Cross-talk between m1 Muscarinic Acetylcholine and $\beta_2$-Adrenergic Receptors

CAMP AND THE THIRD INTRACELLULAR LOOP OF m1 MUSCARINIC RECEPTORS CONFER HETEROLOGOUS REGULATION*

(Received for publication, September 29, 1992)

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Genes encoding the m1 muscarinic (m1 mAChR) and $\beta_2$-adrenergic receptors ($\beta_2$AR) were stably co-expressed into Chinese hamster ovary (CHO) cells to study receptor regulation and cross-talk. Persistent activation of the $\beta_2$AR/adenylate cyclase pathway by isoproterenol leads to heterologous desensitization, internalization, and down-regulation of the m1 mAChR which is comparable, but smaller in magnitude, with that seen with persistent activation of the m1 mAChR by carbachol. This heterologous effect was mimicked by dibutyryl CAMP and forskolin and antagonized by the protein kinase A (PKA) inhibitor H-8. A potential consensus sequence for phosphorylation by PKA (Lys181-Arg-Lys-Thr286) exists on the third intracellular loop of the m1 mAChR, suggesting that receptor phosphorylation by PKA may be involved in heterologous regulation. The loss of m1 mAChRs induced by carbachol was not reversed by H-8, indicating that homologous regulation is not dependent on PKA.

Recent evidence suggests that muscarinic agonist-mediated internalization of the m1 mAChR involves the third intracellular loop (i3) (Maeda, S., Lameh, J., Mallet, W. G., Philip, M., Ramachandran, J., and Saade, W. (1990) FEBS Lett. 269, 386–388). Three deletion mutant receptors were constructed in which the majority, or small regions, of i3 were eliminated but the membrane proximal portions of the loop were left intact. Each of the mutants was co-expressed with the $\beta_2$AR in CHO cells. A small region in i3 was identified which is crucial for carbachol- and isoproterenol-promoted internalization and down-regulation. This region contains a series of 6 serine residues within an 8-amino acid stretch. A similar domain has been identified in the carboxyl tail of the $\beta_2$AR and has been proposed to participate in receptor internalization (Hausdorf, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2979–2983).

Two major signal transduction pathways are those involved in the hydrolysis of inositol lipids by phospholipase C (PLC) and the formation of CAMP by adenylyl cyclase. The cellular effects mediated by CAMP are subserved by protein kinase A (PKA), whereas those of diacylglycerol (a product resulting from the hydrolysis of inositol phosphates) are directed by protein kinase C (PKC). Many reports have indicated that "cross-talk" exists between these two signaling pathways in both neural and non-neural tissues (1). Activation of one pathway has been shown to be stimulatory or inhibitory on the other pathway. For example, treatment of frog erythrocytes with 12-O-tetradecanoyl phorbol-13-acetate, a phorbol ester that activates PKC (2), enhances adenylyl cyclase activity (3). This presumably is due to phosphorylation of adenylyl cyclase by PKC (3). Likewise, for murine L cells transfected with the m5 muscarinic receptor gene, stimulation of this receptor which is coupled to PLC leads to a sensitization of adenylyl cyclase (4). In contrast, PKC activation in NCB20 and NIH 3T3 cells inhibits the CAMP-generating system (5). Stimulation of the adenylyl cyclase pathway has also been reported to interact in a regulatory fashion with the PLC effector system. In rat skeletal myoblasts, exogenously applied forskolin or dibutyryl CAMP (Bt2cAMP) has been shown to impair PKC activity (6). Similarly, activation of PKA impairs the coupling between PLC and a guanine nucleotide-binding protein (G-protein) in NG108-15 cells (7).

Examples of opposite actions involving the adenylyl cyclase system on PLC signaling pathway are those observed in hepatocytes (8).

Less well characterized is the fate of G-protein-coupled receptors during cross-talk of signal transduction pathways. Receptor cross-talk or heterologous regulation refers to that form of regulation in which activation of one receptor by its agonist will functionally modify other receptors (9). By comparison, homologous regulation is agonist-specific, in that only those receptors which interact with the agonist are regulated (9). In both cases, regulation of receptor function can be at the level of transcription or translation. Examples on the molecular mechanisms of receptor cross-talk are limited to adrenergic receptors for the most part. In DDT1 MF-2 vas deferens cells, activation of the $\beta_2$AR/AC pathway elevates $\alpha_1$-adrenergic receptor mRNA and protein levels (10). Similarly, persistent activation of the...
Propranolol were purchased from Sigma. The mammalian expression vector pSP73-ml was digested with PstI and religated. The resulting mutant yield pSP73-ml. To delete amino acids 227-269 from the ml mAChR, reintroduction of nucleotide sequence. Del 275-348 was constructed by digesting pSP73-ml with PvuII and religating with a KpnI linker (5'-CTGGTACCAG-3'). In this deletion mutant, amino acids 275-348 were replaced by a single tyrosine residue. To construct deletion mutants of the P2AR that have deletions in the third intracellular loop (3). A preliminary report of these findings has appeared previously in abstract form (22).

