Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA

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Submitted 28 October 2002; accepted in final form 17 January 2003

Murthy, Karnam S., Huiping Zhou, John R. Grider, and Gabriel M. Makhlouf. Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA. Am J Physiol Gastrointest Liver Physiol 284: G1006–G1016, 2003; 10.1152/ajpgi.00465.2002.—The role of RhoA in myosin light-chain (MLC)20 dephosphorylation and smooth muscle relaxation by PKA and PKG was examined in freshly dispersed and cultured smooth muscle cells expressing wild-type RhoA, constitutively active RhoA(V14), and phosphorylation site-deficient RhoA188. Activation of PKA (5,6-dichloro-1-β-ribofuranosyl benzimidazole [H9252]) or PKG [8-(4-chlorophenylthio)guanosine 3’5’-cyclic monophosphothionate, Sp-isomer; cBIMPS) or both PKA and PKG (VIP) induced phosphorylation of constitutively active RhoA(V14) and agonist (ACh) or GTPγS-stimulated wild-type RhoA but not RhoA188. Phosphorylation was accompanied by translocation of membrane-bound wild-type RhoA and RhoA(V14) to the cytosol and complete inhibition of ACh-stimulated Rho kinase and phospholipase D activities, RhoA/ Rho kinase association, MLC20 phosphorylation, and sustained muscle contraction. Each of these events was blocked depending on the agent used, by the PKG inhibitor KT5823 or the PKA inhibitor myristoylated PKI. Inhibitors were used at a concentration (1 μM) previously shown by direct measurement of kinase activity to selectively inhibit the corresponding kinase. In muscle cells overexpressing the active phosphorylation site-deficient mutant RhoA188, MLC20 phosphorylation was partly inhibited by SNP, VIP, cBIMPS, and 8-pCPT-cGMP, suggesting the existence of an independent inhibitory mechanism downstream of RhoA. Results demonstrate that dephosphorylation of MLC20 and smooth muscle relaxation are preferentially mediated by PKG- and PKA-dependent phosphorylation and inactivation of RhoA.

myosin light chain; myosin light chain phosphatase; regulatory myosin light chain; relaxation

CONTRACTION OF VASCULAR AND VISCERAL SMOOTH MUSCLE IS mediated by phosphorylation of Ser19 on the regulatory myosin light chain (MLC)20 (14, 32) and consists of a transient initial phase followed by a prolonged, sustained phase. The initial phase is mediated by inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]-dependent Ca2+ release and Ca2+/calmodulin-dependent activation of MLC kinase (14, 23, 32). The sustained phase is RhoA dependent and reflects inhibition of MLC phosphatase (6, 32). On activation, RhoA is translocated to the membrane in which it initiates two converging pathways involving activation of Rho kinase and phospholipase D (PLD) (7, 22, 27). Rho kinase inhibits MLC phosphatase via phosphorylation of the 130-kDa regulatory myosin phosphatase targeting subunit-1 (MYPT1) (6, 8, 32, 35). Hydrolysis of phosphatidylycerine by PLD yields phosphatidic acid, which is dephosphorylated to diacylglycerol, leading to activation of PKC; PKC phosphorylates an endogenous 17-kDa inhibitory protein (CPI-17) which binds directly to and strongly inhibits MLC phosphatase (3, 20, 27).

Relaxation of the initial or sustained phase of contraction is mediated by dephosphorylation of Ser19 on MLC20 by the catalytic subunit of MLC phosphatase (21, 37). Relaxant agonists inhibit smooth muscle contraction by activating cAMP- and/or cGMP-dependent protein kinase (PKA and PKG) (1, 15, 21, 29, 34). Low levels of cAMP activate PKA exclusively, whereas higher levels can cross-activate PKG-I (12, 13, 24). When both cyclic nucleotides are generated concurrently, for example, in response to VIP, cAMP activates PKA as well as PKG (24). PKA and PKG inhibit the initial contraction by acting on two critical targets involved in Ca2+ mobilization. Both protein kinases inhibit PLC-β-dependent Ins(1,4,5)P3 formation by phosphorylating RGS-4 and accelerating the inactivation of GTP-bound Goq (28). Only one kinase, PKG-I, inhibits Ins(1,4,5)P3-induced Ca2+ release by direct phosphorylation of the sarcoplasmic Ins(1,4,5)P3 receptor/Ca2+ channel (16, 21).

