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# $Ca^{2+}$-independent, inhibitory effects of cyclic AMP on $Ca^{2+}$ regulation of phosphoinositide 3-kinase C2α, Rho and myosin phosphatase in vascular smooth muscle

Mohammed Ali Azam, Kazuaki Yoshioka, Shinsuke Ohkura, Noriko Takuwa, Naotoshi Sugimoto, Koichi Sato, and Yoh Takuwa

Department of Physiology (MAA, KY, NS, and YT), Kanazawa University Graduate School of Medicine, Kanazawa, Kanazawa 920-8640, Japan

Department of Veterinary Pharmacology (SO, KS), Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

Department of Health and Medical Sciences (NT), Ishikawa Prefectural Nursing University, Kahoku 929-1212, Japan.

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**Running title:** Cyclic AMP inhibits PI3K-C2α-dependent Rho activation

**Corresponding author:** Yoh Takuwa, M.D., Ph.D., Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan. FAX:+81-76-234-4223 TEL:+81-76-265-2165 E-mail:ytakuwa@med.kanazawa-u.ac.jp

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Abbreviations: FSK, forskolin; MLCK, myosin light chain kinase; MLCP, myosin phosphatase; MLC, 20 kDa myosin light chain; PI3K-C2α, phosphoinositide 3-kinase class II α isoform; MYPT1, myosin targeting protein 1; CPI-17, 17-kDa PKC-potentiated inhibitory protein of PP1; EGFP, enhanced green fluorescent protein; ISO, isoproterenol

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## ABSTRACT

We have recently demonstrated in vascular smooth muscle (VSM) that membrane depolarization by high KCl induces $Ca^{2+}$-dependent Rho activation and myosin phosphatase (MLCP) inhibition ($Ca^{2+}$-induced $Ca^{2+}$-sensitization) through the mechanisms involving phosphorylation of MYPT1 and CPI-17. In the present study, we investigated whether and how cyclic AMP affected $Ca^{2+}$-dependent MLCP inhibiton by examining the effects of forskolin, cell permeable dibutyryl cyclic AMP (dbcAMP) and isoproterenol. Forskolin, but not its inactive analogue 1,9-dideoxyforskolin, inhibited KCl-induced contraction and the 20 kDa myosin light chain (MLC) phosphorylation without inhibiting $Ca^{2+}$ mobilization in rabbit aortic VSM. DbcAMP mimicked these forskolin effects. We recently suggested that $Ca^{2+}$-mediated Rho activation is dependent on class II $\alpha$-isoform of phosphoinositide 3-kinase (PI3K-C2$\alpha$). Forskolin inhibited KCl-induced stimulation of PI3K-C2$\alpha$ activity. KCl-induced membrane depolarization stimulated Rho in a manner dependent on a PI3K but not PKC (protein kinase C), and stimulated phosphorylation of MYPT1 at $Thr^{850}$ and CPI-17 at $Thr^{38}$ in manners dependent on both PI3K and Rho-kinase, but not PKC. Forskolin, dbcAMP and isoproterenol inhibited KCl-induced Rho activation and phosphorylation of MYPT1 and CPI-17. Consistent with these data, either forskolin, isoproterenol, a PI3K inhibitor, or a Rho kinase inhibitor, but not a PKC inhibitor, abolished KCl-induced di-phosphorylation of MLC. These observations indicate that cyclic AMP inhibits $Ca^{2+}$-mediated activation of the MLCP-regulating signaling pathway comprising PI3K-C2$\alpha$, Rho, and Rho kinase in a manner independent of $Ca^{2+}$, and point to the novel mechanism of the cyclic AMP actions in the regulation of vascular smooth muscle contraction.

