Differential Regulation by Agonist and Phorbol Ester of Cloned m1 and m2 Muscarinic Acetylcholine Receptors in Mouse Y1 Adrenal Cells and in Y1 Cells Deficient in cAMP-Dependent Protein Kinase†

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ABSTRACT: Cloned muscarinic acetylcholine m1 and m2 receptors were expressed in stably transfected mouse Y1 adrenal cells and in a variant Y1 line, Kin-8, which is deficient in cAMP-dependent protein kinase activity (PKA^-). m1 and m2 receptors were rapidly internalized following exposure of transfected PKA^+ or PKA^- cells to the muscarinic agonist carbachol. Thus, agonist-dependent internalization of m1 and m2 did not require PKA activity. A differential effect of PKA on regulation by agonist of the m2 receptor, but not the m1 receptor, was unmasked in PKA^- cells. The m2 receptor was more sensitive to agonist-dependent internalization, and its rate of internalization was faster in PKA^- cells than it was in PKA^+ cells. Treatment of PKA^+ cells with 8-(4-chlorophenylthio)-cAMP or forskolin did not result in internalization of either m1 or m2 receptors and did not alter the extent of agonist-dependent internalization of m2. These data indicate that the basal activity of PKA may modulate the agonist-dependent internalization of the m2 receptor, but not the m1 receptor. The internalization of the m1 and m2 receptors in both PKA^+ and PKA^- cells was accompanied by desensitization of functional responses. Exposure of PKA^+ cells to 10^{-7} M phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, resulted in a 30 ± 9% decrease in the number of m1 receptors on the cell surface. However, treatment of PKA^- cells expressing the m1 receptor did not result in internalization, suggesting that PKA was required for some aspect of PMA-dependent internalization. The m2 receptor was not internalized following treatment of either PKA^+ or PKA^- cells with PMA. Thus, the m1 and m2 receptors show differential sensitivity to internalization by PMA. Agonist-dependent internalization of the m1 receptor appeared to be independent of activation of PKC because (1) agonist-dependent internalization of m1 was not attenuated in PKA^- cells, (2) the rate and extent of internalization of m1 in cells exposed to PMA were less than those in cells exposed to agonist, and (3) treatment of cells with concanavalin A selectively blocked internalization of m1 in cells exposed to PMA, but not to agonist. The effects of agonist and PMA on receptor internalization were not additive. Exposure of PKA^+ or PKA^- cells to PMA reduced the magnitude of pilocarpine-stimulated PI hydrolysis by about 25%. This did not appear to result from a block of signal transduction at the level of the receptor, but rather downstream in the pathway, perhaps at the level of the G-protein.

Muscarinic acetylcholine receptors (mAChRs)[1] are important neurotransmitter receptors in the central and peripheral nervous systems. Stimulation of mAChRs results in a decrease in the rate and force of contraction in the heart, an increase in contraction in smooth muscle, and an increase in secretion in exocrine glands (Nathanson, 1987). mAChRs are members of a large gene family of hormone and neurotransmitter receptors in which signal transduction is mediated by G-proteins. There are five subtypes of mAChR, each encoded by a separate gene. The m1, -3, and -5 subtypes activate phospholipases A_2 and C and also regulate a certain set of ion channels. The m2 and -4 subtypes inhibit adenylyl cyclase and couple to a different set of ion channels than m1, -3, and -5 (Bonner, 1989).

Exposure of cells or tissues to muscarinic agonists can have both short-term and long-term effects on mAChR number and function [see Nathanson (1987) for review and references]. The number of mAChRs in both cultured cells and tissues in vivo is decreased by long-term exposure (several hours) to agonists. This "down-regulation" of receptor is due to an increased degradation rate of the receptor and results in a decreased functional response. The recovery of mAChR following removal of agonist takes 12-20 h and can be blocked by inhibition of de novo protein synthesis, consistent with the appearance of newly synthesized receptors. Short-term (several minutes) exposure to agonists leads to a decrease ("desensitization") in muscarinic responsiveness without a change in total cellular receptor number. This desensitization in some cases occurs concomitantly with internalization of the mAChR and a decrease in the number of cell surface receptors. The internalized receptors can be recycled to the cell surface in a temperature-sensitive manner in the absence of de novo protein synthesis.

The functions of many members of the G-protein-linked receptor family are regulated by receptor phosphorylation. The β-adrenergic receptor is phosphorylated by a specific kinase (β-adrenergic receptor kinase or BARK) only when it has agonist bound to it, and there is considerable evidence that BARK-mediated phosphorylation is primarily responsible for

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[1] Abbreviations: mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; QNB, quinuclidinyl benzilate.

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homologous desensitization (Lohse et al., 1990). The purified β-adrenergic receptor can also be phosphorylated by cAMP-dependent protein kinase (PKA). Phosphorylation by PKA can also mediate desensitization. Desensitization of the β-adrenergic receptor can also involve (but does not require) sequestration or internalization of the receptor (Sibley et al., 1987). The relative contribution of phosphorylation by BARK, phosphorylation by PKA, sequestration, and internalization to the functional desensitization of the β-adrenergic receptor is complex. Factors influencing the mechanism of desensitization may include the concentration of agonist, the length of time the receptor is exposed to agonist, receptor number, and the cellular environment (Hausdorff et al., 1989; Lohse et al., 1990).

Phosphorylation has also been implicated in the regulation of muscarinic receptors. The purified m1 receptor can be phosphorylated in vitro by both PKA and protein kinase C (PKC) (Haga et al., 1988), and the purified m2 receptor by PKA (Rosenbaum et al., 1987). Incubation of N1E-115 neuroblastoma cells with agonist causes both receptor internalization and activation of PKC; incubation of the cells with phorbol esters causes activation of PKC and receptor internalization with a time course similar to that of agonist (Liles et al., 1986). The best evidence for a role for phosphorylation in the regulation of mAChR is the demonstration that the phosphorylation of the chick and porcine cardiac mAChRs is increased in vivo following exposure to agonist. The increased phosphorylation of the heart mAChR was correlated with both a decrease in affinity for agonist and a decrease in the mAChR-mediated negative chronotropic effect. PKA and PKC were not responsible for the increased phosphorylation, as neither elevation of intracellular cAMP nor treatment with phorbol esters increased receptor phosphorylation (Kwatra et al., 1987, 1989a). The purified cardiac and brain mAChR can be phosphorylated by BARK or a related enzyme in an agonist-dependent manner, consistent with the hypothesis that BARK or an analogous kinase may be responsible for agonist regulation of the mAChR (Kwatra et al., 1989b; Haga & Haga, 1989).

