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Receptor Internalization Delays m4 Muscarinic Acetylcholine Receptor Resensitization at the Plasma Membrane

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SUMMARY

We analyzed the role of receptor internalization and recycling in muscarinic acetylcholine receptor (mAChR) desensitization and resensitization. Incubation of Chinese hamster ovary cells stably expressing the m4 mAChR with 1 mM carbachol for 1 hr reduced cell surface receptor number by 50–60% with no change in total receptor number. Pretreatment of the cells with 450 mM sucrose, which did not affect the ability of m4 receptors to inhibit forskolin-stimulated cAMP accumulation, completely blocked receptor internalization. On the other hand, the carbachol treatment reduced the ability of m4 receptors to inhibit cAMP accumulation in both sucrose-treated and untreated cells, with a similar onset and to a similar extent. The EC_{50} value for carbachol was increased ~10-fold, and maximal inhibition determined at 100 μM carbachol was reduced ~50%. In contrast, thrombin-induced inhibition of cAMP accumulation was not affected. Recycled receptors in cells not treated with sucrose remained refractory to carbachol stimulation for ≥2 hr after agonist removal, even though cell surface receptor number had recovered completely within 1 hr. In contrast, resensitization of receptor function was very rapid in cells treated with sucrose. Ten minutes on removal of agonist, mAChRs in the plasma membrane of sucrose-treated cells were fully resensitized. Also, an internalization-defective m4 mAChR mutant, T399A, that was found to desensitize similar to the wild-type receptor, resensitized more rapidly than the wild-type receptor. We conclude that desensitization and resensitization of m4 mAChRs in Chinese hamster ovary cells can occur at the plasma membrane and that receptor internalization strongly delays the process of resensitization of desensitized receptors.

mAChRs are the main receptors for acetylcholine in the nervous system. They are coupled via heterotrimeric G proteins to various ion channels and enzymes, including adenylyl cyclase and phospholipases involved in the generation of second messengers (1). Like many other receptors, mAChRs are susceptible to desensitization as a result of prolonged interaction with agonists. It is believed that desensitization is associated with phosphorylation of mAChRs by G protein-coupled receptor kinases (2, 3). The phosphorylated receptor may subsequently bind to inhibitory proteins like β-arrestin, which reduce the ability of the receptor to couple to the G protein. Another process that is observed during receptor desensitization is receptor internalization. Within minutes of agonist exposure, 40–60% of the surface receptors are internalized into the interior of the cell and become inaccessible for hydrophilic ligands. The internalized mAChRs may then recycle back to the plasma membrane.

A great deal of effort has been expended in identifying the receptor structures and molecular mechanisms that regulate mAChR internalization (4–7). These studies have indicated that activation of heterotrimeric G proteins and G protein-coupled receptor kinases facilitates m2 and m4 mAChR internalization. Furthermore, the substitution or deletion of serine and threonine residues in the second and third cytoplasmic loops, including those in the putative phosphorylation sites, attenuates internalization of these receptors (4, 7–9). These results have led to the hypothesis that receptor phosphorylation by receptor kinases is involved in but not essential for m2 and m4 mAChR internalization (5). The precise physiological role of receptor internalization and recycling in mAChR function remains uncertain. This question has been difficult to answer in native tissue systems such as primary tissue cultures because radioligand binding measurements made on these complex preparations monitor mAChRs that are coupled not only to one particular signal transduction pathway but also to other effector systems. Similarly, even in tumor cell lines, multiple mAChR subtypes may exist that are linked to separate biochemical effectors. To avoid the complexities that arise from a mixture of mAChRs, we used CHO cells stably expressing the m4 mAChR to address the issue of whether mAChR internalization and recycling possess a functional role. The m4 mAChR

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

was chosen because it is abundantly expressed in the central nervous system, particularly in the cerebral cortex and striatum (10). In the current study, we demonstrate that receptor internalization and recycling represent an important m4 mAChR desensitization mechanism that operates temporally and spatially distinct from the process of receptor desensitization at the plasma membrane.

