A Novel Mechanism for Coupling of m4 Muscarinic Acetylcholine Receptors to Calmodulin-Sensitive Adenylyl Cyclases: Crossover from G Protein-Coupled Inhibition to Stimulation†

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ABSTRACT: Muscarinic m4 acetylchloline receptors are normally coupled through G_i to inhibition of adenylyl cyclases. In the olfactory bulb and some cultured cells, however, m4 receptors can couple to stimulation of adenylyl cyclase activity. In this study, m4 receptors and specific isozymes of adenylyl cyclases were coexpressed in HEK-293 cells to characterize the mechanism(s) for m4 receptor stimulation of adenylyl cyclases. The calmodulin-sensitive type I and type III adenylyl cyclases were chosen for this study because neither enzyme is stimulated by the β/γ complex of G coupling proteins. M4 receptors exhibited either inhibition or stimulation of type I and III adenylyl cyclases depending upon receptor density and agonist concentration. Inhibition of adenylyl cyclase was apparently due to M4 coupling through G_i. Adenylyl cyclase stimulation through m4 receptors was not due to increases in intracellular Ca^{2+} and stimulation of the calmodulin-sensitive enzymes since it was evident in isolated membranes in the absence of free Ca^{2+} and with whole cells preloaded with the Ca^{2+} chelator BAPTA/AM. Stimulation of adenylyl cyclase activities by m4 receptors was apparently mediated via G_s since it was GTP-dependent, was insensitive to pertussis toxin, and was not due to β/γ stimulation. Synthetic peptides derived from a G protein activating region of the m4 receptor mimicked the m4-mediated stimulation of adenylyl cyclase activity. These data demonstrate a novel mechanism for muscarinic regulation of adenylyl cyclases that may involve crossover from inhibitory to stimulatory G protein coupling.

The family of muscarinic acetylcholine receptors (m1–m5) couple via guanine nucleotide binding proteins (G proteins) to a variety of signal transduction systems within cells including adenylyl cyclases, phospholipase C, and ion channels (Nathanson, 1987; Jones et al., 1992). Tissue-specific responses to these receptors are governed by the complex interactions between distinct receptor subtypes, G protein subunits, and effectors expressed in different cell types. Within a given cell, individual muscarinic receptor subtypes can activate multiple G protein/effector pathways adding further complexity to this system. Consequently, a specific muscarinic receptor subtype can initiate divergent intracellular processes when expressed in different cell or tissue types. For example, in most neuronal tissues, m4 muscarinic receptors couple to inhibition of adenylyl cyclase activity, but in the olfactory bulb, m4 activation stimulates adenylyl cyclase activity (Olianas & Onali, 1990, 1991).

What determines the tissue specificity of these responses? While considerable effort has been directed at this question by examining the role of receptor/G protein specificity (Ross, 1989; Hille, 1992), until recently the role of distinct adenylyl cyclase isozymes in determining specific muscarinic responses has been less thoroughly investigated (Tang & Gilman, 1991; Federman et al., 1992; Duzic & Lanier, 1992). With the recent cloning and characterization of six distinct adenylyl cyclase isozymes, however, the breadth of regulatory diversity represented in the adenylyl cyclase family is now being recognized (reviewed in Choi et al., 1993a). Although it was originally believed that only the alpha subunits of G_s or G_i could directly regulate adenylyl cyclases, there is now evidence that the β/γ complex can also modulate adenylyl cyclase activity (Tang & Gilman, 1991; Federman et al., 1992; Taussig et al., 1993). All six adenylyl cyclases cloned to date are sensitive to G_s-α stimulation but vary considerably in their sensitivity to β/γ heterodimers. For example, in the presence of activated G_s-α the type II and IV adenylyl cyclases are stimulated by β/γ released upon receptor activation, while the type I enzyme is inhibited by β/γ. Other adenylyl cyclase isozymes are unaffected by β/γ. Regulation of adenylyl cyclase activity is also controlled by intracellular Ca^{2+} levels; the type I and III adenylyl cyclases are stimulated by calmodulin (CaM)[1] and Ca^{2+} (Krupinski et al., 1989; Choi et al., 1992a), whereas the other four enzymes are insensitive to CaM.

The m4 muscarinic receptor regulates intracellular cAMP through a number of discrete G protein pathways. In general, the activation of m2 and m4 receptors are thought to directly inhibit cAMP production via G_i while the m1, m3, and m5 receptors couple to stimulation of phosphoinositide (PI) hydrolysis (Peralta et al., 1988; Ashkenazi et al., 1989a). Muscarinic receptor stimulated PI turnover increases intracellular Ca^{2+} which can activate the type I adenylyl cyclase (Choi et al., 1992a). In certain cell types, m4 receptors can

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[1] Abbreviations: BAPTA/AM, 1,2-bis(2-amino-5-fluorophenoxy)-ethane *N,N,N′,N′*-tetrakis(acetoxymethyl) ester; CaM, calmodulin; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine; IP3, D-*myo*-inositol 1,4,5-triphosphate; PTX, pertussis toxin; PI, phosphoinositide; [^{3}H]QNB, 1-quinuclidinyl[*phenyl*-4-^{3}H]-benzilate.

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also couple weakly to PI hydrolysis, suggesting that m4 activation might also be able to stimulate adenylyl cyclase activity in tissue expressing Ca^{2+}/CaM sensitive isozymes (Ashkenazi et al., 1989b). The recent demonstration that G_i-coupled receptors can regulate adenylyl cyclase activity via β/γ subunits has suggested a third mechanism by which m4 receptors might regulate intracellular cAMP levels (Federman et al., 1992). In fact, β/γ activation of specific adenylyl cyclase isozymes might explain the recent data indicating that m4 activation can stimulate adenylyl cyclase in a Ca^{2+}-independent manner in the olfactory bulb and certain cultured cell lines (Olianas & Onali, 1990, 1991; Baumgold et al., 1992. Jones et al., 1991).

Since m4 receptor activation can either inhibit and/or stimulate cAMP production in different tissues, we hypothesized that m4 control of cAMP might be determined by differential expression of adenylyl cyclase isozymes. In this study, we expressed the m4 receptor in HEK-293 cells expressing the two known Ca^{2+}/CaM-sensitive adenylyl cyclases, type I and III. We were particularly interested in the CaM-sensitive adenylyl cyclases because they are differentially regulated by a number of distinct pathways including, G_s-α stimulation, G_i-α inhibition, intracellular Ca^{2+} levels, and β/γ subunits (reviewed in Choi et al., 1993a).

In this paper we demonstrate that m4 muscarinic receptors can couple to either stimulation or inhibition of the type I and type III adenylyl cyclases through G protein-mediated processes. The data in this paper is consistent with "crossover" of m4 receptors from G_i inhibition to G_s stimulation of adenylyl cyclase activity.

