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# Elevated Rac1 Activity Changes the $M_3$ Muscarinic Acetylcholine Receptor-Mediated Inhibition of Proliferation to Induction of Cell Death

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## ABSTRACT

Although muscarinic acetylcholine receptors (mAChRs) regulate proliferation in many cell types, the signaling pathways involved are unclear. The participation of the small GTPases Rac1 and RhoA in $M_3$ mAChR-mediated inhibition of proliferation was investigated by activating $M_3$ mAChRs stably transfected in Chinese hamster ovary cells stably coexpressing hemagglutinin (HA)-tagged wild-type or mutant Rac1 or RhoA proteins. Activation of $M_3$ mAChRs activates both Rac1 and RhoA and inhibits cell proliferation in all cell lines tested. mAChR-mediated inhibition of proliferation is diminished in cells expressing dominant-negative HA-Rac1$^{Asn17}$ (m3DNRac) but is enhanced in cells expressing HA-Rac1 (m3WTRac) or constitutively active HA-Rac$^{Val12}$ (m3CARac). The activation of mAChRs in m3WTRac and m3CARac cells also induces apoptosis. Expression of wild-type or mutant RhoA proteins does not alter mAChR-mediated inhibition of proliferation. mAChR-induced inhibition of proliferation is abrogated in all cell lines when $G\alpha_{q/11}$ signaling is terminated by transient expression of the COOH-terminal fragment of phospholipase C (PLC-$\beta$1ct), the $NH_2$-terminal fragment of G protein-coupled receptor kinase, or the regulator of G protein signaling 2. Pretreatment of all cells expressing wild-type or mutant Rac1 proteins with edelfosine, a phosphatidylinositol-specific PLC inhibitor, or Go 6976, which inhibits conventional protein kinase C (PKC) isoforms, diminishes the $M_3$ mAChR's ability to inhibit proliferation. Our results identify $G\alpha_{q/11}$, PLC, and PKC as participants in the $M_3$ mAChR-mediated inhibition of cell proliferation. These findings indicate that in the context of high Rac1 activity, but not RhoA activity, $M_3$ mAChR-mediated activation of these participants triggers cell death.

Muscarinic acetylcholine receptors (mAChRs) are expressed in many different cell types, including carcinoma cells, in which they play a crucial role in the regulation of proliferation of these cells. Activation of $M_3$ mAChRs induces significant proliferation in human colon cancer cell lines, a prostate carcinoma cell line, and primary cells derived from prostate carcinoma (Williams, 2003a). In contrast, activation of endogenous $M_3$ mAChRs inhibits DNA synthesis in several small-cell lung carcinoma cell lines (Williams, 2003a). These contradictory responses also occur in cells stably transfected with mAChRs. Transfected $M_3$ mAChRs can function as both agonist-dependent oncogenes (Gutkind et al., 1991) and receptor-mediated tumor suppressors (Felder et al., 1993) in fibroblasts. Activation of transfected $M_3$ mAChRs inhibits or stimulates cell proliferation, depending on the cellular environment (Nicke et al., 1999; Burdon et al., 2002). Thus, activation of mAChRs initiates diverse proliferative re-

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**ABBREVIATIONS:** mAChR, muscarinic acetylcholine receptor; CHO, Chinese hamster ovary; HA, hemagglutinin; CCh, carbachol; PLC, phospholipase C; GRK, G protein-coupled receptor kinase; RGS, regulator of G protein signaling; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; GPCR, G protein-coupled receptor; BrdU, bromodeoxyuridine; TRITC, tetamethylrhodamine isothiocyanate; 2-APB, 2-aminoethoxydiphenylborate; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester; GST, glutathione *S*-transferase; PBD, p21-activated kinase binding domain; GFP, green fluorescent protein; PBS, phosphate-buffered saline; $IP_3$, inositol 1,4,5-triphosphate; $PLA_2$, phospholipase $A_2$; PC-PLC phosphatidylcholine-specific phospholipase C; CaM kinase II, $Ca^{2+}$/calmodulin-dependent protein kinase II; MEK1/2, mitogen-activated protein kinase kinase 1/2; GEF, guanine nucleotide exchange factor; FACS, fluorescence-activated cell sorting; ECL, enhanced chemiluminescence; GTP$\gamma$S, guanosine 5′-3-*O*-(thio)triphosphate; Go 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; AACOCF3, arachidonyl trifluoromethyl ketone; D609, tricyclodecan-9-yl-xanthogenate; KN-93, 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine); W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; U0126, 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Edelfosine, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine.

sponses, which may be unique to individual cellular environments. The delineation of the signaling pathways participating in the mAChR-mediated inhibition of proliferation will clarify the role of mAChRs in pathophysiological processes, including aberrant cell proliferation.

Events mediated by G protein-coupled receptors (GPCRs), including changes in cell proliferation, often involve the activation of small GTPases of the Rho family (Marinissen and Gutkind, 2001). Signals generated by Rac1 and RhoA can provide both stimulatory and inhibitory signals for cell proliferation (Aznar and Lacal, 2001). Microinjection of Rac1 or RhoA into quiescent fibroblasts stimulates cell-cycle progression and subsequent DNA synthesis (Olson et al., 1995). The expression of dominant-negative Rac1 is associated with arrest in the $G_2$/M phase (Moore et al., 1997) and diminished proliferative responses to GPCR activation (Burstein et al., 1998). However, activation of Rac and Rho proteins may also trigger cell death (Esteve et al., 1998). Therefore, Rac1 or RhoA may act to stimulate cell proliferation or apoptosis depending on the cell context and environment (Aznar and Lacal, 2001).

Our findings that Rac1 and RhoA participate in $M_3$ mAChR-initiated events (Strassheim et al., 1999; Ruiz-Velasco et al., 2002) prompted us to investigate the role of Rac1 and RhoA in the $M_3$ mAChR-mediated inhibition of cell proliferation. Using Chinese hamster ovary (CHO) cells stably expressing $M_3$ mAChRs (CHO-m3) and coexpressing mutant or wild-type Rac1 (Ruiz-Velasco et al., 2002) or RhoA (Strassheim et al., 1999) proteins, we show that Rac1, but not RhoA, participates in the $M_3$ mAChR-mediated inhibition of cell proliferation. Interestingly, activation of the $M_3$ mAChR in cells overexpressing wild-type Rac1 or expressing constitutively active $Rac1^{Val12}$ not only suppresses cell proliferation but also induces cell death.

We also tested whether $G\alpha_{q/11}$, which couples to $M_3$ mAChRs (Wess, 1996), is involved in the $M_3$ mAChR-mediated effects on proliferation. We found that transient transfection with cDNA constructs encoding proteins that inhibit $G\alpha_{q/11}$ activity completely abrogated the $M_3$ mAChR-induced inhibition of proliferation in all cell lines tested. These results indicate that the antiproliferative effects of mAChR activation require the presence of active $G\alpha_{q/11}$. Consistent with this observation, we show that inhibition of the $G\alpha_{q/11}$ signaling cascade using pharmacological inhibitors of phospholipase C (PLC), $Ca^{2+}$ mobilization, or protein kinase C (PKC) diminishes the antiproliferative effects of $M_3$ mAChR activation.

This study identifies $G\alpha_{q/11}$, PLC, and PKC as participants in the $M_3$ mAChR-induced inhibition of proliferation. Our results indicate that in the context of high Rac1 activity, but not RhoA activity, the $M_3$ mAChR-mediated activation of these participants triggers cell death. These findings suggest that specific intracellular cues, such as high Rac activity, can escalate the $M_3$ mAChR-mediated inhibition of cell proliferation to cell death.

## Materials and Methods

**Reagents.** Rabbit polyclonal antibodies to the hemagglutinin (HA) epitope were purchased from Covance (Berkeley, CA), mouse monoclonal anti-bromodeoxyuridine (BrdU) antibody was obtained from Amersham Biosciences Inc. (Piscataway, NJ), and goat anti-mouse IgG labeled with tetramethylrhodamine isothiocyanate (TRITC) was purchased from Southern Biotechnology Associates (Birmingham, AL). Ham's F-12 medium was purchased from Mediatech (Herndon, VA), and fetal calf serum was obtained from Biofluids (Rockville, MD). [$^3$H]Thymidine (specific activity, 74 Ci/mmol) was purchased from Amersham Biosciences (Arlington Heights, IL). Edelfosine, Go 6976, $AACOCF_3$, D609, KN-93, W-7, U0126, LY 294002, Wortmannin, staurosporine, 2-aminoethoxydiphenylborate (2-APB), and BAPTA/AM were obtained from Calbiochem (San Diego, CA). Zeocin was purchased from Invitrogen (Carlsbad, CA). Rnase cocktail (500 U/ml Rnase A, 20,000 U/ml Rnase T1) was purchased from Ambion (Austin, TX). All other reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted in the text.

**Cell Lines.** The CHO-K1 sublines stably transfected with the $M_1$, $M_2$, or $M_3$ mAChR are referred to as CHO-m1, CHO-m2, and CHO-m3, respectively. Although CHO cells express endogenous Rac1 (Ruiz-Velasco et al., 2002) and RhoA (Miao et al., 2002), we have used clonal sublines of CHO-m3 cells stably coexpressing HA-tagged wild-type or mutant Rac1 (Ruiz-Velasco et al., 2002) or RhoA proteins (Strassheim et al., 1999) for our studies. The m3WTRho-1, m3CARho-4, and m3DNRho-2 cells are CHO-m3 sublines stably expressing HA-RhoA, constitutively active HA-$RhoA^{Val14}$, and dominant-negative HA-$RhoA^{Asn19}$, respectively (Strassheim et al., 1999). The m3WTRac, m3CARac, and m3DNRac cells are CHO-m3 sublines stably expressing HA-Rac1, constitutively active HA-$Rac1^{Val12}$, and dominant-negative HA-$Rac1^{Asn17}$, respectively (Ruiz-Velasco et al., 2002). The m3Zeo-2 cells are CHO-m3 cells stably transfected with the pZeoSV2 plasmid. All cells were maintained in complete medium consisting of Ham's F-12 medium supplemented with heat-inactivated fetal calf serum (5%), glutamine (0.3 $\mu$g/ml), penicillin (20 U/ml), and streptomycin sulfate (20 $\mu$g/ml).