Experimental Procedures

Materials. [^{3}H]QNB (43 Ci/mmol) and [^{3}H]NMS (80 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). Thrombin was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. CHO cells expressing the wild-type mouse m4 muscarinic receptor (11) or the internalization-defective m4 mAChR mutant T399A (7) were grown in α medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. Cell media were obtained from GIBCO (Grand Island, NY).

Muscarinic receptor binding assays. Surface receptors were measured by the binding of the membrane-impermeable mAChR antagonist [^{3}H]NMS at a receptor-saturating concentration of 2 nM as described in detail previously (6). The total number of muscarinic receptors was determined by binding of the membrane-permeable muscarinic antagonist [^{3}H]QNB at a receptor-saturating concentration of 600 pM (6). Specific binding of [^{3}H]NMS and [^{3}H]QNB was determined as the amount of binding inhibited by inclusion of 3 μM atropine. Protein concentrations were determined according to the Lowry method.

Desensitization and resensitization of receptor-mediated inhibition of cAMP accumulation. Receptor activity was determined by measuring inhibition of forskolin-stimulated adenylyl cyclase because basal cAMP levels were not altered by carbachol (10 nM to 1 mM). Before the induction of receptor desensitization, CHO cells on 60-mm plates were preincubated in 25 mM HEPES-buffered α medium, pH 7.4, for 20 min at 37° with or without 450 mM sucrose. Then, carbachol (final concentration, 1 mM) or vehicle was added, and cells were incubated for ≤60 min at 37°. During the past 20 min, 5 mM theophylline was present. The inclusion of 5 mM theophylline did not affect receptor internalization. Thereafter, cells were washed three times with warm phosphate-buffered saline to remove sucrose and carbachol and incubated with 2 ml of HEPES-buffered α medium containing 5 mM theophylline, 50 μM forskolin, and the indicated concentrations of carbachol for 5 min at 37°. The reactions were stopped by the addition of 2 ml of trichloroacetic acid. cAMP was isolated and measured as described in detail previously (6). Basal cAMP levels were not altered by 1-hr treatment with carbachol.

Cells that were to be assessed for resensitization after 1-hr incubation with 1 mM carbachol in HEPES-buffered α medium were washed three times and incubated for the indicated periods of time in fresh HEPES-buffered α medium (37°) that was free of agonist. Resensitization of receptor-mediated inhibition of forskolin-stimulated cAMP accumulation was measured as described above in the presence of 5 mM theophylline, which was added to the incubation medium 20 min before the assay. Triplicate plates were performed for each treatment, and each plate was assayed for cAMP levels in triplicate.

Results

m4 mAChR internalization and recycling in CHO cells. Exposure of CHO cells expressing m4 mAChRs to 1 mM carbachol resulted in rapid receptor internalization as measured by binding of the membrane-impermeant radioligand [^{3}H]NMS (Fig. 1). Within 30 min, carbachol caused a 48 ± 5% loss of cell surface receptors, and a steady state level (54 ± 6% reduction) was reached within 1 hr. The loss of cell