**EXPERIMENTAL PROCEDURES**

**Materials—CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). [3H]Quinuclidinyli benzilate ([3H]QNB) (44.9 Ci/mmol), [125I]iodocyanopindolol ([125I]ICYP) (2200 Ci/mmol), and myo-[125I]inositol (15.6 Ci/mmol) were from Du Pont New England Nuclear. Carbachol, atropine, isoproterenol, and propranolol were purchased from Sigma. The mammalian expression vector pSVL was from Pharmacia LKB Biotechnology Inc; pMSVneo was constructed as described previously (23). Restriction enzymes were purchased from New England Biolabs. Dubecko’s modified Eagle’s medium, fetal calf serum, and Geneticin were from Gibco.

**Construction of Deletion Mutant Receptors—**Deletion mutagenesis was employed to identify the region(s) on the third intracellular loop (3) of the ml mAChR that plays a role in receptor internalization during homologous and heterologous regulation. A 1.6-kilobase XhoI-BamHI fragment of the ml mAChR gene (24), containing the entire coding region (plus 8 base pairs 5' to the initiation codon and 260 base pairs of 3'-untranslated region), was subcloned into pSP73 to yield pSP73-ml. To delete amino acids 227-269 from the ml mAChR, pSP73-ml was digested with PstI and religated. The resulting mutant receptor, del 227-269, was in-frame and therefore did not require the reintroduction of nucleotide sequence. Del 275-348 was constructed by digesting pSP73-ml with PvuII and religating with a KpnI linker (5'-CTGGTACCAG-3'). In this deletion mutant, amino acids 275-348 were replaced by a single tyrosine residue. To construct deletion mutant 227-341 (del 227-341), pSP73-ml was digested with PstI and PvuII and religated with a PvuI cohesive end-PvuII blunt end adaptor (5'-AAACCCGGAGGAGGACGAC-3' and complimentary strand 5'-CTGGTACCAGTTTCTCGA-3'). The resulting in-frame construct deletes amino acids 227-341 in ml. Authenticity of all mutations was confirmed by dye sequencing before inserting the mutant receptor genes into the expression vector pSVL.

**Expression of Receptors in CHO Cells—**Genes for the ml mAChR and the β2AR (23) were inserted into the mammalian expression vector pSVL and co-transfected with pMSVneo (which contains the selectable marker aminoglycoside phosphotransferase) into CHO cells using the calcium phosphate precipitation method (25). Deletion mutagenesis of the ml mAChR gene was likewise co-transfected with the β2AR gene into CHO cells. Cells were grown in selective medium containing Geneticin (500 μg/ml) where only those cells stably integrating pMSVneo into their genome survive. Colonies derived from single cells were isolated and expanded. Radioligand binding assays with [3H]QNB or [125I]ICYP were employed to assay for the presence of the ml mAChR and β2AR, respectively (23, 26).

**Receptor Binding Assays with Intact Cells—**To measure the total number of cellular ml mAChR or β2AR, cells expressing both of these receptors were grown in monolayer and incubated without or with different agents for various times. Cells were harvested in PBS minus Ca2+, centrifuged at 250 x g for 1 min at 4 °C, and washed three times in PBS. Intact cells were resuspended in 1 ml of assay buffer (Dubecko’s modified Eagle’s medium supplemented with 25 mM HEPES, pH 7.4) containing [3H]QNB or [125I]ICYP. These were then incubated for 90 min at 15 °C, during which time equilibrium was attained. The binding reaction was terminated by rapid filtration. Specific binding averaged 60-70% for [3H]QNB and 90% of [125I]ICYP.

**Receptor Binding Assays in Plasma Membrane Homogenates—**Two different protocols have been used to measure cell surface receptors. The first employs radioligand binding assays with a plasma membrane preparation (27, 28). In this procedure, cells are lysed by homogenization in ice-cold H2O and centrifuged at low speed. The resulting pellet contains mostly plasma membranes (27, 29). To recover cytoplasmic vesicles containing internalized receptors, the supernatant is subjected to high speed centrifugation (27, 30). If, however, cells are lysed by hypotonic shock in the absence of homogenization, then internalized receptors can be found associated with the plasma membrane fraction (27, 30).

**Cell Surface Protocol—**Utilized to measure cell surface receptors involves the binding of a hydrophilic ligand, such as the mAChR antagonist [3H]N-methylscopolamine, to whole cell preparations (30). We have found both methods equivalent when measuring cell surface ml mAChR in desensitized and nondesensitized CHO cells (27). In the present study, we utilized the plasma membrane protocol (27, 28) because in order to prevent recycling of the receptor, [3H]N-methylscopolamine binding assays must be carried out at 4 °C, necessitating long incubation (>3 h) to achieve equilibrium binding (30).