Mechanisms invoked for relaxation of sustained contraction involve stimulation of MLC phosphatase activity via inhibition of RhoA and/or RhoA-dependent targets, such as MYPT1 and CPI-17 by PKG and/or PKA. Indirect evidence (30) suggests that PKG induces relaxation by phosphorylating RhoA: contraction of permeabilized vascular myocytes by exogenous gergonylgeranylated RhoA was inhibited by exogenous PKG-I and 8-bromo-guanosine 3’5’-cyclic monophosphate (8-Br-cGMP), whereas contraction by a RhoA mutant (RhoA188) that is not susceptible to phosphorylation by PKG was not affected, suggesting the absence of a mechanism of relaxation downstream of RhoA.

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Inhibition of histamine-stimulated contraction and MLC\textsubscript{20} phosphorylation by PKG is accompanied by a decrease in CPI-17 phosphorylation that parallels the increase in MLC phosphatase activity, consistent with inhibition of RhoA activity upstream of the PLD/PKC/CPI-17 pathway (4). However, there is also evidence that PKG stimulates MLC phosphatase activity independently of RhoA (32, 37). The NH\textsubscript{2}-terminal zipper sequence of PKG-I\textalpha binds selectively to and induces phosphorylation of MYPT1. In vitro studies (32) suggest that this phosphorylation has no effect on MLC phosphatase activity; however, blockade of PKG-I\textalpha binding to MYPT1 prevented MLC\textsubscript{20} dephosphorylation by 8-Br-cGMP. Phosphorylation of Thr\textsuperscript{365} in the COOH terminus of MYPT1 by PKA has been demonstrated in vitro only and appears to be associated with an increase in MLC phosphatase activity that could lead to MLC\textsubscript{20} dephosphorylation and relaxation (10, 11).

In the present study, we examined the ability of both PKA and PKG to phosphorylate RhoA in freshly dispersed and cultured smooth muscle cells and determined the effect of this phosphorylation on agonist-stimulated RhoA, Rho kinase, and PLD activities and on RhoA translocation to the cytosol and dissociation from membrane-bound Rho kinase. Parallel studies were done in cultured smooth muscle cells expressing RhoA188,M LC\textsubscript{20} phosphorylation was significantly increased in membranes-bound Rho kinase and PLD at the same time as they inhibited sustained MLC\textsubscript{20} phosphorylation and muscle contraction. In smooth muscle cells expressing Rho\textsuperscript{188}, MLC\textsubscript{20} phosphorylation was significantly inhibited by PKG and PKA in the absence of RhoA phosphorylation, suggesting the existence of a subsidiary mechanism of relaxation downstream of RhoA.

**MATERIALS AND METHODS**

**Preparation of dispersed gastric smooth muscle cells.** Smooth muscle cells were isolated from the circular muscle layer of rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation as described previously (23–26). The partly digested strips were washed, and muscle cells were allowed to disperse spontaneously for 30 min. Cells were harvested by filtration through 500 mm Nitex and centrifuged twice at 350 g for 10 min. For some experiments, the cells were cultured in DMEM containing 10% fetal bovine serum until they attained confluence and were then passaged once for use in various studies (27).

**Expression of dominant-negative Rho\textsuperscript{N19}, constitutively active Rho\textsuperscript{V14}, and phosphorylation site-deficient Rho\textsuperscript{A188}**. Phosphorylation site-deficient Rho\textsuperscript{A188} was subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector (pE7V5), and a myc tag was incorporated into the NH\textsubscript{2} terminus. Recombinant plasmid cDNAs (2 \(\mu g\) each) were transiently transfected into cultured smooth muscle cells using Lipofectamine Plus reagent. The cells were cotransfected with 1 \(\mu g\) of pGreen Lantern-1 DNA to monitor expression. Control cells were cotransfected with 2 \(\mu g\) of pEXV and 1 \(\mu g\) of pGreen Lantern-1 DNA. Transfection efficiency was monitored by the expression of green fluorescent protein using FITC filters. Expression was demonstrable in over 80% of the cells.