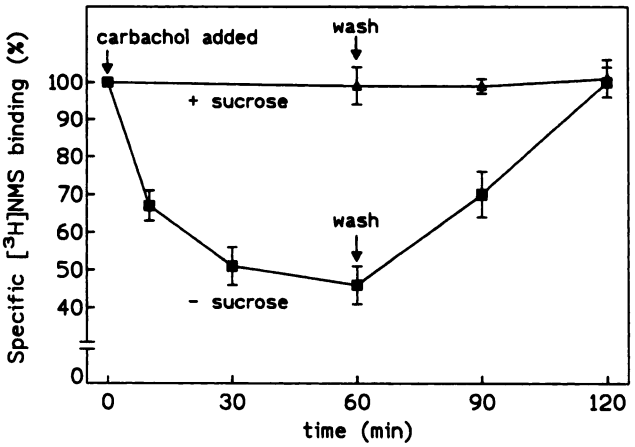

Fig. 1. Kinetics of m4 mAChR internalization and recycling in CHO cells showing the effect of sucrose. CHO cells stably expressing m4 mAChRs were incubated with or without 1 mM carbachol in the absence and presence of 450 mM sucrose for ≤60 min at 37°. Thereafter, cells were washed, and [^{3}H]NMS binding to intact cells was determined either immediately or after 30 or 60 min of incubation in the absence of carbachol. Data are mean ± standard error from three or four separate experiments. Binding data are presented as levels relative to control values at each time point. Control mAChR levels were 186 ± 5 fmol/mg protein and remained constant within experimental error (<10%).

surface receptors took place without a change in total receptor number as determined by [^{3}H]QNB binding to cell homogenates (data not shown). Pretreatment of CHO cells with a hyperosmolar concentration of sucrose (450 mM) was used as a means to inhibit receptor internalization through clathrin-coated pits and vesicles (12, 13). Exposition to 450 mM sucrose for 20 min before the addition of carbachol completely prevented receptor endocytosis (Fig. 1). Recycling of internalized receptors was monitored after removal of carbachol from the medium at 60 min. Within 1 hr after ligand withdrawal, mAChRs had completely reappeared on the cell surface. The rapidity of receptor recycling suggested that *de novo* receptor synthesis was not required. This conclusion was supported by the observation that 350 μM cycloheximide, a concentration sufficient to inhibit protein synthesis in CHO cells effectively, had no effect on m4 mAChR recycling (data not shown).

Desensitization and resensitization of m4 mAChR in CHO cells. We next determined the time course of m4 mAChR desensitization and resensitization in CHO cells. For this, cells were incubated with 1 mM carbachol for 30 or 60 min at 37°. Subsequently, carbachol-induced inhibition of forskolin-stimulated cAMP accumulation in intact cells was determined (Fig. 2). Desensitization of the mAChR response proceeded slowly and became clearly evident after 60 min of incubation with carbachol. As shown in Fig. 3, 1-hr carbachol treatment of the cells resulted in a rightward and upward shift of the concentration-response curve. The EC_{50} for carbachol-induced inhibition of cAMP accumulation was increased from 0.5 to 5.0 μM, and the maximal inhibition determined at 100 μM carbachol was reduced from 62 ± 3% to 25 ± 2% (Table 1). The potency of carbachol to desensitize the maximal inhibition of adenylyl cyclase was 32 ± 4 μM (three independent experiments). We next sought to determine the time course of receptor resensitization in CHO cells after washing the cells free of agonist. After 1 hr, there was only a partial restoration of the maximal inhibitory response but no

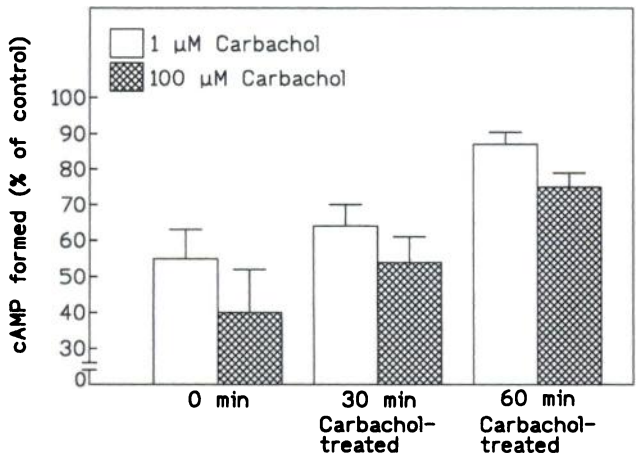

Fig. 2. Desensitization of m4 mAChRs in CHO cells. CHO cells expressing m4 mAChRs were incubated with or without 1 mM carbachol for 30 or 60 min at 37°. Then, the cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation was determined with 1 and 100 μM carbachol as described in Experimental Procedures. Data are mean ± standard error of three experiments and expressed relative to control cAMP levels (545 ± 174 pmol/mg protein) measured in the absence of carbachol.