## INTRODUCTION

$Ca^{2+}$ plays a primary role in the regulation of smooth muscle contraction (Somlyo and Somlyo, 1994). Excitatory receptor agonist stimulation and membrane depolarization induce an increase in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through phospholipase C-catalyzed generation of inositol-1,4,5-trisphosphate ($IP_3$) and/or gating of plasma membrane $Ca^{2+}$ channels, resulting in the activation of the $Ca^{2+}$- and calmodulin-dependent enzyme myosin light chain kinase (MLCK). Receptor agonists also activate Rho and Rho kinase, which leads to inhibition of myosin phosphatase (MLCP) through mechanisms involving Rho kinase-dependent phosphorylation of the MLCP-regulatory proteins MYPT1 and CPI-17 (Noda et al., 1995; Pfitzer, 2001; Somlyo and Somlyo, 2003; Hartshorne et al., 2004). CPI-17 is phosphorylated by the protein kinase C-dependent mechanism as well (Eto et al., 1995; Eto et al., 2001). We and others previously demonstrated in vascular smooth muscle (VSM) that membrane depolarization induces Rho activation and consequent Rho kinase-dependent MLCP inhibition in a $Ca^{2+}$-dependent manner ($Ca^{2+}$-induced $Ca^{2+}$-sensitization) (Mita et al., 2002; Sakurada et al., 2003; Takuwa et al., 2005). Thus, in receptor agonist- and membrane depolarization-stimulated muscle, MLCK activation and MLCP inhibition synergistically induce effective phosphorylation of the 20-kDa myosin light chain (MLC) and a contractile response. We have recently suggested that a phosphoinositide 3-kinase (PI3K) is essential for the $Ca^{2+}$-dependent Rho activation in VSM (Wang et al., 2006). Several lines of evidence implicated PI3K class II α-isoform (PI3K-C2α) in the $Ca^{2+}$-dependent Rho activation; an increase in the $[Ca^{2+}]_i$ induced activation of PI3K-C2α, but not class I p110α;

only PI3K-C2α exhibited a characteristically low sensitivity to the PI3K inhibitors LY294002 and wortmannin compared with the other members of PI3K family (Stein and Waterfield, 2000), and suppression of $Ca^{2+}$-dependent Rho activation and MLCP inhibition required relatively higher concentrations of these PI3K inhibitors; selective PI3K-C2α knockdown by siRNA inhibited contraction.

The second messenger adenosine 3', 5'-cyclic monophosphate (cAMP) mediates relaxation of smooth muscle (Somlyo and Somlyo, 1994). Cyclic AMP was previously shown to induce a reduction in the $[Ca^{2+}]_i$ through multiple mechanisms including inhibition of phospholipase C stimulation, inhibition of $Ca^{2+}$ influx across the plasma membrane and stimulation of $Ca^{2+}$ extrusion from the cell interior, resulting in inhibition of MLCK activation and MLC phosphorylation (Somlyo and Somlyo 2003). On the other hand, cAMP inhibited contraction in permeabilized smooth muscle (Ruegg et al., 1981; Nishimura and van Breemen, 1989), suggesting that cAMP also exerted a relaxing effect that was independent of the $[Ca^{2+}]_i$ lowering effect. Subsequent investigations showed that cAMP as well as guanosine 3', 5'-cyclic monophosphate (cGMP) stimulated MLCP (Wu et al., 1996; Lee et al., 1997; Etter et al., 2001; Bonnevier et al., 2004), thereby mediating inhibition of MLC phosphorylation and contraction. More recent investigations (Sauzeau et al., 2000; Murthy et al., 2003; Wooldridge et al., 2004) suggested that cAMP might stimulate MLCP through mechanisms involving phosphorylation of Rho and/or MYPT1. Very recently, it was shown that forskolin (FSK) inhibited KCl-induced MYPT1 phosphorylation without inhibiting the intracellular $Ca^{2+}$ increase and stimulated relaxation of $Ca^{2+}$-induced contraction (Porter et al., 2006). However, it is unknown whether cAMP

affects the unique $Ca^{2+}$-dependent activation of PI3K-C2α and Rho in smooth muscle.

