Differential signalling by muscarinic receptors in smooth muscle: m2-mediated inactivation of myosin light chain kinase via G_{i3}, Cdc42/Rac1 and p21-activated kinase 1 pathway, and m3-mediated MLC_{20} (20 kDa regulatory light chain of myosin II) phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway

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Signalling via m3 and m2 receptors in smooth muscles involved activation of two G-protein-dependent pathways by each receptor. m2 receptors were coupled via $G\beta\gamma_{i3}$ with activation of phospholipase C-β3, phosphoinositide 3-kinase and Cdc42/Rac1 (where Cdc stands for cell division cycle) and p21-activated kinase 1 (PAK1), resulting in phosphorylation and inactivation of myosin light chain kinase (MLCK). Each step was inhibited by methoctramine and pertussis toxin. PAK1 activity was abolished in cells expressing both Cdc42-DN (where DN stands for dominant negative) and Rac1-DN. MLCK phosphorylation was inhibited by PAK1 antibody, and in cells expressing Cdc42-DN and Rac1-DN. m3 receptors were coupled via $G\alpha_{q/11}$ with activation of phospholipase C-β1 and via RhoA with activation of Rho-associated kinase (Rho kinase), phospholipase D and protein kinase C (PKC). Rho kinase and phospholipase D activities were inhibited by C3 exoenzyme and in cells expressing RhoA-DN. PKC activity was inhibited by bisindolylmaleimide, and in cells expressing RhoA-DN; PKC activity was also inhibited partly by Y27632 (44 ± 5 %). PKC-induced phosphorylation of PKC-activated 17 kDa inhibitor protein of type 1 phosphatase (CPI-17) at Thr^{38} was abolished by bisindolylmaleimide and inhibited partly by Y27632 (28 ± 3 %). Rho-kinase-induced phosphorylation of myosin phosphatase targeting subunit (MYPT1) and was abolished by Y27632. Sustained phosphorylation of 20 kDa regulatory light chain of myosin II (MLC_{20}) and contraction were abolished by bisindolylmaleimide Y27632 and C3 exoenzyme and in cells expressing RhoA-DN. The results suggest that Rho-kinase-dependent phosphorylation of MYPT1 and PKC-dependent phosphorylation and enhancement of CPI-17 binding to the catalytic subunit of MLC phosphatase (MLCP) act co-operatively to inhibit MLCP activity, leading to sustained stimulation of MLC_{20} phosphorylation and contraction. Because Y27632 inhibited both Rho kinase and PKC activities, it could not be used to ascertain the contribution of MYPT1 to inhibition of MLCP activity. m2-dependent phosphorylation and inactivation of MLCK precluded its involvement in sustained MLC_{20} phosphorylation and contraction.

Key words: contraction, myosin light chain, visceral smooth muscle.

INTRODUCTION

Phosphorylation of Ser^{19} on the 20 kDa regulatory light chain of myosin II (MLC_{20}) by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) is a prerequisite for initiating the interaction of actin and myosin, which leads to contraction in visceral and vascular smooth muscles [1,2]. However, the agonist-induced Ca^{2+} transient is dissipated rapidly, and MLCK activity decreases to resting levels, whereas MLC_{20} phosphorylation and contraction are maintained [3–5]. The G-protein-dependent pathway responsible for sustained MLC_{20} phosphorylation and contraction involves inhibition of MLC phosphatase (MLCP, also known as smooth muscle myosin phosphatase 1) and activation of one or more Ca^{2+}-independent MLC kinase(s) [6–17]. Studies of Ca^{2+} sensitization, demonstrating G-protein-dependent augmentation of contractile activity at fixed Ca^{2+} concentrations, have contributed greatly to the discovery of the role of MLCP in sustained MLC_{20} phosphorylation [10–15].

The pathway is initiated by receptor-mediated activation of a heterotrimeric G-protein (G_{13} in intestinal smooth muscle) coupled with activation of the monomeric G-protein RhoA via the p115 Rho GTP exchange factor [18,19]. Activated RhoA is translocated to the plasma membrane where it stimulates a specific Rho kinase and phosphatidylcholine-specific phospholipase D (PLD) [18,20–22]. Hydrolysis of phosphatidylcholine by PLD yields phosphatidic acid, which is dephosphorylated to diacylglycerol, leading to sustained activation of various protein kinase C (PKC) isoenzymes. Rho kinase and PKC, either singly or co-operatively, are involved in the inhibition of MLCP [12–17,23–26]. PKC acts by phosphorylating an endogenous 17 kDa

Abbreviations used: ACh, acetylcholine; Cdc42, cell division cycle 42 kinase; PKC, protein kinase C; CPI-17, PKC-activated 17 kDa inhibitor protein of type 1 phosphatase; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; DN, dominant negative; DTT, dithiothreitol; MLC_{20}, 20 kDa regulatory light chain of myosin II; MLCK, myosin light chain kinase; MLCP, MLC phosphatase; MYPT1, myosin phosphatase targeting subunit; PAK1, p21-activated kinase 1; PEt, phosphatidylethanol; PI, phosphoinositide; PLC-β1, phospholipase C-β1; PLD, phospholipase D; PTx, pertussis toxin; Rho kinase, Rho-associated kinase; TCA, trichloroacetic acid.

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inhibitory protein, PKC-activated 17 kDa inhibitor protein of type 1 phosphatase (CPI-17). Phosphorylation of CPI-17 greatly augments its ability to bind to the catalytic subunit of MLCP, causing inhibition of MLCP activity [24–26]. Although Rho kinase is capable of activating CPI-17 *in vitro*, albeit to a lesser extent when compared with PKC, its predominant effect *in vivo* appears to be via phosphorylation of the large, 110–130 kDa regulatory myosin phosphatase targeting subunit (MYPT1), and inhibition of MLCP activity [27]. In both vascular and visceral smooth muscle, CPI-17 expression varies widely and is most abundant in tonic smooth muscle, consistent with a role for CPI-17 in sustained contraction [28,29]. CPI-17 expression correlated with the ability of phorbol esters to increase contraction and MLC_{20} phosphorylation at submaximal Ca^{2+} concentrations ('Ca^{2+} sensitization'). MLCP expression appears to be related inversely to CPI-17 expression, suggesting that Rho-kinase-dependent phosphorylation of MYPT1 may predominate in tissues where MLCP expression is high. MLC_{20} phosphorylation and contraction induced by MLCP inhibitors in the absence of Ca^{2+} are suppressed by the non-specific kinase inhibitor staurosporine, implying the participation of a Ca^{2+}-independent kinase, probably a zipper-interacting protein kinase or integrin-linked kinase or related kinase(s) [30–33].

In intestinal smooth muscle, the initial (phasic) and sustained (tonic) phases of MLC_{20} phosphorylation and contraction can be distinguished clearly. Activation of G_q-coupled receptors (e.g. cholecystokinin-A) induces a transient, initial contraction mediated by MLCK, followed by a Ca^{2+}-independent sustained contraction [5]. Inhibitors of phosphoinositide (PI) hydrolysis and Ca^{2+} mobilization and inhibitors of calmodulin (calmidazolium) and MLCK (KT-5926) abolish the initial contraction without affecting the sustained contraction, implying that the latter is Ca^{2+}- and MLCK-independent. Sustained contraction is abolished by GDPβS but is not affected by G_q antibody, implying that the contraction is dependent on the activation of a distinct G-protein, shown to be G_{13} in intestinal smooth muscle. Sustained contraction involved sequential activation of G_{13}, RhoA and Rho kinase, PLD and PKC, and is inhibited by the PKC inhibitors, calphostin C and chelerythrine, as well as by selective PKC-ε inhibitors and PKC-ε antibodies [5,18]. The involvement of PKC in sustained contraction is supported by studies in other visceral and vascular smooth muscles [34–37].