Transfected CHO cells expressing β2AR with either wild-type or mutant ml mAChR were incubated without or with receptor agonists, phorbol 12-myristate 13-acetate (PMA), Bt'cAMP, or forskolin for various times. Cells were then washed three times with PBS (pH 7.4) and scraped into ice-cold 50 mM Na+/K+ phosphate buffer (pH 7.4). Cell suspensions were homogenized at 4 °C by Polytron (Brinkmann Instruments, setting 7, 30 s) followed by centrifugation for 20 min at 20,000 x g at 4 °C. The low-speed pellet was resuspended in buffer and used immediately for binding assays. [3H]QNB and [125I]ICYP binding assays were carried out as described previously (23, 26). Reactions, performed in triplicate, were carried out for 60 min at 37 °C and terminated by rapid filtration through Whatman GF/C glass filters using a Brandel cell harvester. Nonspecific binding for [3H]QNB or [125I]ICYP was defined in the presence of 10 μM atropine or 2 μM propranolol, respectively. Ligand binding data were analyzed using the computer program INPLOT (GraphPAD, San Diego, CA). The ratio of cell surface receptors to total receptors ranged from 0.5 to 0.75 for both ml mAChRs and β2ARs in CHO-transfected cells.

**Phosphoinositide Hydrolysis—**Transfected CHO cells expressing the β2AR along with wild-type or mutant ml mAChR were assayed for carbachol (CBC)-stimulated phosphoinositide (PI) hydrolysis as described previously (26). For studies involving agonist preincubation, cells seeded onto 12-well plates were prelabeled with 1 μCi/ml myo-[3H]inositol at 37 °C for 24 h with or without CBC or isoproterenol. This paradigm was employed to prevent the depletion of radiolabeled phosphorylpoliosinositole phosphates during agonist pretreatment. Afterwards, cells were washed three times with PBS (pH 7.4) and rechallenged with CBC. β2ARs were assayed for cAMP formation as described (20). Dose-response curves were analyzed by computerized iterative nonlinear least squares regression (GraphPAD INPLOT).

**RESULTS**

**Pharmacologic Properties of Clonal CHO CHO Cells Co-Expressing the β2AR with Either the Wild-type or Deletion Mutant ml mAChR—**Fig. 1 is a schematic diagram of the deletion mutant receptors constructed by restriction enzyme digestion of the ml mAChR gene. Aliquots of CHO cells retain 19 and 18 amino acids on the amino- and carboxy-terminal portions of i3, respectively, which have been shown previously to be important for ml mAChR/G-protein coupling (31). Del 227-269, del 275-348, and del 227-341 are missing 43, 74, and 115 amino acids out of 156 in i3, respectively.

N. H. Lee and C. M. Fraser, unpublished observation.
CHO cells were stably co-transfected with the genes for the β2AR plus either wild-type or mutant m1 mAChR. Four clonal cell lines were isolated with the following patterns of recombinant receptor expression: β2AR/wild-type m1 (MB-CHO cells), β2AR/del 227-269, β2AR/del 275-348, and β2AR/del 227-341. Saturation binding experiments revealed that all three mutant m1 mAChRs exhibited wild-type binding affinity for antagonists; the Kd value derived from Scatchard analysis of [3H]QNB binding to wild-type m1 mAChR was 26 ± 7 pm (mean ± S.E., n = 3) (Table I). Wild-type and mutant m1 mAChR densities ranged from 1.05 to 2.06 pmol/mg protein in the four clonal cell lines (Table I). In addition, β2AR densities in these cell lines were similar and ranged from 0.80 to 1.53 pmol/mg protein (Table I).

Agonist displacement binding studies revealed that each mutant receptor displayed both high and low affinity sites for CBC; with the Khi and Kli values and the proportion of binding sites present in each affinity state being similar to that seen for the wild-type receptor (Table I). Furthermore, the potency and efficacy of CBC to stimulate PI turnover in clonal cell lines containing the deletion mutants were comparable with those of the wild-type m1 mAChR (EC50 value and -fold stimulation for CBC at the wild-type receptor were 17 μM and 20, respectively, n = 3) (Table I). All responses could be blocked by the muscarinic receptor antagonist, atropine, at a concentration of 10 μM. Hence, the deletion mutant constructs represent functional receptors that retained their ability to couple to PLC.

**Agonist-specific Regulation of m1 mAChR Number in MB-CHO Cells**—CBC-mediated loss of cell surface and total m1 mAChRs in MB-CHO cells was monitored by [3H]QNB binding to plasma membrane homogenates and whole cells, respectively. Cell surface m1 mAChR number steadily declined to 43% of control values during the first 6 h of 1 mM CBC incubation, after which time receptor levels slowly declined to approximately 35% of control levels (Fig. 2A). By comparison, total cellular m1 mAChR content decreased only marginally, by <5%, during 1-6 of h exposure to CBC (Fig. 2A). However, agonist incubations between 12 and 24 h resulted in a 65-75% down-regulation of total m1 mAChRs (Fig. 2A). The receptors remaining after CBC treatment did not exhibit a change in affinity for [3H]QNB. Scatchard analysis revealed that plasma membrane homogenates from CBC-pretreated cells exhibited a single class of binding sites with a Kd of 19 ± 3 pm and a Bmax (maximum binding capacity) of 0.44 ± 0.03 pmol/mg protein (n = 2); by comparison, control cells showed
pretreatment with 1 mM CBC or 1 mM isoproterenol resulted in a 35% decrease in cell surface mAChR number compared with a 50% loss with 1 mM CBC for 5 h (n = 8, **Fig. 2B**). In such cases, the PKA inhibitor H-8 (39) almost completely abolished the observed losses of mAChRs following addition of 0.2 and 2 µM isoproterenol. Unlike isoproterenol treatment, CBC-induced decreases in mAChR number could not be inhibited by H-8 (Fig. 4). Likewise, decreases in β2AR number mediated by 24-h isoproterenol pretreatment, at concentrations of 0.2 and 2 µM, could not be reversed by H-8 (Fig. 4).