**Phosphorylation of RhoA.** Phosphorylation of RhoA was measured from the amount of \(^{32}P\) incorporated into the enzyme after immunoprecipitation with specific RhoA antibody. Smooth muscle cell suspensions (10 ml; 3 \(\times\) 10\textsuperscript{5} cells/ml) were incubated with \(^{32}P\)orthophosphate for 4 h at 31°C. One-milliliter samples were then incubated with various agents for 1 min in the presence or absence of specific PKA or PKG inhibitors. The reaction was terminated with an equal volume of lysis buffer (final concentrations in mM: 150 NaCl, 10 MgCl\textsubscript{2}, 1 PMSF, 10 EDTA, 1 Na\textsubscript{2}PO\textsubscript{4}, 0.2 Na\textsubscript{2}VO\textsubscript{4}, plus 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 \(\mu g/ml\) leupeptin, and 100 \(\mu g/ml\) aprotinin). The cell lysates were separated by centrifugation at 13,000 g for 15 min at 4°C, and they were incubated with polyclonal RhoA antibody for 2 h at 4°C and with 40 \(\mu l\) of protein A-Sepharose for another 1 h. The beads were washed, extracted with Laemmli buffer, and separated by electrophoresis on 10% SDS-PAGE. After transfer to polyvinylidene difluoride membranes, \(^{32}P\)-labeled RhoA was visualized by autoradiography, and the amount of radioactivity in the band was measured by liquid scintillation and expressed as counts/min (cpm)/mg protein.

**Assay for activated RhoA.** Activated RhoA was measured in freshly dispersed or cultured muscle cells incubated for 3 h in low-phosphate medium containing 10 \(\mu l\) \(^{32}P\) orthophosphate and 10 \(\mu l\) of protein A-Sepharose for 1 h. The beads were washed, extracted with Laemmli buffer, and separated by electrophoresis on 10% SDS-PAGE. After transfer to polyvinylidene difluoride membranes, \(^{32}P\)-labeled RhoA was visualized by autoradiography, and the amount of radioactivity in the band was measured by liquid scintillation and expressed as counts/min (cpm)/mg protein.

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**Plasmid construct.** A 414-bp fragment containing the coding sequence for residues 7–89 of RhoA was amplified by PCR with primers (5' and 3') corresponding to the nucleotide sequences for residues 664–687 and 697–713, respectively, of the cDNA sequence for RhoA from mouse kidney (13). The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI/BamHI sites of the mammalian expression vector pEXV, a myc expression vector (pEXV), and a myc tag was incorporated into the NH\textsubscript{2} terminus. Recombinant plasmid cDNAs (2 \(\mu g\) each) were transiently transfected into cultured smooth muscle cells using Lipofectamine Plus reagent. The cells were cotransfected with 1 \(\mu g\) of pGreen Lantern-1 DNA to monitor expression. Control cells were cotransfected with 2 \(\mu g\) of pEXV and 1 \(\mu g\) of pGreen Lantern-1 DNA. Transfection efficiency was monitored by the expression of green fluorescent protein using FITC filters. Expression was demonstrable in over 80% of the cells.

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**Assay for PLD activity.** PLD activity was determined by the formation of the PLD-specific product, phosphatidyl ethanolamine (PEt), as described previously (27). Smooth muscle cells (2 \(\times\) 10\textsuperscript{6} cells/ml) were incubated with \(^{3}H\)myristic acid (2 \(\mu C/ml\)) for 3 h and then with 150 mM ethanolol for 15 min at 31°C in HEPES medium. The cells were centrifuged and resuspended in fresh medium. After stimulation with ACh for 10 min, the reaction was terminated with chloroform/methanol/HCl (100:200:2; vol/vol/vol), and the organic phase was extracted and analyzed for \(^{3}H\)PET by thin-layer chromatography. \(^{3}H\)PET was identified using unlabeled standards and visualized under ultraviolet light at 357 nm. Spots
corresponding to [³H]PET were scraped and counted by liquid scintillation.

**Assay for Rho kinase activity.** Rho kinase activity was determined by immunokinase assay in cell extracts as described previously (27). Rho kinase was immobilized on antibody, and the immunoprecipitates were washed with phosphorylation buffer and incubated for 5 min on ice with 5 μg of myelin basic protein. Kinase assays were initiated by the addition of 10 μCi of [γ-³²P]ATP (3,000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37°C. [³²P]myelin basic protein was absorbed onto phosphocellulose discs, and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the discs was measured by liquid scintillation.

**Immunoblot analysis of RhoA, Rho kinase, and phosphorylated MLC20.** RhoA was measured in membrane and cytosolic fractions by Western blot analysis after treatment with ACh in the presence or absence of PKA and PKG activators. RhoA-Rho kinase association was determined by immunoblot analysis using a phospho-Ser 19–specific antibody. The proteins were resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were incubated for 12 h with appropriate antibody and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. The bands were identified by enhanced chemiluminescence.