Acetylcholine (ACh) interacts with two muscarinic receptors expressed in smooth muscles [37], m3 receptors coupled via $G\alpha_q$ with PI hydrolysis and inositol 1,4,5-trisphosphate-dependent Ca^{2+} release and m2 receptors [the predominant (80 %) receptor species in smooth muscle] coupled via $G\alpha_{i3}$ with inhibition of adenylate cyclase [37–39]. By analogy with other G_{i3}- (or $G_{i1/2}$-) coupled receptors (e.g. adenosine A_1 receptors [40] and purine/pyrimidine $P2Y_2$ receptors [41], opioid μ, δ and κ receptors [42] and somatostatin $sstr_3$ receptors [43]), which stimulate PI hydrolysis and Ca^{2+} mobilization and induce contraction by activating phospholipase C-β3 (PLC-β3) via G$\beta\gamma$ subunits, we postulated that m2 receptors would, similar to m3 receptors, be capable of initiating contraction. Unexpectedly, however, m2 receptors appeared to be inert. The present study examined the pathways initiated by m2 receptors so as to define the mechanisms that disable these receptors. The m2 receptors were shown to initiate two distinct pathways: one pathway involved $G\beta\gamma_{i3}$-dependent stimulation of PI hydrolysis, which did not lead to activation of MLCK or contraction; another distinct pathway involved $G\beta\gamma_{i3}$-dependent sequential activation of PI 3-kinase, Cdc42 (cell division cycle 42) and Rac1, and p21-activated kinase 1 (PAK1), which resulted in a rapid and sustained inhibition of MLCK activity. The pathway initiated by m3 receptors involved transient MLCK-dependent phosphorylation of MLC_{20} and contraction, followed by sustained MLC_{20} phosphorylation and contraction mediated by a RhoA-dependent pathway involving inhibition of MLCP via PKC-dependent phosphorylation and activation of CPI-17 and Rho-kinase-dependent phosphorylation of MYPT1.

EXPERIMENTAL

Dispersion and culture of smooth-muscle cells

Smooth-muscle cells were isolated from the circular muscle layer of rabbit intestine by sequential enzymic digestion, filtration and centrifugation as described previously [41,43]. For some experiments, the cells were placed in a culture in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal bovine serum until they attained confluence [44].

Expression of RhoA-DN (where DN stands for dominant negative), Cdc42-DN or Rac1-DN cDNA in cultured smooth-muscle cells

RhoA-DN, Cdc42-DN or Rac1-DN cDNAs were subcloned into the multiple cloning site (*Eco*RI) of the eukaryotic expression vector pEXV and a *myc* tag was incorporated into the N-terminus. Recombinant plasmid DNAs (2 μg each) were transiently transfected into smooth-muscle cells in primary culture using LIPOFECTAMINE™ Plus reagent for 48 h. In some experiments, muscle cells were co-transfected with Cdc42-DN and Rac1-DN. The cells were co-transfected with 1 μg of pGreen Lantern-1 to monitor expression. Control cells were co-transfected with 2 μg of vector (pEXV) and 1 μg of pGreen Lantern-1 DNA. Transfection efficiency (approx. 85 % of the cells) was monitored by the expression of green fluorescent protein using FITC filters.

Assay for PLD activity

PLD activity was determined by the formation of phosphatidylethanol (PEt) as described previously [45]. Smooth-muscle cells (2×10^6 cells/ml) were incubated with [^{3}H]myristic acid (2 μCi/ml) for 3 h and then with 150 mM ethanol for 15 min at 31 °C in Hepes medium. The cells were then centrifuged at 350 ***g*** for 10 min to remove excess [^{3}H]myristic acid and re-suspended in fresh medium. After stimulation with ACh for 10 min, the reaction was terminated by the addition of 1.8 ml of chloroform/methanol/HCl (50:100:1, by vol.). The organic phase was extracted, dried under N_2 and analysed for [^{3}H]PEt by TLC. [^{3}H]PEt was identified using unlabelled standards and visualized under UV light at 357 nm. The spots corresponding to PEt were scraped and counted by liquid scintillation.

Kinase assays *in vitro* for Rho kinase and PAK1

Rho kinase and PAK1 activities were determined on immunoprecipitates from cell extracts as described previously [18]. Rho kinase and PAK1 were immunoprecipitated with N-terminal antibodies sc-5561, and sc-882 respectively. Immunoprecipitates were washed twice with a phosphorylation buffer containing 10 mM $MgCl_2$ and 40 mM Hepes (pH 7.4) and then incubated for 5 min on ice with myelin basic protein (1 mg/ml) for measurement of PAK1 activity or MLC_{20} (20 μM) for measurement of Rho-kinase activity. Kinase assays were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37 °C. ^{32}P-labelled myelin basic protein or MLC_{20} was absorbed on to phosphocellulose

discs and free radioactivity removed by repeated washing with 75 mM phosphoric acid. The extent of phosphorylation was determined from the radioactivity on phosphocellulose discs by liquid scintillation.

Assay for MLCK activity

MLCK activity was measured by phosphorylation of a smooth-muscle MLC_{20} substrate as described previously [5]. After treatment with ACh for different time periods, the cells were homogenized in a medium containing 50 mM KH_2PO_4, 4 mM EDTA, 15 mM dithiothreitol (DTT), 10 mM NaF, 1 mM PMSF, 0.5 % Triton X-100 and 10 μg/ml aprotinin and centrifuged at 8000 ***g*** for 10 min. The supernatant was added to a mixture containing 0.1 mM Ca^{2+}, 50 mM Mops, 15 mM DTT, 10 mM magnesium acetate, 0.3 μM calmodulin and 18 μM smooth-muscle MLC_{20} or 20 μM myosin. The reaction was initiated with 1 mM [γ-^{32}P]ATP. Aliquots were spotted on Whatman filter paper, rinsed successively with 10 % (v/v) trichloroacetic acid (TCA), 4 % (w/v) PP_i, 95 % (v/v) ethanol and 100 % (v/v) diethyl ether and were dried for measurement of radioactivity.

Assay for PKC activity

PKC activity was measured in the particulate fraction as described previously [45]. The cell suspension (1 ml containing 2×10^6 cells/ml) was incubated with ACh for 5 min in the presence or absence of different inhibitors, and the reaction was terminated by rapid freezing in dry-acetone slurry. After thawing, the cell suspension was homogenized in Tris/HCl (pH 7.5) medium and centrifuged. The membrane pellet was resuspended in Tris/HCl (pH 7.5) medium containing 0.5 % Triton X-100 and centrifuged; the supernatant was taken as the PKC-containing membrane fraction. PKC activity was measured by phosphorylation of the peptide substrate myelin basic protein (250 μM) and the results expressed as c.p.m. $\cdot$ (mg of protein)$^{-1}$ $\cdot$ min^{-1}.

Phosphorylation of MLCK

Phosphorylation of MLCK was determined from the amount of ^{32}P incorporated into the enzyme after immunoprecipitation with MLCK antibody. A 10 ml suspension of smooth-muscle cells (4×10^6 cells/ml) was prelabelled with 0.5 mCi/ml of [^{32}P]P_i for 3 h. Samples (1 ml) were treated with ACh in the presence or absence of various agents. The reaction was terminated with an equal volume of lysis buffer, containing 1 % (v/v) Triton X-100, 0.5 % SDS, 10 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM sodium pyrophosphate, 50 mM NaF and 0.2 mM sodium vanadate. The cell lysates were separated from the insoluble material by centrifugation at 13 000 ***g*** for 15 min at 4 °C, precleared with 40 μl of Protein A–Sepharose and incubated with antibody to MLCK for 2 h at 4 °C. After addition of Protein A–Sepharose (40 μl), the lysates were incubated for 1 h. The immunoprecipitates were washed five times with 1 ml of wash buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.5 % Triton X-100, and extracted with SDS sample buffer. The samples were resolved by SDS/PAGE and ^{32}P-labelled MLCK was visualized by autoradiography, and the amount of radioactivity in the bands was counted.