**Inhibition of CBC-stimulated PI Turnover by Prolonged mAChR and β2AR Activation**—In MB-CHO cells exposed to 1 mM CBC for 24 h, the subsequent ability of the agonist to maximally stimulate PI hydrolysis was reduced by approximately 65% (n = 3). However, there was no apparent change in the EC50 of CBC to stimulate PI turnover in CBC-pretreated cells (25 ± 7 µM) as compared with control cells (19 ± 3 µM) (n = 3 for each treatment group). In addition, basal PI hydrolysis was increased by approximately 20–50% when pretreated with isoproterenol at concentrations ranging from 0.2 nM up to 2 µM (Fig. 3). mAChR loss induced by low isoproterenol concentrations was completely blocked by 2 µM propranolol, indicating that the β2AR activation was responsible for the deficits (Fig. 3).

To determine whether membrane-permeable cAMP analogues as well as other agents which elevate intracellular levels of cAMP could mimic the effects of isoproterenol on mAChR number, MB-CHO cells were incubated with 1 mM BtzcAMP or 100 µM forskolin for 24 h, and [3H]QNB binding assays were performed. In either case, BtzcAMP (n = 3) or forskolin (n = 7) decreased cell surface mAChR number by 38 and 49%, respectively. A comparable decrease in total cellular mAChR number induced by these agents also could be seen in radioligand binding experiments with intact MB-CHO cells (data not shown). These observations suggest a potential role for PKA in heterologous control of mAChR levels. Thus, the effects of inhibiting PKA were investigated. As shown in Fig. 4, pretreatment of MB-CHO cells with the PKA inhibitor H-8 (39) almost completely abolished the observed losses of mAChRs following addition of 0.2 and 2 µM isoproterenol. Unlike isoproterenol treatment, CBC-induced decreases in mAChR number could not be inhibited by H-8 (Fig. 4). Likewise, decreases in β2AR number mediated by 24-h isoproterenol pretreatment, at concentrations of 0.2 and 2 µM, could not be reversed by H-8 (Fig. 4).

**FIG. 2. Time course of homologous and heterologous regulation in MB-CHO cells.** A, homologous regulation of mAChR function. Intact cells were pretreated with 1 mM carbachol for 24 h at 37°C. Cells were then washed three times with PBS and assayed for PI hydrolysis (○), cell surface mAChR number (□), and total mAChR content (■) as detailed under "Experimental Procedures." Values shown are the average ± S.E. of two to ten independent experiments performed in triplicate and are expressed as a percent of control. B, heterologous regulation of mAChR function. Intact cells were pretreated with 2 µM isoproterenol for 24 h at 37°C. Cells were assayed for PI hydrolysis (○), cell surface mAChR number (△), and total mAChR content (△). Values shown are the average ± S.E. of three to ten independent experiments performed in triplicate and are expressed as a percent of control.

The reduction of mAChRs by muscarinic agonists could be mimicked by the phorbol ester PMA, an activator of PKC (2). Hence, pretreatment of MB-CHO cells with 1 µM PMA for 5 h resulted in a 35% decrease in cell surface mAChR number compared with a 50% loss with 1 mM CBC for 5 h (n = 5 for each treatment group). When mAChR number was assessed in intact cells with [3H]QNB after pretreatment with 1 mM CBC or 1 µM PMA for 5 h, total receptor number down-regulated 30 and 35%, respectively (n = 3 for each treatment group).

**Activation of the β2AR Regulates mAChR Number in MB-CHO Cells**—The effects of prolonged β2AR activation on mAChR number and affinity were monitored in MB-CHO cells. Heterologous regulation of mAChR number was time-dependent. Isoproterenol, at 2 µM, steadily decreased the levels of cell surface mAChRs from 3 to 12 h with maximum decreases (45–48%) occurring after 12 h (Fig. 2B). In contrast, total mAChR content did not appreciably decline for the first 6 h (Fig. 2B). This strongly suggests that the initial response to β2AR activation was internalization of mAChRs. Longer incubations with isoproterenol, from 12 to 24 h, led to a 45–50% reduction (down-regulation) in total mAChRs (Fig. 2B). The loss of mAChRls induced by isoproterenol was not accompanied by a change in the KD of [3H]QNB for the remaining receptors in plasma membrane homogenates. Scatchard analysis showed that isoproterenol-pretreated cells (24 h) exhibited a single class of binding sites with a calculated KD of 17 ± 1 µM and a Bmax of 0.74 ± 0.10 pmol/mg protein (n = 3). The loss of [3H]QNB sites induced by isoproterenol was independent of β2AR density over a tested range of 0.4–2.0 pmol/mg protein (data not shown).

