By means of the expression of two chimeric receptors, $\alpha_2/m_3$ and $m_3/\alpha_2$, in which the carboxyl-terminal receptor portions, containing transmembrane (TM) domains VI and VII, were exchanged between the $\alpha_2$ adrenergic and the $m_3$ muscarinic receptor, Maggio et al. (Maggio, R., Vogel, Z., and Wess, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3103–3107) demonstrated that G protein-linked receptors are able to interact functionally with each other at the molecular level to form (hetero-)dimers. In the present study we tested the hypothesis that interaction between receptors might depend on the presence of a long third intracellular (i3) loop and that shortening this loop could impair the capability of receptors to form dimers. To address this question, we initially created short chimeric $\alpha_2$ adrenergic/m3 muscarinic receptors in which 196 amino acids were deleted from the i3 loop ($\alpha_2/m_3$-short and $m_3/\alpha_2$-short). Although co-transfection of $\alpha_2/m_3$ and $m_3/\alpha_2$ resulted in the appearance of specific binding, the co-expression of the two short constructs ($\alpha_2/m_3$-short and $m_3/\alpha_2$-short), either together or in combination, respectively, with $m_3/\alpha_2$ and $\alpha_2/m_3$ did not result in any detectable binding activity. In another set of experiments, a mutant $m_3$ receptor, $m_3/m_2(16aa)$, containing 16 amino acids of the $m_2$ receptor sequence at the amino terminus of the third cytoplasmic loop, which was capable of binding muscarinic ligands but was virtually unable to stimulate phosphatidylinositol hydrolysis, was also mutated in the i3 loop, resulting in the $m_3/m_2(16aa)$-short receptor. Although co-transfection of $m_3/m_2(16aa)$ with a truncated form of the $m_3$ receptor ($m_3$-trunc, containing an in frame stop codon after amino acid codon 272 of the rat $m_3$ sequence) resulted in a considerable carbachol-stimulated phosphatidylinositol breakdown, the co-transfection of $m_3/m_2(16aa)$-short with the truncated form of the $m_3$ receptor did not result in any recovery of the functional activity. Thus, these data suggest that intermolecular interaction between muscarinic receptors, involving the exchange of amino-terminal (containing TM domains I–V) and carboxyl-terminal (containing TM domains VI and VII) receptor fragments depends on the presence of a long i3 loop. One may speculate that when alternative forms of receptors with a different length of the i3 loop exist, they could have a different propensity to dimerize.

Transmembrane receptors recognize and integrate external signals modifying the metabolism or the ionic equilibrium of the cell milieu. Muscarinic receptors belong to the G-protein-coupled class of receptors (2). Molecular cloning studies have revealed the existence of five structurally related muscarinic receptor proteins (m1–m5; Refs. 3 and 4). The five muscarinic receptors are predicted to be composed of seven hydrophobic transmembrane domains (TM domains I–VII) connected by alternating cytoplasmic and extracellular loops, an extracellular amino-terminal domain and an intracellular carboxyl-terminal segment. These receptors couple to a varied group of effectors, including membrane-associated phospholipases, adenylate and guanylate cyclases, and ion channels (5–8). The third intracellular (i3) loop of these receptors confers specificity for G-protein coupling (9). Moreover, it has been suggested that this segment of the receptor is involved in the phenomenon of internalization and down-regulation (10–12).

In a previous article, Maggio et al. (13) showed that muscarinic receptors behave structurally in a fashion analogous to two-subunit receptors. When truncated $m_2$ or $m_3$ receptors (containing TM domains I–V) were co-expressed in COS-7 cells with gene fragments coding for the corresponding carboxyl-terminal receptor portions (containing TM domains VI and VII), functional muscarinic receptors with ligand binding properties similar to the wild type receptors were obtained. These results have been confirmed and extended by Schöneberg et al. (14), who showed that muscarinic receptors consist not only of two but of multiple autonomous folding units.