Western-blot analysis of phosphoproteins (CPI-17, MYPT1 and MLC_{20})

Phosphorylation of CPI-17, MYPT1 and MLC_{20} was measured by Western-blot analysis using phospho-specific antibodies as described previously [25,37,41–43]. Dispersed muscle cells were treated with ACh for different time periods in the presence or absence of diphenylacetoxy-*N*-methylpiperidine (4-DAMP), methoctramine, bisindolylmaleimide or Y27632 and solubilized on ice for 1 h in a medium containing 20 mM Tris/HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5 % SDS, 0.75 % deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin and 100 μg/ml aprotinin. The proteins were resolved by SDS/PAGE and electrophoretically transferred on to PVDF membranes. The membranes were incubated for 12 h with phospho-specific antibodies to CPI-17 (Thr^{38}), MYPT1 (Thr^{696}) or MLC_{20} (Ser^{19}/Thr^{18}) and then for 1 h with a horseradish peroxidase-conjugated secondary antibody. The bands were identified by ECL®.

PI hydrolysis in smooth-muscle membranes

PI hydrolysis was determined in membranes isolated from dispersed muscle cells prelabelled with *myo*[^{3}H]inositol as described previously [38,42,43]. The assay was initiated by addition of 0.4 mg of membrane protein to 25 mM Tris/HCl (pH 7.5), 0.5 mM EGTA, 10 mM $MgCl_2$, 300 nM Ca^{2+}, 100 μM GTP, 5 mM phosphocreatine and 50 units/ml creatine kinase, in a total volume of 0.4 ml. After incubation for 60 s at 31 °C, the reaction was terminated with 0.6 ml of 25 % TCA. The supernatant was extracted four times with diethyl ether, and the amount of labelled inositol phosphates in the aqueous phase was determined by liquid scintillation.

Measurement of contraction in dispersed smooth-muscle cells

Contraction of dispersed muscle cells was measured by scanning micrometry as described previously [42,43]. The mean length of muscle cells treated with ACh in the presence or absence of inhibitors was compared with the mean cell length of untreated cells and contraction was expressed as percentage decrease in the mean cell length.

Materials

[^{3}H]Myristic acid (22.4 Ci/mmol), [γ-^{32}P]ATP and carrier-free [^{32}P]P_i were obtained from NEN Life Science Products (Boston, MA, U.S.A.); polyclonal antibodies to phospho-MYPT1 (Thr^{696}) (07-251) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.); antibodies to RhoA (sc-119), Rho kinase (sc-5561), MLCK (sc-12450) and PAK1 (N-terminal sc-882 and C-terminal sc-881) and phospho-antibody to MLC_{20} (sc-12896) were obtained from Santa Cruz Biotechnology (New York, NY, U.S.A.); all other reagents were from Sigma. RhoA-DN cDNA was a gift from Dr Andrea Todisco (University of Michigan, MI, U.S.A.), Cdc42-DN and Rac1-DN were a gift from Dr Lee Slice (Department of Medicine, CURE: Digestive Diseases Research Center, University of California, Los Angeles, CA, U.S.A.).

RESULTS

Signalling via m3 and m2 receptors during the initial phase of contraction

We [37] and others have shown previously that m3 and m2 receptors are the two muscarinic receptor types expressed in vascular and visceral smooth muscles. RT–PCR and Western-blot analysis of rabbit cultured intestinal smooth-muscle cells used in the present study confirmed the expression of m3 and m2 receptors (results not shown). Previous radioligand binding and

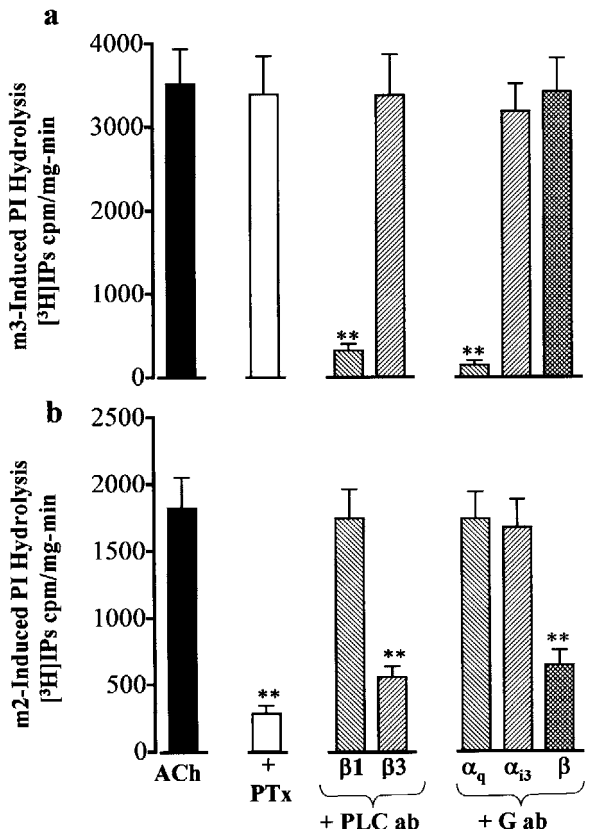

Figure 1 PI hydrolysis induced by m3 and m2 receptors in smooth muscle

Cell homogenates obtained from dispersed muscle cells prelabelled with *myo*-[^{3}H]inositol were treated with 0.1 μM ACh for 60 s in the presence of 0.1 μM methoctramine (**a**) or 0.1 μM 4-DAMP (**b**) to elicit m3- or m2-dependent responses respectively as well as with PTx and various PLC-β and G-protein antibodies. PTx was added for 60 min before the cells were homogenized. The PLC-β and G-protein antibodies (10 μg/ml) were added for 60 min to the homogenates. Results are expressed as total [^{3}H]inositol phosphates (IP; c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1}) above basal level (range: 581 $\pm$ 53 to 600 $\pm$ 61 c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1}). m3 receptor-induced PI hydrolysis was inhibited by PLC-β1 and G$\alpha_{q/11}$ antibodies; m2 receptor-induced PI hydrolysis was inhibited by PTx and by PLC-β3 and Gβ antibodies. Results are expressed as means $\pm$ S.E.M. from five experiments. $^{**}P < 0.001$ for inhibition of PI hydrolysis.

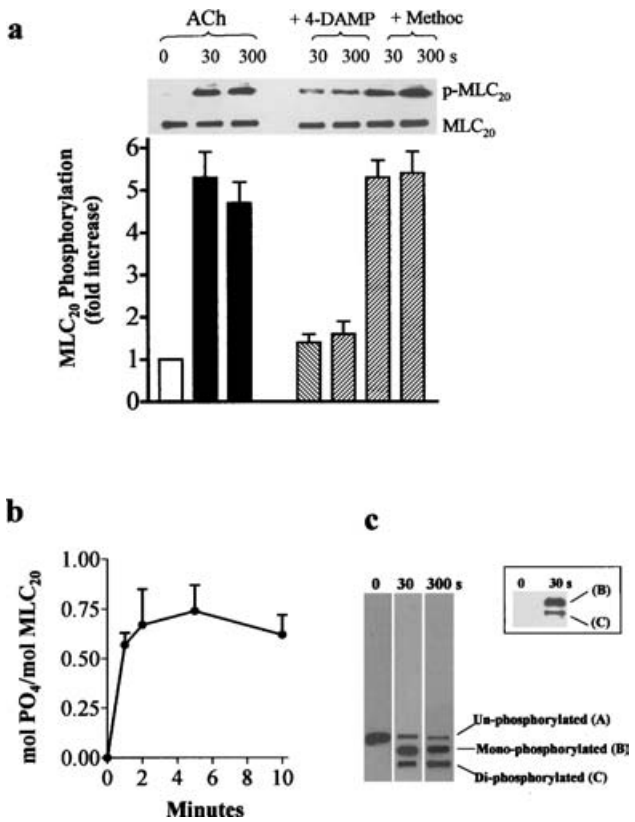

Figure 2 MLC_{20} phosphorylation induced by ACh in smooth-muscle cells

(**a**) Dispersed muscle cells were preincubated for 10 min with 0.1 μM 4-DAMP or 0.1 μM methoctramine and then treated with 0.1 μM ACh. Initial and sustained MLC_{20} phosphorylation measured at 30 and 300 s respectively was inhibited by the m3 antagonist, 4-DAMP ($^{**}P < 0.01$), but not by the m2 antagonist, methoctramine (Methoc). (**b**) Stoichiometry of phosphorylation of MLC_{20}. MLC_{20} (25 μM) was incubated with MLCK in the presence of [γ-^{32}P]ATP for different time periods and the reaction was terminated with TCA. The amount of ^{32}P incorporated into MLC_{20} was measured by scintillation counting. (**c**) Separation of unphosphorylated (A), monophosphorylated (B: Ser^{19}) and diphosphorylated (C: Ser^{19}/Thr^{18}) MLC_{20} by urea/PAGE followed by Western-blot analysis using non-phospho-specific antibody. Inset to Figure 2c: detection of monophosphorylated (B) and di-phosphorylated MLC_{20} (C) by urea/PAGE followed by Western-blot analysis using phospho-specific (Ser^{19}/Thr^{18}) antibody. Results are expressed as means $\pm$ S.E.M from 3–5 experiments.