**Assay of RhoA Activity.** The bacterial expression vector coding for the Rho binding domain of rhotekin tagged to glutathione *S*-transferase (GST) was generously provided by Dr. Martin Schwartz (Scripps Research Institute, La Jolla, CA). The assays were performed as described previously (Varker et al., 2003). Cells were permeabilized by a freeze-thaw cycle and incubated in reaction buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, and 6 mM $MgCl_2$, pH 7.4) in the presence or absence of 100 $\mu$M carbachol (CCh) or 100 $\mu$M GTP$\gamma$S for 30 s at 37°C. The cells were then solubilized in ice-cold lysis buffer (50 mM Tris, 10 mM $MgCl_2$, 500 mM NaCl, 0.2% SDS, 2% Triton X-100, 1% sodium deoxycholate, 10 $\mu$g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2) and centrifuged (14,000*g* for 10 min at 4°C). The resulting supernatants were incubated with the bacterially expressed, GST-tagged Rho binding domain of rhotekin followed by precipitation with glutathione-Sepharose 4B beads, as described previously (Varker et al., 2003). The precipitated HA-tagged RhoA GTP was detected by ECL Western blotting using antibody to HA.

**Assay of Rac1 Activity.** The bacterial expression vector coding for the GST-tagged p21 binding domain of the p21-activated kinase (GST-PBD) was generously provided by Dr. Gary Bokoch (Scripps Research Institute). The assays were performed as described previously (Ruiz-Velasco et al., 2002). Cells were subjected to a freeze-thaw cycle, incubated with 100 $\mu$M CCh or 100 $\mu$M GTP$\gamma$S for 3 min at 37°C, and lysed in lysis buffer. The lysates were incubated with bacterially expressed GST-PBD followed by precipitation with glutathione-Sepharose 4B beads, as described previously (Ruiz-Velasco et al., 2002). The precipitated HA-tagged Rac1 GTP was detected by ECL Western blotting using antibody to HA.

**Transfection of Cells with cDNA Constructs.** The cDNA constructs coding for green fluorescent protein (GFP), GFP-PLC-$\beta$1ct and GFP-RGS2 in the pEGFP-C1 vector and GFP-GRK-nt in the pEGFP-N1 vector (all from BD Biosciences Clontech, Palo Alto, CA), were a generous gift of Dr. Stephen Ikeda (National Institutes of Health, Bethesda, MD). Cells were transfected by suspension in Ham's F-12 medium ($6 \times 10^6$ cells/200 $\mu$l medium) containing 15 $\mu$g

of the indicated cDNA and electroporated by a single electric pulse (200 V for 50 ms) using a BTX Electro Square Porator (Genetronics Inc., San Diego, CA). The electroporated cells were transferred to tissue culture plates and incubated for 24 h (at 37°C in 5% $CO_2$) in complete Ham's F-12 medium.

**BrdU Uptake.** BrdU incorporation was measured using a modification of a previously described assay (Olson et al., 1995). Cells electroporated with different cDNA constructs were plated onto glass coverslips in complete CHO medium ($5 \times 10^4$ cells/ml medium). After 1 h, media with or without CCh (final concentration, 10 $\mu$M) were added to the wells, and the cells were incubated for an additional 16 to 24 h before being incubated with BrdU, according to the manufacturer's specifications (Amersham Biosciences) for 3 h (at 37°C). The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (for 15 min at 4°C) and permeabilized with acid/alcohol (90% ethanol, 5% distilled water, and 5% acetic acid) (90 s at 25°C). After incubating with PBS containing 1% bovine serum albumin (30 min at 25°C), the cells were incubated with mouse antibody to BrdU (90 min at 25°C), followed by incubation with TRITC-labeled anti-mouse IgG (1 h at 25°C). The cells were mounted in PBS containing 90% glycerol and 0.1% *p*-phenylenediamine and examined using an Optiphot fluorescence microscope (Nikon, Melville, NY).

To assess the percentage of BrdU-positive cells, the cells were examined by an investigator without knowledge of the treatment of the cells or the identity of the GFP-tagged protein expressed by the cells. In each assay, investigators examined at least 90 different cells transfected with the same cDNA and subjected to the same treatment.

**DNA Fragmentation Assay.** A DNA fragmentation assay to indicate apoptosis was performed using a modification of a previously reported protocol (Mandlekar et al., 2000). Cells were plated ($2 \times 10^6$ cells/plate) in complete CHO medium. After 1 h, media with or without CCh (final concentration, 10 $\mu$M) were added to the plates, and the cells were incubated for an additional 12 or 24 h. Detached cells suspended in culture supernatants were collected and pooled with adherent cells that were harvested by trypsinization. The cells were washed with PBS and resuspended in a buffer of 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 to lyse the cells. The cell lysates were incubated on ice for 60 min. DNA was extracted by adding an equal volume of neutral phenol/chloroform/isoamyl alcohol mixture, pH 8.0 (Fisher Scientific Co., Fair Lawn, NJ) and precipitated with 2 volumes of 100% ethanol and 0.1 volume of 5 M NaCl at −20°C overnight. The precipitates were dissolved in 10 mM Tris, pH 8.0, and 1 mM EDTA and treated with 1 $\mu$g/ml Rnase A at 37°C for 2 h. DNA fragments were resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

**[$^3$H]Thymidine Uptake.** Uptake of [$^3$H]thymidine by cells was used as an indicator of DNA synthesis as described previously (Varker et al., 2003). Cells were plated in 96-well plates with or without the indicated drugs in complete growth medium to promote exponential proliferation. One hour after plating, additional media with or without the stated concentrations of CCh and [$^3$H]thymidine were added. The cells were incubated for the indicated times in a humidified atmosphere of 5% $CO_2$/95% air. Before harvesting, the cells were incubated (20 min at 37°C) with PBS containing 5 mM EDTA and 5 mM EGTA to induce detachment of the cells from the microtiter plates. The cells were washed and lysed with distilled water and collected on filters using an automatic cell harvester (Skatron, Sterling, VA). The filters were placed in ScintiSafe Econo 1 scintillation fluid (Fisher Scientific) and counted with the use of an LS-6000 $\beta$-counter (Beckman Coulter, Inc., Fullerton, CA).

**Immunofluorescence Assays.** Immunofluorescence assays were conducted by a modification of a previously described protocol (Ruiz-Velasco et al., 2002). Cells electroporated with different cDNA constructs were plated onto glass coverslips in complete CHO medium ($5 \times 10^4$ cells/ml medium). After 2 days in culture, the media were replaced with fresh media with or without CCh (final concentration, 10 $\mu$M), and the cells were incubated for an additional 30 min. The cells were fixed in 4% paraformaldehyde in PBS (15 min,

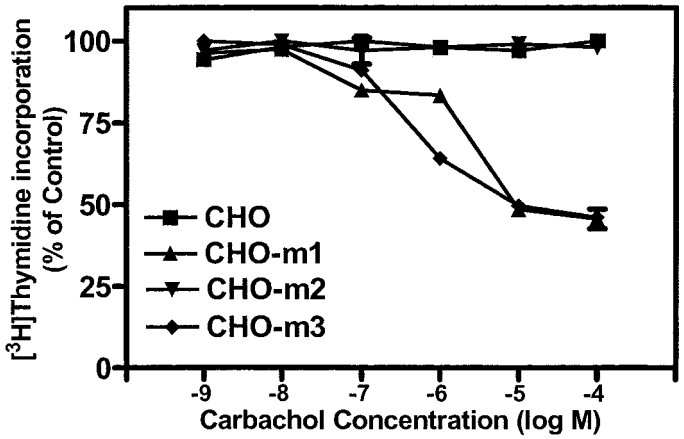


**Fig. 1.** Activation of the $M_1$ or $M_3$ mAChRs inhibits proliferation of CHO cells expressing these receptors. Proliferating CHO cells expressing transfected mAChR subtypes or parental CHO cells were incubated with [$^3$H]thymidine in the absence or presence of CCh. The amount of [$^3$H]thymidine incorporated into nascent DNA was measured 3 h later. Cells incubated without CCh served as controls. Results are the means ± 1 S.E.M. of six determinations from two independent experiments.

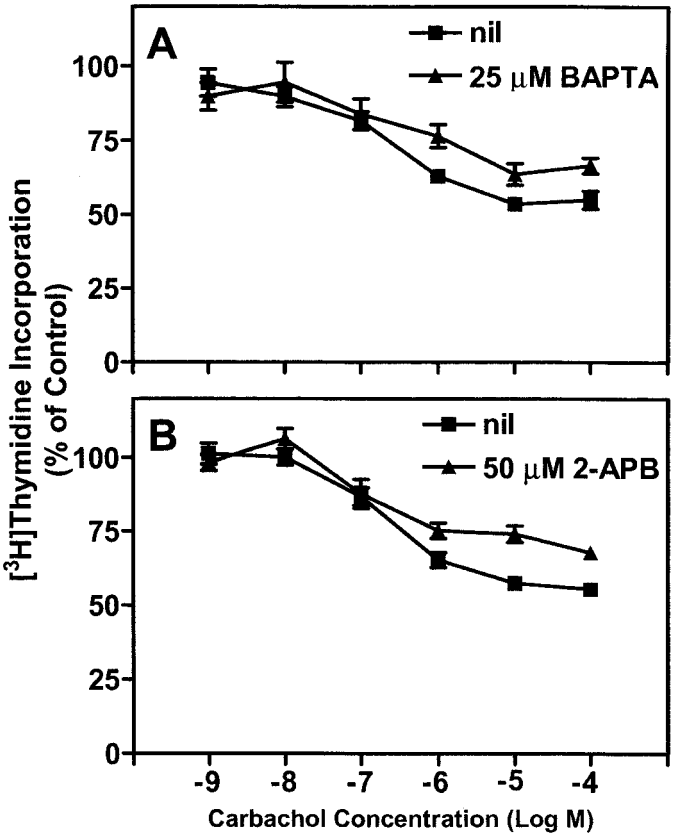


**Fig. 2.** Chelation of intracellular $Ca^{2+}$ with BAPTA/AM or inhibition of $IP_3$-mediated $Ca^{2+}$ mobilization with 2-APB diminishes the $M_3$ mAChR-induced inhibition of proliferation. Uptake of [$^3$H]thymidine by CHO-m3 cells was measured after the cells were cultured for 3 h in the presence or absence of the indicated concentration of carbachol after a 30-min preincubation in the presence or absence of BAPTA/AM (A) or 2-APB (B). CHO-m3 cells incubated with the indicated concentration of BAPTA/AM (A) or 2-APB (B) but in the absence of CCh served as controls. Results shown are the means ± 1 S.E.M. of quadruplicate samples from three independent experiments.