change in the EC_{50} for carbachol (Fig. 3 and Table 1). A recovery period of 2–5 hr was required, during which the EC_{50} for carbachol decreased from 5.0 to 0.9 μM, and the maximal response increased from 25 ± 2% to 63 ± 3% (Fig. 3 and Table 1). Apparently, recovery of receptor-mediated inhibition of cAMP accumulation lagged significantly behind recovery of cell surface mAChR number. Because new protein synthesis may be required for the recovery of physiological sensitivity, we investigated whether inclusion of 350 μM cycloheximide during the first 2 hr of recovery attenuates resensitization of the receptors. (Longer incubation periods with cycloheximide were not possible because treatment of such length caused substantial cell death.) The inclusion of cycloheximide did not block receptor resensitization (control cycloheximide-treated cells: EC_{50} for carbachol, 0.8 ± 0.3 μM; inhibition by 100 μM carbachol, 58 ± 2%; carbachol- plus cycloheximide-treated cells: EC_{50} for carbachol, 4.4 ± 1.5 μM; inhibition by 100 μM carbachol, 48 ± 2%; four independent experiments). This indicates that restoration of receptor function does not require *de novo* protein synthesis.

We next examined desensitization and resensitization of the receptor response in cells in which receptor internalization was blocked by pretreatment with sucrose. This treatment did not affect control cAMP accumulation in response to forskolin or forskolin plus carbachol (Table 1). Furthermore, sucrose pretreatment did not alter the onset of receptor desensitization compared with that observed in untreated cells (Fig. 4). In addition to the similar onset of receptor desensitization, sucrose-treated CHO cells displayed similar changes in EC_{50} for carbachol and reduction in maximal inhibition of cAMP accumulation after 1-hr agonist exposure