**Measurement of relaxation in dispersed smooth muscle cells.** Inhibition of ACh-induced contraction (i.e., relaxation) by sodium nitroprusside (SNP) or VIP was expressed as the decrease in maximal cell contraction induced by 0.1 μM ACh as described previously (24–26). A 0.5-ml aliquot of cell suspension was added to 0.2 ml HEPES medium containing ACh alone and with SNP or VIP, and the reaction was terminated with 1% acrolein. Mean cell length of 50 muscle cells treated with various agents was measured by scanning microscopy and compared with the length of untreated muscle cells (mean control cell length: 116 ± 3 μm).

**Materials.** [³²P]orthophosphate was obtained from Amer sham Pharmacia Biotech (Piscataway, NJ); collagenase and soybean trypsin inhibitor was from Worthington Biochemical (Freehold, NJ); Western blotting and chromatography material was from Bio-Rad Laboratories (Hercules, CA); RhoA antibody (Sc-119), MLC20 phospho-antibody (Sc-12896), and Rho kinase antibody (Sc-119) were from Dr. Lee Slichter, University of California (Los Angeles, CA). RhoA cDNA was a gift from Dr. Lee Slichter, University of California (Los Angeles, CA).

**RESULTS**

**Phosphorylation of RhoA by PKG and PKA.** In the basal state, RhoA is mainly present in the cytosol in inactive form bound to GDP and Rho guanine nucleotide dissociation inhibitor (GDI) and is translocated to the plasma membrane on activation by agonists (7, 17). Treatment of freshly dispersed smooth muscle cells with ACh (0.1 μM), SNP (1 μM), or VIP (1 μM) alone did not induce RhoA phosphorylation (Fig. 1). After treatment with ACh, however, both SNP and VIP induced RhoA phosphorylation, suggesting that the substrate is activated, membrane-bound RhoA (Fig. 1). RhoA phosphorylation induced by SNP was concentration dependent and was abolished by the PKG inhibitor KT5823 but was not affected by PKA inhibitor myristoylated PKI (Figs. 2 and 3). RhoA phosphorylation induced by VIP, which activates both PKA and PKG in gastric smooth muscle cells (24), was partly inhibited by KT5823 (55 ± 6% inhibition) and myristoylated PKI (43 ± 4% inhibition) and abolished by a combination of both kinase inhibitors (93 ± 5% inhibition) (Fig. 3). Nonhydrolyzable membrane-permeable analogs of cGMP [8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate; 8-pCPT-cGMP] and cAMP (5,6-dichloro-

![Fig. 1. Phosphorylation of activated RhoA by activators of PKG and PKA in smooth muscle cells. Freshly dispersed gastric smooth muscle cells labeled with [³²P] were incubated with sodium nitroprusside (SNP; 1 μM) or VIP (1 μM) (A), and with 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP; 10 μM) or 5,6-dichloro-1-β-ribofuranosyl benzimidazole 3',5'-cyclic monophosphonate, Sp-isomer (cBIMPS; 10 μM) (B). The agents were added alone and after stimulation with ACh (0.1 μM) for 5 min. RhoA immunoprecipitates were separated on SDS-PAGE and identified by autoradiography. Radioactivity in the bands was expressed as counts/min (cpm). Immunoblots were performed to determine comigration of radioactive bands with RhoA (not shown). Values are means ± SE of 3 experiments.](image)
1-β-ribofuranosyl benzimidazole 3', 5'-cyclic monophosphothionate, Sp-isomer; cBIMPS) that selectively activate PKG and PKA, respectively, also induced phosphorylation of RhoA (Fig. 1). Phosphorylation by 8-pCPT-cGMP was inhibited by KT5823 only, whereas phosphorylation by cBIMPS was inhibited by myristoylated PKI only (Fig. 3). As previously shown by direct measurements of PKA and PKG activities in gastric smooth muscle cells, KT5823 and PKI, used at concentrations of 1 μM and less, selectively inhibited PKG and PKA activity, respectively (24, 25).