Membrane depolarization by high KCl induces an increase in the $[Ca^{2+}]_i$ through gating of the L-type voltage-dependent $Ca^{2+}$ channel (Sato et al., 1989). Cyclic AMP is much less effective in inhibiting membrane depolarization-induced $Ca^{2+}$ mobilization compared with receptor agonist-induced $Ca^{2+}$ mobilization (Takuwa and Rasmussen, 1987; Sato et al., 1988). In the present study, we employed the experimental condition in which cAMP does not significantly inhibit membrane depolarization-induced $Ca^{2+}$ mobilization but inhibits MLC phosphorylation and contraction in isolated rabbit VSM, and investigated the effects of cAMP on $Ca^{2+}$-dependent activation of PI3K-C2α and Rho and phosphorylation of MYPT1 and CPI-17. Our results show that without significantly inhibiting membrane depolarization-induced $[Ca^{2+}]_i$ increase, cAMP inhibits membrane depolarization-induced activation of PI3K-C2α and Rho, reversing phosphorylation of MYPT1 and CPI-17. These findings together with the recent observations (Porter et al., 2006) indicate that cAMP reverses $Ca^{2+}$-induced MLCP inhibition and suggest that the site of the cAMP action involves the activation of PI3K-C2α.



## MATERIALS AND METHODS

### Materials

Forskolin, 1,9-dideoxy-forskolin (dd-forskolin), PI (phosphatidylinositol), phorbol 12,13-dibutyrate (PDBu), N6, 2'-O-dibutyryl cAMP (dbcAMP) and isoproterenol (ISO)

were bought from Sigma-Aldrich. LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] and GF109203X {2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide} were purchased from Merck-Calbiochem Biosciences (Darmstadt, Germany)). Y27632 [(R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl] was donated by WelFide corporation (Osaka, Japan). LY294002, GF109203X and Y27632 were dissolved in dimethylsulfoxide. Forskolin and dd-forskolin were dissolved in ethanol. The solvent concentrations in the incubation buffers did not exceed 0.1%. Monoclonal anti-MLC antibody (MY-21) and anti–RhoA antibody (26C4) were purchased from Sigma-Aldrich and Santa Cruz Biotechnology, respectively. Rabbit polyclonal anti-phospho (Thr$^{38}$)-CPI-17 (36-006), anti-CPI-17 (07-344), anti-phospho (Thr$^{850}$)-MYPT1 (36-003) and phospho-MYPT1 (Thr$^{695}$) (07-251) were bought from Upstate Biotechnology. Rabbit polyclonal anti-MYPT1 antibody (PRB-457C) was bought from Covance (Berkley, CA, USA). Monoclonal antibody to PI3K-C2α (611046) for western blotting was bought from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal anti-PI3K-C2α antibody was raised in our laboratory as described elsewhere (Wang et al., 2006). Glutathione-*s*-transferase (GST)-Rhotekin was prepared as described previously (Sakurada et al., 2001).

**Tissue preparation and tension measurement**

The Animals were maintained in compliance with the guidelines of the Care and Use of Laboratory Animals in Kanazawa University. Japanese male white rabbits weighing

approximately 2.0 kg were sacrificed by injecting overdoses (60 mg/kg body weight) of sodium pentobarbital into the ear vein (Sakurada et al., 2001). Descending thoracic aortae were removed and immediately placed into ice cold modified Krebs-Henseleit buffer comprising (in mM) NaCl 119, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.5, $CaCl_2$ 1.5, $NaHCO_3$ 25 and Glucose 11 and aerated with 95% $O_2$ and 5% $CO_2$. The aorta was cleaned off of adhering connective tissue and fat and cut into rings of about 4-5 mm length. The endothelial cells were removed by gentle rubbing of the intimal surface with a wooden stick. The rings were mounted under 1gm of resting tension in static incubation muscle chambers, equilibrated in the modified Krebs-Henseleit buffer at 37°C and gasses with 95% $O_2$ and 5% $CO_2$. The tension generated was measured isometrically with force-displacement transducers (UM–203; Kishimoto Medical Instruments, Kyoto, Japan). Before test stimulation, the rings were pre-contracted several times with 60 mM KCl containing buffer followed by washing with the modified Krebs-Henseleit buffer, each time for 5 minutes at one hour interval until a stable contraction was obtained.

