M₃ Muscarinic Acetylcholine Receptors Regulate Cytoplasmic Myosin by a Process Involving RhoA and Requiring Conventional Protein Kinase C Isoforms*

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Derek Strassheim‡, Lisa G. May§, Kimberly A. Varker¶, Henry L. Puhl§, Scott H. Phelps‡, Rebecca A. Porter‡, Robert S. Aronstam§, John D. Noti, and Carol L. Williams‡**

From the Laboratories of Molecular Pharmacology, §Neurobiology, and ¶Molecular Biology, Guthrie Research Institute, and **Department of Surgery, Guthrie Clinic, Sayre, Pennsylvania 18840

Although muscarinic acetylcholine receptors (mAChR) regulate the activity of smooth muscle myosin, the effects of mAChR activation on cytoplasmic myosin have not been characterized. We found that activation of transfected human M₃ mAChR induces the phosphorylation of myosin light chains (MLC) and the formation of myosin-containing stress fibers in Chinese hamster ovary (CHO-m3) cells. Direct activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate (PMA) also induces myosin light chain phosphorylation and myosin reorganization in CHO-m3 cells. Conventional (α), novel (δ), and atypical (ε) PKC isoforms are activated by mAChR stimulation or PMA treatment in CHO-m3 cells, as indicated by PKC translocation or degradation. mAChR-mediated myosin reorganization is abolished by inhibiting conventional PKC isoforms with Go6976 (IC₅₀ = 0.4 μM), calphostin C (IC₅₀ = 2.4 μM), or chelerythrine (IC₅₀ = 8.0 μM). Stable expression of dominant negative RhoA²⁰¹₉ diminishes, but does not abolish, mAChR-mediated myosin reorganization in the CHO-m3 cells. Similarly, mAChR-mediated myosin reorganization is diminished, but not abolished, in CHO-m3 cells which are multi-nucleate due to inactivation of Rho with C3 exoenzyme. Expression of dominant negative RhoA²⁰¹₉ or inactivation of RhoA with C3 exoenzyme does not affect PMA-induced myosin reorganization. These findings indicate that the PKC-mediated pathway of myosin reorganization (induced either by M₃ mAChR activation or PMA treatment) can continue to operate even when RhoA activity is diminished in CHO-m3 cells. Conventional PKC isoforms and RhoA may participate in separate but parallel pathways induced by M₃ mAChR activation to regulate cytoplasmic myosin. Changes in cytoplasmic myosin elicited by M₃ mAChR activation may contribute to the unique ability of these receptors to regulate cell morphology, adhesion, and proliferation.

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** To whom correspondence should be addressed: Molecular Pharmacology Laboratory, Guthrie Research Institute, Sayre, PA 18840. Tel.: 570-882-4650; Fax: 570-882-5151; E-mail: cwilliam@inet.guthrie.org.

† The abbreviations used are: mAChR, muscarinic acetylcholine receptor; BSA, bovine serum albumin; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CHO, Chinese hamster ovary; FCS, fetal calf serum; HA, hemagglutinin; LPA, lysophosphatidic acid; MLC, myosin light chain; MLCK, myosin light chain kinase; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

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(reacted in Refs. 9–12). We found that MLCK antagonists inhibit mACHr-mediated myosin reorganization but only at antagonist concentrations that may affect PKC. Stable expression of dominant negative RhoA\textsubscript{Asn-19} or inactivation of Rho with C3 exoenzyme lessens mACHr-mediated myosin reorganization but does not abolish it.

This study demonstrates that M<sub>4</sub> mACHr activation significantly affects myosin organization in non-muscle cells. Our findings indicate that M<sub>4</sub> mACHr activation induces cytoplasmic myosin reorganization by both PKC- and Rho-dependent mechanisms. These receptor-mediated changes in cytoplasmic myosin may contribute to the unique ability of M<sub>4</sub> mACHr to regulate the adhesion (18–20) and morphology (21, 22) of non-muscle cells.

**Experimental Procedures**

**Reagents**—The SA-2 human IgM monoclonal autoantibody that reacts specifically with myosin heavy chains was obtained from cultures of a thymic lymphocyte clone transformed with Epstein-Barr virus, as described previously (23, 24). Mouse monoclonal antibody to hemagglutinin (HA) was produced from Babco (Berkeley, CA). Mouse monoclonal antibodies to PKC isoforms were purchased from Transduction Laboratories (Lexington, KY). Fluorescein-labeled goat anti-human IgM was obtained from Fisher. Horseradish peroxidase-labeled antibody to mouse immunoglobulins and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech. Reagents for LipofectAMINE-mediated transfection were purchased from Life Technologies, Inc. Carbachol and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Other reagents were obtained from Mediatech (Herndon, VA); fetal calf serum (FCS) was obtained from Biofluids (Rockville, MD); and zeocin was bought from Invitrogen (Carlsbad, CA). Other reagents were obtained from Sigma or from sources listed in the text.