The association of amino-terminal (containing TM domains I–V) and carboxyl-terminal (containing TM domains VI and VII) receptor domains may occur not only intramolecularly but also intermolecularly, thus providing a molecular basis for receptor dimerization. This was demonstrated by creating two chimeric receptor molecules, $\alpha_2/m_3$ and $m_3/\alpha_2$, in which the carboxyl-terminal receptor domains (including TMDs VI and VII) were exchanged between the $\alpha_2$ adrenergic and the $m_3$ muscarinic receptor (1). Although transfection of the two chimeric constructs alone into COS-7 cells did not result in any detectable binding activity, co-expression of the two mutant receptors resulted in a significant number of specific binding sites for the muscarinic ligand N-[^3]H]methylscopolamine and the adrenergic ligand [^3]H]rauwolscine.

Intuitively, the interaction between the two chimeric receptors is due to the exchange of the amino- and carboxyl-terminal receptor fragments held together by the i3 loop. The considerable extension of the i3 loop could free the amino- and carboxyl-terminal receptor domains, promoting intermolecular association. In the present study, we tested this hypothesis by creating several constructs with a short i3 loop.

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The abbreviations used are: TM, transmembrane; i3, third intracellular; aa, amino acid; kb, kilobase; trunc, truncated; IP_1, inositol monophosphate.
Role of the i3 Loop in Muscarinic Receptor Dimerization

**MATERIALS AND METHODS**

**Preparation of Mutant Receptor Constructs**—Rm3pcD (3) and Rα2pRe-cytomegalovirus (15), two mammalian expression vectors containing the entire coding sequence of the rat m3 muscarinic and the α2c adrenergic receptor, respectively, were used to create two chimeric receptors (α2/m3 and m3/α2) in which the carboxyl-terminal receptor domains (including TM domains VI and VII) were exchanged between the α2c adrenergic and the m3 muscarinic receptor (Fig. 1; for details, see the article by Maggio et al. (1)). The construction of the expression plasmid coding for m3/m2(16aa) (α2 receptor in which the first 16 amino acids of the i3 loop have been deleted; the remaining i3 loop was 43 amino acids long. m2- and m3-trunc represent m2 and m3 receptors that have been truncated in the i3 loop; the intracellular carboxyl termini of m2- and m3-trunc consist of 56 and 21 amino acids, respectively.

**FIG. 1. Structure of chimeric α2 adrenergic/m3 muscarinic receptors and various other mutant muscarinic m3 and m2 receptors.**

The amino terminus is located extracellularly, whereas the carboxyl terminus is situated on the cytoplasmic side of the plasma membrane (shaded area). In m3/m2(16aa), the first 16 amino acids of the i3 loop of the m3 receptor have been replaced with the corresponding segment of the m2 muscarinic receptor. The corresponding short construct of each mutant represents a receptor in which 196 amino acids of the i3 loop have been deleted; the remaining i3 loop was 43 amino acids long. m2- and m3-trunc represent m2 and m3 receptors that have been truncated in the i3 loop; the intracellular carboxyl termini of m2- and m3-trunc consist of 56 and 21 amino acids, respectively.

**Ligands**—N-[3H]Methylscopolamine (84 Ci/mmol) was purchased from Amersham Corp., [3H]quinuclidinyl benzilate (43.9 Ci/mmol) and [3H]rauwolscine (82.7 Ci/mmol) were from DuPont NEN. 4-Diphenylacetic acid (DPA; [3H]rauwolscine) from Amersham Corp.; [3H]quinuclidinyl benzilate (43.9 Ci/mmol) and [3H]rauwolscine (82.7 Ci/mmol) were from DuPont NEN. 4-Diphenyla-
Role of the i3 Loop in Muscarinic Receptor Dimerization