EXPERIMENTAL PROCEDURES

Cell Culture and DNA Transfection. Human embryonic kidney 293 cells were grown at 36 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified 95% air/5% CO_2 incubator. Cell culture materials were from Gibco unless otherwise noted. Neomycin-resistant HEK-293 cells stably transfected with an expression vector CDM8 that contained cDNA for type I adenylyl cyclase (CDM8(I-AC), type III adenylyl cyclase (CDM8(III-AC)), or no exogenous DNA were used for this study. Particular clones of each type (1AC43, 3AC3, and 293-NEO) have been previously characterized (Choi et al., 1992b, 1993b) and were used for subsequent cotransfection with m4 muscarinic receptors. Each of these cell lines were stably transfected with either pCD-PS expression vector containing DNA encoding the chick m4 muscarinic receptor or pCD-PS vector alone (Bonner et al., 1988). Briefly, cells were plated in 100-mm dishes at a density of 2×10^6 cells/plate, grown overnight, and transfected with the pCD control vector or pCD(m4) (10 μg of DNA/plate) and a hygromycin resistance vector (1 μg of DNA/plate) by the calcium phosphate method (Chen & Okayama, 1987). Hygromycin-resistant cells were selected in culture medium containing Hygromycin B (Sigma, 460 Units/ml) and 300 μg/mL G418. Clones of hygromycin/neomycin-resistant cells were examined for [^{3}H]QNB binding to detect cells that expressed m4 muscarinic receptor. M4-expressing cells were also examined for adenylyl cyclase activity (via forskolin and/or Ca^{2+}/Cam stimulation) to ensure that CaM-sensitive adenylyl cyclase activities were maintained and that the level of expressed adenylyl cyclase was the same between cell lines expressing varying levels of m4 receptors. After selection, cells were maintained in media containing 230 units/mL hygromycin B and 300 μg/mL G418. Ten hygromycin/neomycin-resistant clones expressing varying levels of m4 receptor and each adenylyl cyclase type were isolated and characterized further. Four hygromycin/neomycin-resistant clones transfected with control vector pCD and each adenylyl cyclase were isolated and characterized. Cell line nomenclature is as follows: cells transfected with pCD control vector were designated "P", cells transfected with m4-pCD were designated "M", cells expressing type I or III adenylyl cyclase were designated "1" or "3" respectively, neomycin resistant cells transfected with control CDM8 vector were designated "N". Thus, for example, neomycin/hygromycin-resistant cells expressing m4 receptors and no exogenous adenylyl cyclase were designated "MN" followed by an arbitrary clone number (e.g. MN-15).

[^{3}H]QNB Binding Assays. Expression levels of m4 muscarinic receptors were determined by measuring specific [^{3}H]-QNB binding to cell membrane homogenates as described by Nathanson et al. (1992). Briefly, cells were grown to confluence in 100-mm culture dishes, washed twice with ice-cold PBS, and harvested in 4 mL of ice-cold 50.0 mM sodium phosphate, pH 7.4. Cells were homogenized by hand with 20 strokes of a ground glass homogenizer and centrifuged at 30000*g*, 4 °C, for 15 min. The pellets were washed once in $NaPO_4$, recentrifuged, and resuspended in 0.5 mL of 50 mM $NaPO_4$. Duplicate samples of 0.1 mL of resuspended membranes were incubated for 90 min at 25 °C with 4.2×10^{-10} M [^{3}H]QNB (Amersham) in a final volume of 1.0 mL. Nonspecific binding was measured in parallel in the presence of 1.0 μM atropine.

Cyclic AMP Accumulation. Changes in intracellular cAMP levels were measured by determining the ratio of [^{3}H]-cAMP to a total ATP and ADP pool in [^{3}H]adenine loaded cells as described by Wong et al. (1991). Confluent cells in 6-well plates were initially incubated in DMEM containing [^{3}H]adenine (2.0 μCi/mL, ICN) for 16–20 h, washed once with 150 mM NaCl, and incubated at 37 °C for 30 min in incubation buffer (118 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10.0 mM glucose, 20.0 mM HEPES, pH 7.4) containing 1.0 mM IBMX, 5.0 μM forskolin, and various effectors as indicated. Reactions were terminated by aspiration, washing cells once with 150 mM NaCl, and adding 1.0 mL of ice-cold 5% TCA containing 1.0 μM cAMP. Culture dishes were maintained at 4 °C for 1–4 h, and acid-soluble nucleotides were separated by ion-exchange chromatography as described (Salomon et al., 1974).

While this assay system affords rapid and extremely sensitive measurements of relative changes in intracellular cAMP levels in response to carbachol, we were initially concerned about reproducibility because we, like others (Federman et al., 1992), noticed that absolute numbers for cAMP accumulation often varied between experiments. It is important to emphasize that relative changes in cAMP were highly consistent between experiments. However, to ensure that these changes in cAMP accumulation were accurate and to assess the relative contribution of transfected adenylyl cyclase vs endogenous adenylyl cyclases expressed in these cells, we used the more laborious method of Gilman (1970) to measure absolute cAMP levels in several cell lines expressing type I, type III, or no exogenous adenylyl cyclase. These results confirmed the relative changes in cAMP levels previously observed in response to carbachol in all cell lines tested. For example, treating forskolin-stimulated type III adenylyl cyclase expressing cell lines P3-2, M3-14, and M3-32 with 1.0 mM carbachol altered intracellular cAMP levels (pmol of cAMP formed/mg of protein) from 4649.9 ± 346.2 to 4677.9 ± 29.4; 4762.0 ± 335 to 2229.6 ± 27.6; and 4268.0 ± 29.4 to 7410.3 ± 282.5, respectively. Furthermore, these experiments demonstrated

that relative changes in cAMP in type I and III adenylyl cyclase cell lines were primarily due to regulation of these exogenously expressed adenylyl cyclase. For example, forskolin stimulated levels of cAMP in control HEK-293 cells (PN-5) were only 551.8 ± 99.5 pmol/mg compared to 3209.1 ± 13.5 and 4649.9 ± 346.2 pmol/mg in type I (P1–6) and III (P3–2) expressing cell lines, respectively. These results indicated that endogenous adenylyl cyclases contributed at most only 10–20% to the relative changes in cAMP levels observed in forskolin-stimulated type I and III expressing cells.

Cell Membrane Preparation. Membranes were prepared as described in Choi et al. (1992a,b). Briefly, confluent cells were washed twice with 150 mM NaCl, harvested into ice-cold membrane buffer (20 mM Tris-HCl, pH 7.4, 2.0 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM dithiothreitol, 5.0 μg/mL leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride), and broken at 4 °C by Dounce homogenization. Membranes were separated from nuclei and unbroken cells by centrifugation at 600*g* for 2 min, and the supernatants were subjected to centrifugation at 30000*g* for 20 min. The resulting membrane pellets were resuspended in membrane buffer (4 °C) to a typical protein concentration of 0.2–0.8 mg/mL and assayed for adenylyl cyclase activity. Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical).

Adenylyl Cyclase Assays. Adenylyl cyclase activity was determined by adding membrane fractions (10–40 μg of protein) to an assay solution containing 1.0 mM α-[^{32}P]ATP (500 cpm/pmol), [^{3}H]-cyclic AMP (20 000 cpm/μmol), 5.0 mM $MgCl_2$, 0.2 mM EGTA, 1.0 mM EDTA, 2.0 mM cyclic AMP, 1.0 mM β-mercaptoethanol, 5.0 mM theophylline, 0.1% bovine serum albumin, 20 mM phosphocreatine, and 100 units/mL creatine phosphokinase in 20 mM Tris-HCl, pH 7.4, in a final volume of 250 μL. After 25 min at 30 °C, reactions were terminated by adding 750 μL of 1.5% SDS, and [^{32}P]-cyclic AMP generated was measured as described by Salomon (1974). Peptide m4:393-411 (RSQVRKKRQMAA-REKKVTR) was synthesized and purified by the University of Washington Molecular Pharmacology Protein Core facility.