4°C) and permeabilized in Triton X-100 (0.2%, 10 min at 25°C). After incubating with PBS containing 1% bovine serum albumin (30 min at 25°C), the cells were incubated with mouse antibody to HA (1 h at 25°C) followed by incubation with TRITC-labeled anti-mouse IgG (1 h at 25°C). The cells were mounted and examined as described above. Digital images of the cells were collected using a Kodak DC 290 zoom digital camera (Eastman Kodak, Rochester, NY) and Adobe Photoshop software (Adobe Systems, Mountain View, CA) .

**Cell-Cycle Analysis.** Cells were plated at $10^6$ cells/10 cm dish with or without 30 $\mu$M edelfosine or 2 $\mu$M Go 6976. One hour after plating, additional media with or without CCh (final concentration, 10 $\mu$M) were added, and the cells were incubated for 24 h. The cells were harvested, washed, and resuspended in 5 mM EDTA in PBS. The cells were fixed in 50% ethanol, incubated with Rnase cocktail (500 U/ml Rnase A, 20,000 U/ml Rnase T1) for 30 min, and stained with propidium iodide. The samples were analyzed using a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA).

## Results

**Activation of the $M_1$ or $M_3$ mAChR Inhibits the Proliferation of CHO Cells.** We tested the ability of transfected $M_1$, $M_2$, or $M_3$ mAChRs to affect the proliferation of CHO cells. Incubation with CCh, which is an agonist for all mAChR subtypes, decreases [$^3$H]thymidine incorporation in CHO-m1 or CHO-m3 cells, which are the sublines transfected with the human $M_1$ or $M_3$ mAChR (Fig. 1). Incubation with CCh does not alter the proliferation of CHO-m2 cells transfected with the human $M_2$ mAChR or untransfected CHO cells (Fig. 1). These results indicate that CHO cell proliferation is inhibited by the activation of mAChRs, which couple to $G\alpha_{q/11}$ subunits, but not by activation of mAChRs, which couple to $G\alpha_{i/o}$.

**Mobilization of Intracellular $Ca^{2+}$ Participates in the $M_3$ mAChR-Induced Inhibition of Proliferation.** The mobilization of intracellular $Ca^{2+}$ is one of the earliest responses to the $M_3$ mAChR-mediated activation of $G\alpha_{q/11}$ (Wess, 1996). The effects of $Ca^{2+}$ mobilization on $M_3$ mAChR-induced inhibition of CHO cell proliferation was investigated using BAPTA/AM, a chelator of intracellular $Ca^{2+}$ (Billman, 1993), and 2-APB, which inhibits inositol 1,4,5-trisphosphate ($IP_3$)-induced $Ca^{2+}$ fluxes (Maruyama et al., 1997). Interestingly, preincubation of CHO-m3 cells with 25 $\mu$M BAPTA/AM (Fig. 2A) or 50 $\mu$M 2-APB (Fig. 2B) reduces the ability of the $M_3$ mAChR to inhibit cell proliferation. Thus, the reduction of intracellular $Ca^{2+}$ levels by chelating intracellular $Ca^{2+}$ or preventing $IP_3$-induced $Ca^{2+}$ mobilization diminishes but does not completely abrogate $M_3$ mAChR-induced inhibition of proliferation.

**Activation of the $M_3$ mAChR Activates Rac1 and RhoA.** Activation of some GPCRs that couple to $G\alpha_{q/11}$ has been shown to result in the activation of small GTPases of the Rho subfamily, including RhoA and Rac1 (Strassheim et al.,

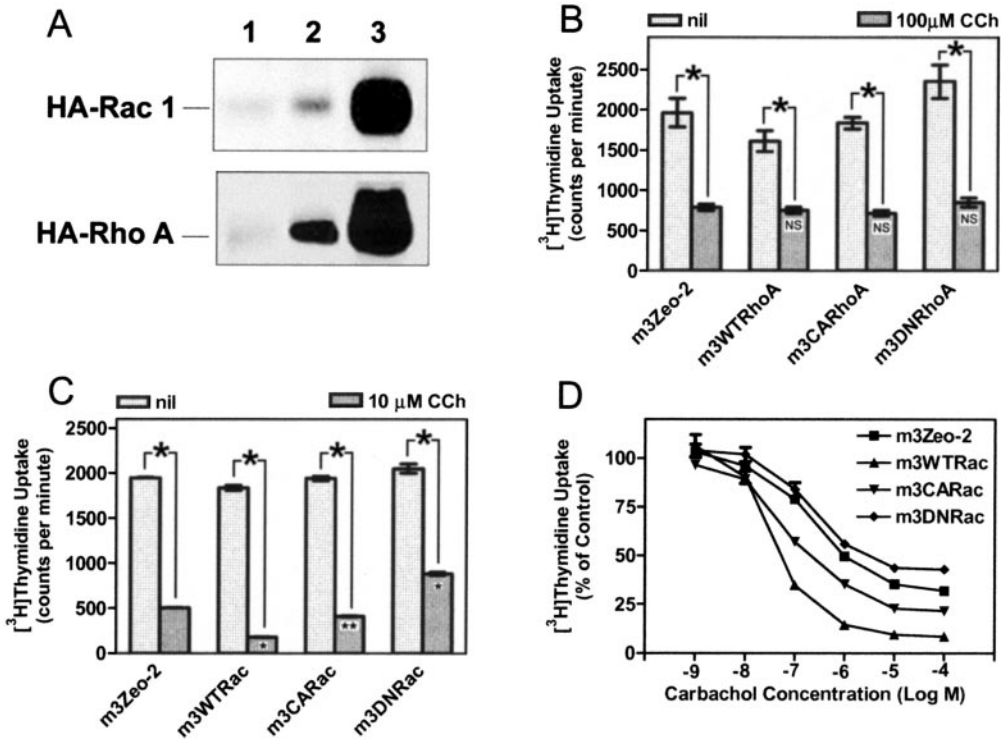


**Fig. 3.** Activation of the $M_3$ mAChR results in HA-Rac1 and HA-RhoA activation, but only Rac1 proteins participate in the $M_3$ mAChR-induced inhibition of proliferation. A, CHO-m3 cells expressing HA-Rac1 (top) or HA-RhoA (bottom) were lysed after 3 min (top) or 30 s (bottom) with no drug (lane 1), 100 $\mu$M CCh to activate the mAChR (lane 2), or 100 $\mu$M GTP$\gamma$S to nonspecifically activate all GTPases (lane 3). HA-Rac1 protein that precipitated with GST-PBD or HA-RhoA protein that precipitated with the GST-tagged Rho binding domain of rhotekin was detected by immunoblotting with HA antibody. Representative immunoblots from at least three independent experiments are shown. Uptake of [$^3$H]thymidine by m3Zeo-2 cells or cells expressing wild-type or mutant RhoA (B) or Rac1 (C and D) proteins was measured after the cells were cultured for 24 h in the absence or presence of 100 $\mu$M CCh (B), 10 $\mu$M CCh (C), or the indicated concentrations of CCh (D). Cells of each indicated cell line incubated in the absence of CCh served as controls for the data presented in D. Results are the means $\pm$ 1 S.E.M. of triplicate samples from two to four independent experiments. Brackets above the columns indicate a statistical comparison between the two bracketed samples. Symbols within a column indicate a statistical comparison between the sample and the sample of m3Zeo-2 cells treated with the indicated dose of CCh (second column); $\star$, $p < 0.001$; $\star\star$, $p < 0.025$; NS, not significant. The means of the measured values of each treatment group were compared by using Student's $t$ test.

1999; Marinissen and Gutkind, 2001; Ruiz-Velasco et al., 2002). The effects of $M_3$ mAChR activation on the activities of HA-tagged Rac1 and HA-tagged RhoA were investigated by precipitating the GTP-bound forms of the proteins using GST-PBD or the Rho-binding domain of rhotekin, respectively. The precipitates were examined by ECL Western blotting using antibody to HA (Fig. 3A). We found that the association of HA-Rac1 with GST-PBD and the association of HA-RhoA with the Rho binding domain of rhotekin are increased by $M_3$ mAChR activation (Fig. 3A). These associations are also increased by the nonspecific activation of GTPases with GTP$\gamma$S (Fig. 3A). Thus, the stimulation of $M_3$ mAChR increases GTP binding to both Rac1 and RhoA, indicating activation of these GTPases.