While these results implicate phosphorylation in the regulation of mAChR, the study of phosphorylation in vivo can be complicated by the presence of multiple subtypes of both mAChR and protein kinases in a single cell or tissue [e.g., see McKinney et al. (1985) and Murphy et al. (1986)]. Furthermore, in vitro phosphorylation experiments may not be biologically relevant because of the nonphysiological assay conditions and high levels of enzyme and receptor required for phosphorylation. We have begun to identify factors affecting internalization and desensitization of the m1 and m2 receptors in order to determine the function of known receptor subtypes in a defined cell line. Cloned muscarinic receptors were transfected into the Y1 cell line (PKA^+) which lacks endogenous mAChRs and a cAMP-resistant variant cell line, Kin-8, in which the regulatory type I subunit of PKA is believed to be defective (PKA^-). Kin-8 cells were selected by mutagenesis and growth in high concentrations of cAMP analogues (Rae et al., 1979). We have found that while both the m1 and the m2 receptors underwent rapid internalization in PKA^+ cells following agonist exposure, only the m1 receptor was internalized following addition of phorbol esters. In PKA^- cells, phorbol ester treatment did not result in internalization of m1 receptor although this receptor internalized normally after agonist exposure. Surprisingly, agonist-induced internalization of the m2 receptor was enhanced in PKA^- cells compared to PKA^+ cells. These results suggest that the presence of a functional PKA is required for PKC-mediated internalization of the m1 receptor and attenuates agonist-induced internalization of the m2 receptor.

Experimental Procedures

Cell Culture and Transfection. Y1 (PKA^+ cells; Yasamura et al., 1966) and Kin-8 (PKA^-, Rae et al., 1979) mouse adrenocarcinoma cell lines were grown in F10 medium supplemented with 15% fetal calf serum, penicillin G (100 units/mL), and streptomycin sulfate (0.1 mg/mL) in an atmosphere containing 5% CO_2. All cell culture reagents were obtained from Gibco.

Cells were transfected by using the calcium phosphate method (Wigler et al., 1979) with either the m1 receptor, a mouse genomic clone (Shapiro et al., 1988), or the m2 receptor, a porcine cDNA clone (Peralta et al., 1987). The coding sequences for the receptors were inserted into the ZEM 228 expression vector (Shapiro et al., 1988), a gift from Eileen Mulvihill, Zymogenetics Inc., Seattle, WA. The vector contains a selectable marker, the neomycin phosphotransferase resistance gene, driven by the constitutive SV40 promoter. Stable transformants were selected by growing the cells in the presence of 500 μg/mL Geneticin (Gibco) and subcloned. Expression of the receptors was driven by the mouse metallothionein-1 promoter, which is induced by exposure of cells to heavy metals. Where indicated, cells were exposed to 120 μM $ZnSO_4$ (cell culture reagent, Sigma) for 24 h prior to testing to increase expression of the receptor. All experiments were performed in zinc-free medium.

Ligand Binding Assays. Total receptor number was determined by binding of the membrane-permeable muscarinic antagonist ^{3}H-labeled quinuclidinyl benzilate, [^{3}H]QNB (Amersam Corp., 36 Ci/mmol), to crude membrane homogenates as described by Halvorsen and Nathanson (1981). Surface receptors were measured by binding of the membrane-impermeable muscarinic antagonist ^{3}H-labeled *N*-methylscopolamine, [^{3}H]NMS (Amersham Corp., 85 Ci/mmol), to intact cells by a modification of previously described methods (Nathanson, 1983; Strader et al., 1987). Cells were grown in 24-well plates (15-mm wells) to 50–70% confluency and incubated at 37 °C with drugs or ligands diluted in F10 medium (250 μL/well). Except for time course experiments, the incubation period with drugs was 1 h. The reaction was stopped by rinsing each well with 1 mL of ice-cold phosphate-buffered saline (PBS: 20 mM NaH_2PO_4/150 mM NaCl, pH 7.4). The cells were incubated with 290 fmol of [^{3}H]NMS in a total volume of 250 μL of PBS containing 0.2% bovine serum albumin for 4 h at 4 °C. To harvest cells, plates were placed on ice, 0.5 mL of ice-cold PBS was added per well, and the cells were scraped. The cell suspension was filtered through GF/C filters (Whatman), the well was rinsed with 0.75 mL of cold PBS, and the filters were rinsed 3 times with 3 mL of cold PBS. Radioactivity was measured by liquid scintillation counting. Total [^{3}H]NMS binding from five wells was averaged per treatment. Nonspecific [^{3}H]NMS binding was measured in a sixth well containing 3 μM atropine and was subtracted from the total binding to yield specific binding. Typically, the nonspecific binding was 50–100 cpm/well compared to a total binding of several hundred to several thousand cpm per well. Each well contained 50–130 μg of total cell protein, averaging 83 ± 13 μg (n = 17).

cAMP Accumulation Assays. cAMP levels were measured by the competition of [^{3}H]cAMP with cellular cAMP for binding to PKA. Cells were grown in 50–80% confluency on 60-mm plates containing about 500 μg of total cell protein. For the assay, cells were incubated for 20 min at 37 °C with

F10 medium buffered with 25 mM HEPES and containing 5 mM theophylline to inhibit phosphodiesterases. Inhibition of cAMP accumulation by 1 mM carbachol was determined in cells stimulated for 5 min by 100 μM forskolin (Calbiochem). The reaction was quenched by the addition of 2 mL of 5% trichloroacetic acid, the cells were scraped, and cAMP was isolated by ion-exchange chromatography as described in Matsuzawa and Nirenberg (1975). cAMP was measured by a protein binding assay as described by Gilman (1970). Triplicate plates were done for each treatment, and each plate was assayed in triplicate in the binding assay.

Phosphatidylinositol Hydrolysis Assay. The ability of carbachol to stimulate phosphatidylinositol (PI) hydrolysis was measured in cells grown to 50–80% confluency on six-well plates (35-mm wells), with approximately 300 μg of total cell protein/well. Cells were labeled by incubation overnight with 1 μCi/mL *myo*-[^{3}H]inositol (Amersham Corp., 16.6 Ci/mmol). For the assay, cells were washed and incubated for 30 min at 37 °C with 1 mL of a saline solution (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$ 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 0.5 mM EDTA, and 20 mM HEPES, pH 7.4) containing 10 mM LiCl to block hydrolysis of [^{3}H]inositol 1-phosphate. PI hydrolysis was stimulated by incubating the cells with the partial muscarinic agonist pilocarpine for 15 min at 37 °C. The reaction was quenched by rinsing the cells with ice-cold PBS and adding 1 mL of cold methanol. Total inositol phosphates were isolated by ion-exchange chromatography as described by Masters et al. (1984). Samples were assayed in triplicate.

Phosphatidylinositol Pool Size. Total labeled inositol phospholipids were determined by extracting the 1-mL methanol samples, prepared as described above, with 0.5 mL of $CHCl_3$ and 0.45 mL of 0.1 M HCl. Samples were sonicated on ice for 10 s using a probe sonicator. Another 0.5 mL of $CHCl_3$ and 0.45 mL of 0.1 M HCl were added and the samples vortexed and centrifuged to separate the phases. The upper phase was discarded, leaving the interface. The interface and lower phase were washed with 0.5 mL of $CHCl_3$/MeOH/0.1 M HCl (3:47:48), vortexed, and centrifuged. The upper phase was discarded, leaving the interface, and the wash was repeated. After centrifugation, the upper phase and interface were discarded, and the lower phase was transferred to scintillation vials and allowed to evaporate at room temperature. The sample was resuspended in scintillation cocktail and counted.

Labeling of phosphatidylinositol, phosphatidylinositol phosphate, and phosphatidylinositol bisphosphate was measured by the deacylation method described by Harden et al. (1987). Cells were grown to 70% confluency on 100-mm plates (about 1.5 mg of total cell protein/plate) and incubated with 3 μCi/mL *myo*-[^{3}H]inositol for 24 h. Two plates were used per point, and each treatment was done in duplicate.