Quantitation of Intracellular Free Ca^{2+}. Cell lines grown on glass cover slips were examined for intracellular free Ca^{2+} using fura-2 by the method of Hassid (1986).

RESULTS

To examine the coupling of m4 muscarinic receptors to type I and III adenylyl cyclases, human embryonic kidney (HEK-293) cells stably expressing either type I or type III adenylyl cyclase were transfected with plasmids containing the m4 muscarinic receptor DNA and a gene conferring resistance to the antibiotic hygromycin. HEK-293 cells were chosen because they contain low levels of endogenous adenylyl cyclases and the regulation of type I and III adenylyl cyclases in these stable lines has been characterized (Choi et al., 1992b, 1993b). Forty independent hygromycin resistant cell lines of each adenylyl cyclase type were screened for stable m4 expression by [^{3}H]QNB binding, and several lines expressing varying levels of m4 receptor were maintained for subsequent studies (Figure 1). Control cell lines transfected with hygromycin resistance and plasmid lacking m4 were also selected. All cell lines were further screened for type specific adenylyl cyclase activity (i.e. Ca^{2+}/Cam sensitivity) to ensure adenylyl cyclase expressions were maintained.

Wild-type HEK-293 cells apparently express low levels of endogenous m1 or m3 muscarinic receptors since carbachol treatment of these cells expressing type I adenylyl cyclase indirectly stimulated cAMP production by increasing intra-

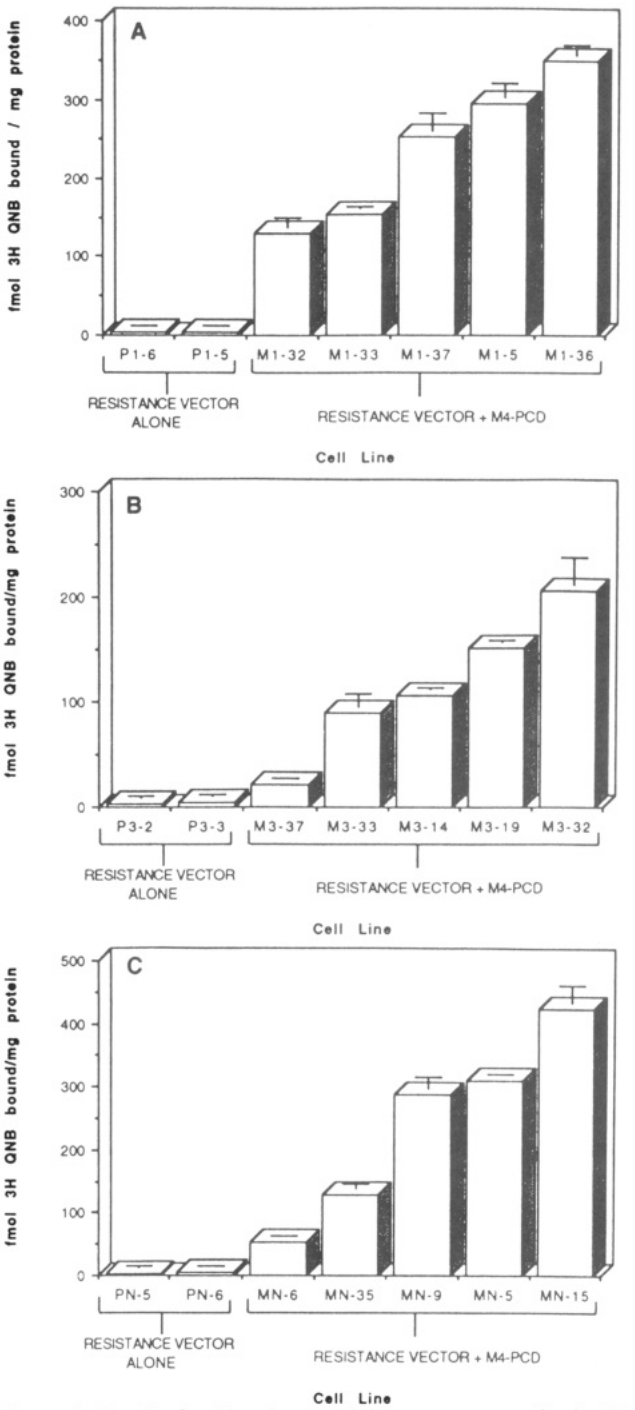

FIGURE 1: Levels of stable m4 muscarinic receptor expression in 293 HEK cells expressing Ca^{2+}/Cam-sensitive adenylyl cyclases. Stable cells line expressing both m4 muscarinic receptors and exogenous adenylyl cyclases were generated by cotransfecting the m4-pCD vector and a hygromycin B resistance vector into stable lines expressing type I or type III adenylyl cyclase, or neomycin resistance alone. M4 receptor expression levels were determined by measuring [^{3}H]QNB binding to membrane homogenates as described in Experimental Procedures. Specific binding was determined by subtracting amount bound in the presence of 1.0 μM atropine. Cell line nomenclature is as follows: cells transfected with pCD control vector were designated "P", cells transfected with m4-pCD were designated "M", cells expressing type I or III adenylyl cyclase were designated "1" or "3" respectively, neomycin resistant cells transfected with control CDM8 vector were designated "N". Thus, for example, neomycin/hygromycin-resistant cells expressing m4 receptors and no exogenous adenylyl cyclase were designated "MN" followed by an arbitrary clone number (e.g. MN-15). (A) Distinct cell lines expressing type 1 adenylyl cyclase and varying levels of m4 muscarinic receptor. (B) Distinct cell lines expressing type III adenylyl cyclase and varying levels of m4 muscarinic receptor. (C) HEK-293 cells expressing no exogenous adenylyl cyclase and varying levels of m4 muscarinic receptor.

cellular free Ca^{2+} (Choi et al., 1992b). However, no specific QNB binding in wild-type cells of hygromycin-resistant control cell lines was detectable, indicating that endogenous muscarinic receptor levels are very low (Figure 1). In cell lines expressing type I or type III adenylyl cyclase, m4 receptor expression varied from 25 to 400 fmol of [^{3}H]QNB bound/mg of protein (Figure 1A,B). Control cells expressing no exogenous adenylyl cyclases had receptor levels ranging from 50 to 450 fmol of [^{3}H]QNB bound/mg protein (Figure 1C). These levels of receptor expression are consistent with m4 levels in several brain tissues (Wall et al., 1992a,b).