 Decorating of mAChRs (Fig. 2B). The loss of mAChRs induced by isoproterenol was not accompanied by a change in the KD of [3H]QNB for the remaining receptors in plasma membrane homogenates. Scatchard analysis showed that isoproterenol-pretreated cells (24 h) exhibited a single class of binding sites with a calculated KD of 17 ± 1 µM and a Bmax of 0.74 ± 0.10 pmol/mg protein (n = 3). The loss of [3H]QNB sites induced by isoproterenol was independent of β2AR density over a tested range of 0.4–2.0 pmol/mg protein (data not shown).

Decreases in mAChR number were dependent on isoproterenol concentration. For example, mAChR number declined by approximately 20–50% when pretreated with isoproterenol at concentrations ranging from 0.2 nM up to 2 µM (Fig. 3). mAChR loss induced by low isoproterenol concentrations was completely blocked by 2 µM propranolol, indicating that the β2AR activation was responsible for the deficits (Fig. 3).

To determine whether membrane-permeable cAMP analogues as well as other agents which elevate intracellular levels of cAMP could mimic the effects of isoproterenol on mAChR number, MB-CHO cells were incubated with 1 mM BtzcAMP or 100 µM forskolin for 24 h, and [3H]QNB binding assays were performed. In either case, BtzcAMP (n = 3) or forskolin (n = 7) decreased cell surface mAChR number by 38 and 49%, respectively. A comparable decrease in total cellular mAChR number induced by these agents also could be seen in radioligand binding experiments with intact MB-CHO cells (data not shown). These observations suggest a potential role for PKA in heterologous control of mAChR levels. Thus, the effects of inhibiting PKA were investigated. As shown in Fig. 4, pretreatment of MB-CHO cells with the PKA inhibitor H-8 (39) almost completely abolished the observed losses of mAChRs following addition of 0.2 and 2 µM isoproterenol. Unlike isoproterenol treatment, CBC-induced decreases in mAChR number could not be inhibited by H-8 (Fig. 4). Likewise, decreases in β2AR number mediated by 24-h isoproterenol pretreatment, at concentrations of 0.2 and 2 µM, could not be reversed by H-8 (Fig. 4).

**Inhibition of CBC-stimulated PI Turnover by Prolonged mAChR and β2AR Activation**—In MB-CHO cells exposed to 1 mM CBC for 24 h, the subsequent ability of the agonist to maximally stimulate PI hydrolysis was reduced by approximately 65% (n = 3). However, there was no apparent change in the EC50 of CBC to stimulate PI turnover in CBC-pretreated cells (25 ± 7 µM) as compared with control cells (19 ± 3 µM) (n = 3 for each treatment group). In addition, basal PI hydrolysis was increased by approximately 20–50% when pretreated with isoproterenol (Iso) at the indicated times in the absence or presence of 2 µM propranolol (Prop) for 24 h at 37°C. Cells were then washed three times with PBS, and the mAChR number was determined by [3H]QNB binding to plasma membranes using a saturating concentration of 400 nM. Data shown are the average ± S.E. of three to five separate experiments performed in triplicate and are expressed as a percent of receptor number in untreated control cells studied in parallel.
labeled PI turnover was reduced by 26 and 60% in cells pre-treated with isoproterenol on basal lipid labeling was observed (data not shown). The time course for desensitization of CBC-stimulated release of inositol phosphates after prolonged activation of the ml mAChR is shown in Fig. 2A. CBC-stimulated PI turnover was reduced by 26 and 60% in cells pre-treated with the agonist for 3 and 24 h, respectively.

Preincubation of MB-CHO cells with 2 μM isoproterenol for 24 h resulted in a subsequent reduction in the ability of CBC to stimulate PI turnover when compared with untreated MB-CHO cells. The loss of responsiveness was such that the efficacy of CBC was reduced by 40% (n = 3). As mentioned above CBC pretreatment led to a 65% decrement in mAChR-stimulated PI hydrolysis. Hence, heterologous desensitization of ml mAChR function was smaller in magnitude as compared with homologous desensitization. As was the case for CBC pretreatment, no change was observed in the EC50 value for CBC-mediated PI hydrolysis in cells pretreated with isoproterenol (33 ± 9 μM) as compared with control cell value (n = 3 for each treatment group). Furthermore, no effect of isoproterenol on basal lipid labeling was observed (data not shown).

The time course for desensitization of CBC-stimulated release of inositol phosphates after prolonged activation of the β2AR is shown in Fig. 2B. CBC-stimulated PI turnover was reduced by 20 and 40% in cells pre-treated with isoproterenol for 3 and 24 h, respectively.