**Cell Lines**—CHO-K1 sublines stably transfected with the M<sub>2</sub>, M<sub>3</sub>, or M<sub>4</sub> subtypes of human mACHr are referred to as CHO-m<sub>1</sub>, CHO-m<sub>2</sub>, and CHO-m<sub>3</sub>, respectively. These sublines, as well as untransfected CHO-K1 cells, were generously provided by Dr. Mark Branum (University of Vermont). Cells were cultured in complete medium consisting of Ham's F-12 medium, heat-inactivated FCS (5%), glutamine (0.3 mg/ml), penicillin (20 units/ml), and streptomycin sulfate (20 \mu g/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 100% humidity.

Establishment and Characterization of CHO-m3 Cell Lines Stably Expressing Wild-type or Mutant RhoA—The pEF-BOS-HA-RhoA and pEF-BOS-HA-RhoA\textsuperscript{Val-14} plasmids coding for HA-tagged dominant negative RhoA Asn-19 was successfully mutated to asparagine, with no significant loss in expression. Stable transfected clonal lines were subjected to DNA sequencing to ensure the successful mutation but does not abolish it.

Immunofluorescent Localization of PKC Isoforms—Cells cultured on glass coverslips in complete medium were incubated in the absence or presence of drugs for the indicated times and fixed for 15 min in 3.7% formaldehyde diluted in PBS containing 0.2% Triton X-100. After nonspecific antibody-binding sites were blocked as described above, the cells were incubated (1 h, 24 °C) with mouse monoclonal antibodies to PKC isoforms diluted in PBS, 1% BSA. The cells were washed and incubated (1 h, 24 °C) with fluorescein-labeled anti-mouse IgG antibody diluted in PBS, 1% BSA. After washing, the cells were mounted and examined by fluorescence microscopy as described above.

Measurement of Myosin-containing Stress Fiber Formation—Stress fibers were fluorescently labeled by propidium iodide (5 \mu g/ml) following the protocol of \textsuperscript{[5]}. The SA-2 human monoclonal autoantibody to the myosin heavy chain was used as described previously to determine the intracellular distribution of myosin (24). Cells were cultured on glass coverslips in complete medium and then fixed with ice-cold acetone and then incubated with the SA-2 antibody, washed in PBS, and incubated (1 h, 24 °C) with fluorescein-labeled anti-human IgM antibody diluted 1:100 in PBS, 1% BSA. In some assays, nuclei were fluorescently labeled by propidium iodide (5 \mu g/ml) that was present during the incubation with the secondary antibody. After washing, the cells were mounted in PBS containing 90% glycerol and 0.1% p-phenylene diamine and examined by fluorescence microscopy using a Nikon Optiphot fluorescence microscope.

The samples were scored for the presence of stress fibers by two investigators without knowledge of the identity nor treatment of the cell lines being examined. The investigators independently assigned scores to the cells in 10 different microscopic fields in each sample, using the following values based on the presence of stress fibers in the cells: 0 = no stress fibers, 1 = few stress fibers, 2 = well defined stress fibers, and 3 = abundant, strongly defined stress fibers. The calculated mean of the scores was used as a measurement of myosin-containing stress fiber formation.

**Measurement of MLC Phosphorylation**—The phosphorylation of MLC in Triton X-100-insoluble lysates was determined as described previously (28). Briefly, CHO-m<sub>3</sub> cells were incubated for 16 h with 5 \mu Ci/ml inorganic \textsuperscript{32}P in phosphate-free Dulbecco's modified Eagle's medium containing 1% heat-inactivated FCS. After incubating with drugs for the appropriate times, the cells were lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 200 \mu M phenylmethylsulfonyl fluoride, 5 \mu g/ml leupeptin, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.2 mM sodium PP<sub>i</sub>, and 10 mM \textbeta-glycerophosphate). The resulting insoluble cytoskeletal pellets were washed twice with Triton X-100 lysis buffer and dissolved by boiling in sample buffer for 30 min. The samples were subjected to ECL Western blotting using a mouse monoclonal antibody to the 20-kDa myosin light chain (Sigma), followed by autoradiography. Densitometry was performed using a Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Preparation and Use of Recombinant C3 Exoenzyme—Recombinant C3 exoenzyme was purified from bacteria transformed with the pGEX<sub>2T</sub>-C3 exoenzyme expression vector, as described previously (29). CHO-m<sub>3</sub> cells were electroporated in the presence of recombinant C3 exoenzyme (15 \mu g/ml) by subjecting them to three electric pulses (capacitance, 0.25 microfarads; voltage, 0.5 kV; duration, 100 \mu s).
CHO-m2 cells transfected with the human M2 mAChR subtype myosin organization in untransfected CHO cells nor in a variety of cell types (4–8,13–17). MLC phosphorylation also occurs in CHO-m3 cells incubated with carbachol or PMA (Fig. 3). Densitometry analysis indicates that MLC phosphorylation is increased by 319 ± 80% in carbachol-treated cells and 199 ± 57% in PMA-treated cells, compared with untreated cells (p < 0.05, n = 3). MLC phosphorylation is more effectively induced by carbachol than by PMA in CHO-m3 cells (Fig. 3), consistent with greater stress fiber formation induced by carbachol than by PMA (Fig. 2).