**Phosphorylation of RhoA in cultured muscle cells overexpressing RhoA mutants.** As shown in freshly dispersed smooth muscle cells, SNP and VIP induced RhoA phosphorylation in cultured smooth muscle cells overexpressing wild-type RhoA but only after stimulation with ACh (Fig. 4A). However, both SNP and VIP induced phosphorylation of RhoA in the absence of ACh in permeabilized cultured smooth muscle cells overexpressing myc-tagged wild-type RhoA after treatment with GTPγS or in cells overexpressing myc-tagged constitutively active RhoV14 (Fig. 4). SNP and VIP did not induce RhoA phosphorylation in cells overexpressing dominant-negative RhoV19 (data not shown). Additional studies were done in cultured muscle cells overexpressing wild-type RhoA or RhoV14. Phosphorylation was induced by 8-pCPT-cGMP or cBIMPS, and immunoprecipitation was performed separately with myc antibody and RhoA antibody. The results with RhoA antibody showed slightly greater phosphorylation indicative of endogenous RhoA (Fig. 4B). The results implied that PKA and PKG preferentially phosphorylated active, membrane-bound RhoA. Inactive, cytosolic RhoA, bound to GDI at its COOH terminus, is probably protected from phosphorylation by PKA or PKG (17).

**Inhibition of RhoA activity by PKA and PKG.** Acetylcholine-stimulated RhoA activity was inhibited by SNP (82 ± 4%) and 8-pCPT-cGMP (92 ± 5%) (Figs. 5 and 6). Inhibition by either agent was completely blocked by KT5823 but was not affected by myristoylated PKI. ACh-stimulated RhoA activity was inhibited also by cBIMPS (73 ± 6%), and the inhibition was completely blocked by myristoylated PKI but was not affected by KT5823 (Fig. 6). VIP inhibited ACh-stimulated RhoA activity by 94 ± 6%; the inhibition was partly blocked by myristoylated PKI and KT5823 and was completely blocked by a combination of both kinase inhibitors, consistent with the ability of VIP to activate both PKA and PKG (Fig. 5).
Absence of PKA/PKG-dependent phosphorylation and inhibition of RhoA activity in phosphorylation site-deficient RhoA188. Previous studies (17, 18, 30, 31) have shown that RhoA can be phosphorylated in vitro by PKA and PKG at Ser188 close to its COOH terminus and that mutation of this residue to Ala prevents phosphorylation by either kinase. In cells overexpressing myc-tagged wild-type RhoA, both SNP and VIP inhibited ACh-stimulated activation of RhoA. Neither SNP nor VIP had any effect on RhoA activation in cells overexpressing RhoA188 (Fig. 7A). Similar results were obtained when RhoA activation was measured directly using the GST-tagged fusion protein Rhotekin (Fig. 7B). The effect of SNP and VIP on RhoA phosphorylation was examined in cells overexpressing myc-tagged RhoA188. No phosphorylation was observed when immunoprecipitation was performed using myc antibody (Fig. 7C). Significant phosphorylation of endogenous wild-type RhoA was observed when immunoprecipitation was performed using RhoA antibody (Fig. 7C). The extent of phosphorylation was small when compared with phosphorylation obtained when wild-type RhoA was overexpressed. Results shown in Fig. 7 confirm that PKA and PKG phosphorylate RhoA at Ser188 in
vivo and that phosphorylation at this site is essential for PKA- and PKG-dependent inactivation of RhoA.

Effects of PKA- and PKG-dependent phosphorylation on translocation of RhoA. Activation of RhoA requires translocation of inactive RhoA to the membrane in which p115RhoGEF mediates the exchange of GTP for GDP (7–9). We examined whether phosphorylation by PKG and PKA accelerated translocation of membrane-bound RhoA back to the cytosol. Treatment of ACh-stimulated cells with SNP or VIP decreased the amount of membrane-bound RhoA, suggesting that RhoA phosphorylation accelerated the translocation of RhoA back to the cytosol (Fig. 8). To examine further whether inhibition of RhoA activity could be distinguished from the effect of accelerated translocation of RhoA back to the cytosol, the effects of SNP and VIP on translocation were measured in cells overexpressing GTPase-resistant RhoV14, which is predominantly membrane bound. Treatment of these cells with SNP and VIP decreased the amount of membrane-bound RhoA, suggesting that translocation was independent of inactivation (exchange of GDP for GTP) (Fig. 8).

Phosphorylation probably decreases the binding of wild-type RhoA or RhoV14 to the membrane by increasing RhoA binding to GDI. The latter appears to extract RhoA from the membrane by competing with membrane lipids for binding to the geranylgeranylated COOH terminus of RhoA (2, 5, 12). Consistent with this notion, SNP and VIP had no effect on the amount of membrane-bound RhoA in cells overexpressing phosphorylation site-deficient RhoA188 (Fig. 8).