**Overexpression of Rac1, or the Expression of Constitutively Active Rac1$^{Val12}$ Enhances the Ability of the $M_3$ mAChR to Inhibit Cell Proliferation.** We investigated the participation of Rac1 and RhoA in the $M_3$ mAChR-induced inhibition of proliferation using CHO-m3 cell lines stably expressing HA-tagged mutant or wild-type Rac1 or RhoA (Strassheim et al., 1999; Ruiz-Velasco et al., 2002). The m3Zeo-2 cells are CHO-m3 cells stably transfected with the pZeoSV2 plasmid. These cells do not exhibit any detectable proliferative differences from the parental CHO-m3 cell line under basal conditions or upon activation of the $M_3$ mAChR. In the absence of drugs, cells expressing wild-type or mutant HA-RhoA or HA-Rac1 proteins exhibit similar rates of proliferation (Fig. 3, B, C, and D), indicating that the transfected

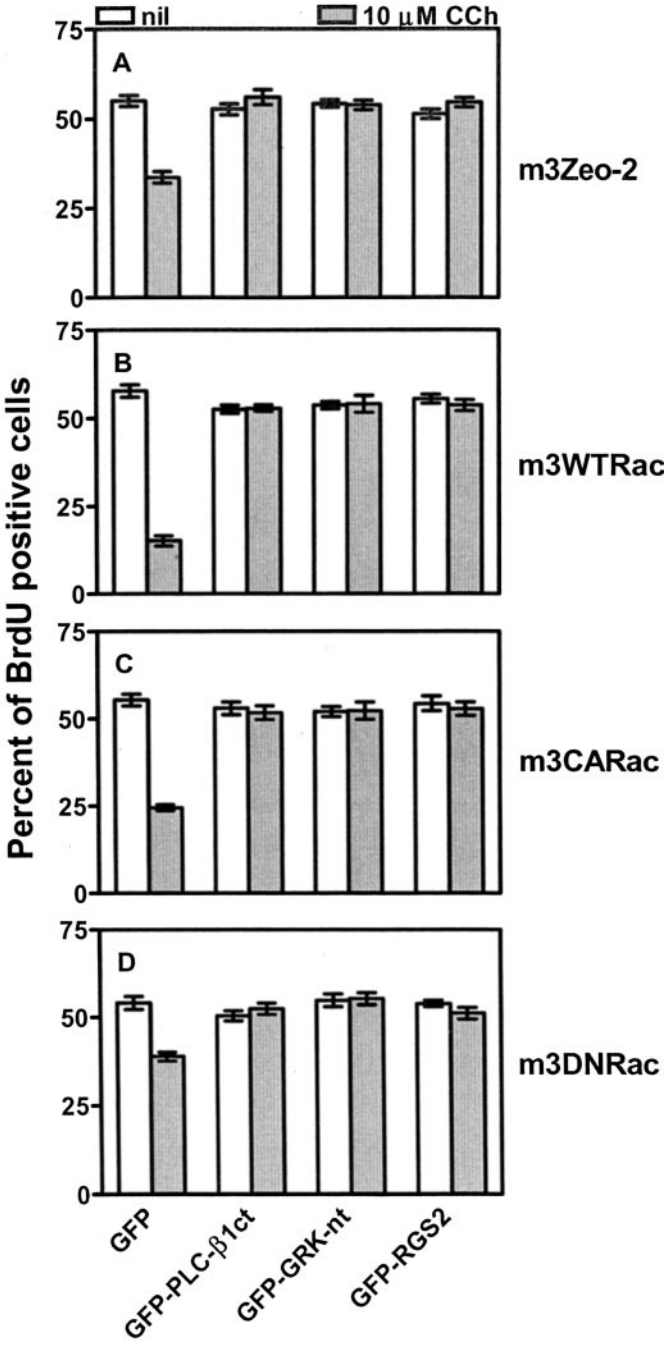


**Fig. 4.** Inactivation of $G\alpha_{q/11}$ signaling abrogates the $M_3$ mAChR-induced inhibition of proliferation. Uptake of BrdU by the indicated cells transiently expressing the identified cDNAs was measured after the cells were cultured for 24 h in the presence or absence of 10 $\mu$M CCh. Investigators scored the BrdU-positive cells without knowledge of the identity or treatment of the cells. The results shown are the means ± 1 S.E.M. from 270 cells scored in three independent experiments.

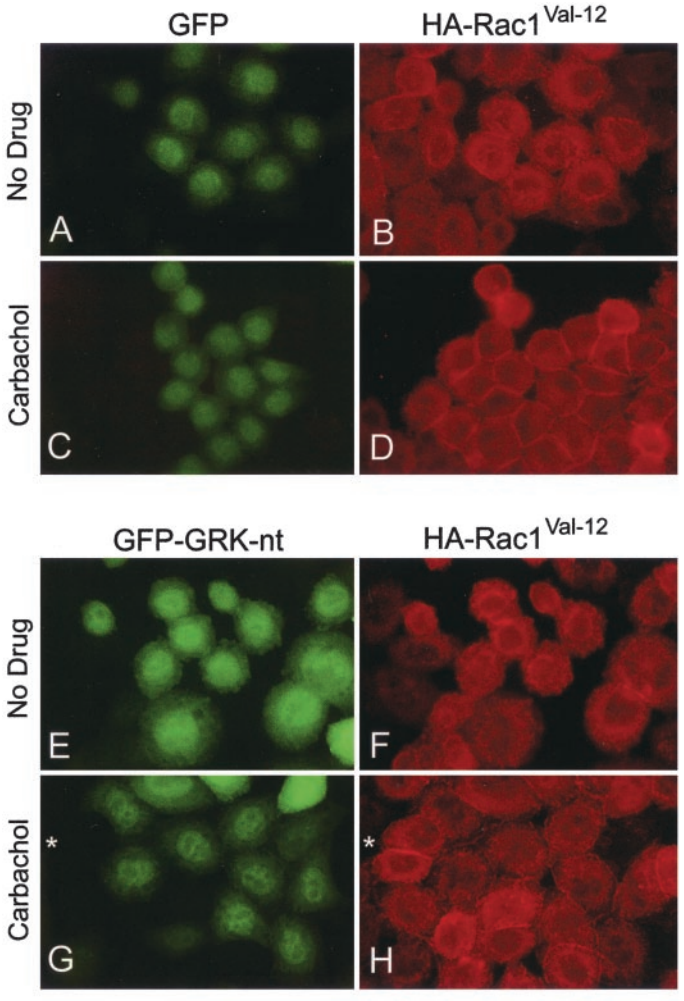


**Fig. 5.** Inactivation of $G\alpha_{q/11}$ signaling diminishes the $M_3$ mAChR-induced accumulation of constitutively active HA-Rac1$^{Val12}$ at cell junctions. m3CARac cells transiently expressing GFP (A-D) or GFP-GRK-nt (E-H) were incubated with no drug (A, B, E, and F) or 10 $\mu$M CCh (C, D, G, and H) for 30 min and were immunofluorescently stained with antibody to HA. The same field of cells is shown in A and B, in C and D, in E and F, and in G and H. ⋆ in H, the accumulation of HA- Rac1$^{Val12}$ at cell junctions of m3CARac cells not expressing GRK-nt. Representative results from three independent experiments are shown. Bar, 10 $\mu$m.

GTPases do not alter normal proliferation. Activation of $M_3$ mAChR significantly diminishes the proliferation of all cell lines investigated (Fig. 3, B and C). Incubation with CCh reduces [$^3$H]thymidine uptake to the same extent in control m3Zeo-2 cells and in cells expressing wild-type RhoA (m3WTRho-1 cells) or mutant RhoA proteins (m3CARho-4 or m3DNRho-2 cells) (Fig. 3B). These findings indicate that RhoA does not participate in the $M_3$ mAChR-induced inhibition of proliferation. In contrast, the expression of HA-tagged wild-type Rac1 in m3WTRac cells or the expression of constitutively active HA-Rac1$^{Val12}$ in m3CARac cells enhances the $M_3$ mAChR-induced inhibition of proliferation compared with that seen in control m3Zeo-2 cells (Fig. 3, C and D). The expression of dominant-negative HA-Rac1$^{Asn17}$ in m3DNRac cells diminishes the ability of the $M_3$ mAChR to inhibit proliferation compared with control m3Zeo-2 cells (Fig. 3, C and D). These findings suggest that Rac1 participates in the antiproliferative effect of $M_3$ mAChR activation.

**G$\alpha_{q/11}$ Activity Is Required for the $M_3$ mAChR-Mediated Inhibition of Proliferation.** The $M_3$ mAChR is known to signal to G$\alpha_{q/11}$ subunits (Wess, 1996). Signaling by G$\alpha_{q/11}$ can be terminated by proteins such as the PLC-$\beta$1ct, which binds to G$\alpha_{q/11}$ (Paulssen et al., 1996), RGS2, which acts as a GTPase-activating protein (Heximer et al., 1997), and GRK-nt, which has an RGS2 domain, allowing it to bind to G$\alpha_{q/11}$ and inactivate it (Carmen et al., 1999). We investigated the involvement of G$\alpha_{q/11}$ in $M_3$ mAChR-induced inhibition of proliferation using m3Zeo-2 (Fig. 3A), m3WTRac (Fig. 3B), m3CARac (Fig. 3C), and m3DNRac (Fig. 3D) cells transiently transfected with cDNA constructs coding for GFP or GFP-tagged PLC-$\beta$1ct, GFP-tagged GRK-nt, or GFP-tagged RGS2. Cells transiently transfected with cDNA coding for GFP exhibit mAChR-induced inhibition of DNA synthesis, as indicated by the decrease in the percentage of BrdU-positive cells (Fig. 4). Transient expression of cDNA constructs coding for GFP-tagged PLC-$\beta$ct, GRK-nt or RGS2 abrogates the $M_3$ mAChR-mediated inhibition of BrdU incorporation in all cell lines examined (Fig. 4). These findings indicate the $M_3$ mAChR-induced inhibition of DNA synthesis, and the resulting decrease in cell proliferation, is dependent on G$\alpha_{q/11}$ signaling.

