the second intracellular loop of mGluR3 elicited consistent responses when expressed in Xenopus oocytes. Although responses were recorded from some oocytes expressing the chimeric receptor R3/1a-(i1,i3,C2) (see "Materials and Methods" and Fig. 2 for the nomenclature of chimeric receptors), these were very small and observed in only 8 out of 55 recorded oocytes. In contrast, many chimeric receptors that possess i2 of mGluR1 activated chloride current upon stimulation with 1 mM Glu (Fig. 2c and d). Taken together, these results suggest that, in contrast to what has been reported for most GPCRs, i2 rather than i3 of mGluR1a plays a critical role in determining the PLC coupling of this receptor. To further support this conclusion, we constructed chimeric mGluR1a receptors with the second or third intracellular loops of mGluR3. The exchange of i3 in mGluR1a did not prevent the receptor from activating the chloride current when expressed in oocytes (Figs. 2e and 5), whereas no responses could be measured with the mGluR1a chimeric receptor with i2 of mGluR3. However, exchanging i2 of mGluR3 with that of mGluR1 was not sufficient to generate a receptor able to activate a chloride current in Xenopus oocytes. Glu-induced responses could be recorded if, in addition to i2, at least another intracellular loop (i1 and/or i3) was simultaneously exchanged (Figs. 2c and 3c). Chimeric receptors that contain i2 and the end of the C-terminal domain (C1 domain) of mGluR1a did not generate responses upon stimulation with Glu (Fig. 2c). However, the exchange of both i2 and the entire C-terminal intracellular domain of mGluR1a generated a chimeric receptor activating PLC in oocytes (Fig. 2c). In contrast to the responses induced by the wild type mGluR1a that were fast and transient (Figs. 2a and 3a), responses generated by this chimeric receptor R3/1a-(i2,C2) had time-to-peak values often greater than 10 s (Figs. 2c and 3d). As shown in Fig. 2c and Fig. 3, e and f, the additional exchange of either i1 or i3 in R3/1a-(i2,C2) generated receptors that induced fast responses in oocytes, similar to those induced by the wild type mGluR1a, regardless of the maximal amplitude of the response (Fig. 3, e and f). This suggests that most intracellular domains of mGluR1a have to be present to recover the functional coupling to the G-protein of the wild type mGluR1a. Similarly, among the chimeric receptors with the C-terminus of mGluR1c, only the R3/1c-(i1,i2,i3,C2) receptor that contains all intracellular domains of mGluR1c, induced responses similar to those generated by mGluR1c (Fig. 2d).

The pharmacological profile of R3/1c-(i2,C2) previously has been studied extensively, and full agonist dose-response curves were constructed (36). This revealed that the chimeric receptors had a pharmacology very similar to that of mGluR3. In the present study, we verified that the new R3/1 chimeric receptors constructed had a pharmacology similar to that of mGluR3, but distinct from that of mGluR1. As shown in Fig. 4, the potent agonist of mGluR1, quisqualate, did activate R1a/3-(i3) chimeric receptors, but not R3/1 receptors. In contrast, L-CCG-I which is a potent agonist at mGluR3, activated R3/1 chimeric receptors, but not R1a/3-(i3). Finally, 1S,3R-ACPD that activates both mGluR1 and mGluR3 activated all chimeric receptors tested.

The expression and coupling to Ca$^{2+}$ signaling of chimeric receptors was also examined in mammalian cells. After transient expression in HEK 293 cells, all chimeric and wild type...
FIG. 3. Analysis of the kinetics of the responses induced by mGluR1a (a), mGluR1c (b), and the chimeric receptors R3/1-(i1,i2,i3) (c), R3/1a-(i2,C2) (d), R3/1a-(i1,i2,C2) (e), and R3/1a-(i2,i3,C2) (f). In each case are presented: a serpentine scheme of the wild type and chimeric receptors, with the mGluR1 sequences in black and the mGluR3 sequence in white; a typical trace obtained upon stimulation with 300 μM Glu. In each graph, the time-to-peak values of individual responses are plotted against I_{max}. Scale bars: vertical, 200 nA; horizontal, 16 s.

FIG. 4. Pharmacological analysis of chimeric receptors. Responses induced by 300 μM Glu, 1 μM quisqualate (Quis), 15 μM L-CCG-I, and 10 μM 15,3R-ACP dehydrogenase on R1a/3-(i3) (a), R3/1-(i1,i2,i3) (b), R3/1a-(i2,C2) (c), and R3/1a-(i1,i2,C2) (d) are presented.
receptors analyzed were found to be expressed at the plasma membrane level as illustrated by immunohistochemistry using antibodies directed against the C terminus of mGluR1a (Fig. 5), mGluR3, or mGluR1c (data not shown). None of these antibodies gave immunostaining in mock-transfected cells or cells expressing a receptor with a different C terminus (Fig. 5 and data not shown). The chimeric receptors that did not activate chloride current in Xenopus oocytes did not induce Ca$^{2+}$ signal in HEK 293 cells when activated with 1 mM Glu (Fig. 6). In contrast, Glu induced clear Ca$^{2+}$ signals in HEK 293 cells expressing chimeric receptors that were functional in oocytes (Fig. 6).