Inhibition of ACh-stimulated RhoA/Rho kinase association by PKA and PKG. Translocation of RhoA back to the cytosol should prevent its association with and activation of two membrane-bound downstream effectors, Rho kinase and PLD. Treatment of dispersed smooth muscle cells with SNP or VIP decreased ACh-stimulated association of RhoA with Rho kinase (Fig. 9). In cultured smooth muscle cells overexpressing RhoV14, Rho kinase was predominantly associated with membrane-bound RhoA. Treatment of these cells with SNP or VIP decreased RhoA/Rho kinase association concurrently with translocation of RhoA back to the cytosol. Inhibition of RhoA/Rho kinase association by

![Fig. 5. Inhibition of ACh-stimulated RhoA activation by SNP and VIP. Freshly dispersed gastric smooth muscle cells labeled with 32P were stimulated with ACh (0.1 μM) and then treated with SNP (1 μM) (A) or VIP (1 μM) (B) in the presence or absence of KT5823 (1 μM) or myristoylated PKI (1 μM). Activity was determined in RhoA immunoprecipitates from the ratio of [32P]GTP/GDP as described in MATERIALS AND METHODS. Results are expressed as %GTP incorporation. Values are means ± SE of 4 experiments.](http://ajpgi.physiology.org/)

![Fig. 6. Inhibition of ACh-stimulated RhoA activation by selective activators of PKA and PKG. Freshly dispersed gastric smooth muscle cells labeled with 32P were stimulated with ACh (0.1 μM) and were then treated with 8-pCPT-cGMP (10 μM) (A) or cbIMPS (10 μM) (B) in the presence or absence of KT5823 (1 μM) or myristoylated PKI (1 μM). Activity was determined in RhoA immunoprecipitates from the ratio of [32P]GTP/GDP as described in MATERIALS AND METHODS. Results are expressed as %GTP incorporation. Values are means ± SE of 4 experiments.](http://ajpgi.physiology.org/)

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SNP and VIP was absent in cultured smooth muscle cells overexpressing phosphorylation site-deficient RhoA188 (Fig. 9).

Inhibition of ACh-stimulated Rho kinase and PLD activities by PKA and PKG. We (27) have previously shown that ACh and other contractile agonists stimulated Rho kinase and PLD activities and that both activities were strongly inhibited in cultured smooth muscle cells overexpressing dominat-negative RhoA188. Here we show that ACh-stimulated Rho kinase and PLD activities in freshly dispersed smooth muscle cells were inhibited by SNP (86 ± 4 and 81 ± 7% inhibition, respectively), and the inhibition was completely reversed by KT5823 but not by myristoylated PKI (Fig. 10). ACh-stimulated Rho kinase and PLD activities were inhibited also by VIP (78 ± 6 and 74 ± 4%, respectively). Inhibition was partly reversed by KT5823 (42 ± 3 and 50 ± 6%) and myristoylated PKI (33 ± 5 and 42 ± 3%) and completely reversed by a combination of both kinase inhibitors (Fig. 10).

Both SNP and VIP inhibited Rho kinase activity in smooth muscle cells overexpressing constitutively active RhoV14 but had no effect in cells overexpressing...
To determine whether PKG and/or PKA could inhibit sustained MLC\textsubscript{20} phosphorylation and muscle contraction by acting on effectors downstream of RhoA, MLC\textsubscript{20} phosphorylation was measured in smooth muscle cells overexpressing wild-type RhoA and phosphorylation site-deficient Rho\textsuperscript{A188}. As shown in Figs. 7, 10, and 11, ACh-stimulated RhoA and Rho kinase activities were similar in smooth muscle cells overexpressing wild-type RhoA and Rho\textsuperscript{A188} suggesting that RhoA-dependent downstream mechanisms were not affected by the mutation. VIP and SNP, as well as cBIMPS and 8-pCPT-cGMP, virtually abolished ACh-stimulated MLC\textsubscript{20} phosphorylation in cells overexpressing wild-type RhoA but only partly inhibited MLC\textsubscript{20} phosphorylation in cells overexpressing Rho\textsuperscript{A188} (Fig. 14). Complete inhibition of MLC\textsubscript{20} phosphorylation in cells overexpressing wild-type RhoA was accompanied by complete inhibition of RhoA and Rho kinase activities (Figs. 7 and 10), precluding the involvement of mechanisms downstream of RhoA. However, partial inhibition by PKG and PKA in muscle cells overexpressing Rho\textsuperscript{A188} suggested the existence of a subsidiary inhibi-
The crucial role of RhoA in mediating sustained MLC\textsubscript{20} phosphorylation and muscle contraction has focused attention on the ability of PKA and/or PKG to induce relaxation by inactivating RhoA and/or RhoA-dependent downstream effectors (4, 30). Two RhoA-dependent convergent pathways that lead to inhibition of MLC phosphatase and enhanced phosphorylation of MLC\textsubscript{20} have been characterized. The two major effectors within these pathways are the regulatory subunit of MLC phosphatase MYPT1 and the PKC-dependent inhibitor of MLC phosphatase CPI-17 (3, 6, 32, 35). Here we show that, whereas RhoA is the main target of PKG and PKA, there exists a subsidiary target downstream of RhoA normally masked by inactivation of RhoA.