Conventional PKC Isoforms Are Activated by M₃ mAChR Stimulation and Are Required for mAChR-mediated Myosin Reorganization—CHO-m3 cells were immunofluorescently labeled with the SA-2 myosin antibody. The presence of myosin-containing stress fibers in the cells was scored by two independent investigators without knowledge of the identity nor treatment of the cells. Results are the means ± 1 S.E. calculated from three independent experiments.

**RESULTS**

**Myosin Reorganizes into Stress Fibers upon Activation of M₃ or M₂ mAChR**—We tested the ability of transfected M₃ mAChR to regulate myosin organization in CHO cells. Treatment with carbachol, which is an agonist for all mAChR subtypes, induces myosin reorganization in the CHO-m3 subline transfected with human M₃ mAChR (Fig. 1). Myosin is diffusely distributed in untreated CHO-m3 cells (Fig. 1, panel A) and becomes peripherally localized within 15 min of carbachol exposure (Fig. 1, panel B). The close proximity of the cells to one another after treatment with carbachol for 15 min reflects cell-cell adhesion induced by mAChR activation (20). Myosin-containing stress fibers appear within 30 min of mAChR activation and remain prominent for several hours in the presence of carbachol (Fig. 1, panel C). The carbachol-treated cells initially exhibit increased cell spreading but become elongated with less detectable myosin-containing stress fibers after prolonged exposure to the agonist (Fig. 1, panel D). These findings indicate that M₃ mAChR activation profoundly affects myosin organization.

Carbachol also induces myosin reorganization in CHO-m1 cells transfected with human M₁ mAChR but does not alter myosin organization in untransfected CHO cells nor in CHO-m2 cells transfected with the human M₂ mAChR subtype (Fig. 2). These results indicate that only mAChR subtypes which activate PKC induce myosin reorganization. Direct activation of PKC with PMA induces myosin reorganization in all CHO sublines (Fig. 2), indicating that PKC activation reorganizes myosin. Stimulation of M₁ or M₂ mAChR induces the formation of myosin-containing stress fibers to a greater extent than does treatment with PMA (Fig. 2).

Phosphorylation of MLC alters myosin organization in a variety of cell types (4–8,13–17). MLC phosphorylation also occurs in CHO-m3 cells incubated with carbachol or PMA (Fig. 3). Densitometry analysis indicates that MLC phosphorylation is increased by 319 ± 80% in carbachol-treated cells and 199 ± 57% in PMA-treated cells, compared with untreated cells (p < 0.05, n = 3). MLC phosphorylation is more effectively induced by carbachol than by PMA in CHO-m3 cells (Fig. 3), consistent with greater stress fiber formation induced by carbachol than by PMA (Fig. 2).

**FIG. 1.** Activation of M₃ mAChR induces myosin reorganization in CHO-m3 cells. CHO-m3 cells were incubated in the absence (A) or presence of 10 μM carbachol for 15 min (B), 90 min (C), or 24 h (D) and were immunofluorescently labeled with the SA-2 antibody to myosin. Bar represents 15 μm.

**FIG. 2.** Myosin reorganization is induced by carbachol in CHO-m1 and CHO-m3 cells and by PMA in all CHO sublines. CHO-m1 and CHO-m3 cells were incubated with 10 μM carbachol (B, E, H, and K), or 10 nM PMA (C, F, I, and L) for 90 min and immunofluorescently labeled with the SA-2 myosin antibody. Bar represents 15 μm. b, the indicated CHO sublines were incubated with 10 μM carbachol, 10 nM PMA, or no drug for 90 min and immunofluorescently labeled with the SA-2 myosin antibody. The presence of myosin-containing stress fibers in the cells was scored by two independent investigators without knowledge of the identity nor treatment of the cells. Results are the means ± 1 S.E. calculated from three independent experiments.
stained with antibodies to different PKC isoforms to determine whether mAChR agonists or PMA induce PKC translocation, which indicates PKC activation (30). Immunofluorescent staining of PKC-β, -γ, -ε, and -ι in CHO-m3 cells was undetectable, and immunofluorescent staining of PKC-δ and -μ produced a diffuse cytosolic pattern that was not detectably altered by PMA or carbachol treatment (data not shown). In contrast, PKC-α is diffusely distributed in the cytosol of untreated CHO-m3 cells (Fig. 4a, panel A), and localizes to cell–cell junctions within 15 min of exposure to carbachol (Fig. 4a, panel B). PKC-α remains at cell–cell junctions for over 24 h in the continuous presence of carbachol (Fig. 4a, panel D). Treatment with PMA increases PKC-α at cell–cell junctions and at regions of membrane ruffling (Fig. 4a, panel E). However, PKC-α is undetectable at cell–cell junctions or membrane ruffles after 24 h of PMA treatment (Fig. 4a, panel F).