FIG. 5. Immunofluorescence detection of different wild type and chimeric receptors expressed in HEK 293 cells using an antibody directed against the C terminus of mGluR1a. a, mGluR3; b, mGluR1a; c, R3/1a-(C2); d, R3/1a-(i1,C2); e, R3/1a-(i1,i3,C2); f, R3/1a-(i2,C2); g, R3/1a-(i1,i2,C2); h, R3/1a-(i2,i3,C2).

**DISCUSSION**

The present study was aimed at examining the role of all intracellular domains of mGluR1a and mGluR1c in determin-
ing their coupling to PLC-activating G-proteins. For that purpose, the different intracellular domains of the adenylyl cyclase-coupled mGluR3 were systematically exchanged by their mGluR1a or mGluR1c equivalent. Our results revealed that all chimeric receptors able to strongly activate the chloride current in *Xenopus* oocytes, or to induce Ca\(^{2+}\) signals in transfected HEK 293 cells, possess the second intracellular loop of mGluR1. The importance of i2 in determining the transduction mechanism of mGluR1 was strengthened by showing that the exchange of i2 of mGluR1a with that of mGluR3 abolishes its coupling to PLC. Although the absence of a radioactive ligand with high enough affinity prevented us from estimating the receptor density in the transfected cells, immunofluorescence studies indicated that all chimeric receptors including those that did not show a Glu-induced Ca\(^{2+}\) signal were properly targeted to the plasma membrane of HEK 293 cells. Whether some of these receptors retained their ability to inhibit adenyl cyclase has not been examined yet.

These data indicate that, within the intracellular domains of mGluR1, i2 plays a critical role in the activation of PLC-coupled G-protein. Considering this and our previous studies (36), our data indicate that, within i2, the 16 C-terminal residues are necessary for optimum coupling to PLC. That such a short segment plays a critical role in G-protein coupling in mGluRs is supported by our recent observation that only few residues in the \(\alpha\) subunit of G-proteins play a critical role in their selective interaction with mGluRs. As observed with other \(\gamma\)i-coupled GPCRs (45), \(\gamma\)i-coupled mGluRs can activate PLC when co-expressed with a chimeric Go16 subunit that has its 5 C-terminal residues replaced by those of Go12. Finally, it is interesting to note that the C-terminal portion of i2 is likely to fold into an amphipathic \(\alpha\) helix (36). Such a secondary structure has been proposed to be important for the receptor domains involved in G-protein activation (46–51), although this may not be a general rule (52).

As previously reported, the exchange of the second intracellular loop of mGluR3 with that of mGluR1 is not sufficient to obtain a chimeric receptor able to activate the chloride current in *Xenopus* oocytes. The additional exchange of another intracellular domain is necessary. We previously reported that the additional exchange of the C terminus of mGluR1c was sufficient to allow the chimeric receptor to activate PLC in oocytes (36). Similar results were obtained with the chimeric receptor with i2 and the entire C-terminal intracellular domain of mGluR1a. Our data also revealed that the simultaneous exchange of i2 and either i3 or i1 plus i3 also generated chimeric receptors able to activate chloride currents in oocytes, even though the C-terminal domain was not exchanged. Therefore, all intracellular domains of mGluRs play a role in G-protein activation by facilitating the action of i2. However, none of the specific residues found in i1, i3, or the C-terminal intracellular domain of PLC-coupled mGluRs are absolutely required for the activation of PLC. In agreement with this conclusion, the sequences of i1 and i3 of the Ca\(^{2+}\)-sensing receptors (that activate PLC) are almost identical with those of adenylyl cyclase-coupled mGluRs (Fig. 1).

As reported for other receptors expressed in *Xenopus* oocytes (7, 11, 37, 42–44), the responses elicited by the R3/1 chimeric receptors can be either fast (small time to peak values) or more slowly generated (larger time to peak values). This difference is observed whatever the maximal amplitude of the responses and is therefore unrelated to the level of expression of the receptors. The kinetic of the response appears therefore as a good measure of the efficacy of coupling of a given receptor to this transduction cascade (7, 11, 44). Interestingly, most of the intracellular domains have to be present in the R3/1 chimeras to recover the time to peak value of the corresponding wild type

\[^{2}\] J. Gomeza, C. Joly, I. Brabet, J. Bockaert, and J.-P. Pin, unpublished results.
mGluR1a or mGluR1c. Taken together, these results suggest that the coupling efficacy of mGluRs is determined by the combination of all their intracellular domains. Accordingly, a cooperation between several intracellular domains in controlling either the specificity or the efficacy of coupling to G-proteins has already been reported for other GPCRs (8, 14, 16, 19).

In conclusion, our results indicate that i2 of mGluR1 plays a critical role in the G-protein coupling selectivity of this receptor and may therefore be regarded as the equivalent of i3 of most critical role in the G-protein coupling selectivity of this receptor. Accordingly, we suggest that the coupling efficacy of mGluRs is determined by receptor (mGluR1a or mGluR1c).
The Second Intracellular Loop of Metabotropic Glutamate Receptor 1 Cooperates with the Other Intracellular Domains to Control Coupling to G-proteins
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