Protein Kinase C Activity. PKC activity was determined in the presence of calcium, phosphatidylserine, and diolein by measuring the incorporation of [^{32}P]P_i into histone III as described by Liles et al. (1986), with the following modifications. Cells were grown to 70% confluency on 100-mm plates. Two plates were combined per assay, and each treatment was done in duplicate. To assay activity in the membrane fraction, the pellet was solubilized by incubation on ice for 30 min with buffer containing 1% Nonidet P-40 (Sigma) and applied directly to the DEAE-Sephacel column, bypassing the centrifugation step. The column bed volume was reduced from 1.5 to 0.5 mL. The cytosolic fraction was eluted in 2.5 mL of buffer containing 0.1 M NaCl. The membrane fraction was eluted in 2.5 mL of buffer containing 0.15 M NaCl. PKC activity in the absence of calcium was subtracted from activity in the presence of calcium to obtain calcium-dependent activity.

Table I: Receptor Levels in Transfected Cells[a]

cell line	$ZnSO_4$	[^{3}H]NMS binding (fmol/well)	[^{3}H]QNB binding (fmol/mg of membrane protein)
m1Y	–	4 ± 2 (7)	143
	+	36 ± 11 (49)	1634
m2Y	–	ND	45
	+	6 ± 2 (36)	129
m1K	–	13 ± 2 (16)	501
	+	45 ± 11 (18)	1411
m2K	–	13 ± 3 (6)	285
	+	48 ± 8 (14)	1056

[a] The total and surface levels of m1 and m2 receptors in PKA$^+$ and PKA$^-$ cells were determined by the binding of [^{3}H]QNB to membrane homogenates, or [^{3}H]NMS to intact cells. Receptor levels were measured in cells that were exposed to 120 μM $ZnSO_4$ for 24 h to increase the level of receptor expression, and in cells not treated with $ZnSO_4$. Data for [^{3}H]NMS binding represent the mean femtomoles per well for the number of experiments indicated parenthetically. For each experiment, the specific binding to cells in 5 wells of a 24-well plate was averaged. [^{3}H]NMS binding to m2Y cells not exposed to $ZnSO_4$ was too low to be readily determined. The average total cell protein per well was 83 ± 13 μg (n = 17). [^{3}H]QNB data represent the mean of two experiments with samples assayed in duplicate. ND is not determined.

Chemicals and Data Presentation. Chemicals were all of analytical grade and obtained from commercial suppliers. All cell culture reagents were obtained from Gibco. Concanavalin A, type IV, NaF (A-573), $AlCl_3$ (N-5299), and phorbol esters were from Sigma. Phorbol esters were dissolved in Me_2SO. Results are expressed as the mean ± standard error. Student's t test was used to indicate the significance at the 0.05 probability level. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Expression of m1 and m2 in PKA$^+$ and PKA$^-$ Cells. The m1 and m2 receptors were transfected into Y1 (PKA$^+$) and Kin-8 (PKA$^-$) cells, and individual subclones of each transfection were isolated and screened for expression of mAChRs by [^{3}H]QNB binding after exposure of cells to 120 μM $ZnSO_4$ for 24 h. There was no detectable mAChR in untransfected cells (<5 fmol/mg of membrane protein). Subclones were chosen for further testing on the basis of expressing similar levels of receptor after 24-h exposure of cells to 120 μM $ZnSO_4$ and identified by the receptor type, m1 or m2, and the cell line, Y (Y1 cells) or K (Kin-8 cells). In initial experiments, at least two subclones of each transfection were examined to assure that the response of the subclone selected for further study was representative. In the case of the m2Y cells, the subclone expressing the highest number of receptors was chosen.

The surface and total receptor levels of the subclones used in this study, as indicated by binding of [^{3}H]NMS or [^{3}H]-QNB, respectively, are shown in Table I. [^{3}H]NMS is a quaternary ammonium lipophobic ligand and thus will only label mAChR on the surface of intact cells, while [^{3}H]QNB is lipophilic ligand that that recognize both cell surface and internalized receptors in intact cells [see Nathanson (1987) for review and references]. We determined receptor number in cells exposed to $ZnSO_4$, as well as cells not exposed to $ZnSO_4$. This allowed us to determine the effect of receptor number on the response and also to control for possible effects of $ZnSO_4$ on the physiology of the cell. Receptor number

Table II: Receptor Internalization following Exposure of PKA$^+$ and PKA$^-$ Cells to Carbachol[a]

cell line	$ZnSO_4$	[^{3}H]NMS binding (% control)
m1Y	−	40 ± 5 (3)
	+	52 ± 6 (16)
m2Y	−	ND
	+	47 ± 5 (24)
m1K	−	42 ± 5 (6)
	+	66 ± 6 (13)
m2K	−	14 ± 1 (2)
	+	19 ± 3 (12)

[a] Surface levels of m1 and m2 receptors in PKA$^+$ and PKA$^-$ cells were determined by the binding of [^{3}H]NMS to intact cells that were exposed to 1 mM carbachol for 1 h. Receptor levels were determined in cells that were exposed to 120 μM $ZnSO_4$ for 24 h to increase the level of receptor expression, and in cells not treated with $ZnSO_4$. Data are presented as a percentage of specific [^{3}H]NMS binding to control cells not exposed to carbachol. The number of independent experiments upon which each mean is based is indicated parenthetically. ND is not determined.

increased 2–3 times in cells exposed to $ZnSO_4$ compared to the level in untreated cells, except in the m1Y line in which there was about a 10-fold induction. The receptor number in m2Y cells not treated with $ZnSO_4$ was too low to readily permit assays on intact cells.

The fraction of total receptors present on the cell surface was determined by comparing the binding of the hydrophobic ligand, [^{3}H]QNB, and the hydrophilic ligand, [^{3}H]NMS, on matched 60-mm plates of cells. The specific binding of [^{3}H]QNB to membranes prepared from duplicate plates, or of [^{3}H]NMS binding to three aliquots of intact cells from triplicate plates, was normalized to the level of protein in membranes prepared from each plate as described for the [^{3}H]QNB binding assay. Essentially all the expressed m1 and m2 receptors were present at the cell surface in both PKA$^+$ and PKA$^-$ cells. The average ratio of [^{3}H]NMS to [^{3}H]QNB binding in two or three experiments was 1.04 ± 0.16 for m1Y cells, 0.96 ± 0.01 for m2Y cells, 1.05 ± 0.15 for m1K cells, and 1.07 ± 0.10 for m2K cells. In general, natively expressed mAChRs are all present on the cell surface (Nathanson, 1983). This result differs from the distribution of the cloned β-adrenergic receptor expressed in these cells. Allen et al. (1989) reported that 85% of the β-adrenergic receptor was present on the surface of PKA$^-$ cells but less than 10% was expressed on the surface of PKA$^+$ cells.

We previously showed that the m1 and m2 receptors expressed in Y1 cells couple to their usual effectors, stimulating phospholipase C and inhibiting adenylyl cyclase, respectively (Shapiro et al., 1988). Data presented in this report will show that the receptors also functioned normally in PKA$^-$ cells.