PKG and PKA induced relaxation by phosphorylating activated, membrane-bound RhoA and accelerating its inactivation (i.e., exchange of GDP for GTP) and its dissociation from membrane-bound substrates (i.e., translocation back to the cytosol). In cultured smooth muscle cells overexpressing wild-type RhoA, PKA and PKG abolished Rho kinase activity and MLC\textsubscript{20} phosphorylation in cells overexpressing wild-type RhoA, but they had little or no effect on Rho kinase activity in cells overexpressing Rho\textsuperscript{A188}, yet they partly inhibited MLC\textsubscript{20} phosphorylation. The partial inhibition of MLC\textsubscript{20} phosphorylation suggested the existence of an independent inhibitory mechanism downstream of RhoA normally masked in cells expressing wild-type RhoA by PKA and PKG-dependent phosphorylation and inactivation of RhoA (29, 33).

These results differ from those of Sauzeau et al. (30), who used a different experimental approach in which endogenous RhoA was not activated by agonists but was added exogenously. In these experiments, both exogenous RhoA and Rho\textsuperscript{A188} induced contraction in permeabilized vascular muscle strips, but only contraction induced by RhoA was inhibited by 8-Br-cGMP, suggesting the absence of mechanisms distal to RhoA. In contrast, Surks et al. (33) detected a PKG-dependent inhibitory mechanism downstream of RhoA normally masked by upstream inactivation of RhoA.

**DISCUSSION**

The crucial role of RhoA in mediating sustained MLC\textsubscript{20} phosphorylation and muscle contraction has focused attention on the ability of PKA and/or PKG to induce relaxation by inactivating RhoA and/or RhoA-dependent downstream effectors (4, 30). Two RhoA-dependent convergent pathways that lead to inhibition of MLC phosphatase and enhanced phosphorylation of MLC\textsubscript{20} have been characterized. The two major effectors within these pathways are the regulatory subunit of MLC phosphatase MYPT1 and the PKC-dependent inhibitor of MLC phosphatase CPI-17 (3, 6, 32, 35). Here we show that, whereas RhoA is the main target of PKG and PKA, there exists a subsidiary target downstream of RhoA normally masked by inactivation of RhoA.

PKG and PKA induced relaxation by phosphorylating activated, membrane-bound RhoA and accelerating its inactivation (i.e., exchange of GDP for GTP) and its dissociation from membrane-bound substrates (i.e., translocation back to the cytosol). In cultured smooth muscle cells overexpressing wild-type RhoA, PKA and PKG abolished Rho kinase activity and MLC\textsubscript{20} phosphorylation in cells overexpressing wild-type RhoA, but they had little or no effect on Rho kinase activity in cells overexpressing Rho\textsuperscript{A188}, yet they partly inhibited MLC\textsubscript{20} phosphorylation. The partial inhibition of MLC\textsubscript{20} phosphorylation suggested the existence of an independent inhibitory mechanism downstream of RhoA normally masked in cells expressing wild-type RhoA by PKA and PKG-dependent phosphorylation and inactivation of RhoA (29, 33).

These results differ from those of Sauzeau et al. (30), who used a different experimental approach in which endogenous RhoA was not activated by agonists but was added exogenously. In these experiments, both exogenous RhoA and Rho\textsuperscript{A188} induced contraction in permeabilized vascular muscle strips, but only contraction induced by RhoA was inhibited by 8-Br-cGMP, suggesting the absence of mechanisms distal to RhoA. In contrast, Surks et al. (33) detected a PKG-dependent inhibitory mechanism downstream of RhoA normally masked by upstream inactivation of RhoA.