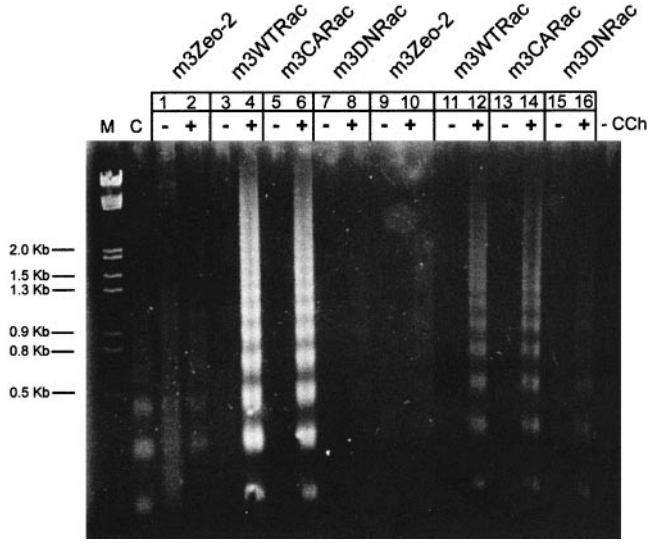


**Fig. 6.** Activation of the $M_3$ mAChR induces apoptosis. Cells were incubated in the presence (even lanes) or absence (odd lanes) of 10 $\mu$M CCh for 12 h (lanes 1–8) or 24 h (lanes 9–16). Cellular DNA was extracted and resolved by agarose gel electrophoresis. Lane M indicates size markers. Lane C indicates m3Zeo-2 cells treated with 0.5 $\mu$M staurosporine for 12 h that served as a positive control. A representative gel from two independent experiments is shown.

**Inhibition of G$\alpha_{q/11}$ Signaling Diminishes the $M_3$ mAChR-Mediated Translocation of Rac1 to Cell Junctions.** Activation of $M_3$ mAChR initiates numerous Rac1-dependent cellular responses, including the translocation of Rac1 from membrane ruffles to cell junctions, which is an indication of Rac1 activation (Ruiz-Velasco et al., 2002). We investigated the role of G$\alpha_{q/11}$ signaling in the activation of Rac1 by examining the $M_3$ mAChR-induced changes in the intracellular distribution of HA-Rac1 proteins. Incubation of m3CARac cells with CCh for 30 min induces the accumulation of constitutively active HA-Rac1$^{Val12}$ at cell junctions during mAChR-induced cell-cell compaction (Ruiz-Velasco et al., 2002) (Fig. 5D). Transfection of m3CARac cells with the cDNA construct coding for GFP-tagged GRK-nt diminishes $M_3$ mAChR-induced cell-cell compaction and the translocation of HA-Rac1$^{Val12}$ to cell junctions (Fig. 5H). In contrast, there is strong junctional localization of HA-Rac1$^{Val12}$ in CCh-treated cells not expressing GRK-nt (*, Fig. 5H). The expression of GFP-tagged PLC-$\beta$1ct or GFP-tagged RGS2 by m3CARac cells also diminishes the $M_3$ mAChR-induced translocation of HA-Rac1$^{Val12}$ to cell junctions (data not shown). These findings indicate that G$\alpha_{q/11}$ signaling is involved in the $M_3$ mAChR-mediated translocation of HA-Rac1 proteins, suggesting that G$\alpha_{q/11}$ participates in Rac1 activation by the $M_3$ mAChR.

**$M_3$ mAChR Activation Induces Apoptosis.** The $M_3$ mAChR-induced reduction of [$^3$H]thymidine uptake could be caused by a diminished entry or progression through S phase, an increase in cell death, or a combination of both responses. We investigated the possibility that the $M_3$

TABLE 1

Drugs that do not alter the $M_3$ mAChR-induced inhibition of proliferation

Uptake of [$^3$H]thymidine by m3Zeo-2 and cells expressing wild-type or mutant HA-Rac1 proteins was measured after the cells were cultured for 24 h in the absence or presence of the indicated concentrations of the listed drugs and in the absence or presence of 10 $\mu$M CCh. The results of three independent experiments using each drug were used to compile data for this table. Enzymes listed in parentheses are considered secondary targets of the indicated drug (Davies et al., 2000).

| Drug | Target Enzyme | Concentrations Tested |
|---|---|---|
| | | $\mu M$ |
| $AACOCF_3$ | Phospholipase $A_2$ | 6.25–200 |
| D609 | Phosphatidylcholine-specific phospholipase C (mitogen-activated protein kinase) | 2.5–80 |
| KN-93 | CaM kinase II | 0.625–20 |
| W-7 | CaM kinase II MLC kinase | 3.125–100 |
| U0126 | MEK1 and MEK2 (p38, protein kinase B) | 1.56–50 |
| LY 294002 | Phosphatidylinositol 3-kinase (casein kinase 2) | 1.56–50 |
| Wortmannin | Phosphatidylinositol 3-kinase (myosin light chain kinase, $PI_4$ kinase) | 0.313–50 |

mAChR-mediated inhibition of proliferation might be caused by the induction of apoptosis by examining DNA fragmentation in cells exposed to CCh for 12 h (Fig. 6, lanes 1–8) or 24 h (Fig. 6, lanes 9–16). Exposure of m3WTRac cells (Fig. 6, lane 4) or m3CARac cells (Fig. 6, lane 6) to CCh for 12 h results in large increases in cell death, primarily caused by apoptosis. The response of the m3Zeo-2 cells to the apoptotic effects of CCh is less marked (Fig. 6, lane 2); no evidence of apoptosis is seen in m3DNRac cells exposed to CCh for 12 h (Fig. 6, lane 8). DNA fragmentation is evident in all cell lines after 24 h of CCh treatment (Fig. 6, lanes 10, 12, 14, and 16). These findings indicate that $M_3$ mAChR activation induces apoptosis in all cell lines studied. This response is most marked in the m3WTRac and m3CARac cells, which are the cell lines most responsive to mAChR-induced inhibition of proliferation (Fig. 3, C and D).

**PI-PLC Participates in the $M_3$ mAChR-Induced Inhibition of Proliferation.** $M_3$ mAChR stimulation alters the activity of numerous enzymes that may participate in the inhibition of proliferation. We examined the participation of different proteins in the $M_3$ mAChR-induced inhibition of proliferation by treating m3Zeo-2, m3WTRac, m3CARac, and m3DNRac cells with a number of selective antagonists (Table 1). Treatment of all the sublines with a range of concentrations of inhibitors of phospholipase $A_2$ ($PLA_2$), phosphatidylcholine-specific PLC (PC-PLC), $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II), myosin light chain kinase, mitogen-activated protein kinase kinase 1 and 2 (MEK1/2), and phosphatidylinositol 3 kinase do not affect the $M_3$ mAChR-induced inhibition of proliferation.

Agonist binding to the $M_3$ mAChR causes $G\alpha_{q/11}$ to associate with PLC-$\beta$, resulting in activation of the enzyme (Marinissen and Gutkind, 2001). The PI-PLC inhibitor edelfosine (Powis et al., 1992) was used to investigate the role of PLC-$\beta$ in the inhibition of proliferation induced by $M_3$ mAChR activation. Preincubation of all cell lines with edelfosine diminishes the mAChR-induced inhibition of proliferation (Fig. 7). Although this response occurs at all tested concentrations of CCh, edelfosine is most effective at inhibiting the antiproliferative effects of 10 $\mu$M CCh (Fig. 7, C, F,

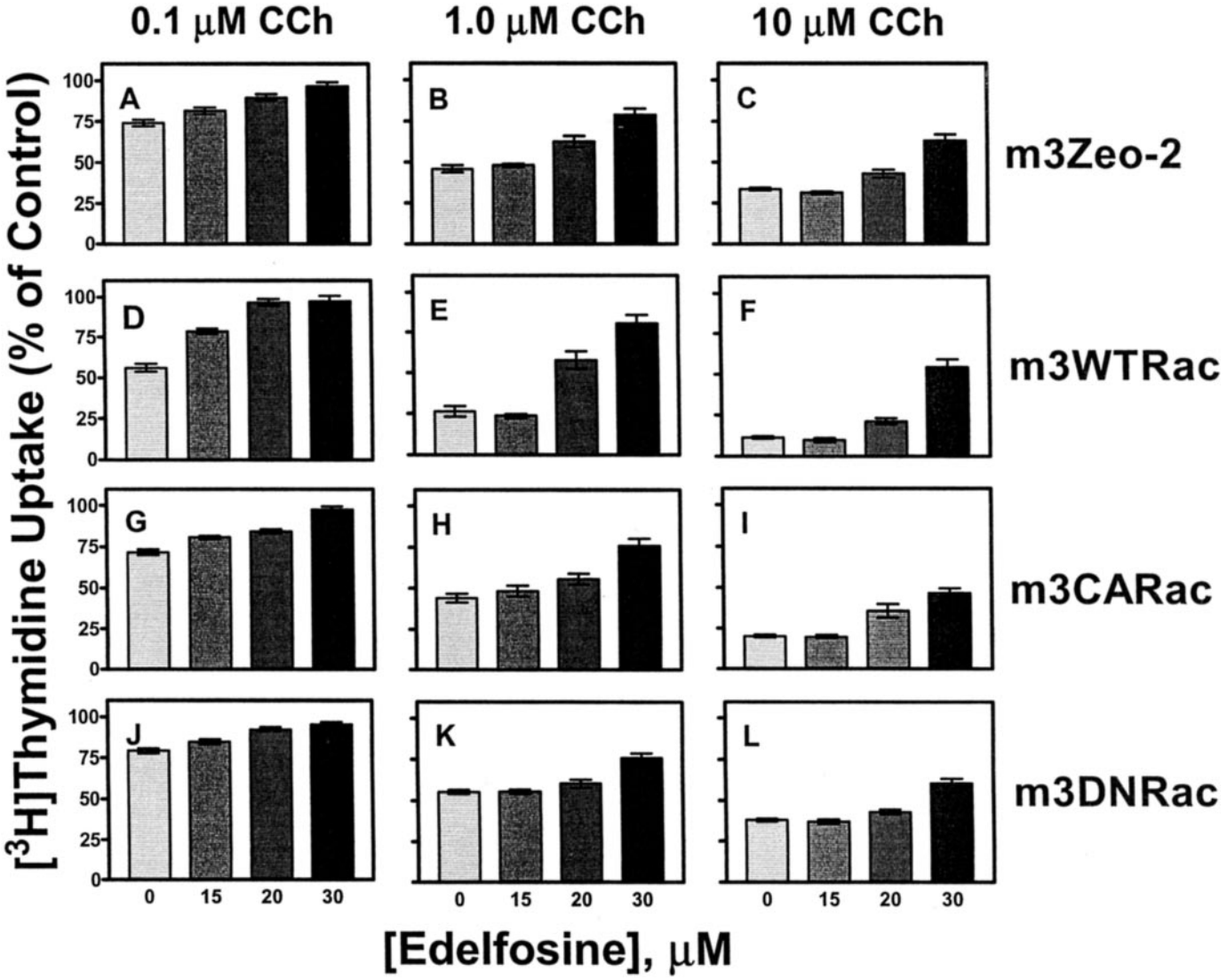


**Fig. 7.** Inactivation of PLC with edelfosine diminishes the $M_3$ mAChR-induced inhibition of proliferation. Uptake of [$^3$H]thymidine by m3Zeo-2 cells or cells expressing wild-type or mutant Rac1 proteins was measured after the cells were cultured for 24 h in the presence or absence of the indicated concentration of drugs. Cells of each cell line incubated with the indicated concentration of edelfosine but in the absence of CCh served as controls. Results shown are the means ± 1 S.E.M. of quadruplicate samples from four to five independent experiments.