Activation of PKC results in degradation of the enzyme; this effect is most evident when PKC is activated by phorbol esters (31, 32), but it can also occur when PKC is activated by Ca²⁺ and diacylglycerol (33). Degradation of PKC isoforms was measured to characterize further which isoforms are activated by PMA or M₃ mAChR stimulation (Fig. 4, b and c). PKC-α, -δ, -ε, and -μ were detected by Western blotting of CHO-m3 cell lysates. Levels of PKC-α are significantly diminished by PMA but only minimally affected by carbachol (Fig. 4, b and c). This finding is consistent with the sustained presence of translocated PKC-α at cell membranes in carbachol-treated CHO-m3 cells (Fig. 4a, panel D) and the loss of translocated PKC-α in PMA-treated cells (Fig. 4a, panel F). Prolonged exposure to PMA or carbachol significantly diminishes the levels of PKC-δ and -ε but does not diminish PKC-μ (Fig. 4, b and c). These results indicate that conventional (α), novel (δ), and atypical (ε) PKC isoforms are activated by PMA or carbachol in CHO-m3 cells.

The effects of specific PKC antagonists on myosin reorganization were investigated to determine the participation of different PKC isoforms in carbachol-induced cytoskeletal reorganization (Fig. 5). Previous in vitro studies using purified PKC isoforms indicate that conventional and novel PKC isoforms are inhibited by calphostin C (IC₅₀ = 0.05 μM) and chelerythrine (IC₅₀ = 0.66 μM) (34, 35), whereas only the conventional PKC isoforms are inhibited by Go6976 (IC₅₀ = 0.006 μM) (36). These PKC antagonists also inhibit other purified kinases such as PKA when the antagonist concentrations exceed 50–170 μM (34–36). Carbachol-mediated stress fiber formation in CHO-m3 cells is inhibited by calphostin C (IC₅₀ = 2.4 ± 0.3 μM), chelerythrine (IC₅₀ = 8.0 ± 0.5 μM), and Go6976 (IC₅₀ = 0.4 ± 0.02 μM) (Fig. 5). Calphostin C and chelerythrine inhibit both cell

Fig. 3. Phosphorylation of MLC is enhanced by treatment with carbachol or PMA. CHO-m3 cells labeled with inorganic ³²P were incubated with 10 μM carbachol, 10 nM PMA, or no drug for 60 min and lysed in 1% Triton X-100. The phosphorylated 20-kDa MLC in the Triton X-100-insoluble pellet from cells treated with no drug (lane 1), carbachol (lane 2), or PMA (lane 3) was identified by anti-MLC antibody in Western blots (a) and by autoradiography (b). The Western blot has smaller lane widths than the autoradiograph because the channels in which the antibodies were applied in the Western blot were a smaller width than the wells in which proteins were applied to the polyacrylamide gel. Results shown are representative of three independent experiments which produced similar results.

Fig. 4. Translocation and degradation of PKC isoforms occurs in CHO-m3 cells treated with carbachol or PMA. a, CHO-m3 cells were untreated (A) or incubated with 10 μM carbachol for 15 min (B), 45 min (C), or 24 h (D) or incubated with 10 nM PMA for 15 min (E) or 24 h (F). All cells were immunofluorescently labeled with an antibody to PKC-α. Bar represents 15 μm. b, CHO-m3 cells were untreated, or incubated with 1 μM carbachol or 1 μM PMA for 24 h and lysed. The lysates were probed by ECL Western blotting using PKC isoform-specific antibodies. Densitometry of the ECL Western blots was performed to determine the percent of PKC isoform levels remaining in the drug-treated cells compared with untreated control cells. Results are the means ± S.E. from three independent experiments. c, representative ECL Western blots from the experiments described in b.
were immunofluorescently labeled with the SA-2 antibody to myosin. The presence of myosin-containing stress fibers in CHO-m3 cells.

Before being exposed to carbachol, the cells were preincubated for 30 min with 4 \(\mu\)M calphostin C (C), 10 \(\mu\)M chelerythrine (D), 2 \(\mu\)M Go6976 (E), 2 \(\mu\)M KT5926 (F), 40 \(\mu\)M ML-7 (G), or 100 \(\mu\)M ML-7 (H). All cells were immunofluorescently labeled with the SA-2 antibody to myosin.

spreading and stress fiber formation (Fig. 5a, panels C and D), whereas Go6976 inhibits stress fiber formation but not cell spreading (Fig. 5a, panel E). The ability of Go6976 to inhibit mACHR-mediated stress fiber formation indicates that conventional PKC isoforms are required for mACHR-mediated stress fiber formation.

Intracellular Ca\(^{2+}\) mobilized by M\(_3\) mACHR stimulation may enhance activation of conventional PKC isoforms, which differ from other PKC isoforms by being sensitive to Ca\(^{2+}\) (reviewed in Ref. 30). Other Ca\(^{2+}\)-dependent kinases, such as Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) or MLCK, may also participate in myosin reorganization. The role of CaMKII in mACHR-mediated stress fiber formation was tested using the antagonists KN-62 and KT5926. KN-62 specifically inhibits the in vitro activity of CaMKII (IC\(_{50}\) = 0.90 \(\mu\)M) (37), whereas KT5926 inhibits the in vitro activities of several kinases, including CaMKII (IC\(_{50}\) = 0.004 \(\mu\)M), MLCK (IC\(_{50}\) = 0.018 \(\mu\)M), and PKC (IC\(_{50}\) = 0.72 \(\mu\)M) (38, 39). Myosin reorganization induced by M\(_3\) mACHR activation is not affected by KN-62 at concentrations up to 20 \(\mu\)M, but it is inhibited by KT5926 (IC\(_{50}\) = 0.6 \(\pm\) 0.04 \(\mu\)M) (Fig. 5). Interestingly, KT5926 induces the same morphological effects as the PKC antagonist Go6976; the cells are well spread but lack detectable stress fibers after M\(_3\) mACHR activation (Fig. 5a, panel F).