pharmacological studies in these cells had demonstrated a high selectivity of methoctramine for m2 receptors and 4-DAMP for m3 receptors [37]. This made it possible to use these antagonists to examine signalling pathways initiated by m3 and m2 receptors. Since m3 and m2 receptors are coupled with G_q and G_{i3} respectively, responses mediated by m2 receptors only (e.g. inhibition of adenylate cyclase) are pertussis toxin (PTx)-sensitive [37]. In the present study, PTx was used as an additional tool to identify pathways initiated by m2 receptors.

ACh was shown previously to stimulate PI hydrolysis, inositol 1,4,5-trisphosphate formation and Ca^{2+} release in dispersed intestinal smooth-muscle cells [38,39]. In the present study, we show that PI hydrolysis was induced by both m3 and m2 receptors: PI hydrolysis induced by m3 receptors was mediated by Gα_q-dependent activation of PLC-β1, whereas PI hydrolysis induced by m2 receptors was mediated by G$\beta\gamma_{i3}$-dependent activation of PLC-β3 (Figure 1). PI hydrolysis via m3 receptors was inhibited by PLC-β1 and G$\alpha_{q/11}$ antibodies, whereas PI hydrolysis via m2 receptors was inhibited by PTx, and PLC-β3 and Gβ antibodies (Figure 1). Activation of PLC-β3 followed a well-established pattern of activation of this isoenzyme in smooth muscles by $\beta\gamma$ subunits of G_{i1}, G_{i2} and G_{i3} [39–43]. The effectiveness of PLC-β1 and -β3 antibodies and antibodies to Gα and Gβ subunits in blocking responses in muscle cells has been characterized previously [39–43]. ACh induced a transient activation of MLCK and a sustained phosphorylation of MLC_{20} (Figures 2 and 3) and muscle contraction (Figure 3). MLCK activity attained a peak within 30 s, decreased rapidly thereafter and was virtually absent within 2 min (Figure 3). The initial contraction paralleled the time course of MLCK activity and was inhibited selectively by the MLCK inhibitor ML-9 (Figure 3). Initial MLC_{20} phosphorylation was inhibited selectively by ML-9 (inset to Figure 3b). The time course and stoichiometry of MLC_{20} phosphorylation *in vitro* are depicted in Figure 2(b). Stoichiometry *in vivo* was closely similar (0.55 ± 0.05 and 0.56 ± 0.06 mol P/mol MLC_{20} at 30 and 300 s respectively). Both initial and sustained MLC_{20} phosphorylation induced by ACh consisted of mono- and diphosphorylated MLC_{20} at Ser^{19} and Thr^{18}/Ser^{19} respectively (Figure 2c). The inset in Figure 2(c) shows that the phospho-antibody used recognized both mono- and diphosphorylated MLC_{20}.

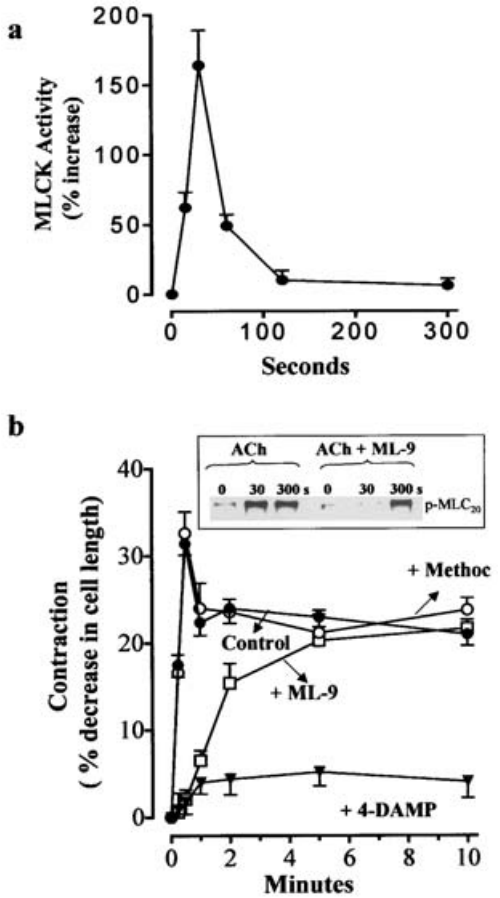

Figure 3 MLCK activity and contraction induced by ACh in smooth-muscle cells

(**a**) Dispersed smooth-muscle cells were treated with ACh for different time periods and MLCK activity measured as described in the Experimental section was expressed as percentage above basal activity (2462 ± 257 c.p.m./mg of protein). (**b**) Dispersed smooth-muscle cells were treated with 0.1 μM ACh for different time periods in the presence or absence of 0.1 μM 4-DAMP, 0.1 μM methoctramine or 10 μM MLCK inhibitor ML-9. Contraction was measured by scanning microscopy. Mean control cell length, 115 ± 6 μm; initial peak decrease in cell length at 30 s, 36.8 ± 2.3 μm or 32 ± 2 %. Antagonists had no effect on control cell length. Inset to Figure 3b: ACh-induced MLC_{20} phosphorylation at 0.5 and 5 min in the presence or absence of the MLCK inhibitor ML-9. Results are expressed as means ± S.E.M. from four experiments.

Both initial and sustained MLC_{20} phosphorylation and contraction were abolished by 4-DAMP, but were not affected by methoctramine (Figures 2 and 3). Thus m2 receptors did not mediate contraction and, in this respect, they differed from other G_i-coupled receptors that activate PLC-β3 and stimulate Ca^{2+} release and contraction. We postulated that m2 receptors initiated a distinct pathway that led to inactivation of MLCK.

Pathway mediating m2 receptor-induced phosphorylation and inactivation of MLCK

In the presence of the CaM-kinase II inhibitor, KN-62 (10 μM), ACh acting via m2 receptors stimulated MLCK phosphorylation in a time- and concentration-dependent fashion (Figures 4a and 4b). MLCK phosphorylation was significant within 30 s, increasing progressively for 5 min. The stoichiometry of MLCK phosphorylation after 5 min treatment of the cells with ACh was 0.48 ± 0.05 mol P/mol MLCK. The time course and stoichiometry of MLCK phosphorylation *in vitro* by exogenous PAK1 is shown in the inset to Figure 4(a). In primary cultures of smooth-muscle cells permeabilized with saponin, MLCK phosphorylation was inhibited by preincubation for 60 min with antibody sc-881 directed against the C-terminus of PAK1 (5 μg/ml; Figure 4d). Permeabilization in the absence of C-terminal PAK1 antibody (1156 ± 205 c.p.m.) or in the presence of N-terminal PAK1 antibody (1067 ± 175 c.p.m.) had no effect on MLCK phosphorylation. ACh-induced MLCK phosphorylation in freshly dispersed muscle cells was inhibited by methoctramine but not by 4-DAMP (Figure 4c). MLCK phosphorylation was also inhibited by preincubation of cultured intestinal smooth-muscle cells for 60 min with PTx, and in cultured muscle cells co-expressing Cdc42-DN and Rac1-DN, but not in cells expressing RhoA-DN (Figure 4d). Phosphorylation of basal MLCK was accompanied by inhibition of MLCK activity and MLC_{20} or myosin phosphorylation (Figure 5).