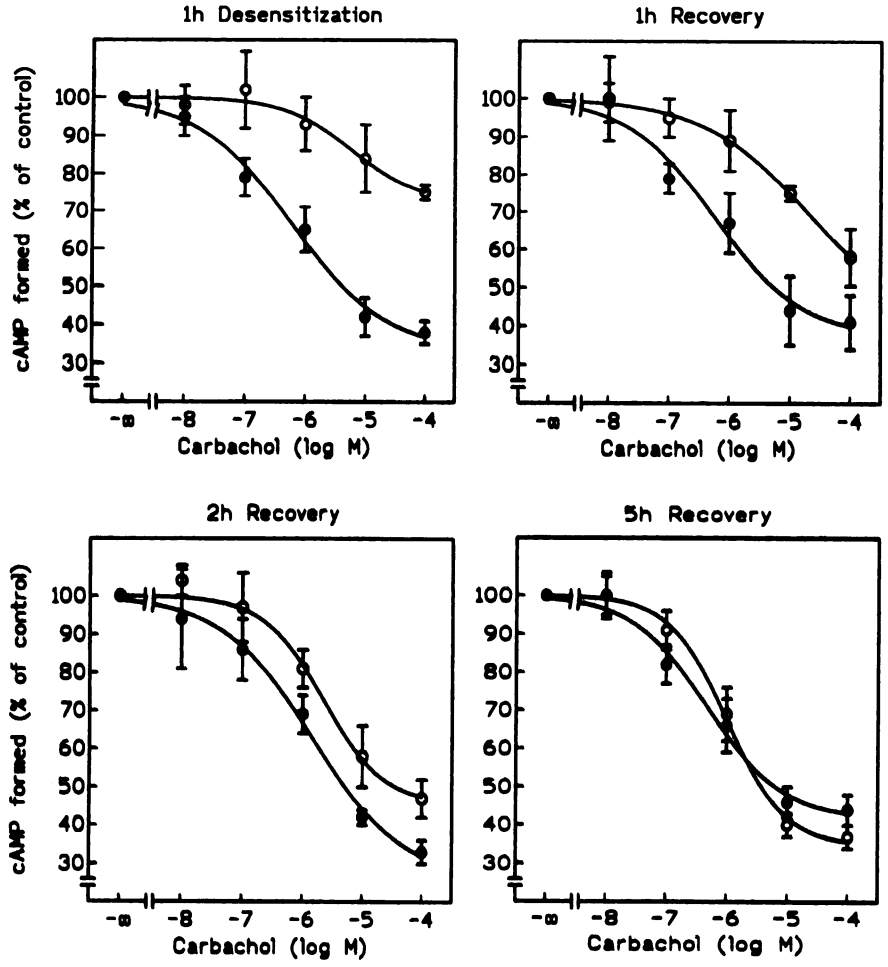

Fig. 3. Desensitization and resensitization of m4 mAChRs in CHO cells. CHO cells expressing m4 mAChRs were incubated with (○) or without (●) 1 mM carbachol for 1 hr at 37°. Then, the cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation was determined either immediately (*1h Desensitization*) or after 1, 2, and 5 hr of incubation in media free of agonist (*Recovery*). Data used to construct the curves are mean ± standard error of three to six experiments and expressed relative to control cAMP levels measured in the absence of carbachol. Analysis of the concentration-response curves is given in Table 1.

TABLE 1

Functional characteristics of desensitized and resensitized m4 mAChRs in CHO cells

CHO cells expressing m4 mAChRs were stimulated for 1 hr with or without 1 mM carbachol in the absence (control cells) or presence (sucrose-treated cells) of 450 mM sucrose. After washing of the cells, the ability of receptors to inhibit forskolin (50 μM)-stimulated cAMP accumulation was determined either immediately (1-hr Desensitization) or after 10 min or 1, 2, or 5 hr of incubation of the cells in medium without carbachol (Recovery). Basal cAMP levels in CHO cells (95–126 pmol/mg protein) were not altered by either sucrose or carbachol treatment. Values are mean ± standard error. Parentheses show the number of independent experiments.

Treatment conditions		Inhibition by carbachol		Control cAMP accumulation
		EC_{50}	at 100 μM	
		μM	%	pmol/mg
Control cells				
1-hr desensitization (6)	Control	0.5 ± 0.2	62 ± 3	886 ± 111
	Carbachol treated	5.0 ± 3.0	25 ± 2	851 ± 123
1-hr recovery (4)	Control	0.5 ± 0.3	59 ± 8	1139 ± 304
	Carbachol treated	5.6 ± 3.1	42 ± 8	973 ± 276
2-hr recovery (3)	Control	1.0 ± 0.6	67 ± 3	941 ± 70
	Carbachol treated	2.0 ± 0.9	53 ± 5	937 ± 47
5-hr recovery (6)	Control	0.4 ± 0.4	56 ± 4	1244 ± 238
	Carbachol treated	0.9 ± 0.3	63 ± 3	1190 ± 295
Sucrose-treated cells				
1-hr desensitization (4)	Control	0.6 ± 0.3	56 ± 4	894 ± 46
	Carbachol treated	5.6 ± 3.9	25 ± 10	943 ± 167
10-min recovery (4)	Control	0.9 ± 0.3	55 ± 7	1142 ± 178
	Carbachol treated	0.6 ± 0.2	50 ± 8	885 ± 104