**Measurement of the $[Ca^{2+}]_i$ in aortic VSM**

The method for determination of the $[Ca^{2+}]_i$ was previously described in detail (Sato et al., 1988; Sato et al., 2000). Briefly, rabbit thoracic aorta was removed and cut into helical strips, followed by removal of endothelium by gently rubbing with a cotton swab. VSM strips were loaded with the acetoxymethyl ester of fura-PE3 (fura-PE3-AM) (TEFLABS, Austin, TX, USA) by incubating in the normal HEPES-buffered solution comprising (in mM) NaCl 125.4, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, Glucose 11, and HEPES 10 (pH7.4)) in

the presence of 10 μM fura-PE3-AM and 0.02% Cremophor EL for 4-6 h at room temperature in the dark room. VSM strips were placed in a tissue bath containing the HEPES-buffered solution aerated with 100% $O_2$ at 37°C. Strips were illuminated alternately with 340 and 380 nm light and the fluorescence strength of 500 nm emission at 340 and 380 nm excitation (F340 and F380, respectively) was detected with a CAF-100 spectrofluorimeter (Japan Spectroscopic, Tokyo, Japan). A change in the $[Ca^{2+}]_i$ was determined by monitoring the ratio of F340/F380. KCl stimulated $Mn^{2+}$ quenching of fura-PE3 fluorescence at 360 nm excitation in the presence of 0.5 mM $Mn^{2+}$ in the buffer, indicating that KCl stimulated $Ca^{2+}$ influx across the plasma membrane. VSM strips were first stimulated with 40 mM KCl in the absence of FSK, then treated or non-treated with 30 μM FSK for 20 min, and stimulated with KCl. Because the ratio of F340/F380 at the non-stimulated basal condition and KCl-stimulated condition was gradually reduced due to leak and bleaching of fura-PE3 during the observation periods, the ratio of F340/F380 at the 2nd KCl stimulation was expressed as a percentage of the value at the first KCl stimulation.

**Rho assay**

Aortic rings contracted isometrically were frozen by quickly immersing into liquid nitrogen. Frozen rings were homogenized with a homogenization buffer comprising 50 mM Tris/HCl (pH 7.2), 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 20 μg/ml each of leupeptin and aprotinin, and 1mM phenylmethylsulfonyl fluoride (PMSF), as described previously (Sakurada et al., 2001). The homogenates were

centrifuged at 15,000 rpm at 4°C for 10 minutes, and the resultant supernants (300μg protein) were incubated with GST-Rhotekin immobilized onto glutathione-Sepharose 4B beads (Amersham Biosciences) at 4°C for 30 minutes. Portions of the supernatants were employed for a protein assay by Lowry's method and total Rho assay by western blotting using anti-RhoA antibody. The beads were washed twice with a washing buffer comprising 50mM Tris/HCl (pH 7.2), 1% TritonX-100, 150 mM NaCl and 10mM $MgCl_2$. RhoA bound to beads were solubilized with Laemmli's SDS sample buffer and boiled for 5 minutes, followed by separation on a 15% acrylamide gel, electro-transfer onto polyvinylidene difluoride membrane and detection using anti-RhoA antibody and alkaline phosphatase-conjugated secondary antibody. Specific bands were visualized with alkakline phosphatase colorigenic reaction and densities of bands were quantitated with a densitometry using the Quantity One software. The amount of GTP-bound RhoA (GTP-Rho) was normalized for total amount of RhoA in each sample, and the quantitative data of normalized amounts of GTP-Rho are expressed as multiples over a value in unstimulated tissues, which is expressed as 1.0

**Determinations of MYPT1 and CPI-17 phosphorylation**

Aortic rings contracted isometrically were fixed in acetone dry ice slurry containing 20 mM dithiothreitol (DTT) and 10% trichloroacetic acid (TCA), and washed in acetone containing 10mM DTT at room temperature, as described previously (Wang et al., 2006). Fixed tissue were homogenized in a homogenization buffer comprising 20 mM Tris/HCl (pH 7.5), 100 mM NaF, 1 mM $Na_3Vo_4$, 0.1% SDS, 2 mM EGTA, 0.5% NP-40, 20 μg/ml each of leupeptin

and aprotinin and 1 mM PMSF. A portion of homogenates was taken for determination of a protein concentration by Lowry's method. The homogenates were mixed with 4 times concentrated Laemmli's SDS sample buffer and boiled for 5 minutes. The samples (40 μg protein) were separated on 8% SDS-PAGE, followed by western blot analysis using phospho-protein specific antibodies and antibodies that recognize both phospho- and nonphospho-proteins. The amounts of phospho-MYPT1 and phospho-CPI-17 quantitated by densitometry were normalized for total amounts of MYPT1 and CPI-17 in each sample, and the quantitative data of normalized amounts of the phospho-proteins were expressed as multiples over a value in unstimulated tissues, which is expressed as 1.0.