We initially examined cells expressing different levels of m4 receptor with or without the type I or III adenylyl cyclases by monitoring changes in intracellular cAMP production after exposure to the muscarinic agonist, carbachol. Carbachol treatment of cell lines expressing type I adenylyl cyclase but no exogenous m4 receptor (e.g. P1-6, Figure 2A) caused a small increase in cAMP production with an EC_{50} of approximately 50 μM. These data are consistent with the results of Choi et al. (1992b) who demonstrated a Ca^{2+}-dependent carbachol stimulation of the type I adenylyl cyclase expressed in HEK-293 cells, presumably via IP3 mediated Ca^{2+} increases. Intracellular cAMP levels declined in response to carbachol in most cell lines expressing type I adenylyl cyclase with low to moderate levels of the m4 receptor (e.g. M1-32, M1-37), presumably via m4 activation of G_i (Figure 2A). Maximal inhibition for each cell line in the presence of 1.0 mM carbachol varied from 40% (cell line M1-37) to 80% (cell line m1-33) (i.e. 20–60% of cAMP accumulation in the absence of carbachol). In cell line M1-36, which expressed type I adenylyl cyclase and relatively higher levels of m4, carbachol stimulated cAMP levels 1.5–2.5-fold (Figure 2A).

Cell lines expressing the type III adenylyl cyclase and varying levels of m4 receptor showed similar responses to carbachol (Figure 2B). Carbachol treatment of control cells expressing no exogenous muscarinic receptor had no effect on cAMP production (e.g. cell line P3-2, Figure 2B). These results are consistent with our previous findings which indicated that endogenous muscarinic receptors in HEK-293 cells do not affect type III adenylyl cyclase activity (Choi et al., 1992a,b). Like type I adenylyl cyclase-containing cells, cAMP production was inhibited approximately 50% by carbachol in type III cell lines expressing low to moderate levels of m4 receptors (e.g. M3-37, M3-14, Figure 2B). However, in cell line M3-32, which expressed the highest levels of m4, carbachol treatment increased cAMP production approximately 1.5-fold. Maximal stimulation of cAMP production in M3-32 was generally slightly lower than M1-36, varying from 120 to 200% of control levels in four experiments performed.

Cyclic AMP levels in wild-type HEK-293 cells expressing no exogenous adenylyl cyclase and no exogenous muscarinic receptor were unaffected by carbachol treatment (e.g. PN-5, Figure 2C). In m4 expressing lines, cAMP accumulation was inhibited approximately 40–60% by carbachol in a dose-dependent manner (e.g. MN-5, Figure 2C). In all cell lines, 1.0 μM atropine, a muscarinic antagonist, blocked the effects of 1.0 mM carbachol on cAMP production.

The concentration dependence for carbachol effects on intracellular cAMP in M1-36 and M3-32 cells was examined in greater detail over an expanded range of carbachol concentrations (Figure 3). Interestingly, treatment of both M1-36 and M3-32 with low carbachol concentrations decreased cAMP production to 50–60% while higher concentrations of carbachol stimulated cAMP levels approximately 1.5-fold. In both cell lines, maximal cAMP inhibition was observed at 0.1–0.5 μM carbachol with an EC_{50} of approximately 0.1 μM while the EC_{50} for stimulation ($\sim$5.0 μM) was approximately 100-fold higher. In comparison, the EC_{50} for carbachol inhibition in cell lines that only demonstrated cAMP inhibition (e.g. M1-37, M3-14, M3-37) ranged between

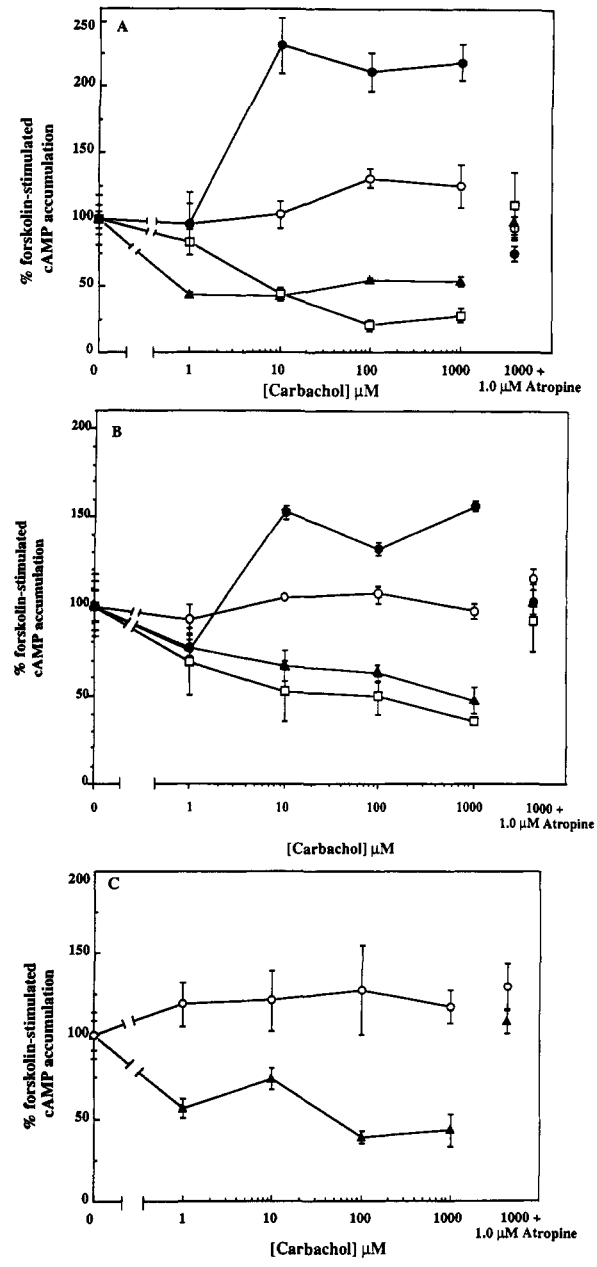

FIGURE 2: Effect of carbachol on cAMP accumulation in representative HEK-293 cells expressing Ca^{2+}/Cam-sensitive adenylyl cyclases and varying levels of m4 muscarinic receptors. Cell lines stably expressing varying levels of m4 receptor and (A) type I adenylyl cyclase [cell line P1–6, ○ (no m4); M1-32, ▲ (low m4); M1-37, □ (med m4); M1-36 ● (high m4)]; (B) type III adenylyl cyclase [cell line P3-2, ○ (no m4); M3-37, ▲ (low m4); M3-14, □ (med m4); M3-32, ● (high m4)]; or (C) neomycin resistance alone [cell line PN-5, ○ (no m4); MN-5, ▲ (high m4)] were exposed for 30 min to incubation buffer containing 5.0 μM forskolin, 1.0 mM IBMX, and increasing concentrations of the muscarinic agonist carbachol. To ensure the effects of carbachol were mediated via muscarinic activation, each cell line was also treated with the highest carbachol concentration (1.0 mM) in the presence of 1.0 μM atropine. Cyclic AMP accumulations were determined as described in Experimental Procedures. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation for each cell line in the absence of carbachol. These data represent triplicate determinations in one experiment; three additional experiments gave similar results. Taken together, data from four independent experiments indicated that there is no significant difference in the dose-dependence of cAMP inhibition between cell lines M1-32 and M1-37 as suggested by the representative data presented in Figure 2A.