Table I

| Receptor | [3H]NMS | [3H]Rauwolscine |
|----------|---------|-----------------|
|          | B_max  | K_d  | n_H | B_max  | K_d  | n_H |
| m3 wild type | 843 ± 33 | 27 ± 3 | 1.02 ± 0.09 | 792 ± 47 | 1.70 ± 0.21 | 1.09 ± 0.10 |
| α2 wild type | 43 ± 5 | 21 ± 2 | 1.07 ± 0.05 | 38 ± 5 | 1.93 ± 0.42 | 1.03 ± 0.09 |
| α2/m3 | No specific [3H]NMS or [3H]rauwolscine binding detectable | No specific [3H]NMS or [3H]rauwolscine binding detectable |
| α2/m3-short + m3α2-short | No specific [3H]NMS or [3H]rauwolscine binding detectable | No specific [3H]NMS or [3H]rauwolscine binding detectable |
| m3/m2(16aa)-short | 115 ± 13 | 24 ± 3 | 1.90 ± 0.07 |
| m2-trunc + α2/m3 | No specific [3H]NMS binding detectable |
| m2-trunc + α2/m3-short | No specific [3H]NMS binding detectable |

RESULTS

Lack of Intermolecular Interaction with Short Chimeric α2 Adrenergic/m3 Muscarinic Receptors—Co-transfection of the two chimeric α2/m3 and m2/α2 receptors in COS-7 cells resulted in the expression of specific binding sites for N-[3H]methylscopolamine and [3H]rauwolscine (Table I), in accordance with previous reports by Maggio et al. (1). The B_max observed was 43 ± 5 and 38 ± 5 fmol/mg proteins, respectively, for N-[3H]methylscopolamine and [3H]rauwolscine (Table I). Binding was also observed with the co-transfection of the chimeric α2/m3 receptor together with m3-trunc or m2-trunc receptor fragments. Interestingly, the number of binding sites obtained with the combination α2/m3 and m2-trunc (153 ± 12 fmol/mg proteins) was the highest we observed, indicating that the interaction between these two receptors was the most efficient. The co-transfected chimeric receptors displayed binding properties similar to those of the two wild type α2 adrenergic and m3 muscarinic receptors (Table I).

To assess whether the length of the i3 loop was crucial in allowing the (hetero)dimerization between the chimeric receptors, short chimeric α2 adrenergic/m3 muscarinic receptors were prepared and transfected in COS-7 cells with α2/m3 and m3/α2. The co-transfection of α2/m3-short and m3/α2-short, respectively, with m3/α2 and α2/m3 was ineffective in recovering detectable binding activity. Likewise, co-transfection of the two chimeric α2/m3-short and m3/α2-short receptors together did not result in any detectable binding sites. The same absence of binding activity was observed with the co-transfection of the two truncated (m2-trunc and m3-trunc) receptors with α2/m3-short (Table I).

Lack of Carbachol-stimulated Phosphatidylinositol Hydrolysis after Co-expression of Short Chimeric Receptors—To better define the role of the i3 loop in receptor dimerization, additional experiments were performed with mutant m3 muscarinic receptors severely impaired in their ability to mediate stimulation of phosphatidylinositol hydrolysis. The chimeric receptor m3/m2(16aa) has been shown to bind muscarinic ligands with wild type affinity but is virtually unable to mediate stimulation of phosphatidylinositol hydrolysis when transfected alone (9). When this mutated receptor was co-transfected with a truncated m3 muscarinic receptor (m3-trunc), there was a considerable increase in intracellular IP1 levels after carbachol stimulation (1). These findings were confirmed in our experiments (Fig. 2A and Tables II and III), as the maximum increase in IP1 levels was 93 ± 8% above basal levels, and the carbachol EC50 was 0.68 ± 0.33 μM. The chimeric m3/m2(16aa)-short receptor in which the i3 loop was considerably shortened, similarly to m3/m2(16aa), bound muscarinic antagonists with wild type affinities (N-[3H]methylscopolamine Kd, 29.08 ± 4.37 pm; 4-diphenylacetoxy-N-methylpiperidine methiodide Ki, 1.93 ± 0.11 nm) and agonists slightly more efficiently than the m3 muscarinic receptor (IC50, 2.80 ± 0.46 μM and 10.20 ± 2.62 μM, respectively, for acetylcholine and carbachol; Table II). Like m3/m2(16aa), m3/m2(16aa)-short failed to increase phosphatidylinositol hydrolysis when transfected alone (maximum increase in IP1 levels, 25 ± 6% above baseline). Despite the similarities with m3/m2(16aa), m3/m2(16aa)-short failed to increase phosphatidylinositol hydrolysis when co-transfected with m3-trunc (maximum increase in IP1 levels, 21 ± 4% above baseline; Fig. 2B and Table III), showing that intermolecular interaction was prevented in this case by the reduced length of the i3 loop.