Agonist-Dependent Internalization of m1 and m2. Exposure of PKA$^+$ cells containing m1 or m2 receptors to 1 mM carbachol for 1 h resulted in a loss of about 50% of the surface receptors (Table II) relative to the level of specific [^{3}H]NMS binding to cells not exposed to carbachol. When m1Y cells not exposed to $ZnSO_4$ were tested, the level of internalization was slightly greater than in $ZnSO_4$-treated cells. The PKA$^-$ cells transfected with the m1 receptor responded similarly as the PKA$^+$ cells. These data show that agonist-dependent internalization of the m1 receptor was similar in PKA$^+$ and PKA$^-$ cells, suggesting that internalization was independent of PKA activity. There was 53% decline in specific [^{3}H]NMS binding to m2Y cells following exposure to 1 mM carbachol (Table II). This was similar to the level of internalization of m1 in Y1 cells. In contrast to the m1 receptor, which was internalized to a similar extent in Y1 and PKA$^-$ cells, the m2 receptor was internalized to a greater extent in PKA$^-$ cells than

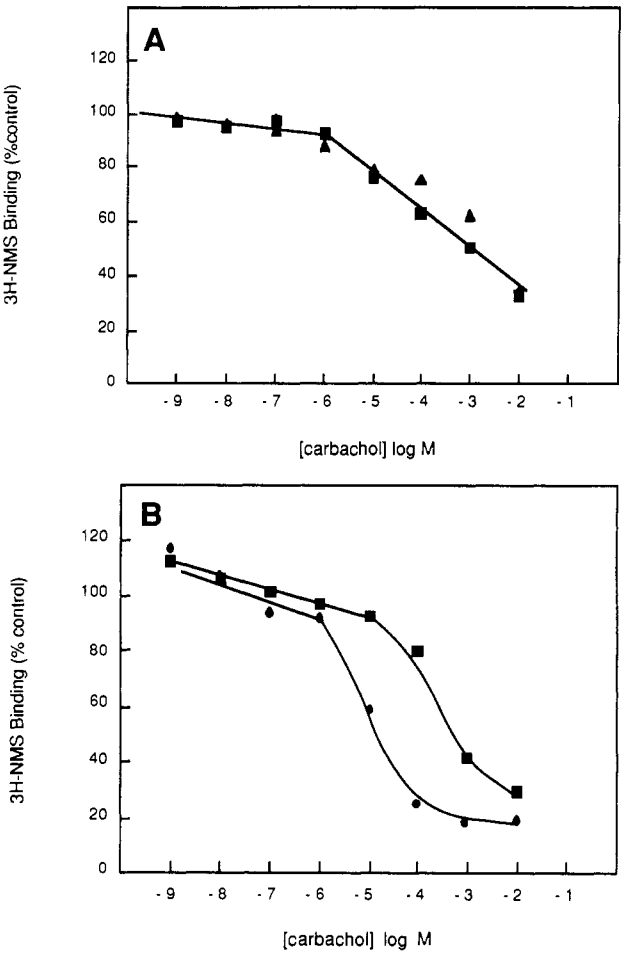

FIGURE 1: Dependence of internalization of m1 and m2 on the concentration of carbachol. Cells grown on 24-well plates were treated with zinc and exposed to the indicated concentrations of carbachol for 1 h, and cell surface receptor number was determined by specific [^{3}H]NMS binding. The data represent the mean of two experiments having less than 10% difference between them. (A) Response of m1Y cells (■) and m1K cells (▲); (B) response of m2Y cells (■) and m2K cells (●).

in Y1 cells. This suggests that the presence of an active PKA may render the m2 receptor less sensitive to agonist-induced internalization. Activation of PKA by exposure of PKA$^+$ cells to 150 μM 8-(4-chlorophenylthio)-cAMP or 100 μM forskolin did not result in internalization of either the m1 or the m2 receptors; specific [^{3}H]NMS binding was 93 ± 5% of control (n = 4) in m1Y cells and 102 ± 3% (n = 3) in m2Y cells. Also, exposure of m2Y cells to carbachol in the presence of 150 μM 8-(4-chlorophenylthio)-cAMP or 100 μM forskolin did not significantly alter the number of receptors internalized compared to carbachol alone, 46 ± 7% versus 56 ± 8% of control (n = 5), respectively. Thus, the effect of PKA is likely to be due to tonic phosphorylation of the m2 receptor, or some other protein involved in receptor internalization.

Concentration Dependence of Internalization of m1 and m2 by Agonist. The carbachol dose response curve for internalization of the m1 receptor was identical in PKA$^+$ and PKA$^-$ cells (Figure 1A). The carbachol dose response curve was characteristically steeper for m2 than for m1 receptors (Figure 1B). Also, the m2 receptor was more sensitive to internalization by carbachol in PKA$^-$ cells than in PKA$^+$ cells. The increased sensitivity of the m2 receptor to internalization in PKA$^-$ cells was not due to a difference in the affinity for carbachol. The apparent affinity of carbachol for binding to the m2 receptor was the same in PKA$^+$ and PKA$^-$ cells, as

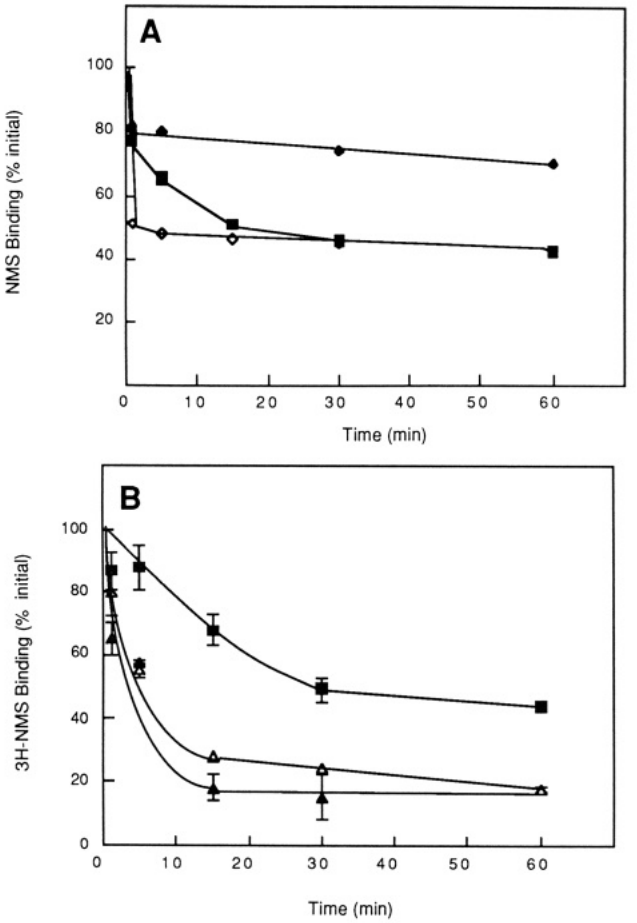

FIGURE 2: Rate of internalization of m1 and m2. Cells grown on 24-well plates were treated with zinc and incubated with 1 mM carbachol for the indicated period of time, and the surface receptor number was determined by specific binding of [^{3}H]NMS. Data are presented as a percent of binding to cells not incubated with carbachol. Points with an error bar represent the mean ± standard error of three experiments; points without a bar represent the mean of two experiments. (A) Data for zinc-treated m1Y cells (■) and and zinc-treated (♦) and untreated (◇) m1K cells; (B) data for m2Y (■) and zinc-treated (▲) and untreated m2K (△) cells.

carbachol/[^{3}H]QNB competition curves were similar in membrane homogenates prepared from both cell types (data not shown).