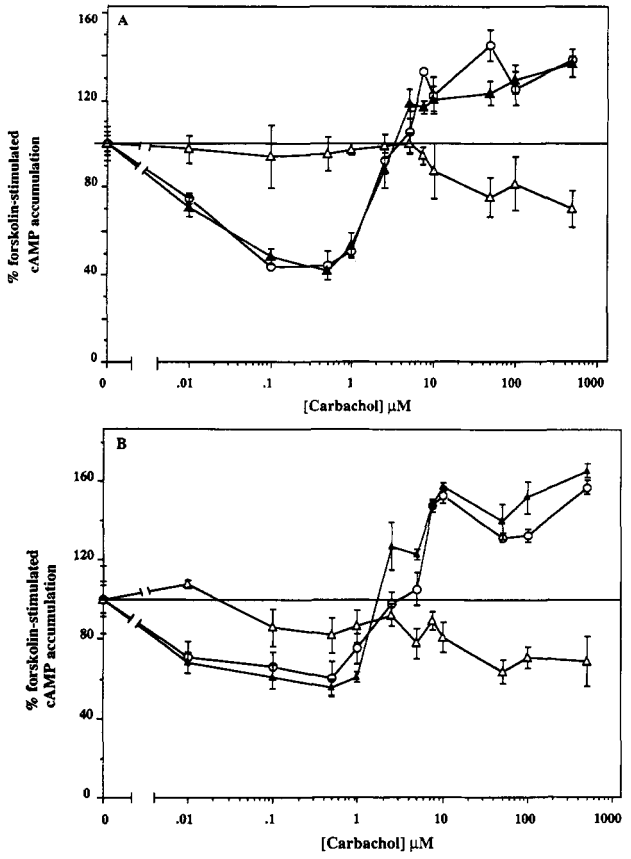

FIGURE 3: Effects of BAPTA/AM on carbachol-mediated increases in cAMP accumulation in HEK-293 cells expressing Ca^{2+}/Cam-sensitive adenylyl cyclases and m4 muscarinic receptors. Cell lines expressing m4 receptors and either (A) type I adenylyl cyclase (M1-36) or B) type III adenylyl cyclase (M3-32) were exposed to carbachol in the presence (△) or absence (▲,○) of 1.0 μM atropine for 30 min in incubation buffer containing 5.0 μM forskolin and 1.0 mM IBMX. Relative cAMP accumulations were determined as described in Experimental Procedures. Immediately prior to carbachol exposure, cells were incubated for 30 min in the presence (▲) or absence (△,○) of 100.0 μM BAPTA/AM in nonsupplemented DMEM and wahsed twice with 150 mM NaCl. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation for each cell line and treatment in the absence of carbachol. These data represent triplicate determinations in one experiment; one additional experiment gave similar results.

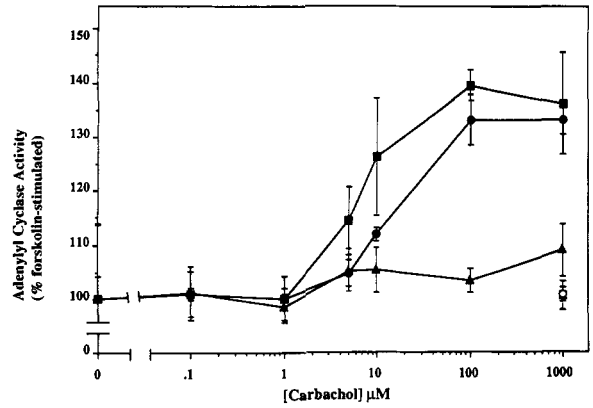

FIGURE 4: Effect of muscarinic agonist on adenylyl cyclase activity in membranes isolated from HEK-293 cells expressing type I adenylyl cyclase and m4 muscarinic receptors. Membranes isolated from cell lines expressing varying levels of m4 muscarinic receptor and type I adenylyl cyclase (P1-6, ▲; M1-37, ●, ○; M1-36, ■, □) were assayed for adenylyl cyclase activity in the presence of 10.0 μM GTP, 5.0 μM forskolin, and increasing concentrations of carbachol. To ensure the effects of carbachol were mediated via muscarinic activation, membranes were also assayed at the highest carbachol concentration (1.0 mM) in the presence of 1.0 μM atropine (○, □). Results are expressed as a percentage of forskolin-stimulated adenylyl cyclase activity for each cell line and treatment in the absence of carbachol. Specific activities (pmol cAMP formed/min/mg) of forskolin-stimulated adenylyl cyclase activity in isolated membranes were P1-6, 149.82 ± 20.67; M1-37, 148.53 ± 6.49; M1-36, 134.40 ± 18.68. Membrane isolation and adenylyl cyclase assay are described under Experimental Procedures. These data represent triplicate determinations in one experiment; two additional experiments gave similar results.

1.0 and 10.0 μM carbachol (Figure 2). Atropine treatment blocked both the carbachol-mediated inhibition and stimulation of cAMP accumulation in these cells, although at high agonist concentrations, cAMP levels were slightly lowered.

In most m4 expressing cell lines, we observed classical m4 receptor-mediated inhibition of cAMP, presumably via G_i activation. We were particularly interested, however, in understanding the mechanisms by which m4 receptors stimulated the accumulation of cAMP in cell lines M1-36 and M3-32. Two primary hypothesis have been proposed to explain muscarinic receptor stimulation of adenylyl cyclases. The first suggests that muscarinic stimulation of PI turnover and subsequent increases in intracellular Ca^{2+} activate Ca^{2+}/Cam-sensitive adenylyl cyclases (Felder et al., 1989; Jansson et al., 1991; Choi et al., 1992b). The second hypothesis proposes direct activation of β/γ-sensitive isozymes of adenylyl cyclase (Federman et al., 1992; Baumgold, 1992). Since the type I and III adenylyl cyclases are not stimulated by β/γ (Tang & Gilman, 1991), we investigated the role of Ca^{2+} in m4-mediated stimulation of cAMP in cell lines M1-36 and M3-32.

To determine the role of Ca^{2+} in carbachol stimulation of intracellular cAMP, we preloaded cells with the intracellular Ca^{2+} chelator BAPTA/AM to block any carbachol-stimulated increases in $[Ca^{2+}]_i$. Preliminary experiments using fura-2-loaded M1-36 and M3-32 cells indicated that treatment with 0.5 mM carbachol elicited a rapid 4–5-fold increase in $[Ca^{2+}]_i$ from approximately 40 to 160 μM. Pretreatment of these same cells with 100.0 μM BAPTA/AM for 30 min completely buffered any Ca^{2+} increases during a 30-min exposure to 0.5 mM carbachol (data not shown). We then examined the effect of BAPTA/AM treatment on carbachol-stimulated cAMP increases. In both cell lines, carbachol inhibition at low concentrations and stimulation at high concentrations were unaffected by BAPTA buffering of $[Ca^{2+}]_i$, suggesting that Ca^{2+} was not required for any of the carbachol-mediated effects on cAMP accumulation (Figure 3A,B).