I, and L). The "rescue" effect is greatest in the cell lines most susceptible to CCh-induced inhibition of proliferation, the m3WTRac cells, which overexpress wild-type Rac1 (Fig. 7, D-F), and the m3CARac cells, which express constitutively active $Rac1^{Val12}$ (Fig. 7, G-I). These results indicate that PI-PLC enzymes participate in the $M_3$ mAChR-mediated inhibition of proliferation.

To examine the effects of mAChR activation on cell-cycle progression, we defined the proportion of the cell population in each phase of the cell cycle and in the sub-$G_o/G_1$ cell population after incubation of the cells with 10 $\mu$M CCh for 24 h (Fig. 8). Treatment of m3Zeo-2 and m3DNRac cells with CCh significantly depresses the proportion of the cell population in the S phase (Fig. 8, B and N) compared with that seen in untreated cells (Fig. 8, A and M). Very little CCh-induced increase in the proportion of these cells in the sub-$G_o/G_1$ population is observed (Fig. 8, B and N). These results indicate that in the m3Zeo-2 and m3DNRac cell lines, activation of the $M_3$ mAChR results in inhibition of DNA synthesis and not in a concomitant massive increase in cell death, although some apoptosis is evident (Figs. 6 and 8, B and N). In contrast, incubation of m3WTRac and m3CARac cells with CCh for 24 h results in a massive increase in cells in the sub-$G_o/G_1$ population, indicating a large increase in cell death (Fig. 8, F and J) compared with that of untreated cells (Fig. 8, E and I). The proportion of CCh-treated m3WTRac cells (Fig. 8F) and m3CARac cells (Fig. 8J) in the S phase is greatly reduced compared with cells incubated without CCh (Fig. 8, E and I) and compared with CCh-treated m3Zeo-2 (Fig. 8B) and m3DNRac (Fig. 8N) cells. Thus, activation of the $M_3$ mAChR diminishes cell-cycle progression in the m3Zeo-2 cells (Fig. 8B) and m3DNRac cells (Fig. 8N); in contrast, activation of the $M_3$ mAChR induces cell death in the m3WTRac (Fig. 8F) and m3CARac (Fig. 8J) cells.

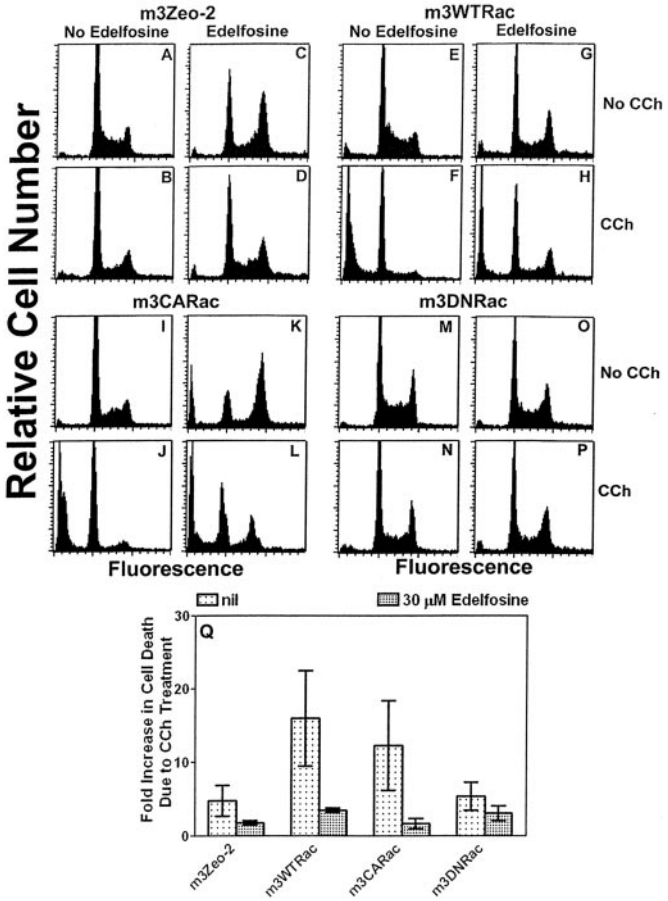


**Fig. 8.** Incubation of cells with edelfosine induces $G_2/M$ arrest and rescues the cells expressing wild-type or constitutively active Rac1 proteins from $M_3$ mAChR-induced cell death. Control m3Zeo-2 cells (A-D) or cells expressing wild-type (E-H), constitutively active $Rac^{Val12}$ (I-L), or dominant-negative $Rac^{Asn17}$ (M-P) were subjected to FACS analysis after being cultured for 24 h in the absence (A, B, E, F, I, J, M, and N) or presence (C, D, G, H, K, L, O, and P) of 30 $\mu$M edelfosine and in the absence (A, C, E, G, I, K, M, and O) or presence of 10 $\mu$M CCh (B, D, F, H, J, L, M, and P). Results shown are representative of three independent experiments. The fold increase in cell death (Q) was determined by defining the percentage of cells in the sub-$G_o/G_1$ population compared with that in the $G_1$, S, and $G_2/M$ phases of the cell cycle. Results shown are the means ± 1 S.E.M. calculated from three independent experiments.

Treatment of cells with edelfosine increases the proportion of cells in the $G_2/M$ phase of the cell cycle (Fig. 8). This effect is greater in the m3Zeo-2 (Fig. 8C), m3WTRac (Fig. 8G), and m3CARac (Fig. 8K) cells than in m3DNRac (Fig. 8O) cells. Pretreatment of m3WTRac and m3CARac cells with edelfosine before the addition of CCh decreases the sub-$G_o/G_1$ population of cells and increases the proportion of cells in the S and $G_2/M$ phases of the cell cycle (Fig. 8, H and L). Thus, inhibition of PI-PLC activity leads to a decrease in $M_3$ mAChR-induced cell death (Fig. 8Q).

**Conventional Isoforms of PKC Participate in the $M_3$ mAChR-Induced Inhibition of Proliferation.** Activation of PI-PLC by the $M_3$ mAChR results in the generation of diacylglycerol, which in turn activates PKC. We investigated the role of PKC in the $M_3$ mAChR-induced inhibition of proliferation using Go 6976, an inhibitor of conventional PKC isoforms (Martiny-Baron et al., 1993). Preincubation of all the cell lines with Go 6976 diminishes the ability of $M_3$ mAChR activation to inhibit cell proliferation (Fig. 9). Treatment of m3Zeo-2, m3WTRac, m3CARac, and m3DNRac cells with 10 $\mu$M CCh results in a reduction of [$^3$H]thymidine uptake to 27 ± 0.9, 9 ± 0.7, 18 ± 1, and 42 ± 1.5% of control, respectively (Fig. 9, C, F, I, and L). Incubation of m3Zeo-2, m3WTRac, m3CARac, and m3DNRac cells with 2 $\mu$M Go 6976 followed by 10 $\mu$M CCh results in the reduction of [$^3$H]thymidine uptake to 63 ± 2.5, 21 ± 1.7, 46 ± 3.7, and 60 ± 1.5% of control, respectively (Fig. 9, C, F, I, and L). These results indicate that conventional PKC isoforms may participate in the $M_3$ mAChR-induced inhibition of proliferation.

Cell-cycle studies indicate that treatment of cells with Go 6976 moderately diminishes the proportion of the cell population in the S phase of the cell cycle (Fig. 10, C, G, K, and O). In contrast to edelfosine, which profoundly increases the proportion of $G_2/M$ cells (Fig. 8), Go 6976 does not significantly affect $G_2/M$ progression. However, Go 6976 does protect cells from CCh-induced cell death. Preincubation of m3WTRac and m3CARac cells with 2 $\mu$M Go 6976 reduces the ability of $M_3$ mAChR activation to induce cell death in these two cell lines (Fig. 10, H, L, and Q). Thus, inhibition of PKC activity decreases $M_3$ mAChR-induced cell death (Fig. 7Q) in the m3WTRac and m3CARac cells.

## Discussion

We found that CHO cell proliferation is markedly diminished by the activation of transfected $M_1$ or $M_3$ mAChRs that couple to $G\alpha_{q/11}$ but not by activation of the $M_2$ mAChR that

couples to $G\alpha_{i/o}$, thus confirming previous findings (Detjen et al., 1995; Burdon et al., 2002; Williams, 2003a). The mAChR-induced inhibition of proliferation was completely abrogated in cells transiently expressing PLC-$\beta$1ct (Paulssen et al., 1996), RGS2 (Heximer et al., 1997), or GRK-nt (Carmen et al., 1999), which are proteins that terminate $G\alpha_{q/11}$ signaling. Many studies implicate $G\beta\gamma$ heterodimers in GPCR-mediated changes in cell proliferation (Marinissen and Gutkind, 2001). However, our findings indicate that the $M_3$ mAChR signal which inhibits proliferation is transduced by the $G\alpha_{q/11}$ subunit.