The effects of the MLCK antagonists ML-7 and ML-9 on mACHR-mediated stress fiber formation were also determined (Fig. 5). ML-7 and ML-9 were previously found to inhibit MLCK activity (IC\(_{50}\) = 0.3 and 3.8 \(\mu\)M, respectively) as well as PKC activity (IC\(_{50}\) = 42 and 54 \(\mu\)M, respectively) (40–42). We found that mACHR-mediated stress fiber formation is inhibited by ML-7 (IC\(_{50}\) = 75 \(\pm\) 6.9 \(\mu\)M) and by ML-9 (IC\(_{50}\) = 82 \(\pm\) 5.8 \(\mu\)M) (Fig. 5). Cells treated with 40 \(\mu\)M ML-7 continue to exhibit carbachol-induced stress fibers even though cell spreading is diminished (Fig. 5a, panel G). Carbachol-induced stress fibers are lost only when the cells are treated with high enough concentrations of ML-7 to cause the cells to detach from the substratum (Fig. 5a, panel H). Similar morphological effects were induced by comparable concentrations of the MLCK antagonist ML-9 (data not shown).

Inactivation of RhoA Diminishes but Does Not Abolish Stress Fiber Formation Induced by M\(_3\) mACHR Activation—The involvement of RhoA in myosin organization was investigated using CHO-m3 cells stably transfected with HA-tagged wild-type or mutant RhoA. Two independent clonal CHO-m3 cell lines expressing HA-RhoA were generated and named m3WTRho-1 and m3WTRho-11. Two independent clonal CHO-m3 cell lines expressing constitutively active HA-RhoA-Val\(^{14}\) were named m3CARho-1 and m3CARho-4, and three independent clonal lines expressing dominant negative HA-RhoA-Val\(^{14}\) were named m3DNRho-2, m3DNRho-4, and m3DNRho-6. These cell lines express similar levels of HA-tagged wild-type or mutant RhoA (Fig. 6a). Two independent clonal CHO-m3 cell lines stably transfected with only the pZeoSV2 plasmid, named m3Zeo-1 and m3Zeo-2, do not express HA (Fig. 6a). Similar levels of M\(_3\) mACHR are expressed by these cell lines and parental CHO-m3 cells, as indicated by spreading (Fig. 5a, panels C and D).

Bar represents 15 \(\mu\)M. h, CHO-m3 cells were preincubated for 30 min with no antagonist (control cells) or with antagonists selective for PKC (A), CaMKII (B), or MLCK (C). The cells were then incubated with 10 \(\mu\)M carbachol for 90 min and immunofluorescently labeled with the SA-2 antibody to myosin. The presence of myosin-containing stress fibers in the cells was scored by two independent investigators without knowledge of the cell treatments. These scores were used to calculate the percent of carbachol-induced stress fiber formation. The percent of carbachol-induced stress fiber formation in cells exposed to antagonist, compared with cells that were not exposed to antagonists, is shown (Fig. 5a, panel E). The results are the means \(\pm\) 1 S.E. calculated from three to six independent experiments.
[\textsuperscript{3}H]-methylscopolamine binding (data not shown). Proximal signal transduction by M\textsubscript{3} mAChR is also similar in these cell lines, as indicated by carbachol-induced Ca\textsuperscript{2+} mobilization (Fig. 6b).

Expression of constitutively active RhoA\textsuperscript{Val-14} induces the formation of myosin-containing stress fibers (Fig. 7a, panel D, and b). Carbachol increases stress fiber formation in all the sublines (Fig. 7). However, carbachol-induced stress fiber formation is diminished in cells expressing dominant negative RhoA\textsuperscript{Asn-19}, compared with the other cell lines (Fig. 7a, panel G, and b).

Since expression of dominant negative RhoA\textsuperscript{Asn-19} diminishes carbachol-induced stress fiber formation (Fig. 7), we investigated the effects of expressing carboxyl terminal RhoA\textsuperscript{C-terminus} on PMA-induced stress fiber formation (Fig. 8). Interestingly, PMA treatment induces similar increases in stress fiber formation in cells expressing dominant negative RhoA\textsuperscript{Asn-19} and in the control cell lines (Fig. 8). These results indicate that expressing dominant negative RhoA\textsuperscript{Asn-19} does not alter PMA-induced stress fiber formation.