To further characterize the role of Ca^{2+} in m4 stimulation of adenylyl cyclase, we directly measured adenylyl cyclase activity in membranes in the absence of Ca^{2+} (Figure 4). Because m4-coupled G_i-mediated inhibition of adenylyl cyclase in isolated membranes requires high NaCl concentrations (Jakobs et al., 1979; Lichtshtein et al., 1979), we performed assays both in the presence and absence of NaCl. Since we were primarily interested in the mechanism of m4 stimulation of adenylyl cyclase, we initially performed assays in the absence of NaCl to eliminate inhibitory coupling. In the presence of 10.0 μM GTP, 5.0 μM forskolin, 200 μM EGTA, and no added Ca^{2+} or NaCl, carbachol did not inhibit adenylyl cyclase activities in membranes isolated from any cell line tested (Figure 4). Under these conditions, type I adenylyl cyclase activity in M1-36 membranes was stimulated approximately 1.5-fold by carbachol with a concentration dependence similar to that in whole cells (EC_{50} = 5–10 μM). In the absence of NaCl, adenylyl cyclase activity in membranes isolated from cell line M1-37, which exhibited only carbachol-mediated cAMP inhibition in whole cell experiments, was also stimulated in a dose-dependent manner by carbachol. In type III adenylyl cyclase expressing cell lines, only membranes isolated from

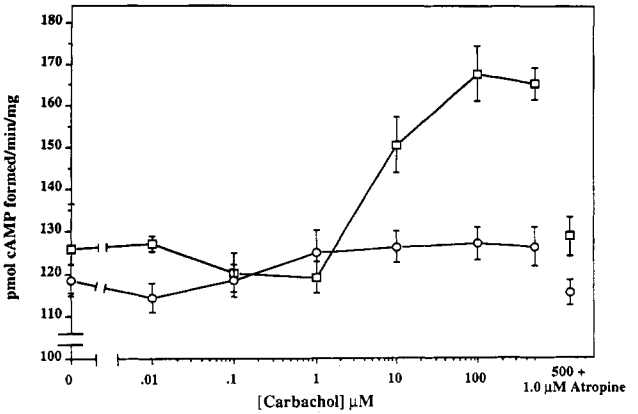

FIGURE 5: Effect of GDP-β-S on carbachol-stimulated adenylyl cyclase activity in membranes expressing type I adenylyl cyclase and m4 receptors. Membranes isolated from cell line M1-36 were assayed for adenylyl cyclase activity in the presence of 10.0 μM GTP, 5.0 μM forskolin, and increasing concentrations of carbachol either in the presence (○) or absence (□) of 50.0 μM GDP-β-S. To ensure the effects of carbachol were mediated via muscarinic activation, membranes were also assayed at the highest carbachol concentration (0.5 mM) in the presence of 1.0 μM atropine. Membrane preparation and adenylyl cyclase assay are described under Experimental Procedures. These data represent triplicate determinations in one experiment; one additional experiments gave similar results.

cell line M3-32 demonstrated carbachol stimulation of adenylyl cyclase activity (data not shown). In all cases, 1.0 μM atropine blocked carbachol stimulation of adenylyl cyclase activity. In the presence of high NaCl (100 mM), carbachol stimulation of adenylyl cyclase activity in cell lines M1-37 and M1-36 was reduced 66 and 75%, respectively, presumably due to competing inhibitory actions of G_i (data not shown). In cell lines, expressing lower m4 receptor levels (e.g. M3-14), 1.0 mM carbachol inhibited adenylyl cyclase activity in membranes 20–30% in the presence of 100 mM NaCl. The addition of low concentrations of forskolin was required to observe m4-mediated inhibition of adenylyl cyclase activity (e.g. Jakobs et al., 1979; Lichtshtein et al., 1979) and improved the ability of m4 to stimulate adenylyl cyclase.

Since m4 stimulation of either type I or III adenylyl cyclase did not require Ca^{2+}, we wondered if this stimulatory coupling was dependent on activation of a G protein. To examine this question, we measured carbachol regulation of adenylyl cyclase activity in membranes isolated from cell line M1-36 in the presence or absence of GDP-β-S, a GDP analogue which blocks G protein activation. In the presence of 10.0 μM GTP, 50.0 μM GDP-β-S completely inhibited carbachol mediated stimulation of type I adenylyl cyclase, but basal adenylyl cyclase activity was unaffected (Figure 5).

Since m4 receptors are known to couple to G_i and G protein activation was apparently required for carbachol stimulation of adenylyl cyclase in these cells, we evaluated the role of G_i for m4 stimulation using pertussis toxin (PTX). We also anticipated that inhibition of G_i activity with PTX might unmask G_s coupling to m4 receptors that is not normally seen when G_i is functional. PTX treatment of cell line M1-36, in which cAMP accumulation was inhibited at low carbachol concentrations and stimulated at high carbachol concentrations, completely eliminated carbachol inhibition of cAMP and revealed a dramatic increase in cAMP accumulation in response to carbachol (Figure 6A). Toxin treatment did not shift the apparent EC_{50} for carbachol stimulation; however, cAMP production was stimulated 10-fold over basal levels. Interestingly, in cell line M1-37, which exhibited only cAMP inhibition in response to carbachol, PTX treatment abolished cAMP inhibition and unmasked a 2-fold carbachol-mediated stimulation of cAMP. Similar results were seen in other m4/

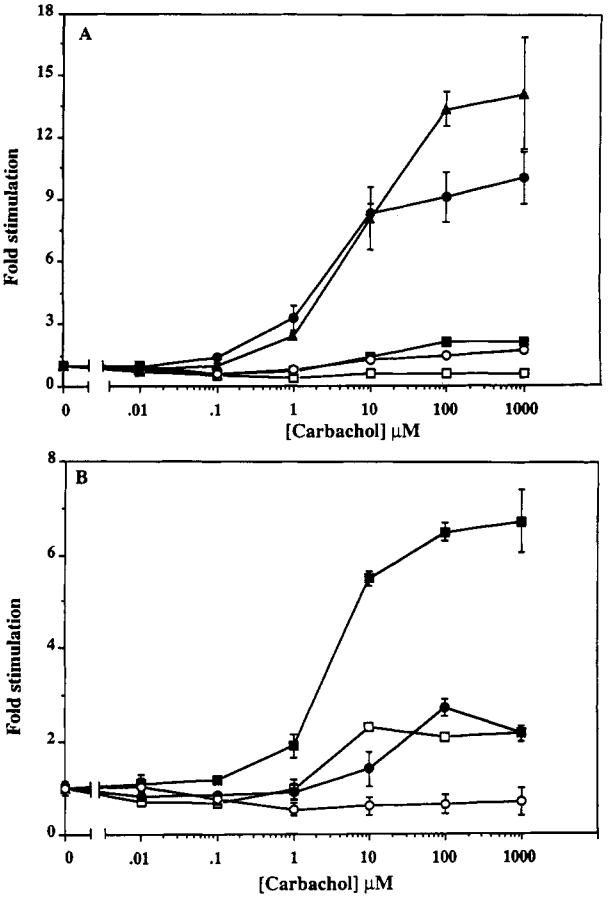

FIGURE 6: Carbachol-mediated stimulation of cyclic AMP accumulation in cells treated with PTX. Monolayers of cells expressing m4 receptors and either (A) type I adenylyl cyclase (M1-37 (□, ■), M1-36 (○, ●, ▲)) or (B) type III adenylyl cyclase (M3-14 (○, ●), M3-32 (□, ■)) were incubated for 16–20 h in the presence (■, ●, ▲) or absence (□, ○) of 100 ng/mL PTX in DMEM containing [^{3}H]adenine. Immediately prior to carbachol exposure, PTX-containing media was removed, cells were incubated for 30 min in the presence (▲) or absence (□, ■, ○, ●) of 100.0 μM BAPTA/AM in nonsupplemented DMEM and washed twice with 150 mM NaCl. Cells were exposed to increasing concentrations of carbachol for 30 min in incubation buffer containing 5.0 μM forskolin and 1.0 mM IBMX, and relative cAMP accumulations were determined as described in Experimental Procedures. Results are expressed as fold-stimulation of forskolin-stimulated cAMP accumulation for each cell line and treatment in the absence of carbachol. These data represent triplicate determinations in one experiment; one additional experiment gave similar results.

type I adenylyl cyclase expressing cell lines that were examined. cAMP production in type I adenylyl cyclase expressing cells, which expressed no exogenous muscarinic receptor, was not affected by PTX treatment (data not shown).