m2 receptor-induced activation of PAK1

ACh-stimulated PAK1 activity was not affected when immunoprecipitation was obtained with the N-terminal PAK1 antibody sc-882, but was inhibited by 80 ± 4 % when it was obtained with the C-terminal PAK1 antibody sc-881. In freshly dispersed muscle cells, ACh-stimulated PAK1 activity was inhibited selectively by methoctramine and by preincubation with PTx for 60 min, but was not affected by 4-DAMP (Figure 6a). PAK1 activity was also inhibited partly in primary cultures of smooth-muscle cells expressing Cdc42-DN or Rac1-DN, and abolished in cells expressing both Cdc42-DN and Rac1-DN, but was not affected in cells expressing RhoA-DN (Figure 6b). PAK1 activity was not affected by *Clostridium botulinum* C3 exoenzyme (2 μg/ml), the RhoA kinase inhibitor Y27632 (1 μM), the p38 mitogen-activated protein kinase inhibitor SB203580 (1 μM), the MAP kinase/extracellular-signal-regulated kinase inhibitor PD98059 (10 μM) and the PKC inhibitor bisindolylmaleimide (1 μM). The pattern of PAK1 activation suggested that m2 receptors mediated inhibitory phosphorylation of MLCK via sequential activation of G_{i3}, Cdc42 and Rac1, and PAK1.

m3 receptor-induced activation of Rho kinase, PLD and PKC

Studies in vascular and visceral smooth muscles have shown that sustained contraction is G-protein-dependent and involves activation of RhoA and its translocation to the plasma membrane [20]. In intestinal smooth-muscle cells, cholecystokinin octapeptide activated sequentially G_{13}, RhoA, PLD and PKC [18]. In the present study, we show that ACh stimulated Rho-kinase activity in a time-dependent fashion (Figure 7). ACh-stimulated activity was inhibited by 4-DAMP, but was not affected by methoctramine or PTx (Figure 7). Rho-kinase activity in these cells was also abolished by Y27632, but was not affected by SB203580, PD98059 or bisindolylmaleimide (results not shown). Rho-kinase activity was inhibited in primary cultures of intestinal smooth-muscle cells expressing RhoA-DN, but not in cells expressing Cdc42-DN and Rac1-DN (results not shown).

Sustained PLD activity, which was downstream of RhoA, was inhibited by 4-DAMP and C3 exoenzyme but not by methoctramine or PTx in freshly dispersed smooth-muscle cells (Figure 8a). ACh-stimulated PLD activity was inhibited in primary cultures of smooth-muscle cells expressing RhoA-DN, but not in cells expressing Cdc42-DN and Rac1-DN (Figure 8b).

Sustained PKC activity measured 10 min after treatment of cultured smooth-muscle cells with ACh was abolished by

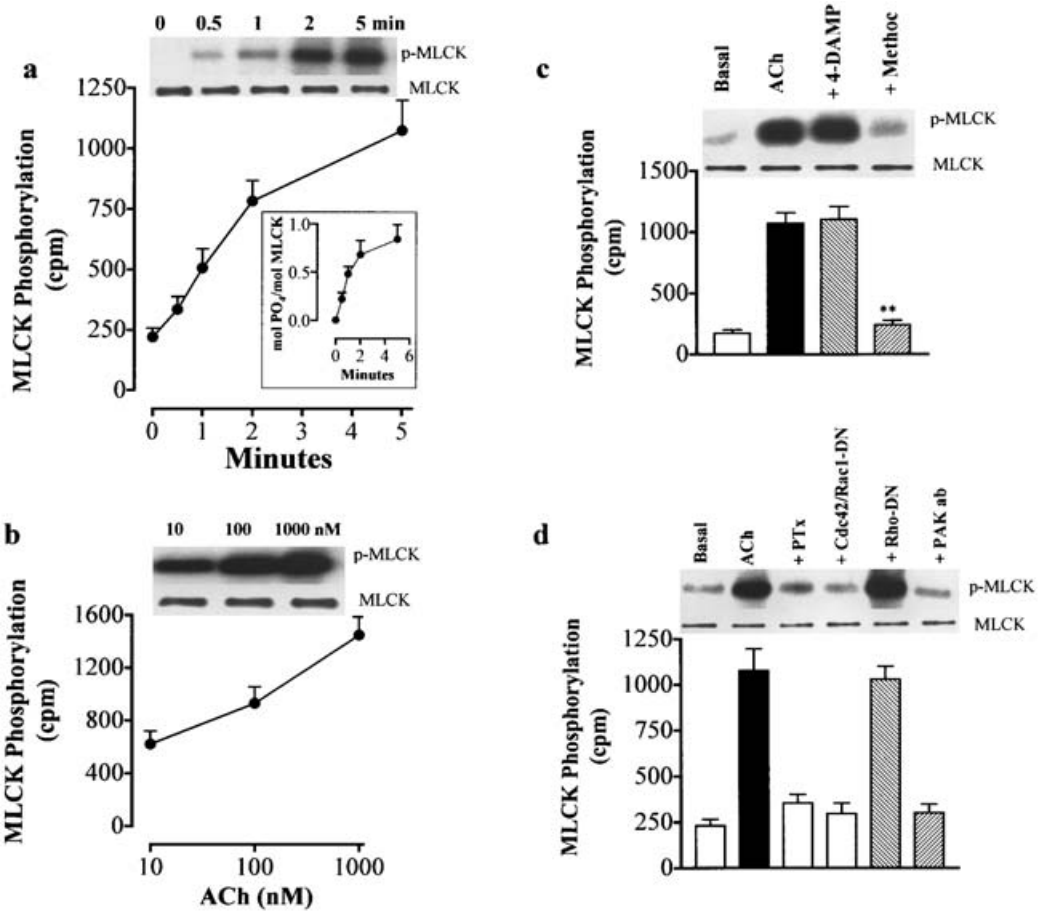

Figure 4 m2 receptor-dependent phosphorylation of MLCK

MLCK phosphorylation in freshly dispersed or cultured smooth-muscle cells was measured in the presence of CaM kinase inhibitor KN-62 (10 μM). Mean basal phosphorylation at zero time was 220 $\pm$ 38 c.p.m. Dispersed smooth-muscle cells were treated (**a**) with 0.1 μM ACh for different time periods, or (**b**) with different concentrations of ACh for 2 min; (**c**) dispersed muscle cells were also treated with 0.1 μM ACh in the presence or absence of 0.1 μM methoctramine or 0.l μM 4-DAMP. Experiments depicted in (**d**) were done on primary cultures of control smooth-muscle cells and cells expressing RhoA-DN or co-expressing Cdc42-DN and Rac1-DN. ACh was added to control cultures after preincubation for 60 min with 400 ng/ml PTx or 5 μg/ml PAK1 antibody. For experiments with PAK1 antibody, the cells were first permeabilized with 35 μg/ml saponin for 5 min. The inset to (**a**) shows the time course of MLCK phosphorylation induced by PAK1. MLCK (10 μM) was incubated with PAK1 (5 μg) in the presence of [γ-^{32}P]ATP for different time periods and the reaction was terminated with TCA. The amount of ^{32}P incorporated was measured by scintillation counting. $^{**}P < 0.01$ for inhibition of MLCK phosphorylation. Results are expressed as means $\pm$ S.E.M from three experiments.

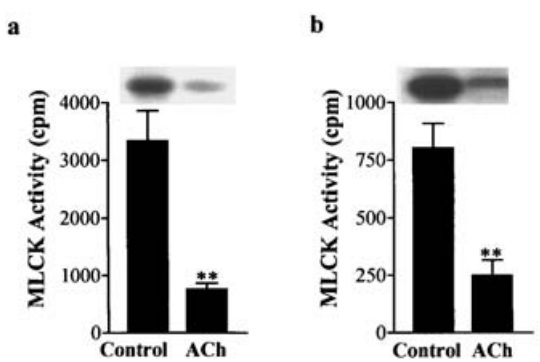

Figure 5 m2 receptor-mediated phosphorylation of MLCK inhibits MLCK activity

MLCK immunoprecipitates were obtained from control cells and cells treated with 0.1 μM ACh for 2 min in the presence of 0.1 μM 4-DAMP. MLCK activity was measured using exogenous MLC_{20} (**a**) or myosin (**b**) as substrate. $^{**}P < 0.01$ for inhibition of MLCK activity. Results are expressed as means $\pm$ S.E.M. from three experiments.

bisindolylmaleimide and inhibited by Y27632 (44 $\pm$ 5%) and in cells expressing RhoA-DN (61 $\pm$ 4%).