Rate of Agonist-Dependent Internalization of m1 and m2. The m1 receptor was rapidly internalized after exposure of either PKA$^+$ or PKA$^-$ cells to 1 mM carbachol (Figure 2A). Internalization of the m1 receptor was essentially complete after 15-min exposure of PKA$^+$ cells to agonist, or 5-min exposure of PKA$^-$ cells. The m2 receptor was internalized more slowly in PKA$^+$ than in PKA$^-$ cells, with a $t_{1/2}$ of 15 min, compared to 5 min in PKA$^-$ cells (Figure 2B). Agonist-dependent internalization of m2 was essentially complete within 30 min in PKA$^+$ cells, with a 51% loss of specific [^{3}H]NMS binding. In PKA$^-$ cells, internalization was complete by 15 min, and there was an 85% loss of [^{3}H]NMS binding. These data show that the extent, rate, and sensitivity of the m2 receptor to agonist-dependent internalization were diminished in cells with a normal level of PKA compared to PKA$^-$ cells.

PMA-Dependent Internalization. The m1 and m2 receptors differed in their response to PKC activation by phorbol 12-myristate 13-acetate (PMA). There was no decrease in specific [^{3}H]NMS binding to m2 receptors after 1-h exposure of m2Y or m2K cells to 10^{-7} M PMA (Figure 3). In contrast, exposure of m1Y cells to PMA resulted in a 30 ± 9% (n = 31) decline in binding. The biologically inactive phorbol, 4α-phorbol 12,13-dideconoate, was without effect. PMA-dependent internalization was greatly attenuated or blocked in PKA$^-$ cells. Specific [^{3}H]NMS binding to PMA-treated m1K cells was 99 ± 4% (n = 5) of control cells not exposed to PMA. This suggests that the presence of a functional PKA was required for the PKC-dependent internalization of the m1 receptor. A dose response curve for PMA-dependent internalization of the m1 receptor in Y1 cells in shown in Figure 4.

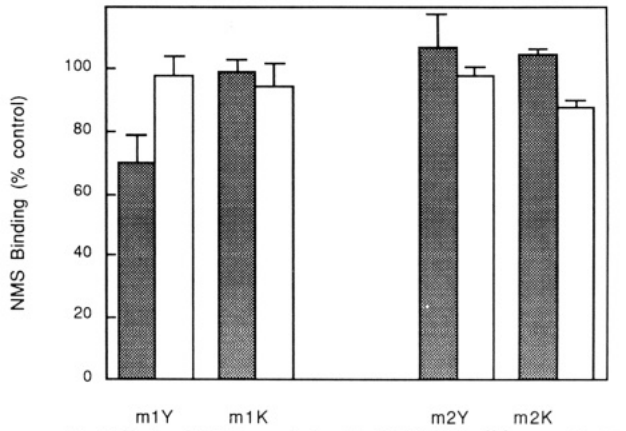

FIGURE 3: Effect of PMA and 4α-phorbol 12,13-dideconoate on internalization of m1 and m2 receptors. PKA$^+$ and PKA$^-$ cells grown on 24-well plates were exposed to 10^{-7} M PMA (solid bars) or 4α-phorbol 12,13-dideconoate (open bars) for 1 h, and surface receptor number was determined by the specific binding of [^{3}H]NMS to intact cells. Data are presented as the percentage of [^{3}H]NMS binding to cells not exposed to drugs and represent the mean ± SEM of 3–31 experiments.

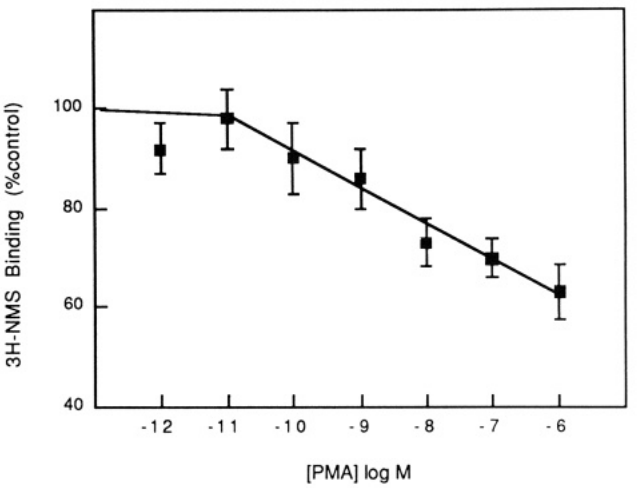

FIGURE 4: Dependence of internalization of m1 on the concentration of PMA. Y1 cells were exposed to the indicated concentrations of PMA for 1 h, and the number of receptors on the cell surface was determined by the binding of [^{3}H]NMS. The data represent the mean ± standard error of four experiments.

The observation that agonist-dependent internalization occurred in cells in which PKC activation was without effect suggests that internalization resulting from exposure to agonist did not require the activation of PKC. We exposed PKA$^+$ and PKA$^-$ cells to both carbachol and PMA for 1 h to determine whether the effects of agonist and PKC activation on receptor internalization were additive. In three experiments, there was less than 3% difference in the level of internalization of the m1 receptor in either PKA$^+$ cells or PKA$^-$ cells exposed to PMA and carbachol compared to cells exposed to carbachol alone. Thus, although agonist-dependent internalization does not appear to involve activation of PKC, the response to carbachol and PMA was not additive.

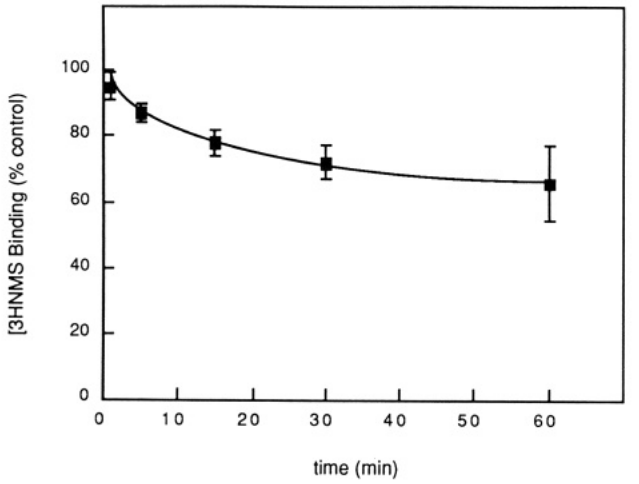

FIGURE 5: Rate of internalization of m1 by PMA. Y1 cells grown on 24-well plates were exposed to 10^{-7} M PMA for the indicated period of time, and the surface receptor number was determined by the specific binding of [^{3}H]NMS. Data are presented as a percent of the binding to cells not exposed to PMA. Points represent the mean ± SEM of three to five experiments.