In all type III adenylyl cyclase cell lines, PTX treatment blocked m4-mediated inhibition of cAMP accumulation. Carbachol stimulation of cAMP in M3-32 cells was increased from 2- to 7-fold by PTX treatment (Figure 6B). As in type I adenylyl cyclase cell lines, PTX treatment of type III cell lines inhibited by carbachol uncovered a carbachol-mediated stimulation of cAMP at higher agonist concentrations (e.g. M3-14, Figure 6B). PTX treatment of type III adenylyl cyclase cell lines which expressed no exogenous m4 receptors did not affect cAMP metabolism. To ensure that the increased cAMP stimulation caused by PTX was still not due to increases in $[Ca^{2+}]_i$, we preloaded PTX-treated M1-36 cells with BAPTA/AM prior to carbachol exposure and determined that this increased cAMP accumulation was unaffected by Ca^{2+} chelation (Figure 6A).

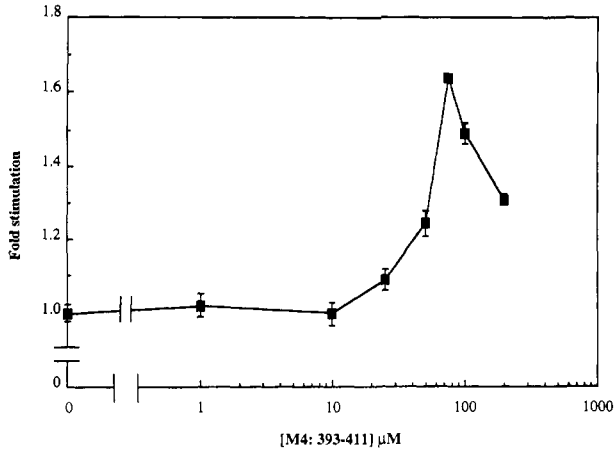

FIGURE 7: Effect of G protein activating peptide m4:393–411 on adenylyl cyclase activity in PTX-treated membranes expressing type III adenylyl cyclase. Monolayers of cells expressing type III adenylyl cyclase and no exogenous m4 receptor (P3-2) were incubated for 16–20 h in the presence of 100 ng/mL PTX. Membranes isolated from these cells were assayed for adenylyl cyclase activity in the presence of 10.0 μM GTP and increasing concentrations of m4:393–411. Membrane preparation and adenylyl cyclase assay are described under Experimental Procedures. These data represent triplicate determinations in one experiment; one additional experiments gave similar results.

Because pertussis toxin treatment of cells uncoupled G_i-mediated inhibition and revealed a dramatic increase in cAMP accumulation, we wondered if the differential coupling by m4 receptors to inhibition and stimulation of adenylyl cyclase might be due to different affinities of the m4 receptor for G_i and G_s. Recently, Okamoto and Nishimoto (1992) described a polypeptide sequence within the human m4 muscarinic receptor which apparently represents the G_i activating region for this receptor. Synthetic peptides corresponding to residues 375–400 of the human m4 receptor were able to directly activate G_i at nanomolar concentrations in a magnesium-dependent manner (Okamoto & Nishimoto, 1992). Interestingly this peptide was also able to activate G_s but at 100-fold higher concentrations. Since stimulation of adenylyl cyclase by the m4 receptor required approximately 100-fold higher concentrations of carbachol than inhibition (Figure 3), we tested whether a peptide corresponding to this region in the chick m4 receptor (M4:393-411) could directly mimic the differential effects of the activated receptor on adenylyl cyclase activity. Using membranes isolated from cell line P3-2, which stably expressed the type III adenylyl cyclase and no exogenous muscarinic receptor, we measured adenylyl cyclase activity in response to increasing concentrations of peptide m4:393-411. Initial experiments with forskolin-stimulated activity indicated that m4:393-411 elicited a biphasic response similar to that observed with agonist-activated m4 receptor: slight inhibition (10%) at low peptide concentrations (1.0 μM) and increasing adenylyl cyclase activity at higher concentrations (~50 μM). As with intact m4 receptor, the inhibitory phase of this response was only observed in the presence of 100 mM NaCl. To more carefully examine peptide stimulation of adenylyl cyclase, we treated P3–2 cells with PTX to eliminate potential G_i-mediated competing effects and measured adenylyl cyclase activity in isolated membranes with increasing concentrations of m4: 393-411 (Figure 7). In PTX-treated membranes, M4:393-411 did not inhibit adenylyl cyclase activity at any concentration but stimulated adenylyl cyclase activity 1.6-fold with an EC_{50} of 50 μM. At high peptide concentrations (≥100 μM), stimulation of adenylyl cyclase activity was reduced. These results are consistent with the crossover of m4 receptor G protein activating peptides from G_i to G_s activation observed by Okamoto and Nishimoto (1992) and suggest that stimulation of adenylyl cyclase by m4 receptors occurs via G_s activation.

If m4 receptor activation of G_s is responsible for adenylyl cyclase stimulation, one might expect that cholera toxin pretreatment of M1-36 or M3-32 cells would reduce the available pool of G_s which can couple to m4 and reduce the level of adenylyl cyclase stimulation at high carbachol concentrations. Indeed, pretreatment of M1-36 cells overnight with cholera toxin decreased the carbachol-mediated stimulation of adenylyl cyclase activity (data not shown). These results are difficult to interpret, however, because cholera toxin treatment may directly and maximally activate G_s-mediated adenylyl cyclase activity.

DISCUSSION

Jones et al. (1991) have shown that m4 receptors in transfected CHO cells display a biphasic response; at low agonist concentrations cAMP accumulation is inhibited while at higher agonist concentrations cAMP accumulation is stimulated. The mechanism underlying this Ca^{2+}-independent m4 stimulation of adenylyl cyclase is not known, although it has been hypothesized that this phenomenon may be due to stimulation of β/γ-sensitive adenylyl cyclase isozymes (Federman et al., 1992; Baumgold, 1992; Bourne & Nicoll, 1993). It has been difficult to define the role of β/γ in this phenomenon because coupling of m4 receptors to specific forms of adenylyl cyclase with known sensitivities to β/γ had not been examined until this study.