**FIG. 4. H-8 impairs isoproterenol-induced, but not carbachol-induced, effects on ml mAChR number.** Intact cells were pretreated with the indicated concentrations of carbachol (CBC) or isoproterenol (ISO) in the absence (□) and presence (●) of 20 μM H-8 for 24 h at 37 °C. Cells were then washed three times with PBS, ml mAChR and β2AR numbers were determined by [3H]QNB and [3H]ICIY binding to plasma membranes, respectively, using a saturating concentration of 400 pM for each radioligand. Data shown are the average ± S.E. of three to eight separate experiments performed in triplicate and are expressed as a percent of receptor number in untreated control cells studied in parallel. *, significantly different from cells not treated with H-8.

**FIG. 5. Homologous-induced losses of wild-type and deletion mutant ml mAChRs.** Intact cells containing the β2AR and either the wild-type or deletion mutant ml mAChR were incubated with 1 mM carbachol at the indicated times at 37 °C. Cells were washed three times with PBS, plasma membranes prepared, and receptor binding assays performed with a saturating concentration of 400 pM [3H]QNB. Data shown are the average ± S.E. of three to ten independent experiments performed in triplicate and are expressed as a percentage of receptor number in untreated cells studied in parallel. *, significantly different when compared with the pretreated wild-type ml mAChR. A, wild-type ml mAChR. B, mutant ml mAChR missing amino acids 227–269 (del 227–269). C, mutant ml mAChR missing amino acids 275–348 (del 275–348). D, mutant ml mAChR missing amino acids 227–341 (del 227–341). Insets, at the top are schematic representations of the fifth and sixth transmembrane domains and the third intracellular loop of various ml mAChR constructs. The darkened area indicates the portion of the third intracellular loop that was deleted.

Deletion of amino acids 227–341 produced a mutant ml mAChR (del 227–341) that did not internalize significantly following CBC pretreatment for up to 6 h, as compared with wild-type receptor which exhibited a 50 and 62% loss after 3- and 6-h CBC exposure, respectively (Fig. 5, A and D). Longer CBC incubations of 24 h resulted in a decline of cell surface del 227–341 which mimicked wild-type losses (Fig. 5, A and D). By comparison, the same i3 mutation essentially abolished the ability of β2AR activation to trigger ml mAChR internalization for up to 24 h (Fig. 6, A and D). These findings suggest that a region within amino acids 227–341 may control CBC- and isoproterenol-mediated ml mAChR internalization.

Smaller deletions were constructed to better define the domain(s) on i3 which mediates ml mAChR internalization. Del 227–269, like the large deletion mutant del 227–341, was refractory to CBC-induced receptor internalization (Fig. 5, A and B). This was especially true for CBC incubations of 3 and 6 h where del 227–269 was essentially unresponsive. After 12-h CBC pretreatment, deficits in cell surface del 227–269 levels approached wild-type losses; however, internalization was still significantly (p < 0.05) impaired as compared with wild-type receptor (Fig. 5, A and B). Also of note was the finding that the same deletion completely impaired ml mAChR internalization triggered by heterologous receptor activation for up to 24 h (Fig. 6, A and B). In contrast to the results obtained with del 227–269, deletion of amino acids 275–348 from the ml sequence had no effect on homologous- or heterologous-mediated internalization (Figs. 5, A and C, and 6, A and C). These results suggest the motif(s) responsible for ml mAChR internalization mediated by homologous and heterologous receptor activation is the same or at the very least localized within amino acids 227–269.

**Heterologous Regulation of the ml mAChR**

7953
Homologous- and Heterologous-induced Uncoupling of the m1 mAChR—Persistent activation of m1 mAChRs or βARs in MB-CHO cells desensitizes CBC-stimulated PI turnover which closely parallels m1 mAChR internalization (Fig. 2). The molecular basis for G-protein-coupled receptor desensitization (uncoupling) has been shown to involve receptor phosphorylation and/or internalization away from the effector enzyme (9). However, it is unlikely that desensitization of the PI response is due to the loss of cell surface m1 mAChRs (Fig. 7, A and B). The reduced magnitude of CBC rechallenge to maximally stimulate PI turnover does not appear to be due to a defect in the coupling of G-protein and PLC, since the efficacy and potency of NaF to stimulate PI turnover was not modified by CBC or isoproterenol pretreatment (Fig. 8). Hence, amino acids 227–269 of the m1 mAChR sequence appear to be involved in receptor internalization but not uncoupling. Therefore, these results suggest that a separate motif outside of residues 227–341 may be responsible for agonist-induced uncoupling (see “Discussion”).

**DISCUSSION**

Cross-talk Involving the m1 mAChR—Although numerous reports have appeared on the interactions of multiple signaling pathways at the post-receptor level, information on cross-talk at the level of the receptor is limited. This is especially true for the m1 mAChR gene family. To date, mAChRs in cardiac tissue (m2 subtype), intestinal smooth muscle (m2 and m3 subtypes), and SK-N-SH neuroblastoma cells (m3 subtype) have been shown to desensitize upon activation of the βAR/adenylate cyclase system (33–35). However, the exact loci and molecular mechanisms of cross-talk between the mAChR and βAR are not yet resolved.