Some reports indicate that ADP-ribosylation of Rho by C3 exoenzyme more effectively produces an altered phenotype than expression of dominant negative RhoA\textsuperscript{Asn-19} (43, 44). CHO-m3 cells were electroporated with C3 exoenzyme to determine the effects of inactivating Rho by ADP-ribosylation. The ability of C3 exoenzyme to ribosylate Rho in CHO-m3 cells was tested in \textsuperscript{32}P]ADP-ribosylation assays (Fig. 6c). Ribosylation by C3 exoenzyme slows the migration of RhoA in Western blots (lanes 1 and 3) or presence (lane 2) of C3 exoenzyme. The lysates were incubated in vitro with [\textsuperscript{3}P]NAD in the absence (lane 3) or presence (lane 1 and 2) of C3 exoenzyme. The samples were probed with RhoA antibody (Western blot) and subjected to autoradiography (Autoradiograph). The striped and open rectangles indicate the positions of RhoA that migrated slower and faster, respectively.

\textsuperscript{[3]H]N-methylscopolamine binding (data not shown). Proximal signal transduction by M\textsubscript{2} mAChR is also similar in these cell lines, as indicated by carbachol-induced Ca\textsuperscript{2+} mobilization (Fig. 6b).

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Inactivation of Rho with C3 exoenzyme inhibits CHO-m3 cytokinesis, resulting in multinucleate cells (Fig. 9, panel B). Although these cells are abnormally spread and elongated, they exhibit a diffuse cytosolic distribution of myosin similar to cells electroporated in the absence of C3 exoenzyme (Fig. 9). Activation of M\textsubscript{2} mAChR induces the formation of stress fibers in cells electroporated with C3 exoenzyme (Fig. 9, panel D), although stress fiber formation is somewhat diminished compared with untreated cells. These results indicate that myosin
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DISCUSSION

This study demonstrates that M₁ or M₃ mACHR activation profoundly alters myosin organization in CHO cells. Conventional PKC isoforms and Rho proteins participate in the mACHR-mediated formation of myosin stress fibers, as depicted in the model shown in Fig. 10. Similar pathways of mACHR-mediated myosin reorganization occur in CHO cells and smooth muscle cells, although some differences exist. Comparing these pathways provides insight into the mACHR-mediated mechanisms controlling myosin organization.

Role of PKC in mACHR-mediated Myosin Reorganization—PKC participates in mACHR-mediated myosin reorganization in CHO cells. This conclusion is supported by our finding that M₃ mACHR stimulation or PMA treatment activates several PKC isoforms and induces myosin stress fiber formation in these cells. Our finding that myosin reorganization is induced by the M₃ but not the M₁ mACHR subtype also supports this conclusion, since the M₁ but not the M₃ mACHR subtype activates PKC (2, 3).

The effects of the PKC antagonists Go6976, calphostin C, and chelerythrine provide compelling evidence that conventional PKC isoforms are required for myosin reorganization. Both mACHR-mediated stress fiber formation and cell spreading are inhibited by calphostin C or chelerythrine, which antagonize conventional and novel PKC isoforms (34, 35) In contrast, only mACHR-mediated myosin reorganization is inhibited by Go6976, which specifically antagonizes conventional PKC isoforms (36). It is generally believed that PKC activation is required for cell spreading, and other signals are required for cytoskeletal reorganization (45–51). Our findings suggest that while the novel PKC isoforms are needed for cell spreading, the conventional PKC isoforms are needed for myosin reorganization.

The concentrations of Go6976, calphostin C, or chelerythrine which inhibit myosin reorganization in CHO cells are approximately 10–70-fold higher than those that inhibit PKC in vitro (34–36). Higher concentrations of these drugs may be needed in vivo for the antagonists to cross the cell membrane and gain access to intracellular PKC, in contrast to in vitro studies in which the antagonists interact directly with purified PKC. Despite this requirement for higher drug concentrations in vivo, these antagonists still inhibit myosin reorganization more effectively in vivo than they inhibit other kinases such as PKA in vitro. This finding indicates that these antagonists inhibit myosin reorganization by inactivating PKC.

The ability of PKC to regulate myosin organization in CHO cells is consistent with the effects of PKC activation on smooth muscle myosin. Activation of PKC with phorbol esters enhances MLC phosphorylation in smooth muscle cells and induces sustained contraction of different smooth muscle tissues (reviewed in Ref. 10). Phosphorylation of MLC increases actin-myosin interactions by inducing the formation of bipolar myo-
sin filaments and exposing actin-binding sites on myosin (reviewed in Refs. 9–12). PKC can directly phosphorylate MLC (52, 53) or enhance MLC phosphorylation by inhibiting MLC phosphatase (54). It is believed that smooth muscle contraction induced by phorbol esters involves the PKC-dependent inhibition of MLC phosphatase, rather than direct phosphorylation of MLC by PKC (reviewed in Refs. 9–11). PKC activation may similarly enhance MLC phosphorylation in CHO cells by inhibiting MLC dephosphorylation, as depicted in Fig. 10.

Although PKC is required for carbachol-induced myosin reorganization in CHO-m3 cells, the role of PKC in the mAChR-mediated regulation of smooth muscle myosin is less clear. PKC antagonists inhibit carbachol-induced contractions in some types of smooth muscle (55) but not in others (56). These variable responses may be due to the expression of different mAChR subtypes or PKC isoforms by different types of smooth muscle.