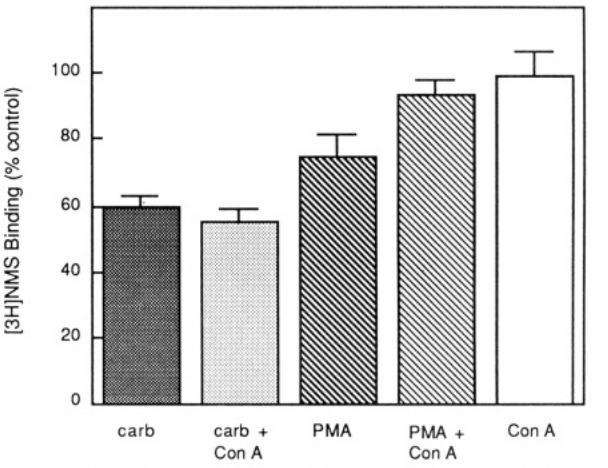

FIGURE 6: Concanavalin A blocks internalization of m1 by PMA. Y1 cells grown on 24-well plates were exposed to 1 mM carbachol, carbachol plus 10^{-7} M PMA, PMA, PMA plus 0.25 mg/mL concanavalin A, or concanavalin A alone. Concanavalin A was added 45 min before the PMA. The number of surface receptors was determined by the specific binding of [^{3}H]NMS. Data are presented as a percent of the binding to cells not exposed to drugs. Points represent the mean ± SEM of five experiments.

The inability of PMA to induce internalization of m1 receptors in PKA$^-$ cells could result from a loss of PKC activity in these cells. Y1 cells are reported to contain high levels of PKC (Widmaier & Hall, 1985; Papadopoulos & Hall, 1989); however, there are no reports on the activity of PKC in Kin-8 cells. We observed that Y1 and Kin-8 cells both contract after 30-min exposure to 10^{-7} M PMA, suggesting that both cell lines contain active PKC. We measured PKC activity in the membrane and cytosolic fractions of m1K cells. In three experiments, exposure of the cells to 10^{-7} M PMA for 1 h increased the PKC activity in the membrane fraction by (4.8 ± 0.3)-fold over the activity in unstimulated cells, and there was a corresponding decrease in the activity of the cytosolic fraction. This indicates that PMA activated PKC in Kin-8 cells and caused it to be translocated from the cytosol to the membrane. Activation of PKC by PMA was rapid, with a 2.5-fold increase in membrane-associated PKC activity after 10-min exposure of cells to PMA.

Rate of Internalization of m1 Receptors Exposed to PMA. The previous data suggest that agonist- and PMA-dependent internalizations of the m1 receptor occur by separate mechanisms. We determined the rate of internalization of the m1 receptor by PMA to see if it was different from the rate of internalization by carbachol. In m1Y cells, about 50% of the m1 receptor is internalized following exposure of cells to 1 mM carbachol for 1 h, and the $t_{1/2}$ is less than 5 min (Figure 2A). In contrast, internalization of the m1 receptor following exposure of cells to 10^{-7} M PMA occurs more slowly, with a $t_{1/2}$ of about 10 min, and the magnitude was less than for agonist-dependent internalization (Figure 5). The difference in the rate of internalization by agonist and PMA is consistent with the hypothesis that agonist-dependent internalization does not invovle activation of PKC.

Concanavalin A Selectively Blocks PMA-Dependent Internalization of m1 in Y1 Cells. Agonist- and PMA-induced internalization of the m1 receptor could also be disassociated by treatment of cells with concanavalin A. PKC-dependent internalization of m1 was blocked by exposing m1Y cells to PMA in the presence of 0.25 mg/mL concanavalin A. In five experiments, exposure of cells to 10^{-7} M PMA resulted in surface receptor number decreasing to 75 ± 7% of untreated cells. Cells exposed to concanavalin A during treatment with PMA had receptor levels 93 ± 5% of control (Figure 6). Concanavalin A alone was without effect (99 ± 7% of control) and did not significantly affect carbachol-dependent internalization (carbachol alone was 60 ± 3% of control compared to 55 ± 4% with carbachol and concanavalin A). Concanavalin A is reported to block PMA-dependent internalization of the β_2-adrenergic receptor in C6 cells by inhibiting translocation of PKC from the cytosol to the membrane (Fishman et al., 1987). This mechanism may also explain the selective block of PMA-dependent internalization of m1 by concanavalin A, as the m1 receptor does not bind concanavalin A (Rauth et al., 1986).

Agonist-Dependent Desensitization of m1 and m2. The coupling of m1 to phospholipase C was determined after pretreating the cells with 1 mM carbachol for 1 h in the presence of *myo*-[^{3}H]inositol and subsquently measuring the ability of the muscarinic agonist pilocarpine to stimulate PI hydrolysis. Internalization of m1 receptors was accompanied by desensitization of PI hydrolysis in both PKA$^+$ and PKA$^-$ cells. Stimulation of PI hydrolysis by maximal (10^{-4} M) and submaximal (10^{-5} M) levels of pilocarpine was decreased by about 25% in both cell lines in cells exposed to 1 mM carbachol for 1 h compared to untreated cells (Figure 7A). Thus, desensitization of m1 receptors was independent of PKA activity. In these experiments, pretreatment with carbachol resulted in a decrease in specific [^{3}H]NMS binding of 52 ± 4% in PKA$^+$ cells and 59 ± 3% in PKA$^-$ cells compared to control.

To control for possible changes in the labeling of inositol phospholipids due to pretreatment of cells with carbachol, we measured [^{3}H]inositol incorporation into total phospholipids and phosphorylated lipid fractions. In two experiments, pretreatment of m1K cells with carbachol did not significantly alter labeling of total inositol phospholipids relative to untreated cells. The level of total labeled inositol phospholipids in cells pretreated with carbachol was 117% of control. Pretreatment of cells with carbachol also had no effect on labeling of phosphatidylinositol, phosphatidylinositol phosphate, or phosphatidylinositol bisphosphate. In three experiments, labeling of phosphatidylinositol, phosphatidylinositol phosphate, and phosphatidylinositol bisphosphate in cells pretreated with carbachol was 114 ± 16%, 116 ± 18%, and 135 ± 34% of control, respectively.