In the previous article (1) we have excluded that homologous recombination events may have led to the recreation of wild type receptor plasmid DNA. This eventuality has been definitely ruled out in the present studies by the fact that the two short chimeric α2 adrenergic/m3 muscarinic receptors did not show any binding even though they share sequence homology. Furthermore, in the functional experiments the homology shared by m3/m2(16aa) and m3/m2(16aa)-short beyond the m2 sequence with the m3-trunc plasmid DNA is the same (12 bases), but only the transfection of the first construct with m3-trunc resulted in a substantial recovery of phosphatidylinositol hydrolysis after carbachol stimulation.

Shortening of the i3 Loop Does Not Alter m3 Muscarinic Receptor Characteristics—Our results show that shortening the i3 loop of the wild type m3 muscarinic receptor (resulting in the m3-short) did not modify the binding parameters (Table II) or the functional activity (Fig. 2B and Table III) of the receptor expressed in COS-7 cells; furthermore, the ability of this receptor to be internalized (tested on stably transfected CHO-K1 cell lines) was not impaired (Fig. 2C). Note that the same binding parameters and phosphatidylinositol hydrolysis activity were obtained for m3 wild type and m3 short in stably transfected CHO-K1 cells (data not shown). These data suggest that the large deletion of the i3 loop did not alter the overall structure of the receptor.

DISCUSSION

Despite the commonly presented models of G-protein-coupled receptors that depict the protein as a closed packed structure, several reports indicate that G-protein-coupled receptors are in fact formed by multiple autonomous folding domains. In 1988 Kobilka et al. (19) described that "split" β2 adrenergic receptors co-expressed in Xenopus oocytes bind adrenergic li-
at 37°C, and the resulting increase in intracellular IP1 levels was
found. Cells were incubated with increasing concentrations of carbachol for 1 h
and sequestered receptors were incubated above basal levels determined in the absence of carbachol. Basal IP1 levels were similar in all experiments. For internalization, CHO-K1 cells stably transfected with m3 and m3-short receptors were incubated for up to 1 h at 37 °C, and sequestered receptors were assayed with N-[3H]methylscopolamine and [3H]quinuclidinyl benzilate. Data are expressed as percentages of controls. Each curve represents the mean of three experiments, each carried out in duplicate.

FIG. 2. Carbachol induces phosphatidylinositol hydrolysis (A and B) and internalization (C) of various mutant m3 muscarinic receptors. For phosphatidylinositol hydrolysis, transfected COS-7 cells were incubated with increasing concentrations of carbachol for 1 h at 37 °C, and the resulting increase in intracellular IP1 levels was determined. Responses are expressed as percentages of increases in IP1, above basal levels determined in the absence of carbachol. Basal IP1 levels were similar in all experiments. For internalization, CHO-K1 cells stably transfected with m3 and m3-short receptors were incubated with carbachol for up to 1 h at 37 °C, and sequestered receptors were assayed with N-[3H]methylscopolamine and [3H]quinuclidinyl benzilate. Data are expressed as percentages of controls. Each curve represents the mean of three experiments, each carried out in duplicate.

m3 muscarinic receptors may consist not only of two but of multiple autonomous folding domains. Splitting the m3 muscarinic receptor in all three intracellular and three extracellular loops, they demonstrated that some of these split receptors have the ability to maintain muscarinic binding and functional activity. Furthermore, they demonstrated that proper intracellular trafficking and plasma membrane insertion does not require the presence of the full-length receptor protein. In fact, even quite short polypeptides that contain only the first two or three TM domains are properly transported to the plasma membrane.