Activation of PI-PLC by $G\alpha_{q/11}$ generates two signaling cascades: the $IP_3$ signaling pathway leading to mobilization of intracellular $Ca^{2+}$, and the diacylglycerol pathway that activates PKC (Wess, 1996). We have shown that buffering of $G\alpha_{q/11}$ signaling, inhibition of PI-PLC, inhibition of $Ca^{2+}$ mobilization, or inhibition of PKC all serve to diminish the ability of the $M_3$ mAChR to inhibit cell proliferation. Thus, all of these signaling components downstream of $G\alpha_{q/11}$ participate in the $M_3$ mAChR-mediated inhibition of proliferation.

Our findings indicate that activation of $M_3$ mAChR inhibits its cell proliferation by Rac1-dependent and Rac1-independent means. Expression of dominant-negative $Rac1^{Asn17}$ in m3DNRac cells lessens the $M_3$ mAChR-induced inhibition of proliferation, indicating that Rac1 participates in this event. However, the mAChR-mediated inhibition of proliferation may also occur by a Rac1-independent mechanism, because the expression of $Rac1^{Asn17}$ does not completely abrogate the antiproliferative response. Interestingly, activation of mAChRs in cells expressing increased levels of Rac1 (m3WTRac cells) or in cells expressing constitutively active $Rac1^{Val12}$ (m3CARac cells) results not only in inhibition of proliferation but also in apoptosis. Thus, a single signal, activation of mAChR, induces the inhibition of proliferation in these cells. In the presence of a second signal, overexpression of active Rac1, cell death is triggered (Fig. 11). Our hypothesis that two signals are required to induce Rac1-

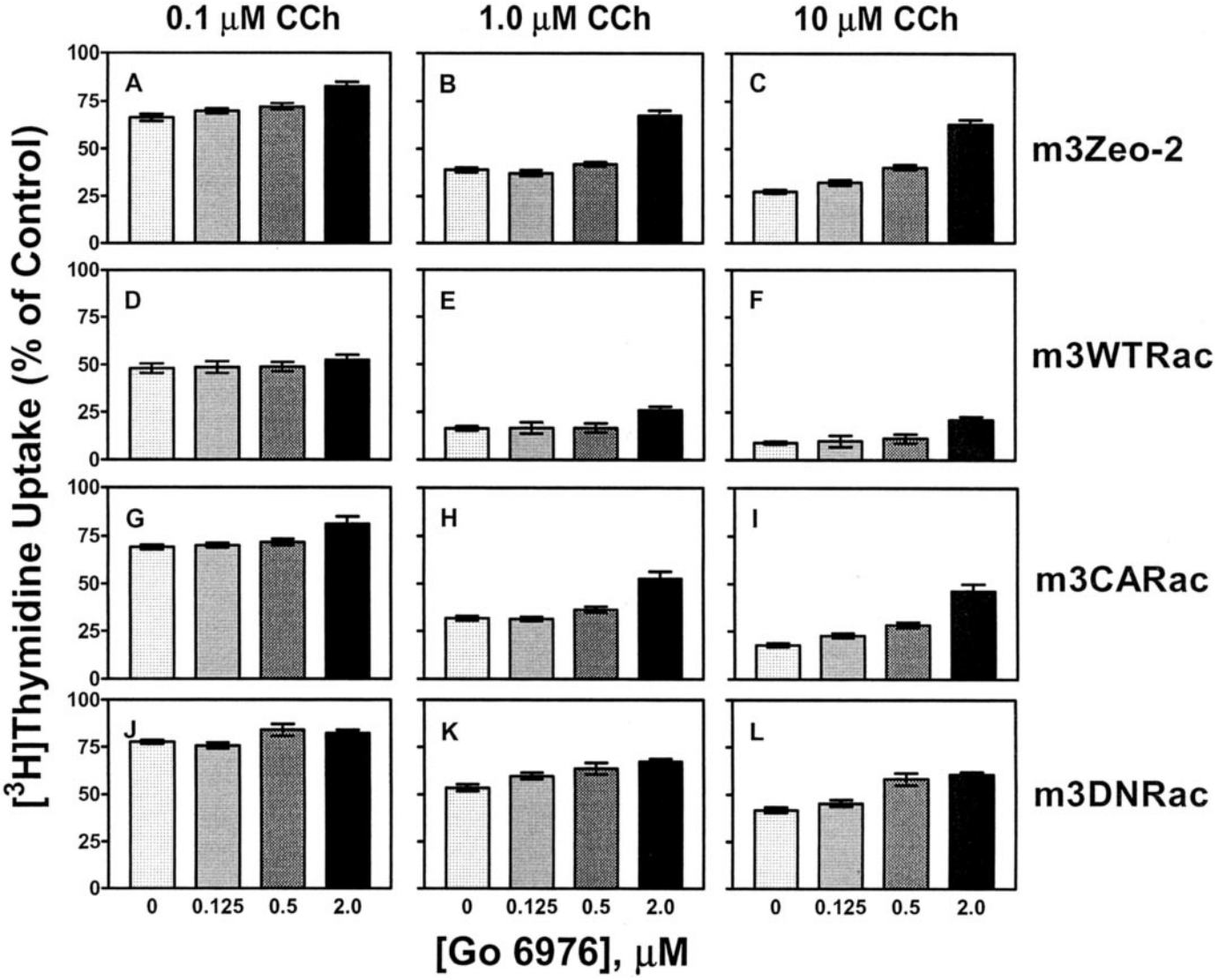


**Fig. 9.** Inactivation of conventional isoforms of PKC with Go 6976 diminishes the $M_3$ mAChR-induced inhibition of proliferation. Uptake of [$^3$H]thymidine by m3Zeo-2 cells or cells expressing wild-type or mutant Rac1 proteins was measured after the cells were cultured for 24 h in the presence or absence of the indicated concentrations of drugs. Cells of each cell line incubated with the indicated concentration of Go 6976 but in the absence of CCh served as controls. Results shown are the means ± 1 S.E.M. of quadruplicate samples from three to four independent experiments.

mediated cell death is consistent with previously reported findings. Overexpression of Rac1 induces apoptosis only upon serum deprivation in NIH 3T3 fibroblasts and human erythroleukemia K562 cells (Esteve et al., 1998; Embade et al., 2000). Introduction of constitutively active *OsRac1,* a rice homolog of human Rac1, induces apoptosis only in cells that also carry a lesion mimic mutant of rice (Kawasaki et al., 1999). Thus, the presence of at least two cooperative signals is required for the induction of cell death.

Interestingly, our results indicate that only Rac1 participates in the mAChR-induced inhibition of proliferation even though stimulation of $M_3$ mAChRs activates both RhoA and Rac1. These findings are consistent with previous observations that both RhoA and Rac1 activity levels are increased after treatment of U937 cells with tumor necrosis factor-$\alpha$, but only Rac1 participates in the inhibition of cell growth and induction of apoptosis (Esteve et al., 1998).

We present findings that support the role of $G\alpha_{q/11}$ in regulating Rac1-mediated activity. Activation of $M_3$ mAChR induces the accumulation of HA-$Rac1^{Val12}$ at cell junctions, a process that involves the activated form of Rac1 (Ruiz-Velasco et al., 2002). In m3CARac cells transiently expressing GRK-nt, the translocation of HA-$Rac1^{Val12}$ to cell junctions is greatly diminished. Our results cannot determine whether signaling through $G\alpha_{q/11}$ directly activates Rac1. However, dampening of $G\alpha_{q/11}$ activity does interfere with the ability of $M_3$ mAChR to regulate Rac1 activity. This finding suggests that $G\alpha_{q/11}$ participates in the activation of Rac1 analogous to the $G\alpha_{q/11}$-mediated activation of RhoA (Vogt et al., 2003).

We observed that the antiproliferative effects of $M_3$ mAChR activation are greater in cell populations expressing wild-type Rac1 than in those expressing constitutively active $Rac1^{val12}$ proteins. It is intriguing to speculate that this response occurs because wild-type Rac1 is more effective than constitutively active $Rac1^{Val12}$ in competitively interacting with a guanine nucleotide exchange factor (GEF) that activates other small GTPases required for cell proliferation. One such GEF may be SmgGDS (Williams, 2003b). Because GEFs only associate with the GDP-bound forms of small GTPases, GEFs should associate more often with wild-type Rac1 than with constitutively active Rac1, which remains in the GTP-bound form for prolonged periods. In the absence of mAChR stimulation, wild-type Rac1 would not compete with other small GTPases for GEFs. However, mAChR activation may stimulate the interaction of wild-type Rac1 with certain GEFs, diminishing the interaction of these GEFs with other small GTPases. If the interaction of these GEFs with other small GTPases is required for cell proliferation or survival, then mAChR activation would have a greater antiproliferative effect in m3WTRac cells than in m3CARac cells. Consistent with these findings, other studies have reported that wild-type Rac1 is more deleterious than constitutively active Rac1 under certain conditions (Esteve et al., 1998). Additional studies are needed to clarify these somewhat surprising findings.

We investigated the role of several enzymes, including PC-PLC, $PI_3$ kinase, $PLA_2$, CaM kinase II, and MEK1/2, which have been identified as possible participants in mAChR- or Rac1-initiated pathways, to determine whether they are involved in mAChR-induced inhibition of proliferation. PC-PLC, which is involved in some CHO cell signaling pathways (Wen et al., 1995), transduces apoptotic signals in a variety of cell types (Civone et al., 1995). Activation of $PI_3$ kinase induces Rac-mediated events (Rodriguez-Viciana et al., 1997). $PLA_2$ activity is necessary for the synthesis of arachidonic acid, which can initiate several Rac-mediated events such as membrane ruffling (Shin et al., 1999) and c-fos serum response element activation (Kim and Kim, 1997). CaM kinase II is necessary for activation of the Rac GEF Tiam1, (Fleming et al., 1999) and for activation of Rac itself (Lian et al., 2001). Activated Rac1 can stimulate MEK1/2 in a wide variety of cells (Eblen et al., 2002). Our studies, using a wide range of selective inhibitor concentrations, indicate that these enzymes are not required for the $M_3$ mAChR-initiated inhibition of proliferation. This finding is intriguing because all of these enzymes have been implicated in GPCR-signaling events and have been reported to be activators or downstream targets of Rac-mediated processes.