Phosphorylation of CPI-17 and MYPT1

The linkage of PKC to MLC_{20} phosphorylation involved phosphorylation of CPI-17 expressed in intestinal smooth-muscle cells. ACh induced CPI-17 phosphorylation at Thr^{38} in freshly dispersed smooth-muscle cells: phosphorylation was abolished by 4-DAMP and bisindolylmaleimide, and was inhibited partly (28 $\pm$ 3%) by Y27632 (Figure 9). CPI-17 phosphorylation was not affected by methoctramine (Figure 9).

ACh phosphorylated MYPT1 on Thr^{696} in a concentration-dependent fashion (Figure 10). MYPT1 phosphorylation was abolished by 4-DAMP and Y27632, but was not affected by methoctramine or bisindolylmaleimide (Figure 10b).

Sustained MLC_{20} phosphorylation and muscle contraction

As shown in Figure 2, sustained MLC_{20} phosphorylation measured at 5 min after treatment with ACh was abolished by 4-DAMP,

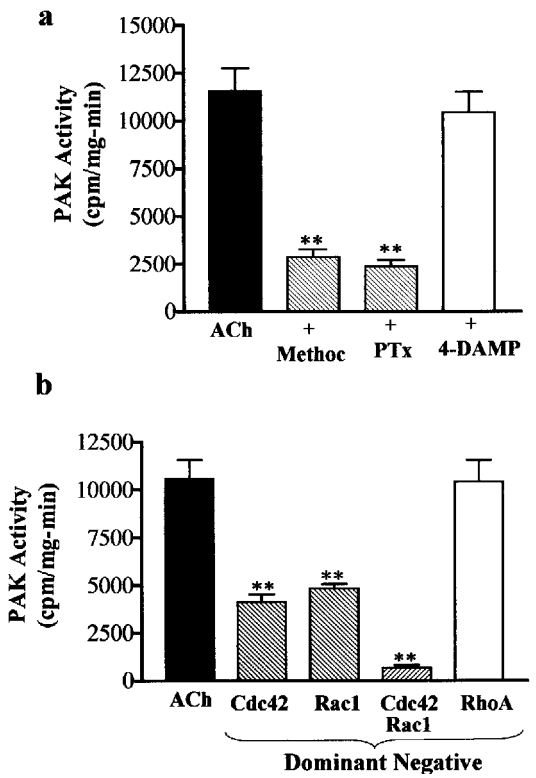

Figure 6 m2 receptor-dependent activation of PAK1 in freshly dispersed smooth-muscle cells and in cells expressing RhoA-DN, Cdc42-DN and/or Rac1-DN

(**a**) Freshly dispersed muscle cells were treated with 0.1 μM ACh for 5 min in the presence or absence of 0.1 μM 4-DAMP or 0.1 μM methoctramine and PAK1 activity was measured as described in the Experimental section. (**b**) Cultures of control smooth-muscle cells and cells expressing RhoA-DN, Cdc42-DN, Rac1-DN or co-expressing Cdc42-DN and Rac1-DN were treated with 0.1 μM ACh for 5 min and PAK1 activity was measured. Results are expressed as c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1} above basal level (range of basal level in freshly dispersed cells: 2390 $\pm$ 265 to 2646 $\pm$ 278 c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1}; range in cultured cells: 2362 $\pm$ 282 to 2961 $\pm$ 345 c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1}). Control ACh-stimulated PAK1 activities in freshly dispersed and cultured smooth-muscle cells were similar. Results are expressed as means $\pm$ S.E.M. from four experiments. $^{**}P < 0.01$, significant inhibition.

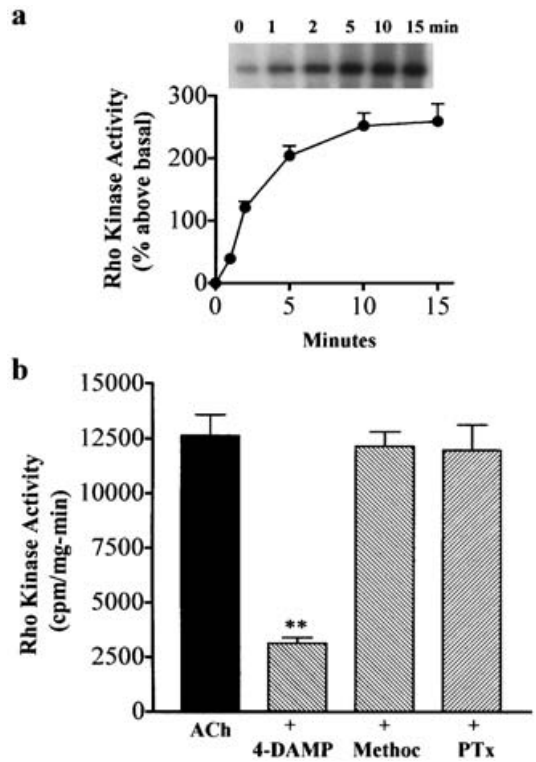

Figure 7 Time course of ACh-stimulated Rho-kinase activity and sensitivity to m3 receptor antagonists

(**a**) Dispersed smooth-muscle cells were treated with 0.1 μM ACh for different time periods, and rho-kinase activity was measured in immunoprecipitates as described in the Experimental section. (**b**) Smooth-muscle cells were preincubated with 0.1 μM 4-DAMP or 0.1 μM methoctramine for 10 min or with 400 ng/ml PTx for 60 min, and then stimulated with 0.1 μM ACh. Rho-kinase activity was expressed as percentage above basal activity (basal level: 2514 $\pm$ 302 to 2972 $\pm$ 205 c.p.m. $\cdot$ mg^{-1} $\cdot$ min^{-1}). Results are expressed as means $\pm$ S.E.M. from five experiments. $^{**}P < 0.01$ for inhibition of Rho-kinase activity.

but was not affected by methoctramine. Sustained MLC_{20} phosphorylation was also abolished by Y27632 and by bisindolylmaleimide in freshly dispersed smooth-muscle cells, and by expression of RhoA-DN in cultured muscle cells (Figure 11). Initial MLC_{20} phosphorylation measured at 30 s was not affected by Y27632 or bisindolylmaleimide (Figure 11).

Unlike initial contraction, sustained contraction was inhibited strongly by C3 exoenzyme, bisindolylmaleimide and Y27632 (Figure 12). The pattern suggests that a RhoA-mediated pathway involving inhibition of MLCP via Rho-kinase-dependent phosphorylation of MYPT1 and/or via PKC-dependent phosphorylation of CPI-17 is required for optimal inhibition of MLCP and sustained phosphorylation of MLC_{20}.

DISCUSSION

The present study characterized the signalling pathways mediated by muscarinic m3 and m2 receptors in smooth muscles, and provided evidence that m2 receptors mediate inhibitory phosphorylation of MLCK. Although capable of stimulating PI hydrolysis, m2 receptors were not capable of initiating or sustaining smooth-muscle contraction, because of downstream inactivation of Ca^{2+}/calmodulin-dependent MLCK. This ability to inactivate MLCK may distinguish m2 receptors from other receptors coupled with various isoforms of G_i in smooth muscles (opioid μ, δ and κ receptors, adenosine A_1 receptors, somatostatin $sstr_3$ receptors and $P2Y_2$ receptors) that stimulate PI hydrolysis and Ca^{2+} release and induce contraction [40–43]. Methoctramine-sensitive receptors were uncoupled selectively from G_{i3} by PTx [37]. The latter was used as an additional tool to identify signalling pathways initiated by m2 receptors.