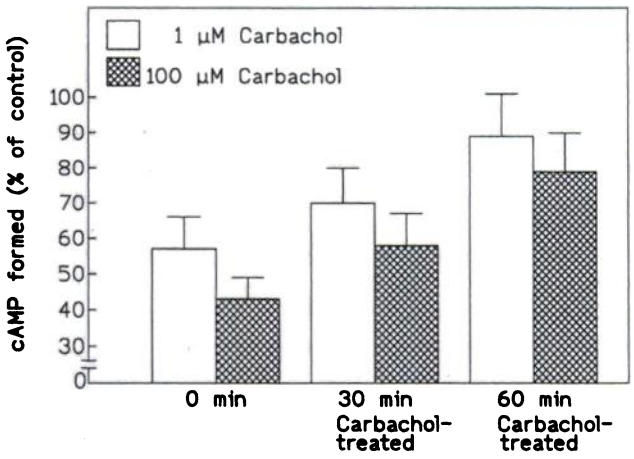

Fig. 4. Time course of m4 mAChR desensitization in sucrose-treated CHO cells. CHO cells expressing m4 mAChRs were pretreated with 450 mM sucrose and then incubated with or without 1 mM carbachol for 0, 30, or 60 min at 37°. Then, the cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation by 1 and 100 μM carbachol was determined. Data are mean ± standard error of three experiments and expressed relative to control cAMP levels (877 ± 348 pmol/mg protein) measured in the absence of carbachol.

as observed in control cells (Fig. 5 and Table 1). However, resensitization of the receptors proceeded much faster in sucrose-treated than in untreated cells. As early as 10 min after agonist washout, there was complete restoration of m4 mAChR function (Fig. 5). The addition of 450 mM sucrose to the cells for a period of 20 min after 1-hr carbachol treatment did not accelerate receptor resensitization (data not shown). Thus, the rapid acquisition of full responsiveness in sucrose-treated cells is not caused by a direct "stimulatory" effect of sucrose on the resensitization process.

To investigate whether the observed mAChR desensitization was homologous or heterologous, we determined the functional response of endogenous thrombin receptors in carbachol-pretreated and control cells. As shown in Fig. 6, inhibition of cAMP accumulation by thrombin receptors, which, like m4 mAChRs, couple to pertussis toxin-sensitive G proteins, was not affected by 1-hr pretreatment with 1 mM carbachol.

On the basis of the present results, one would expect impairment of receptor internalization by site-directed mutagenesis of the receptor to result in a more rapid resensitization after receptor desensitization. We previously reported the construction of a mutant m4 mAChR in which T399 in the membrane proximal segment of the third cytoplasmic loop was replaced with alanine (7). Substitution of this residue with alanine did not affect receptor-mediated inhibition of adenylyl cyclase but strongly reduced the rate and extent of receptor internalization; i.e., internalization was only 16% and 46% of that of the wild-type receptor after 10- and 60-min treatment with 1 mM carbachol, respectively (7). The findings in the current study prompted us to use the internalization-defective T399A to test that hypothesis. As shown in Fig. 7, T399A underwent desensitization in response to 1-hr treatment with 1 mM carbachol much like the wild-type receptor. However, unlike the wild-type receptor, T399 was apparently fully resensitized 1 hr after removal of the agonist (Fig. 7).

Discussion

In many cells expressing mAChRs, prolonged exposure to muscarinic agonists results in receptor desensitization and internalization of the receptors into the cells. In the current study, desensitization of m4 mAChR-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells proceeded relatively slowly despite the attendant loss of cell surface receptors. The sustained receptor function is likely to be due to a large signal transduction reserve in the m4 receptor-mediated inhibition of cAMP formation. Previous studies in native cell lines and tissues have indicated that m4 mAChRs are coupled very efficiently to inhibition of adenylyl cyclase (14, 15). For example, in the rat striatum, in which m4 mAChR expression accounts for 30–45% of the total number of mAChRs (10, 16), carbachol can still produce a 50% maximal response (inhibition of adenylyl cyclase) at concentrations that occupy only 3% of the total striatal mAChRs (14).