Treatment of all the cell lines with the PI-specific PLC

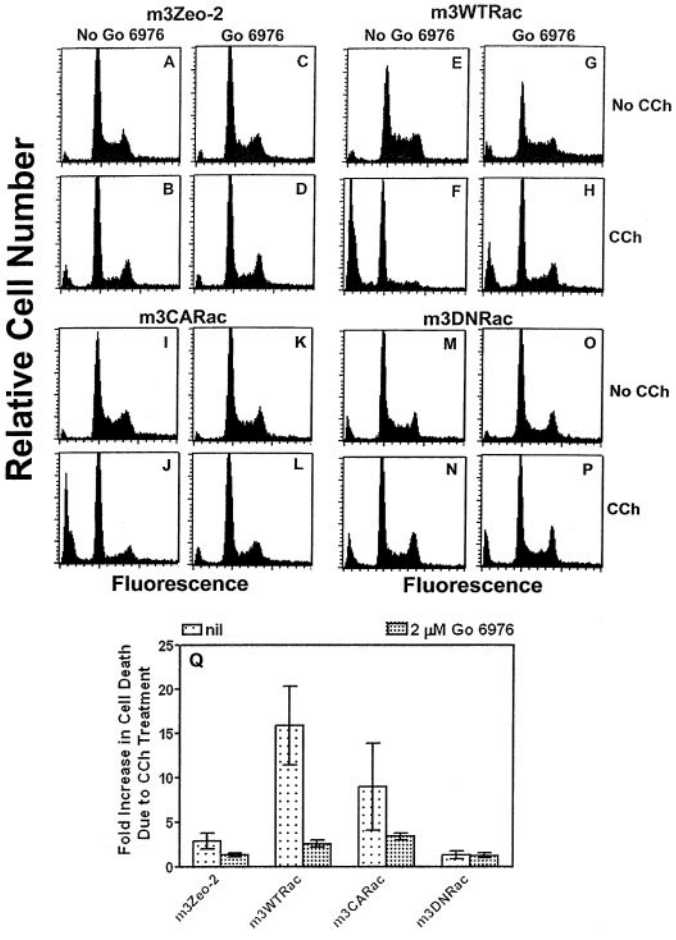


**Fig. 10.** Incubation of cells with Go 6976 rescues the cells expressing wild-type or constitutively active Rac1 proteins from $M_3$ mAChR-induced cell death. Control m3Zeo-2 cells (A-D) or cells expressing wild-type (E-H), constitutively active $Rac^{Val12}$ (I-L), or dominant-negative $Rac^{Asn17}$ (M-P) were subjected to FACS analysis after being cultured for 24 h in the absence (A, B, E, F, I, J, M, and N) or presence (C, D, G, H, K, L, O, and P) of 2 $\mu$M Go 6976 and in the absence (A, C, E, G, I, K, M, and O) or presence of 10 $\mu$M CCh (B, D, F, H, J, L, M, and P). Results shown are representative of three independent experiments. The fold increase in cell death (Q) was determined by defining the percentage of cells in the sub-$G_o/G_1$ population compared with that in the $G_1$, S, and $G_2$/M phases of the cell cycle. Results shown are the means ± 1 S.E.M. calculated from three independent experiments.

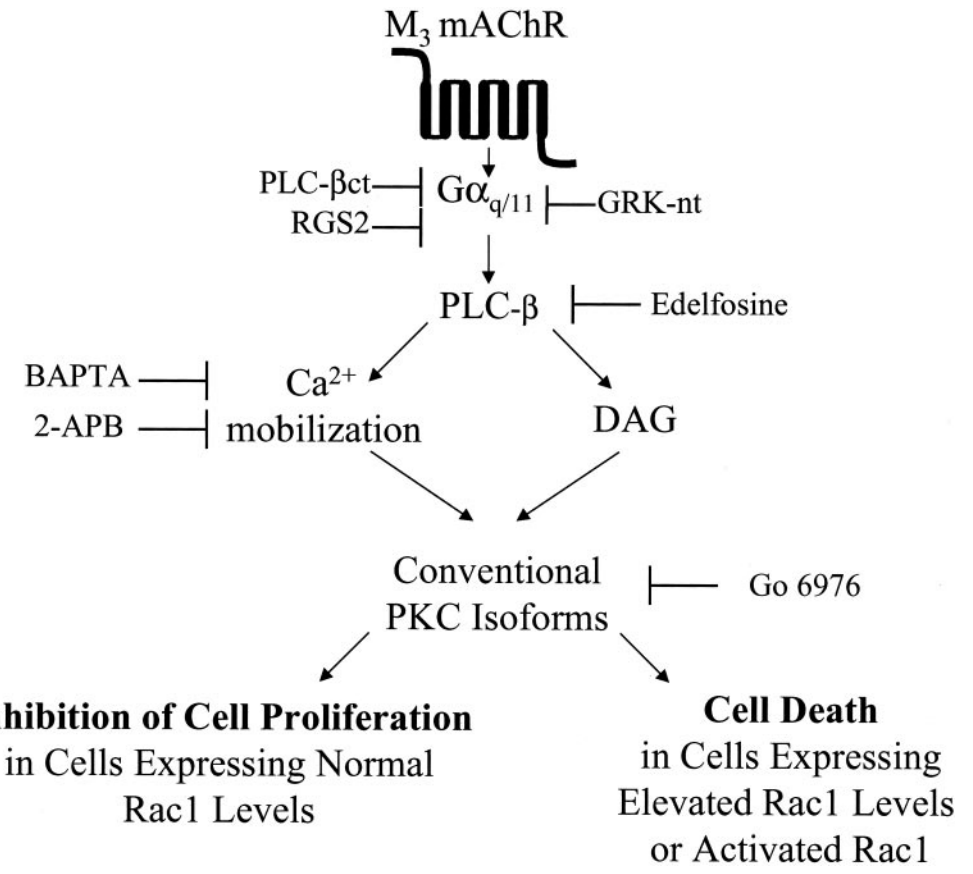


**Fig. 11.** A model depicting the involvement of Rac1, PLC, and PKC in the $M_3$ mAChR-induced inhibition of proliferation. Inhibition of $G\alpha_{q/11}$ signaling by PLC-$\beta$ct, GRK-nt, or RGS2 completely abrogates the $M_3$ mAChR antiproliferative effects. Activation of the $M_3$ mAChR in cells expressing normal levels of Rac1 leads to inhibition of proliferation. In the presence of high levels of Rac1 activity, activation of the $M_3$ mAChR triggers cell death. $Ca^{2+}$ mobilization, PLC, and PKC may contribute to both the $M_3$ mAChR-mediated inhibition of proliferation and induction of cell death, because inhibition of these signaling components by the indicated pharmacological agents diminishes these $M_3$ mAChR-mediated responses.

inhibitor edelfosine (Powis et al., 1992) diminishes the ability of the $M_3$ mAChR to inhibit proliferation and partially rescues the m3WTRac and m3CARac cells from mAChR-induced cell death. Treatment with edelfosine slows the progression through $G_2$/M in numerous cell types (Shafer and Williams, 2003) including the m3Zeo-2, m3WTRac, and m3CARac cells studied here. The accumulation of edelfosine-treated cells in $G_2$/M phases may prevent them from entering another phase of the cell cycle in which they are more susceptible to the $M_3$ mAChR-antiproliferative effects.

Interestingly, we observed a greater proportion of m3DNRac cells in the $G_2$/M phase compared with the other cell lines (Fig. 8M). This agrees with previous reports that Rac1 regulates progression through $G_2$/M phases (Olson et al., 1995). The m3DNRac cells are the least responsive to the $M_3$ mAChR-mediated inhibition of proliferation. It is intriguing to speculate that the slowed progression of these cells through $G_2$/M phases makes them less vulnerable to mAChR-induced inhibition of proliferation.

Inhibition of conventional isoforms of PKC with Go 6976 diminishes the $M_3$ mAChR-induced inhibition of proliferation. This finding indicates that PKC activation contributes to the $M_3$ mAChR-mediated inhibition of proliferation. PKC activity may be important for mAChR-induced inhibition of proliferation because PKC may have a role in activating Rac1. Interestingly, PKC is known to regulate the activity of small GTPases by regulating their association with RhoGDI (Mehta et al., 2001). Other investigators have shown that the PKC-mediated phosphorylation of RhoGDI causes the release of Rho from RhoGDI, thereby activating the GTPase (Mehta et al., 2001). By analogy, the PKC-mediated phosphorylation of RhoGDI may release Rac1 from RhoGDI; this is a plausible mechanism because Rac1 associates with RhoGDI in the CHO cells we used (Ruiz-Velasco et al., 2002). Inhibition of conventional isoforms of PKC by Go 6976 may prevent the release of Rac1 from RhoGDI. This event may prevent activation of Rac1 and thus diminish the mAChR-induced inhibition of proliferation.

Our findings indicate that the presence of elevated Rac1 activity alters the response of these cells to $M_3$ mAChR stimulation. The stably transfected cells used in this study tolerate the expression of excess wild-type or mutant Rac1 proteins without affecting normal cell-cycle progression or increasing cell death. $M_3$ mAChR activation inhibits the proliferation of cells expressing normal Rac1 levels but promotes the death of cells expressing high Rac1 levels or activity. These findings suggest that the apparent contradictory responses of different cell systems to $M_3$ mAChR activation involve differential expression of signaling molecules such as Rac1.

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