**DISCUSSION**

The crucial role of RhoA in mediating sustained MLC\textsubscript{20} phosphorylation and muscle contraction has focused attention on the ability of PKA and/or PKG to induce relaxation by inactivating RhoA and/or RhoA-dependent downstream effectors (4, 30). Two RhoA-dependent convergent pathways that lead to inhibition of MLC phosphatase and enhanced phosphorylation of MLC\textsubscript{20} have been characterized. The two major effectors within these pathways are the regulatory subunit of MLC phosphatase MYPT1 and the PKC-dependent inhibitor of MLC phosphatase CPI-17 (3, 6, 32, 35). Here we show that, whereas RhoA is the main target of PKG and PKA, there exists a subsidiary target downstream of RhoA normally masked by inactivation of RhoA.

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dent mechanism that could potentially function downstream of RhoA. They showed that blocking the interaction of the NH2-terminal leucine zipper sequence of PKG-I/H9251 with MYPT1 partly reversed the dephosphorylation of MLC20 by 8-Br-cGMP, but they did not determine whether phosphorylation of RhoA contributed to dephosphorylation of MLC20.

The ability of RhoA phosphorylation to abolish MLC20 phosphorylation precludes the necessity but does not eliminate the possibility for inactivation of targets downstream of RhoA by PKG or PKA. A likely candidate is the Rho kinase target, MYPT1, which binds selectively to PKG-I/H9251 but can be phosphorylated by PKA at Thr853 (10, 33). Phosphorylation by both kinases is assumed to enhance MLC phosphatase activity by uncoupling the catalytic subunit from membrane-bound MYPT1. The possibility that CPI-17, which is also located downstream of RhoA and is known to regulate MLC phosphatase activity, may, in turn, be regulated by PKG and/or PKA has not been explored.

It is not clear how phosphorylation of RhoA at Ser188 accelerates its inactivation and translocation from the membrane. In the resting state, inactive GDP-bound RhoA is mainly present in the cytosol bound to GDI for which it has high affinity (7, 17). GDI binds to RhoA via its geranylated COOH terminus, close to the site of phosphorylation (Ser188) by PKA and PKG (17). Consequently, little or no phosphorylation by PKA or PKG was observed in the present study in the basal state. Inactivation of membrane-bound RhoA involves GTP hydrolysis and translocation of RhoA back to the cytosol via a noncatalytic association with GDI; the latter appears to compete with membrane phospholipids for binding to inactivated RhoA (5, 17). Though a distinct process, translocation of RhoA back to the cytosol was tantamount to inactivation, since it diverted RhoA from its membrane-bound substrates, chiefly, Rho kinase and PLD. It is possible that other components involved in the process of inactivation or translocation are susceptible to regulatory phosphorylation by PKG and PKA. Phosphorylation of GDI, a known substrate for PKG, is reported to stabilize the inactive Rho-GDI complex in the cytosol (2).

In summary, this study shows that both PKG and PKA act preferentially on RhoA to inhibit sustained MLC20 phosphorylation and muscle contraction. Both kinases phosphorylate RhoA, causing its prompt inactivation and translocation back to the cytosol away from its membrane-bound substrates, Rho kinase and PLD. The two pathways, Rho kinase/MYPT1 and PLD/PKC/CPI-17, that converge to inhibit MLC phospho-

![Fig. 13. Inhibition of ACh-induced sustained muscle contraction by selective activators of PKG and PKA. Freshly dispersed muscle cells were treated with ACh (0.1 μM) for 5 min and then treated with 8-pCPT-cGMP (10 μM) or cBIMPS (10 μM) in the presence or absence of KT5823 or myristoylated PKI. Muscle contraction expressed as % decrease in control cell length: 106 ± 4 μm; decrease in muscle cell length at 5 min: 24 ± 3 μm or 21 ± 2%. Values are means ± SE of 4–5 experiments.](http://ajpgi.physiology.org/)

![Fig. 14. Inhibition of ACh-induced myosin light-chain (MLC)20 phosphorylation by SNP and VIP. Cultured smooth muscle cells overexpressing wild-type RhoA or RhoA188 were stimulated with ACh (0.1 μM) and then treated with SNP (1 μM) or VIP (1 μM) (A) and with 8-pCPT-cGMP (10 μM) or cBIMPS (10 μM) (B). MLC20 phosphorylation was determined using phosphospecific antibody. ACh-stimulated sustained MLC20 was partly inhibited in muscle cells expressing RhoA188. Values are means ± SE of 3 experiments.](http://ajpgi.physiology.org/)

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tase are blocked, resulting in dephosphorylation of MLC20 and muscle relaxation. The direct inactivation of RhoA masks a possible action of PKG and PKA on targets downstream of RhoA.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-28300.

REFERENCES