It has been demonstrated that short term activation of mAChRs with muscarinic agonists (<1 h) leads to their rapid internalization/sequstration away from the cell surface and into an intracellular compartment (36). This agonist-specific process (homologous regulation) is not accompanied by a

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**Fig. 6.** Heterologous-induced losses of wild-type and deletion mutant m1 mAChRs. Intact cells containing the βAR and either the wild-type or deletion mutant m1 mAChR were incubated with 2 μM isoproterenol at the indicated times at 37°C. Cells were washed three times with PBS, plasma membranes prepared, and receptor binding assays performed with a saturating concentration of 400 μM [3H]QNB. Data shown are the average ± S.E. of three to ten independent experiments performed in triplicate and are expressed as a percentage of receptor number in untreated cells studied in parallel. *p < 0.05 significantly different when compared with the pretreated wild-type m1 mAChR. A, wild-type m1 mAChR. B, mutant m1 mAChR missing amino acids 227–269 (del 227–269). C, mutant m1 mAChR missing amino acids 227–341 (del 227–341). D, mutant m1 mAChR missing amino acids 227–341 (del 227–341). Insets, at the top of each panel are schematic representations of the fifth and sixth transmembrane domains and the third intracellular loop of various m1 mAChR constructs. The darkened area indicates the portion of the third intracellular loop that was deleted.

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**Fig. 7.** Time course of wild-type and deletion mutant m1 mAChR uncoupling induced by homologous and heterologous receptor activation. Cells, seeded in 12-well plates, were preincubated with ymo- [3H]inositol for 24 h in the absence and presence of 1 mM carbachol (A) or 2 μM isoproterenol (B), for the indicated times at 37°C. Cells were washed three times with PBS and rechallenged with 3 mM carbachol for 30 min in the presence of 10 mM LiCl for PI hydrolysis assays. Values shown are the average ± S.E. of three independent experiments performed in triplicate and expressed as a percentage of control.
these studies, which is in agreement with the number of PKA phosphorylation sites on the m1 mAChR based on primary incorporation was ~1 mol of phosphate/mol of receptor in membrane-permeable cAMP analogue, mimic the effects of isoproterenol-induced, but not CBC-induced, losses in m1 mAChR number (39). Cells were washed three times with PBS and rechallenged with the indicated concentrations of NaF plus 10 mM AlCl3 in the presence of 10 mM LiCl. Values shown are the average ± S.E. of three independent experiments performed in triplicate and expressed as a fold basal stimulation of PI hydrolysis.

In this report, we describe a similar series of events that occur during heterologous regulation, where m1 mAChRs initially internalize and then down-regulate as a consequence of β2AR activation. For both homologous and heterologous receptor activation, down-regulation of m1 mAChRs did not occur for at least 6 h, after which time losses of total m1 mAChRs proceeded in a time-dependent manner. A recent study has indicated that agonist-mediated internalization of mAChRs is a prerequisite for down-regulation in SK-N-SH neuroblastoma cells (38) and requires G-protein involvement (38). We do not know at this time whether a similar chain of events is obligatory for heterologous down-regulation of mAChRs. Experiments are currently underway to address this issue.

Heterologous Control of mAChR Number by cAMP May Involve PKA-β2AR-Mediated Regulation of m1 mAChR Number Appears to Involve PKA—This premise is supported by the following findings. First, forskolin, a diterpene that directly stimulates adenylate cyclase, and Bt,cAMP, a membrane-permeable cAMP analogue, mimic the effects of isoproterenol pretreatment. Second, prior exposure of MB-CHO cells to H-8, a PKA inhibitor (39), reversed isoproterenol-induced, but not CBC-induced, losses in m1 mAChR number (Fig. 4). Consistent with this notion is the finding that mAChRs purified from brain tissue, which is abundant in the m1 subtype (40), can be phosphorylated directly by the catalytic subunit of PKA in vitro (19). The stoichiometry of 32P incorporation was ~1 mol of phosphate/mol of receptor in these studies, which is in agreement with the number of PKA phosphorylation sites on the m1 mAChR based on primary structure analysis (see below). It is noteworthy that PKA also has been demonstrated to covalently modify other muscarinic receptor subtypes such as the m2 mAChR (21). Primary structure analysis of the m1 mAChR reveals a potential PKA phosphorylation sequence motif Lys354-Arg-Lys-Thr356 on i3 (Fig. 9) (41), in an area proposed to be important for receptor-G-protein coupling (31). Sites for PKA are also present in the second intracellular loop of the m2 and m4 subtypes and i3 of the m3 and m5 subtypes (Fig. 9).

The role of PKA-mediated phosphorylation has been extensively examined in the hamster β2AR (42, 43). Two PKA phosphorylation motifs are found in the receptor, one of these sites being located on i3 and the other on the COOH-terminal domain (Fig. 9). Removal of these motifs by deletion or site-directed mutagenesis produces mutant receptors that are no longer phosphorylated by PKA and exhibit an impaired ability to undergo rapid heterologous desensitization and down-regulation (42, 43). Taken together, these results suggest that PKA may serve a similar role in m1 mAChR function. Identification of these motifs in the m2 and m3 mAChRs, previously shown to undergo modulation upon βAR activation (33–35), underscores PKA's potential involvement in cross-regulating these subtypes; an unappreciated mechanism at the time these studies were performed. However, direct confirmation of PKA phosphorylation of these consensus sites during heterologous regulation of mAChRs awaits future mutagenesis experiments.