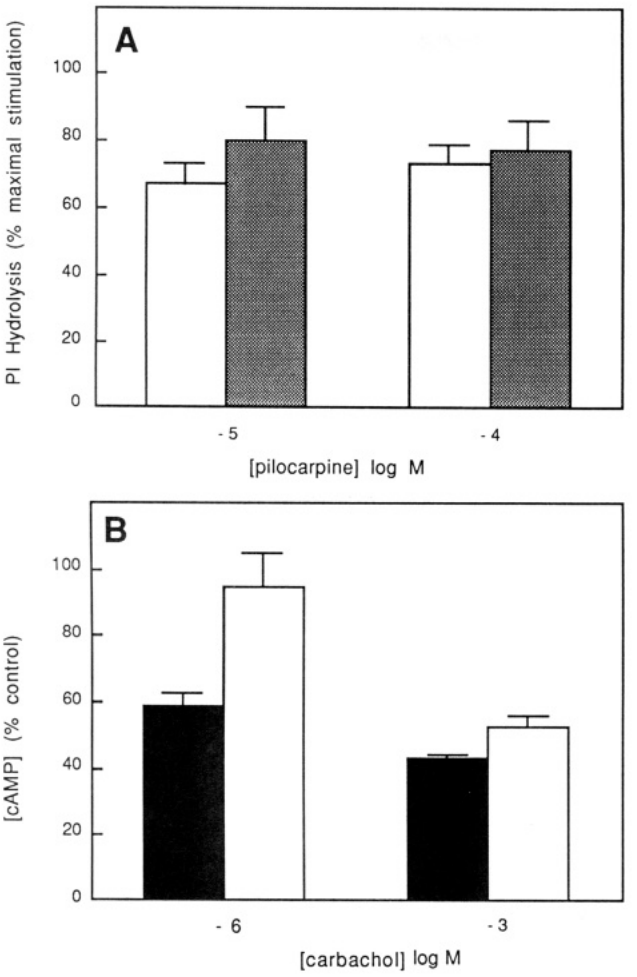

FIGURE 7: Desensitization of m1 and m2 receptors. (A) Effect of pretreatment of m1Y (open bars) and m1K (solid bars) cells with 1 mM carbachol on the subsequent stimulation of PI hydrolysis by 10^{-5} or 10^{-4} M pilocarpine is shown. m1Y and m1K cells pretreated with carbachol lost 52 and 59% of surface receptors, respectively, compared to untreated m1Y cells exposed to zinc expressing 899 ± 121 fmol of receptor/mg of total cell protein and m1K cells not exposed to zinc expressing 198 ± 23 fmol of receptor/mg of total cell protein. Data represent the mean ± standard error of three experiments for m1Y cells and five experiments for m1K cells. PI hydrolysis is presented as a percent of the maximal stimulation of PI hydrolysis in cells not pretreated with carbachol, which was 3068 + 850 cpm/plate for m1Y cells and 1749 ± 273 cpm/plate for m1K cells. (B) Ability of m2 receptors exposed to 10^{-6} or 10^{-3} M carbachol to inhibit forskolin-stimulated cAMP accumulation was determined in m2K cells pretreated with 1 mM carbachol for 1 h (open bars) and cells not pretreated (solid bars). After pretreatment, plates were washed 3 times with 3 mL of warm PBS, and HEPES-buffered F10 medium was added as described in the text. Data are presented as a percent of the cAMP levels in cells not exposed to carbachol during the assay. In control cells not pretreated with carbachol, the average cAMP level was 713 ± 140 pmol/mg (n = 8); with pretreatment, it was 565 ± 76 pmol/mg (n = 5).

Internalization of the m2 receptor in PKA^- cells was accompanied by desensitization of carbachol-dependent inhibition of adenylyl cyclase. In m2Y cells pretreated with carbachol, cell surface receptor number declined from 603 ± 134 fmol/mg of total cell protein (n = 7) in untreated cells to 82 ± 20 fmol/mg of total cell protein (n = 5). Under these conditions, there was a small but significant attenuation of inhibition of cAMP accumulation in response to stimulation of the receptor with 1 mM carbachol (53 ± 3% of control, n = 3, compared to 43 ± 1%, n = 4) (Figure 7B). However, when cells were stimulated with a submaximal concentration of carbachol (10^{-6} M), there was almost complete loss of inhibition of cAMP accumulation in pretreated cells. Thus, desensitization of m2 was characterized by a small decline in maximal response but a large decrease in potency.

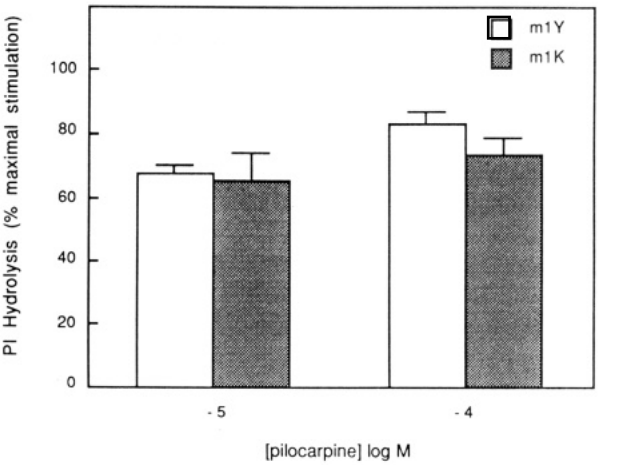

FIGURE 8: PI hydrolysis is reduced in cells exposed to PMA. The effect of pretreatment of Y1 (open bars) or PKA^- (solid bars) cells with 10^{-7} M PMA for 1 h prior to the subsequent stimulation of PI hydrolysis by 10^{-5} or 10^{-4} M pilocarpine is shown. Specific [^{3}H]NMS binding declined by 20 and 6% in m1Y and m1K cells pretreated with PMA, respectively. PI hydrolysis is presented as a percent of the maximal stimulation in cells not exposed to PMA, which was 25-fold in m1Y cells expressing 899 ± 121 fmol of receptor/mg of total protein and 11-fold in m1K cells expressing 174 ± 15 fmol of receptor/mg of total protein. Data are presented as the mean ± SEM or three to four experiments.

Desensitization of Pilocarpine-Stimulated PI Hydrolysis by PMA. PKA^+ and PKA^- cells were labeled with *myo*-[^{3}H]inositol for 24 h and exposed to 10^{-7} M PMA in the presence of label for 1 h, and the ability of pilocarpine to stimulate PI hydrolysis was determined. Under these conditions, pretreatment of cells with PMA resulted in about a 20% decline in the level of pilocarpine-stimulated PI hydrolysis compared to the level in control cells not exposed to PMA. The magnitude of PMA-dependent desensitization was similar in PKA^+ and PKA^- cells (Figure 8). Thus, desensitization was not dependent on PKA and was independent of receptor internalization, as specific binding of [^{3}H]NMS to m1Y cells exposed to 10^{-7} M PMA declined 20 ± 4% (n = 4) relative to control, while in m1K cells binding declined by only 6 ± 1% (n = 3). Moreover, PMA-dependent desensitization was independent of the concentration of pilocarpine. There was no significant difference in the extent of internalization at maximal and submaximal levels of pilocarpine. These data suggest that the block of signal transduction in cells exposed to PMA occurred distal to the m1 receptor or that desensitization did not involve internalization.

We then determined the ability of AlF_4^- to stimulate PI hydrolysis in PMA-treated cells. AlF_4^- is thought to directly activate G-proteins (Blackmore & Exton, 1986). If the block in signal transduction occurred at the level of the G-protein rather than the receptor, a similar level of attenuation of AlF4$^-$-stimulated PI hydrolysis should be observed in PKA^+ and PKA^- cells. In cells exposed to 10^{-7} M PMA, there was a 37 ± 10% (n = 3) decline in PI hydrolysis stimulated by 5 mM NaF/10 μM $AlCl_3$ in m1Y cells and a 36 ± 11% (n = 4) decline in m1K cells compared to the level of stimulation in untreated cells. Thus, the decrease in mAChR-mediated PI hydrolysis in Y1 cells treated with PMA appears to occur at the level of the G-protein.

To control for possible changes in labeling of the inositol phospholipids in cells pretreated with PMA, we measured the effect of pretreatment on the total pool of labeled inositol

phospholipids and labeling of the phospholipid fractions. In two experiments, pretreatment of cells with 10^{-7} M PMA for 1 h in the presence of *myo*-[^{3}H]inositol did not decrease the total pool of inositol phospholipids (109% of control) nor labeling of the lipid fractions (compared to labeling in control cells, phosphatidylinositol was 118 ± 9%, phosphatidylinositol phosphate was 107 ± 10%, and phosphatidylinositol bisphosphate was 101 ± 13%).