Muscarinic m2 receptors were shown previously to inhibit adenylate cyclase activity via $G\alpha_{i3}$ [37]. In the present study, the receptors were shown to activate two additional pathways via $G\beta\gamma_{i3}$, one of which involved activation of PLC-β3 and stimulation of PI hydrolysis, whereas the other involved sequential activation of Cdc42/Rac1 and PAK1, resulting in inhibitory phosphorylation of MLCK. ACh-stimulated MLCK phosphorylation was inhibited by methoctramine, PTx and PAK1 antibody. PAK1 activation was downstream of Rac1 and Cdc42, since it was inhibited partly in muscle cells expressing Rac1-DN or Cdc42-DN mutants and abolished in cells co-expressing both mutants. The involvement of Rho family GTPases in cytoskeletal organization and cell motility, particularly the ability of Rac1 (and probably Cdc42) to activate PAK1 and induce inhibitory phosphorylation

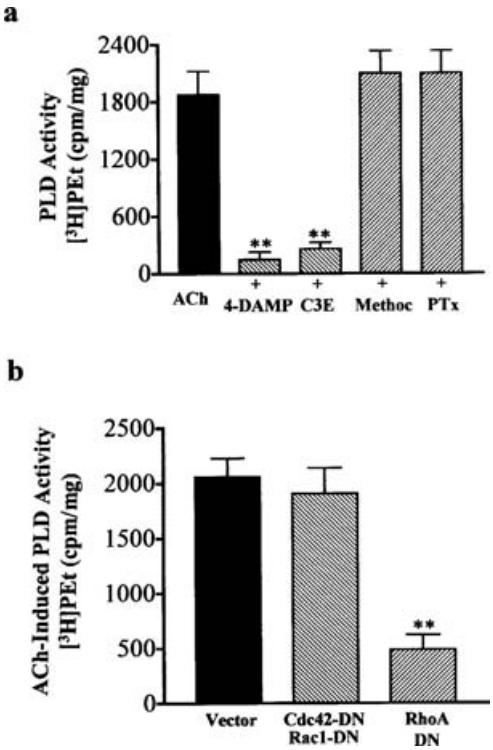

Figure 8 m3 receptor-dependent activation of PLD in freshly dispersed smooth-muscle cells and in cultured cells expressing RhoA-DN or co-expressing Cdc42-DN and Rac1-DN

(**a**) Dispersed muscle cells were treated with 0.1 μM ACh for 5 min after preincubation with 400 ng/ml PTx or 2 μg/ml C3 exoenzyme for 60 min and 0.1 μM 4-DAMP or 0.1 μM methoctramine for 10 min. PLD activity was measured as described in the Experimental section and expressed as [^{3}H]PEt [c.p.m. · (mg of protein)$^{-1}$] above basal activity (range: 686 $\pm$ 109 to 795 $\pm$ 83 c.p.m./mg). (**b**) Primary cultures of control smooth-muscle cells and cells expressing RhoA-DN or co-expressing Cdc42-DN and Rac1-DN were treated with 0.1 μM ACh for 5 min. PLD activity was measured and expressed in c.p.m./mg of protein above basal activity (range: 588 $\pm$ 98 to 716 $\pm$ 72 c.p.m./mg). Results are expressed as means $\pm$ S.E.M. from four experiments. $^{**}P < 0.01$ for inhibition of PLD activity.

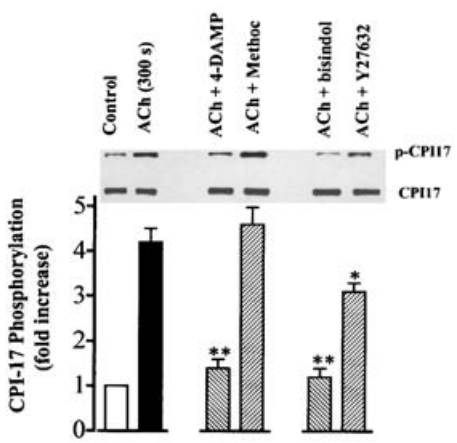

Figure 9 m3 receptor-dependent, PKC-mediated phosphorylation of CPI-17

Freshly dispersed muscle cells were preincubated for 10 min with 0.1 μM 4-DAMP, 0.1 μM methoctramine, 1 μM bisindolylmaleimide (bisindol) or 1 μM Y27632, and then treated for 5 min with 0.1 μM ACh. Cell lysates were analysed for CPI-17 phosphorylation at Thr^{38} using a phospho-Thr^{38}-specific antibody. Inhibition of phosphorylation was obtained with the m3 receptor antagonists and the PKC inhibitor. Partial inhibition by Y27632 reflected the ability of this compound to inhibit PKC activity. Results are expressed as means $\pm$ S.E.M. from three experiments. $^{*}P < 0.01$ and $^{**}P < 0.001$ for inhibition of CPI-17 phosphorylation.

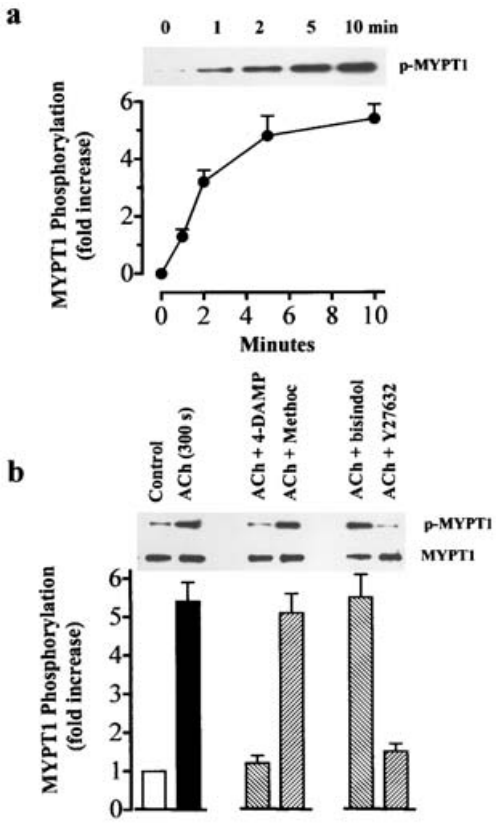

Figure 10 m3 receptor-dependent, Rho-kinase-mediated phosphorylation of MYPT1

(**a**) Freshly dispersed smooth-muscle cells were treated with 0.1 μM ACh for different time periods and cell lysates were analysed for MYPT1 phosphorylation at Thr^{696} using a phospho-Thr^{696}-specific antibody. Results are expressed as means $\pm$ S.E.M. from three experiments. (**b**) Dispersed smooth-muscle cells were preincubated for 10 min with 0.1 μM 4-DAMP, 0.1 μM methoctramine, 1 μM bisindolylmaleimide or 1 μM Y27632, and then treated with 0.1 μM ACh for 5 min. Cell lysates were analysed for MYPT1 phosphorylation at Thr^{696} using a phospho-Thr^{696}-specific antibody. Complete inhibition of phosphorylation was obtained with the m3 receptor antagonists and Y27632 ($^{**}P < 0.001$). Results are expressed as means $\pm$ S.E.M. from three experiments.

of MLCK, has been reported in some cell systems [46,47]. MLCK phosphorylation by PAK1 decreased maximal MLCK activity and was independent of calmodulin binding to the enzyme, in contrast with MLCK phosphorylation by cAMP-dependent protein kinase and by Ca^{2+}/calmodulin-dependent protein kinase II, which alters the affinity of MLCK for calmodulin [2,48]. The probable participation of PI 3-kinase in upstream activation of Rac1 and Cdc42 is supported by our studies [18] showing m2-dependent, PTx-sensitive activation of PI 3-kinase and by evidence of muscarinic activation of PI 3-kinaseγ in smooth muscles from canine colon and equine trachea [49].