Agonist-specific Regulation of mAChR Number Does Not Involve PKA—mAChRs coupled to PLC are known to weakly stimulate cAMP formation in CHO cells (44), presumably via a calmodulin-dependent process (45), although this may not be the sole mechanism. Inasmuch as CBC-mediated down-regulation was not blocked by H-8, PKA appears not to be required for homologous regulation of the m1 mAChR in MB-CHO cells. Likewise, PKA activity was not required for isoproterenol-induced losses of βARs in the present study, not an unexpected finding (46).

Role of i3 in Receptor Cross-talk—The third intracellular loop (i3) of the m1 mAChR contains 156 amino acids, of which only 11 and 21 residues at the NH2- and COOH-termini of the receptor. The sequence motif for the m1 mAChR represents amino acids 351–354 of the rat sequence (24).

Fig. 8. Carbachol and isoproterenol treatment does not modify NaF stimulation of phosphoinositide hydrolysis. Intact cells, containing the β2AR and wild-type m1 mAChR, were prelabeled with [3H]phosphatidylinositol for 24 h in the absence and presence of 1 mM carbachol (CBC) or 2 μM isoproterenol at 37 °C. Cells were washed three times with PBS and rechallenged with the indicated concentrations of NaF plus 10 mM AlCl3 in the presence of 10 mM LiCl. Values shown are the average ± S.E. of three independent experiments performed in triplicate and expressed as a fold basal stimulation of PI hydrolysis.

Fig. 9. Consensus sequence motifs for PKA phosphorylation on the mAChR subtypes and the β2AR. PKA sequence motif is boxed and generalized as follows: B-B-X-S/T, where B represents the basic amino acids arginine or lysine, X represents any amino acid, and S/T represents the phosphoacceptor site of either serine or threonine. These sites are located on the second intracellular loop (i2), third intracellular loop (i3), or the carboxyl terminal (COOH) of the receptor. The sequence motif for the m1 mAChR represents amino acids 351–354 of the rat sequence (24).

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1 D. Gurwitz, C. M. Fraser, A. Fisher, unpublished observations.
terminal ends, respectively, are necessary for conferring receptor-G-protein coupling (31). The central portion of \( i_3 \) appears to play a role in muscarinic agonist-mediated internalization, as deletion of up to 130 amino acids impaired this process (32). The results presented here, where we have deleted amino acids 227–341 but left intact the membrane proximal portions of \( i_3 \), are in agreement with the above studies. Moreover, our results reveal that the middle portion of \( i_3 \) is also required for m1 mAChR internalization during heterologous regulation. By deleting smaller regions within amino acids 227–341, we have identified a 43-residue domain (amino acids 227–269) that is crucial for both homologous- and heterologous-promoted internalization. Of interest was the finding that del 227–341 exhibited impaired ability to internalize during the first 12 h of CBC incubation but not at 24 h. This was not the case during \( \beta_2 \)AR activation, where del 227–341 exhibited impaired ability to internalize for up to 24 h. Recent findings in our laboratory indicate that long term exposure to CBC (>6 h) destabilizes m1 mAChR mRNA. Such a regulatory event explains the loss of m1 mAChRs at later CBC incubations.

In terms of homologous internalization, del 275–348 was indistinguishable from wild-type m1 mAChR. This result was somewhat surprising in light of a recent report implicating amino acids 264–286 in the homologous-mediated internalization of the human m1 mAChR (47). It should be noted that the sequence identity of \( i_3 \) in the rat m1 mAChR (present study) and its human homolog (47) is 100%. We do not have an explanation at this time for the discrepancies between our results and those of Lameh et al. (47). It is possible that the different cell lines utilized in the transfection experiments in the two studies are a contributing factor. Another possibility is that an additional domain located inside residues 275–348 participates in receptor recycling (47). Regardless, these two studies when taken together implicate multiple \( i_3 \) domains in m1 mAChR internalization.

Residues 227–269 and 284–292, as postulated by Lameh et al. (47), contain a series of polar residues that may contribute to receptor uncoupling. With the exception of amino acids 351–354, do not appear to participate in agonist-mediated m1 mAChR internalization. Specifically, amino acids 227–269 (present study) and 284–292 (47) are involved in homologous internalization, whereas amino acids 227–269 and 351–354 (PKA site) are required for heterologous internalization. These domains, with the exception of amino acids 351–354, do not appear to participate in agonist-mediated m1 mAChR uncoupling. Hence, it seems likely that additional domains (e.g. PKC sites) are responsible for receptor uncoupling. Clearly, the role, if any, of in situ phosphorylation of m1 mAChR in receptor uncoupling remains to be clarified and awaits future experiments.

Acknowledgments—We thank T. Miller and E. Cheng for expert technical assistance.

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Heterologous Regulation of the m1 mAChR