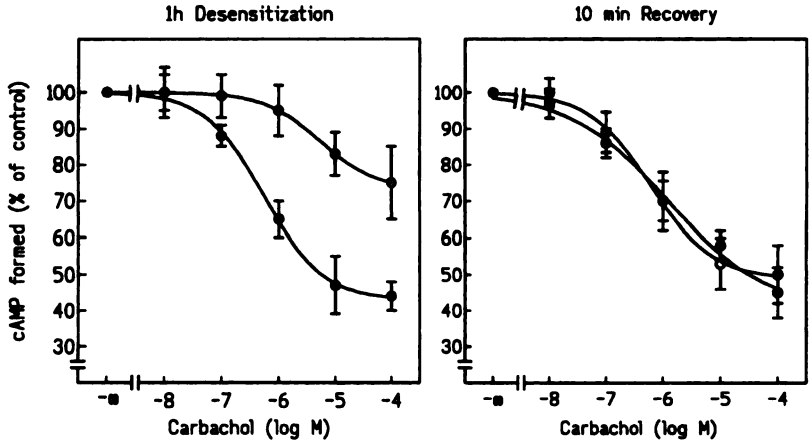

Fig. 5. Desensitization and resensitization of m4 mAChRs in sucrose-treated CHO cells. CHO cells expressing m4 mAChRs and pretreated with 450 mM sucrose were incubated with (○) or without (●) 1 mM carbachol for 1 hr at 37°. Then, the cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation was measured either immediately (*1h Desensitization*) or after 10 min incubation in media free of agonist (*10 min Recovery*). Data used to construct the curves are mean ± standard error of four experiments and expressed relative to control cAMP levels measured in the absence of carbachol. Analysis of the concentration-response curves is given in Table 1.

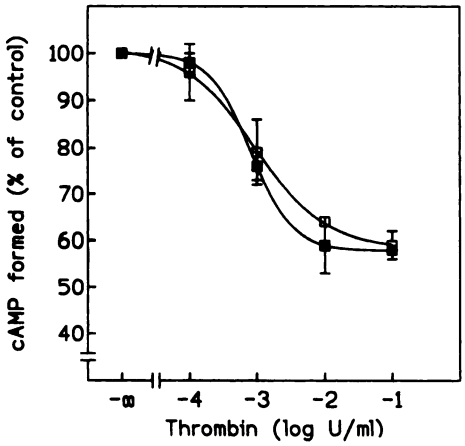

Fig. 6. Lack of heterologous desensitization in carbachol-treated CHO cells. CHO cells expressing m4 mAChRs were incubated with (○) or without (●) 1 mM carbachol for 1 hr at 37°. Then cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation by thrombin was determined. Data are mean ± standard deviation of two independent experiments. Control forskolin-stimulated cAMP levels after incubation without and with 1 mM carbachol were 866 ± 171 and 972 ± 208 pmol/mg protein, respectively.

The first major finding of the current study is that internalization is not required for m4 mAChR desensitization to occur. This reasoning is supported by several independent findings. First, treatment of the cells with hypertonic sucrose solution, which completely blocked internalization, did not affect receptor desensitization. Second, although after 30 min of carbachol treatment receptor internalization had almost reached its maximum level, desensitization of receptor-mediated inhibition of cAMP accumulation was hardly apparent. Third, the potency of carbachol to induce m4 mAChR internalization (3 μM) (6) is 10-fold higher than the potency of the agonist to induce desensitization of m4 mAChR-mediated inhibition of adenylyl cyclase (32 μM). Fourth, desensitization of the internalization-defective m4 mutant T399A occurred to a similar extent as seen with the wild-type receptor. Our results are in agreement with a recent study of m2 mAChR desensitization and internalization. Overexpression of a dominant-negative G protein-coupled receptor kinase 2 mutant in human embryonic kidney 293 cells completely abolished acute agonist-induced receptor desensitization but did not affect receptor internalization (9). These observations provide evidence that internalization of the G_i-coupled m2 and m4 mAChRs is not a mechanism responsible for initiation of agonist-induced receptor desensitization. Interestingly, a recent report suggested that internalization is involved in m3 mAChR desensitization in human embryonic kidney 293 cells (17). Mutant m3 mAChRs that did not internalize were less desensitized than wild-type receptors. Also, treatment of the wild-type receptor-bearing cells with concanavalin A prevented both internalization and desensitization (17).