We have discovered that m4 receptors can couple to inhibition or stimulation of CaM-sensitive adenylyl cyclases. Inhibition of the type I and III adenylyl cyclase activities was most likely mediated via G_i activation since PTX treatment completely eliminated inhibition. While inhibition of the type I enzyme might have been due to β/γ inhibition, the carbachol concentration dependence for inhibition of the type I and III adenylyl cyclases was similar. Furthermore, β/γ regulation could not explain inhibition of the type III adenylyl cyclase since it is not affected by β/γ. In isolated cell membranes, inhibition of adenylyl cyclase was only apparent in the presence of high NaCl concentrations. This NaCl requirement for G_i coupling may explain the seemingly paradoxical stimulation of adenylyl cyclase in membranes isolated from cell line M1-37, which exhibited cAMP inhibition in intact cells. In the absence of NaCl, G_i-mediated inhibition is uncoupled, and competing stimulatory responses may be revealed.

While in most cell lines m4 activation inhibited cAMP accumulation, in cell lines expressing the highest levels of m4, carbachol elicited a biphasic response. The stimulatory component was apparent in all cell lines after PTX treatment, suggesting that there may be competition between PTX-sensitive and -insensitive G proteins for receptor recognition. The type I enzyme appeared to be slightly more sensitive to carbachol stimulation compared to the type III adenylyl cyclase, but this may reflect higher levels of m4 expression in type I expressing cells.

What is the mechanism underlying m4-mediated increases in cAMP? Experiments with BAPTA-treated cells and adenylyl cyclase assays in isolated membranes demonstrated that stimulation of the type I or III adenylyl cyclases by m4 receptors did not require Ca^{2+}. These results are consistent with those reported in the olfactory bulb, where both the type I and III adenylyl cyclases are expressed (Xia et al., 1991; Glatt and Snyder, 1993), but differ from results in SK-N-SH cells where Ca^{2+}-independent muscarinic stimulation of

adenylyl cyclase was only apparent in whole cells (Baumgold et al., 1992). In m4-transfected CHO cells, m4 induced increases in cAMP with a carbachol concentration dependence similar to what we observed, but the mechanism for stimulation in this system was not determined and the types of adenylyl cyclases expressed in these cells is unknown (Jones et al., 1991).

Several lines of evidence indicate that the stimulation of type I and III adenylyl cyclases by m4 receptors was not due to m4-mediated release of β/γ from G_i or G_o. β/γ stimulation seems unlikely since the type I adenylyl cyclase is inhibited and the type III enzyme is unaffected by β/γ subunits (Tang & Gilman, 1991; Taussig et al., 1993). Furthermore, PTX treatment of these cells enhanced the m4 stimulation of adenylyl cyclase rather than blocking stimulation as would be expected if β/γ released from G_i or G_o was responsible. Stimulation of the type I and III adenylyl cyclases in membranes required GTP, suggesting that stimulation was mediated via a stimulatory G protein, presumably G_s.

Recently, Nishimoto and his colleagues described a polypeptide sequence motif within several G protein coupled receptors which apparently represents specific G protein activator regions (Okamoto et al., 1991; Okamoto & Nishimoto, 1992; Ikezu et al., 1992). Within the human m4 receptor, amino acids sequences 130–147, 217–240, and 375–400 are potential G protein interaction domains and peptides corresponding to these sequences stimulate GTP binding to sepcific G proteins. Synthetic peptides corresponding to residues 130–147 and 375–400 were able to activate G_i/G_o, but only peptide 375-400 could activate at nanomolar concentrations (Okamoto & Nishimoto, 1992). Since the activation domain corresponding to residues 130–147 is conserved across all known muscarinic receptors and activator region 375–400 only occurs in m2 and m4 receptors, the 375–400 sequence is most likely the G_i/G_o activation site (Okamoto & Nishimoto, 1992). Interestingly peptides 130–147 and 375–400 were also able to active G_s but at concentrations 30–100 times greater than required for G_i activation (Okamoto & Nishimoto, 1992). In the chick m4 receptor, which was used in this study, all three G protein activator regions present in human m4 are conserved (Tietje et al., 1990). Peptides derived from two of these domains had significant inhibitory and stimulatory effects on adenylyl cyclase activity while the third had no effect (Dittman and Storm, unpublished observation). Peptide m4:393-411 (corresponds to peptide 375–400 in the human m4 receptor) was able to mimic the dose-dependent biphasic inhibitory and stimulatory actions of activated m4 receptor on adenylyl cyclase activity. Therefore, the ability of m4 receptors to either inhibit or stimulate CaM-sensitive adenylyl cyclases may reflect the differential affinities of these domains for G_i vs G_s. Since the affinity of these domains are apparently higher for G_i than G_s, m4 stimulation of adenylyl cyclases via G_s may only be evident when m4 receptor density is high, or when G_i is inhibited with PTX. It is interesting to note that the stimulation by the M4 peptide was lower than with M4 agonist under the same conditions, presumably because of the lower efficacy of an added peptide compared to the holoreceptor expressed in the plasma membrane.

The human α_2C10 adrenergic receptor also contains several putative G protein activator regions including residues 131–148 in the second cytoplasmic loop which specifically activates G_s with a similar EC_{50} for G_s activation as domain 375–400 of m4 (Okamoto & Nishimoto, 1992). It is interesting that this adrenergic receptor subtype can also functionally couple to both G_i-mediated inhibition and G_s-mediated stimulation of adenylyl cyclase (Fraser et al., 1989; Eason et al., 1992).

What role does m4 receptor coupling to G_s play in cellular signal transduction? In the olfactory bulb and retina (Olianas & Onali, 1990, 1991; Brown & Rietow, 1982), Ca^{2+}-independent stimulation of adenylyl cyclase by muscarinic receptors has been reported, although stimulation is apparently sensitive to PTX in the olfactory bulb (Olianas & Onali, 1992). M4 activation of G_s may act directly to stimulate adenylyl cyclases or alternatively the dual G_s and G_i coupling properties of m4 might facilitate β/γ stimulation of type II and IV adenylyl cyclases. Since activated G_s-α is required for β/γ stimulation of type II and IV adenylyl cyclase, receptors such as m4 or α_2C10, which can activate both G_s-α and stimulate β/γ release from G_i or G_o, may provide signal integration through a single receptor (Tang & Gilman, 1991; Bourne & Nicoll, 1993).

In summary, we have demonstrated that the m4 muscarinic acetylcholine receptors can couple to G_i-mediated inhibition or G_s-mediated stimulation of CaM-sensitive adenylyl cyclases, depending upon receptor density. These results and the recent demonstration of G_s activating domains in m4 and α_2 adrenergic receptors suggests that the m4 receptors may belong to a subclass of dual functioning receptors that can show crossover from G_i- to G_s-mediated regulation of adenylyl cyclases. We can now define four coupling mechanisms for muscarinic receptor regulation of adenylyl cyclases which include direct inhibition through G_i, indirect stimulation of CaM sensitive enzymes by mobilization of intracellular Ca^{2+}, modulation of adenylyl cyclases by released β/γ, and direct stimulation through Gs coupling. All of these mechanisms may play important regulatory roles in specific areas of the nervous system.

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