Initial and sustained MLC_{20} phosphorylation and muscle contraction were exclusively a property of m3 receptors. These receptors initiated two distinct signalling pathways in smooth muscles, one of which involved G_q-dependent activation of PLC-β1 and stimulation of PI hydrolysis, whereas the other involved sequential activation of G_{13} and RhoA [18]. Stimulation of PI hydrolysis resulted in Ca^{2+}/calmodulin-dependent activation of MLCK, and was responsible for the initial, transient MLC_{20} phosphorylation and contraction. As shown with other G_q-dependent receptors (e.g. cholecystokinin-A), initial contraction

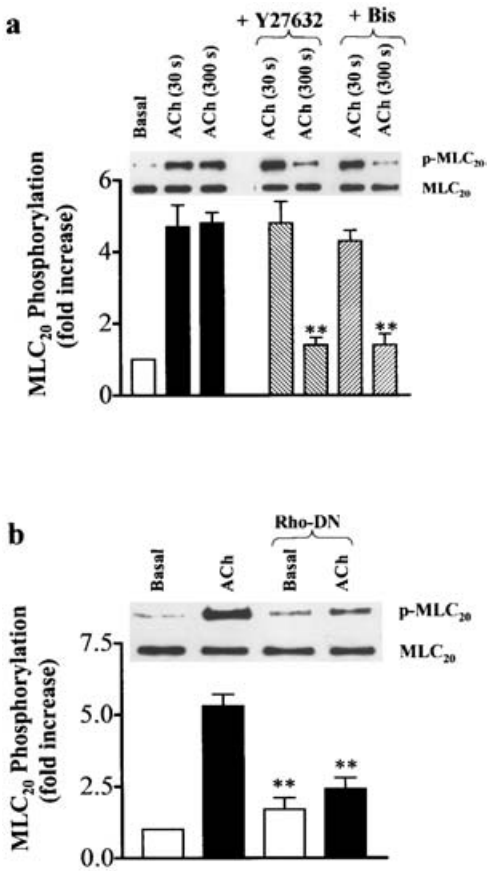

Figure 11 m3 receptor-dependent phosphorylation of MLC_{20}

(**a**) Freshly dispersed muscle cells were treated for 30 and 300 s with 0.1 μM ACh after addition of 1 μM bisindolylmaleimide (Bis) or 1 μM Y27632. (**b**) Cultured smooth-muscle cells expressing vector alone or RhoA-DN were treated with 0.1 μM ACh for 300 s. Cell lysates were analysed for MLC_{20} phosphorylation using a phospho(Ser^{19}/Thr^{18})-specific antibody. ACh-stimulated MLC_{20} phosphorylation at 300 s was inhibited by bisindolylmaleimide and Y27632 and in cells expressing RhoA-DN. Results are expressed as means $\pm$ S.E.M. from three experiments. $^{**}P < 0.01$ for inhibition of MLC_{20} phosphorylation.

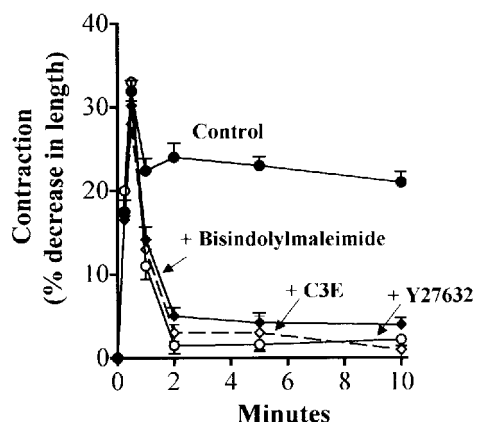

Figure 12 m3 receptor-dependent RhoA- and PKC-mediated sustained contraction

Freshly dispersed muscle cells were treated with 0.1 μM ACh for various time periods after addition of 2 μg/ml C3 exoenzyme for 60 min, 1 μM bisindolylmaleimide for 10 min or Y27632 for 10 min. Initial contraction was not affected, whereas sustained contraction was inhibited by all three agents. Mean control muscle cell length, 115 μm; peak decrease in muscle cell length at 30 s, 39 $\pm$ 3 μm or 31 $\pm$ 2 %. Results are expressed as means $\pm$ S.E.M. from 4–5 experiments.

was abolished by inhibitors of PI hydrolysis and inhibitors of calmodulin and MLCK, but was not affected by PKC inhibitors [5]. Initial contraction and MLC_{20} phosphorylation were inhibited selectively by the MLCK inhibitor ML-9, but were not affected by bisindolylmaleimide or Y27632 (Figures 2, 11 and 12).

The RhoA-dependent pathway was responsible for sustained MLC_{20} phosphorylation and contraction. RhoA activated two effector enzymes, Rho kinase and PLD, the latter eventually leading to activation of PKC [45]. Sustained phosphorylation of MLC_{20} and contraction were abolished by bisindolylmaleimide and Y27632 (Figures 11 and 12). The effectiveness of both inhibitors could mean that Rho kinase and PKC act in series at different downstream loci or that both Rho-kinase-dependent phosphorylation of MYPT1 and PKC-dependent phosphorylation of CPI-17 and stimulation of CPI-17 binding to MLCP are essential for complete suppression of MLCP activity *in vivo*. In the present study, ACh induced Rho-kinase-dependent phosphorylation of MYPT1 at Thr^{696} and PKC-dependent phosphorylation of CPI-17 at Thr^{38} *in vivo*.

Previous studies in these cells have shown that sustained PKC activity was dependent largely on diacylglycerol derived from dephosphorylation of phosphatidic acid, the primary product of PLD [45]. Both Rho kinase and PLD activities stimulated by ACh were inhibited by 4-DAMP and C3 exoenzyme, and in cells expressing Rho-DN, but were not affected by methoctramine or PTx or in cells expressing Cdc42-DN and Rac1-DN. Consistent with this, ACh-stimulated PKC activity was inhibited strongly in cells expressing RhoA-DN and was abolished by bisindolylmaleimide. However, PKC activity was also inhibited strongly by Y27632, probably reflecting the ability of Y27632 to inhibit Ca^{2+}-independent PKC isoenzymes [14,50]. This raises the possibility that inhibition of sustained MLC_{20} phosphorylation and contraction by Y27632 could reflect in part its ability to inhibit PKC activity.

Studies in vascular and visceral smooth muscles have demonstrated the ability of PKC, particularly agonist-stimulated Ca^{2+}-independent PKC-ε and PKC-δ, to phosphorylate CPI-17 and greatly enhance the ability of CPI-17 to bind to and inactivate MLCP [50]. We have shown previously that sustained contraction induced by G-protein-coupled receptors is inhibited by calphostin C and chelerythrine, as well as by a specific PKC-ε antibody or a selective pseudosubstrate inhibitor of PKC-ε [5]. Recent studies have demonstrated the ability of Rho kinase to phosphorylate CPI-17 *in vitro* and of Y27632 to block this phosphorylation [27]. As shown in Figure 9, Y27632 inhibited CPI-17 phosphorylation *in vivo*, albeit to a lesser extent than bisindolylmaleimide. The inhibition could reflect the ability of Y27632 to inhibit PKC activity rather than Rho-kinase-induced phosphorylation of CPI-17. This interpretation is supported by the fact that inhibition of PKC activity by bisindolylmaleimide abolishes CPI-17 phosphorylation *in vivo*. Kitazawa et al. [25] have shown clearly that in rabbit femoral artery strips, Y27632 and a derivative of bisindolylmaleimide inhibit histamine-stimulated CPI-17 phosphorylation and muscle contraction in parallel. The ability of Y27632 to inhibit PKC activity and Rho-kinase activity *in vivo* makes it difficult to ascertain the precise contribution of Rho-kinase-dependent phosphorylation of MYPT1 to inhibition of MLCP and stimulation of sustained MLC_{20} phosphorylation and contraction.

Recent studies have demonstrated that the temporal shift from MLCK- to RhoA-dependent phosphorylation of MLC_{20} in smooth-muscle cells is accompanied by a spatial shift of phosphorylated MLC_{20} from a central to a cortical (peripheral) location as monitored by a phospho-Ser^{19}-specific antibody [51]. The initial centrally located MLC_{20} phosphorylation was suppressed

by the MLCK inhibitor ML-9, whereas the sustained peripherally located MLC_{20} phosphorylation was suppressed by Y27632 and by expression of a RhoA-DN, providing further evidence for the dependence of sustained MLC_{20} phosphorylation and contraction on activation of RhoA.

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