It was previously shown that PKC activation induces or enhances the formation of actin-containing stress fibers in some types of non-muscle cells (45, 47, 57) but not in others (58). These results indicate that PKC activation has cell type-specific effects on stress fiber formation. These specific effects may be due to altered PKC isoform expression or dissimilarities in PKC-mediated signaling pathways among different cell types.

Role of RhoA in mAChR-mediated Myosin Reorganization—Increased stress fiber formation in cells expressing constitutively active RhoAVal-14 indicates that active RhoA contributes to myosin reorganization in CHO-m3 cells. However, active RhoA is not essential for mAChR-mediated myosin reorganization in CHO-m3 cells. This conclusion is supported by our
finding that carbachol-induced stress fiber formation is diminished but not abolished in CHO-m3 cells expressing dominant negative RhoAAsn-19 or treated with C3 exoenzyme. Expression of dominant negative RhoAAsn-19 may specifically inhibit RhoA activity because dominant negative RhoAAsn-19 competitively interacts with RhoA regulatory proteins. In contrast, C3 exoenzyme can ADP-ribosylate several forms of Rho, including RhoA and RhoB (59), resulting in potentially greater Rho inactivation than that produced by expressing dominant negative RhoAAsn-19. Consistent with these possibilities, we found that the morphology of CHO-m3 cells is altered more drastically by C3 exoenzyme than by expression of dominant negative RhoAAsn-19.

Treatment with C3 exoenzyme causes CHO-m3 cells to become multi-nucleate, which is an indication of Rho inactivation (60–62). If Rho is inactive in dividing cells, the actomyosin contractile ring at the cleavage furrow does not function properly and cytokinesis is inhibited, resulting in multi-nucleate cells (61, 62). The ability of carbachol to induce stress fiber formation in multi-nucleate, C3 exoenzyme-treated CHO-m3 cells provides strong evidence that mAChR-mediated stress fiber formation still occurs even when Rho is inactive.

Although Rho is apparently not essential for stress fiber formation induced by mAChR activation, Rho may be required for stress fiber formation induced by other agonists. Rho must be active for bombesin or lysophosphatidic acid (LPA) to induce stress fiber formation in serum-starved Swiss 3T3 cells (58, 63). Bombesin- or LPA-dependent stress fiber formation is significantly diminished by 27 μM genistein and is completely abolished by 110 μM genistein, indicating that a tyrosine kinase is required for stress fiber formation induced by these agonists (58). In contrast, we found that genistein concentrations up to 180 μM do not alter mAChR-mediated stress fiber formation (data not shown). These findings indicate that different Rho-mediated signaling pathways leading to stress fiber formation are induced by bombesin or LPA in serum-starved Swiss 3T3 cells and by mAChR activation in exponentially proliferating CHO-m3 cells.

Rho inactivation diminishes the agonist-induced contraction of smooth muscle (17, 64–68). Carbachol-induced contraction of tracheal (66), ileal (68), and longitudinal intestinal (17, 67) smooth muscle strips is diminished by treatment with C3 exoenzyme. Phosphorylation of MLC induced by carbachol (17) or other agonists (reviewed in Ref. 10) is also diminished by inactivating Rho in smooth muscle. These and other findings support the model that Rho participates in contraction by enhancing MLC phosphorylation (reviewed in Refs. 10–12). Active Rho proteins can enhance MLC phosphorylation by inhibiting MLC phosphatase (69) or by activating Rho kinase, which directly phosphorylates MLC (70, 71). We are investigating the possibility that Rho proteins similarly regulate myosin organization in CHO cells by altering MLC phosphorylation, as depicted in Fig. 10. This possibility is supported by studies demonstrating that Rho inactivation diminishes MLC phosphorylation in non-muscle cells (8, 72).

Rho inactivation in CHO-m3 cells does not diminish PMA-induced myosin reorganization, even though it diminishes mAChR-mediated myosin reorganization. These findings are consistent with a report that Rho inactivation does not affect phorbol ester-induced contraction of cerebrovascular smooth muscle but inhibits serotonin-induced contraction of the same tissue (64). These results may occur because Rho and PKC participate in separate signaling pathways to regulate myosin organization, as depicted in Fig. 10. According to this model, PKC induces myosin reorganization independently of Rho. This model explains why Rho inactivation does not affect PMA-induced myosin reorganization but diminishes mAChR-mediated myosin reorganization. This model also explains why Rho inactivation does not completely abolish mAChR-mediated stress fiber formation; mAChR-mediated activation of PKC induces myosin reorganization even when Rho is inactive.

We found that PKC antagonists completely abolish mAChR-mediated stress fiber formation. This finding indicates that PKC inactivation inhibits both Rho- and PKC-dependent myosin reorganization induced by mAChR stimulation. This result may occur because PKC inactivation inhibits mAChR-mediated signaling to Rho. PKC may act in parallel with several other effectors to activate Rho following mAChR stimulation. If this possibility is correct, then PKC antagonists should inhibit mAChR-mediated activation of Rho and subsequent Rho-dependent myosin reorganization. This model does not predict that PKC activation can independently activate Rho, because other mAChR-mediated signals in addition to PKC activation may be needed to stimulate Rho activity.