Discussion

In this study, we compared agonist-dependent internalization and desensitization of cloned m1 and m2 muscarinic receptor subtypes transfected in PKA$^+$ and PKA$^-$ cells. Both the m1 and m2 receptors were internalized following exposure to agonist, and internalization was accompanied by desensitization in both cell lines. This is the first demonstration that PKA activity was not required for agonist-dependent regulation of either the m1 or m2 receptors.

The rate, extent, and sensitivity to agonist for internalization of the m1 receptor were similar in PKA$^+$ and PKA$^-$ cells (Figures 1 and 2). However, the m2 receptor was internalized more slowly and to a lesser extent and was less sensitive to agonist when it was expressed in PKA$^+$ cells compared to PKA$^-$ cells (Figures 1 and 2). This result indicates that basal PKA activity modulates, but is not necessary for, agonist-dependent internalization of the m2 receptor. Inhibition of basal PKA activity has also been shown to block other physiological responses in cells (Mellon et al., 1989). The decreased sensitivity to regulation of the m2 receptor in PKA$^+$ cells by agonist could be due to tonic phosphorylation of the receptor. This hypothesis is supported by the observations that the m2 receptor can be phosphorylated by PKA in vitro (Rosenbaum et al., 1987) and exists as a phosphoprotein in intact tissue (Kwatra et al., 1989a). We found that increasing cellular cAMP concentrations above the basal level did not affect agonist-dependent internalization of the m2 receptor and did not cause internalization of either the m1 or the m2 receptors. These findings are consistent with previous reports that activation of PKA in vivo did not increase the level of phosphorylation of the avian cardiac or porcine atrial receptors (Kwatra & Hosey, 1986, Kwatra et al., 1987, 1989a). Alternatively, PKA may phosphorylate some other protein involved in receptor internalization.

To determine whether the decrease in receptor number following exposure of receptors to agonist was accompanied by desensitization, we measured the ability of the m1 receptor to stimulate PI hydrolysis and the m2 receptor to inhibit cAMP accumulation in cells pretreated with 1 mM carbachol for 1 h. There was a desensitization of functional responses for both m1 and m2 receptors following exposure to agonist. The magnitude of the desensitization was less than the decline in specific [^{3}H]NMS binding, consistent with the presence of spare receptors capable of coupling to effectors. For the m1 receptor in PKA$^+$ and PKA$^-$ cells, there was a decline in efficacy for pilocarpine-stimulated PI hydrolysis but no change in potency (Figure 7A). In contrast, the m2 receptor in PKA$^-$ cells showed a small but significant decline in efficacy but a large decrease in potency for carbachol-dependent inhibition of cAMP accumulation (Figure 7B). Thus, PKA activity was not necessary for either the agonist-dependent internalization or the desensitization of the m1 or m2 receptors.

Exposure of cells transfected with the mAChR to 10^{-7} M pMA resulted in internalization of the m1, but not the m2, receptor (Figure 3). The sensitivity of the m1 receptor to regulation by PKC is consistent with previous reports that the mAChR purified from porcine cerebrum, a tissue which contains predominantly the m1 receptor (Haga & Haga, 1989), is phosphorylated by purified PKC (Haga et al., 1988). The lack of sensitivity of the m2 receptor to regulation by PKA is consistent with reports that the purified receptor from porcine atria is not phosphorylated by purified PKC (Haga et al., 1988; Rosenbaum et al., 1987).

An unexpected finding was that PMA-dependent internalization of the m1 receptor was greatly attenuated or absent in cells which were deficient in PKA. Thus, it appears that the PKC-dependent internalization of the m1 receptor requires PKA for some step in the process. Other processes mediated by PKC may also depend on PKA, as activation of PKC stimulates secretion in Y1 cells, but is ineffective in Kin-8 cells (Estensen et al., 1983).

Stimulation of the m1 receptor activates phospholipase C and results in activation of PKC due to the formation of diacylglycerol. Thus, agonist-dependent internalization of the m1 receptor might be due to activation of PKC. However, several observations suggest that agonist-dependent internalization can occur independently of activation of PKC: (1) PMA-dependent internalization occurred only in PKA$^+$ cells while agonist-dependent internalization occurred similarly in PKA$^+$ and PKA$^-$ cells; (2) the rate and extent of internalization of m1 by agonist and PMA differed; and (3) concanavalin A selectively blocked PMA-dependent internalization of the m1 receptors, while agonist-dependent internalization was unaffected. Although agonist-induced internalization did not appear to involve activation of PKC, the effects of carbachol and PMA on internalization of receptors were not additive. PKC activation also results in internalization of the β-adrenergic receptor, and the mechanism of PKC-dependent internalization has been proposed to differ from that of agonist-dependent internalization (Limas & Limas, 1983; Kassis et al., 1985; Towes et al., 1987; Fishman et al., 1987; Lohse et al., 1990). Whether or not the effects of agonist and PKC on internalization of the β-adrenergic receptor were additive varied with the cell type studied.

Exposure of cells to PMA was accompanied by desensitization of pilocarpine-stimulated PI hydrolysis in both PKA$^+$ and PKA$^-$ cells. In both cases, pretreatment of cells with 10^{-7} M PMA resulted in a 25% decline in stimulation of PI hydrolysis by 10^{-4} M pilocarpine (Figure 8). The extent of desensitization was not significantly different at submaximal concentrations of pilocarpine (10^{-5} M). Desensitization was independent of receptor internalization, as pretreatment of PKA$^+$ cells with PMA resulted in a 20% decline in specific [^{3}H]NMS binding while pretreatment of PKA$^-$ cells resulted in a 6% decline. These data suggest that PMA-dependent desensitization did not occur at the level of the m1 receptor, but rather at the level of the G-protein or phospholipase C. The finding that AlF_4^--dependent stimulation of PI hydrolysis was attenuated to the same extent in both PMA-pretreated PKA$^+$ and PKA$^-$ cells suggests that PKC blocks signal transduction at the level of the G protein. PKC has been previously reported to inactivate the G-protein that couples to phospholipase C (Katada et al., 1985; Orellana et al., 1987) and to inhibit phospholipase C directly (Rhee et al., 1989).

It is also possible that desensitization in PMA-treated cells results from inactivation of the receptor without its internalization. If this were the case, then PKA is required for PKC-dependent internalization, but not for desensitization. This seems unlikely in light of the findings of Conklin et al. (1988). They report that exposure of the m1 receptor in A9 L cells to PMA resulted in desensitization of PI hydrolysis but potentiation of arachidonic acid hydrolysis. This suggests that

the desensitization of PI hydrolysis did not involve inactivation of the receptor but was due to a block somewhere distal in the transduction pathway.

In summary, these results show that PKA is not required for the m1 or m2 receptors to couple to effectors or to be regulated by agonist. The two receptor subtypes differ in the rate and sensitivity of agonist-induced internalization and in susceptibility to internalization by treatment with PMA. Thus, differential regulation of the m1 and m2 receptors might occur within the same cell. The internalization of the m2 receptor by agonist was increased, and the internalization of the m1 receptor by PMA was reduced in PKA^- cells relative to wild-type PKA^+ cells. The results suggest that PKA modulates receptor function, perhaps by tonic phosphorylation of the receptor or some other protein involved in its internalization. Therefore, different mAChR subtypes expressed in the same cell may be differentially regulated in response to exposure to agonist, activation of PKC, and alterations in basal levels of intracellular cAMP.

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