The second major finding of our study is that m4 mAChR resensitization apparently occurs at the plasma membrane and that receptor internalization is not required for but, on the contrary, delays m4 mAChR resensitization. These ob-

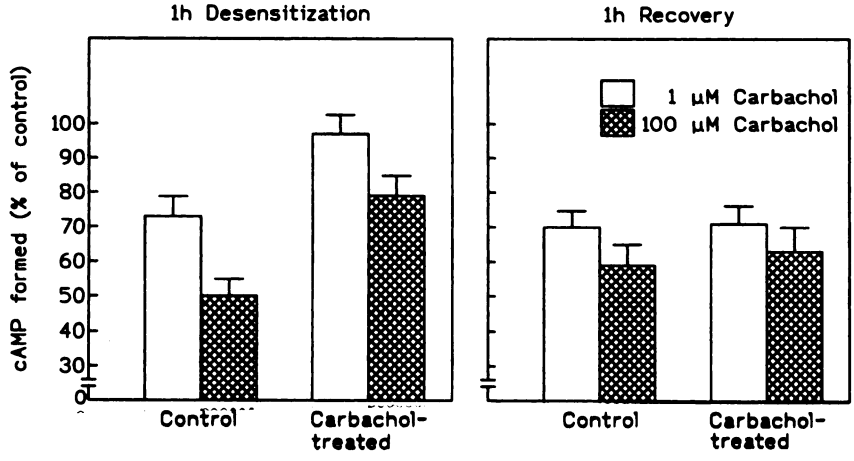

Fig. 7. Desensitization and resensitization of stably expressed T399A m4 mAChR mutant in CHO cells. CHO cells expressing T399A m4 mAChRs were incubated with or without 1 mM carbachol for 1 hr at 37°. Then, either the cells were washed, and inhibition of forskolin (50 μM)-stimulated cAMP accumulation by 1 and 100 μM carbachol was determined (A) or cells were washed and incubated in the absence of carbachol for 1 hr, after which inhibition of cAMP accumulation by 1 and 100 μM carbachol was measured (B). Data are mean ± standard deviation of three independent experiments. Control forskolin-stimulated cAMP levels were 651–808 pmol/mg protein.

servations are in contrast to what is described for β_2-adrenoceptors. In the β_2-adrenoceptor system, the internalization-recycling pathway seems to be required for resensitization of the receptors (13, 18). Inhibition of β_2-adrenoceptor internalization by hyperosmolar sucrose completely blocked the recovery of β_2-adrenoceptor function in CHO cells. In the present study, inhibition of internalization had the opposite effect on m4 mAChR function. Resensitization of noninternalized receptors in sucrose-treated cells proceeded much faster than that of internalized receptors in untreated cells. Furthermore, noninternalized receptors fully resensitized at the plasma membrane. Similarly, the internalization-defective T399A mutant m4 mAChR resensitized more rapidly than the wild-type receptor. Thus, internalization delays the acquisition of full responsiveness of the receptors after reappearing on the cell surface. The mechanism that underlies this protracted desensitization state of the receptors is not understood. However, two observations suggest that the recycled receptors themselves are the primary site of change. First, desensitization of m4 mAChR function was found to be homologous in nature. Second, recovery of receptor function did not require *de novo* receptor synthesis. There is a precedent for such refractoriness in the recovery of functional responsiveness after receptor internalization. Lohse *et al.* (18) reported that although β_2-adrenoceptor internalization and recycling in human A431 cells serve to restore the function of desensitized receptors, receptor function recovered only after the full reappearance of receptors at the cell surface. The sustained desensitization state of the receptors may relate to the rearrangement of receptors with G proteins or other signal transduction factors in the plasma membrane after internalization and recycling (18). Alternatively, mAChRs may have been modified during the internalization-recycling process, and conversion to the original state of functional activity is slow. Regardless of the mechanism, our results suggest that desensitization of m4 mAChRs in CHO cells involves multiple components (i.e., agonist-induced receptor desensitization at the plasma membrane as well as prolongation of the desensitized state after receptor internalization and recycling). Future studies may provide more information about the molecular mechanism that is responsible for the slow resensitization of receptor function after agonist-induced receptor internalization.

Acknowledgments

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