Role of MLCK in mAChR-mediated Myosin Reorganization—Many studies indicate that MLCK plays a central role in regulating myosin activity in muscle and non-muscle cells (reviewed in Refs. 9–12). Intracellular Ca2+ mobilized by M1 or M3 mAChR stimulation can activate MLCK, resulting in MLC phosphorylation and subsequent contraction of smooth muscle (13–16). Activation of MLCK in non-muscle cells similarly affects myosin activity (5, 6, 73). Thus, it is reasonable to assume that MLCK participates in mAChR-mediated myosin reorganization in CHO-m3 cells. However, the effects of the MLCK antagonists do not support the assumption that MLCK contributes to mAChR-mediated myosin reorganization in CHO-m3 cells. Very high concentrations of the MLCK antagonists ML-7 and ML-9 are needed to inhibit mAChR-mediated stress fiber formation in CHO-m3 cells. Carbachol-induced stress fiber formation in CHO-m3 cells is half-maximally inhibited by 75 μM ML-7 or 82 μM ML-9 and maximally inhibited by 100 μM ML-7 or 120 μM ML-9. These concentrations exceed those needed to inhibit MLCK, PKA, or PKC activity in vitro (40–42). The high antagonist concentrations needed to inhibit CHO-m3 microfilament formation do not simply reflect an inability of the drugs to enter the cells. Other studies demonstrated that concentrations as low as 3 μM ML-7 or 5 μM ML-9 can significantly affect in vivo processes (74). We found that 40 μM ML-7 diminishes mAChR-mediated cell spreading (Fig. 5a, panel G), indicating that effective intracellular concentrations of ML-7 and ML-9 accumulate in CHO-m3 cells even when the cells are exposed to relatively low antagonist concentrations. Thus, the higher ML-7 and ML-9 concentrations needed to inhibit mAChR-mediated stress fiber formation (100 and 120 μM, respectively) indicate that these MLCK antagonists may affect myosin organization by inactivating other kinases, such as PKC.

Incubation of CHO-m3 cells with 2 μM KT5926 abolishes mAChR-mediated stress fiber formation but not cell spreading (Fig. 5a, panel F). The effects of KT5926 are probably not mediated by CaMKII, since the CaMKII antagonist KN-62 does not affect mAChR-mediated myosin reorganization. Instead, KT5926 probably inhibits myosin reorganization by diminishing MLCK or PKC activity. Choi and colleagues (73) concluded that KT5926 inactivates both PKC and MLCK, based on their finding that 1 μM KT5926 inhibits both PKC-dependent and MLCK-dependent phosphorylation of MLC in rat basophilic RBL-2H3 cells. This result is consistent with our observation that KT5926 induces the same effects as the PKC antagonist Go6976 in CHO-m3 cells (Fig. 5a, panels E and F). Thus, the effects of KT5926 on mAChR-mediated myosin reorganization may involve inactivation of PKC.

Although MLCK can be activated by mAChR-mediated in-
increases in intracellular Ca$^{2+}$, it is possible that other kinases diminish MLCK activity following mAChR stimulation. CaMII can phosphorylate MLCK and reduce the affinity of MLCK for Ca$^{2+}$/calmodulin, diminishing MLCK activity (reviewed in Refs. 9–11). Thus, it is conceivable that mAChR stimulation does not significantly increase MLCK activity in CHO-m3 cells, due to concomitant mAChR-mediated activation of CaMII. Previous studies demonstrated that mAChR or PKC stimulation can induce smooth muscle contraction in the absence of MLCK activity, indicating that MLCK activity is not absolutely required for mAChR- or PKC-dependent activation of myosin (75, 76).

The potential lack of MLCK participation in mAChR-mediated myosin reorganization may explain why myosin-containing stress fibers form in CHO-m3 cells only after prolonged exposure to carbachol. If mAChR stimulation does not activate MLCK, MLC phosphorylation may only slowly increase due to a PKC- and Rho-mediated inhibition of MLC phosphatase, resulting in a gradual increase in myosin-containing stress fibers. This possibility is consistent with reports that much slower smooth muscle contractions are induced by carbachol or PMA when MLCK is inactive, compared when MLCK is active (75, 76).

Conclusions—This study provides the first evidence that $M_1$ or $M_3$ mAChR activation induces myosin reorganization in non-muscle cells. Both PKC and Rho participate in mAChR-mediated myosin reorganization in CHO-m3 cells. PKC and Rho also participate in the mAChR-mediated regulation of smooth muscle myosin. These findings indicate that mAChR regulate myosin activity by similar mechanisms in CHO cells and smooth muscle. The CHO sublines transfected with mAChR subtypes provide a useful system for elucidating receptor-mediated signals affecting myosin activity in both muscle and non-muscle cells.

The $M_1$ and $M_3$ mAChR subtypes have a unique ability to modulate the morphology (21, 22), adhesion (18–20), and proliferation (25, 77–80) of non-muscle cells. Changes in myosin activity also significantly alter the morphology, adhesion, and division of non-muscle cells (reviewed in Ref. 12). The ability of $M_1$ or $M_3$ mAChR to regulate non-muscle myosin suggests that some of the unique effects of activating these receptors may involve changes in cytoplasmic myosin activity.

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