In their report, Schöneberg et al. (14) showed that among the split receptors, only the receptor split in the i3 loop retained wild type affinity for all muscarinic ligands tested, suggesting that the i3 loop does not exert indirect conformational effects on the proper arrangement of the transmembrane receptor core (formed by TM1–VII). The long extension of the i3 loop may explain why this segment of the protein does not restrain the rest of the receptor in any particular conformation. Moreover, this characteristic of the i3 loop is probably the basis that allows chimeric α2/m3 and m3/α2 receptors to exchange domains leading to (hetero)dimerization (1).

It is reasonable to think that shortening the i3 loop could impair the ability of receptors to interact. In agreement with this hypothesis, the data presented in this article show that chimeric α2 adrenergic/m3 muscarinic receptors with a large deletion in the i3 loop (α2/m3-short and m3/α2-short) are not able to interact anymore. For these short α2 adrenergic/m3 muscarinic receptor constructs we cannot exclude the possibility that the absence of intermolecular interaction could be due to an alteration in proper protein folding. However, this explanation seems to be unlikely, because other short constructs such as m3-short and m3/m2(16aa)-short (which have the same deletion in the i3 loop) do not display any alteration in their ability to fold and to be inserted into the plasma membrane. Rather, it seems that this deletion favors receptor expression in some way, as is suggested by the slightly but consistently higher number of binding sites observed for these two short constructs compared with the analogous receptors bearing the normal i3 loop (m3 wild type and m3/m2(16aa), respectively; Table II). Epitope-tagged receptors could have been constructed to address whether these two receptors are indeed present on the cell surface, but this experiment would not have excluded the possibility of misfolded protein not able to interact even when present on the membrane.

In another set of experiments designed to overcome this problem, we used functionally impaired m3 muscarinic receptors. Like m3/m2(16aa), the short form of this receptor (m3/m2(16aa)-short) displays binding affinities similar to the wild type m3 receptor (besides a slight increase in agonist affinity). Nonetheless, the co-transfection of m3/m2(16aa)-short with m3-trunc was unable to restore phosphatidylinositol hydrolysis activity, indicating that the short i3 loop considerably limits the independence of the amino- and carboxyl-terminal fragments of the m3/m2(16aa)-short receptor and, consequently, their ability to interact with foreign receptor domains.

Our data lead to the conclusion that the length of the i3 loop plays a critical role in the regulation of receptor dimerization. Although muscarinic receptors physiologically do not have variants with a shorter i3 loop, dopamine receptors do. For example, an alternative splicing in the i3 loop of the dopamine D2 receptor leads to a short and a long form that differ in length by 29 amino acids (20). Even though in our experiments we deleted a very large portion of the protein (196 amino acids) to prevent receptor interaction, it is possible that smaller deletions could be equally effective, if the i3 loop is near a border-
line length for intermolecular interaction between receptors. It has been demonstrated that the i3 loop of muscarinic receptors is involved in physiological activities like sequestration and down-regulation. For example, Lameh et al. (10), using a series of deletion mutants of the i3 loop of the m1 muscarinic receptor, demonstrated that these deletions left the phosphatidylinositol turnover activity unchanged but impaired the ability of the mutated receptors to internalize. Furthermore, they defined a narrow domain in the middle of the i3 loop (apparently maintained in all five muscarinic receptor subtypes), which is likely to be involved in receptor sequestration.

Our experiments performed with the short form of the wild type m3 muscarinic receptor (m3-short) demonstrate that the large deletion of the i3 loop leaves the binding characteristics of the receptor, the phosphatidylinositol hydrolysis activity, and the ability of the receptor to internalize unvaried. These data indicate that the large portion of the i3 loop that has been deleted, at least in the rat m3 muscarinic receptor, is not essential for sequestration, but like the mutant receptor m3/m2(16aa)-short, it might prevent receptor dimerization. As the portion of the i3 loop removed contains several residues that are potential sites of phosphorylation (2), and evidence indicates that phosphorylation of the receptors at serine and threonine residues may be involved in the desensitization of G-protein-linked receptors (21, 22), it remains to be established whether this deletion of the i3 loop impairs receptor down-regulation, as has been reported by Shapiro and Nathanson (12) for the human m1 muscarinic receptor.

At the moment we do not know what the physiological role of receptor dimerization is, and only speculations are possible. For example, interaction between different receptor subtypes could lead to stimulation of different G proteins. We have demonstrated that the amino-terminal domain of the m2 muscarinic receptor can efficiently interact with the carboxyl-terminal domain of the m3 receptor and form a hybrid m2/m3 receptor complex. Co-localization of m2 and m3 muscarinic receptor subtypes in the same cells could lead to the formation of an m2/m3 receptor heterodimer, which could stimulate other G proteins than the wild type m2 and m3 receptors. Alternatively, the intermolecular interaction might promote aggregation of receptors and a consequent compartmentalization of second messenger increase. In support of this view, a recent article by Wreggett and Wells (23) based on binding studies and gel electrophoresis experiments on solubilized receptors from porcine atria has clearly demonstrated a cooperativity of an m2/m3 receptor heterodimer, which could stimulate other G proteins than the wild type m2 and m3 receptors. Further comparative studies between the wild type m3 muscarinic receptor and the mutant m3-short receptor could reveal the physiological role of the dimerization.

In conclusion, we have demonstrated that removal of a large fragment of the i3 loop from the m3 muscarinic receptor does not modify its ability to bind ligands, to stimulate phosphatidylinositol hydrolysis, and to internalize; however, the same deletion alters the intermolecular interaction between receptors, showing that receptor dimerization depends on the length of the i3 loop. Based on the high structural homology found among all G-protein-coupled receptors, our findings should be of general importance for the entire class of integral membrane proteins.

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### Table II

| Receptor                  | B<sub>max</sub> | K<sub>d</sub> | 4-DAMP, K<sub>i</sub> | Acetylcholine, IC<sub>50</sub> | Carbachol, IC<sub>50</sub> |
|---------------------------|-----------------|--------------|------------------------|-----------------------------|----------------------------|
| m3 wild type              | 699 ± 30        | 25.55 ± 4.32 | 2.76 ± 0.19            | 9.50 ± 2.08                 | 79.15 ± 14.19              |
| m3-short                  | 748 ± 15        | 22.39 ± 1.82 | 2.82 ± 0.18            | 12.68 ± 2.37                | 84.34 ± 18.49              |
| m3/m2(16aa)               | 325 ± 21        | 28.32 ± 3.25 | 2.52 ± 0.18            | 5.36 ± 0.81                 | 50.22 ± 7.53               |
| m3/m2(16aa)-short         | 385 ± 15        | 29.08 ± 4.37 | 1.93 ± 0.11            | 2.80 ± 0.46                 | 10.20 ± 2.62               |

### Table III

| Receptor                  | Amount of transfected DNA | B<sub>max</sub> | Phosphatidylinositol hydrolysis |
|---------------------------|---------------------------|-----------------|-------------------------------|
| m3 wild type              | 5                         | 202 ± 17        | 198 ± 15                      |
| m3-short                  | 5                         | 253 ± 22        | 194 ± 24                      |
| m3-trunc                  | 20                        | 232 ± 15        | 18 ± 7                        |
| m3/m2(16aa)               | 20                        | 187 ± 12        | 95 ± 8                        |
| m3/m2(16aa) + m3-trunc    | 10 + 10                   | 295 ± 22        | 25 ± 0.6                      |
| m3/m2(16aa)-short + m3-trunc | 10 + 10                | 156 ± 14        | 21 ± 4                        |

a ND, not determinable with sufficient accuracy.
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