**Measurement of MLC phosphorylation**

Contracted aortic rings were fixed as described for determinations of MYPT1 and CPI-17 phosphorylation, and solubilized in the urea sample buffer. For determination of total MLC phosphorylation, the extracts were separated on urea-glycerol PAGE, followed by western blotting using anti-MLC antibody (MY-21) (Noda et al., 1995; Nagumo et al., 2000). Densities of bands corresponding to non-phosphorylated, mono-phosphorylated, and di-phosphorylated forms of MLC were quantitated, and the ratio of mono-phosphorylated and di-phosphorylated forms of MLC over total MLC were calculated. Because the amount of di-phosphorylated MLC was small (less than 3% of total MLC) in KCl-stimulated muscle, it was difficult to quantitatively evaluate the amount of di-phosphorylated MLC by the urea-glycerol PAGE and western blotting. Therefore, we employed western blot analysis using anti-di-phosphorylated ($Thr^{18}$ and $Ser^{19}$)

MLC-specific antibody, which was more sensitive than the urea-glycerol PAGE method, for quantitative determination of MLC di-phosphorylation. Three parts of urea-extracted samples were mixed with 1 part of 4 times concentrated Laemmli's SDS sample buffer and the extracts were separated on 15% SDS-PAGE, followed by western analysis using anti-MLC antibody (MY-21) and anti-di-phosphorylated MLC-specific antibody (Sakurada et al., 1998), which was donated by Dr. Seto (Asahi Chemical Industry, Fuji, Japan). For quantitation of MLC di-phosphorylation, a density of a band detected by anti-diphosphorylated MLC antibody was corrected by MLC protein amounts, and the results were expressed as multiples over a value in the non-treatment.

**PI3K-C2α kinase assay**

Contracted aortic rings were rapidly frozen in liquid nitrogen and homogenized in a homogenization buffer comprising 20 mM Tris/HCl (pH7.5), 150 mM NaCl, 10 mM NaF, 1% Nonidet-40, 1 mM $Na_3VO_4$, 5 mM EDTA, 20 μg/ml each of leupeptin and aprotinin and 1mM PMSF (Wang et al., 2006). The homogenates were clarified by centrifugation at 15,000 rpm at 4°C for 10 min. Portions of the supernatants were taken for determination of protein contents by Lowry's method and PI3K-C2α protein amounts by western blotting. The resultant supernatants (400 μg protein) were subjected to immunoprecipitation using specific polyclonal anti-PI3K-C2α antibody. The anti-PI3K-C2α immunoprecipitates were incubated with 50 μl of the kinase assay buffer (20 mM Tris/HCl (pH7.5), 100 mM NaCl, 0.5 mM EGTA, 20 mM $MgCl_2$, 10 μM ATP, 1.8 MBq/ml of [$\gamma$-$^{32}$P]ATP (Amersham

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Biosciences), 200 μg/ml PI) at 25°C for 30 min. The reaction was terminated by adding 100 μl of $CH_3OH/CHCl_3/HCl$ (2:1:0.02 (V:V:V)). Extracted lipid fraction was separated by thin layer chromatography using Silica gel 60 plates and the solvent system of $CH_3OH/CHCl_3/25\%NH_4OH/H_2O$ (43:38:5:7 (V:V:V)). Radioactivity in the spot corresponding to PI 3-phosphate was quantitated by using FujiBAS Bioimage analyzer 2000 (Fuji, Tokyo, Japan). The activity of PI3K-C2α was normalized for the PI3K-C2α protein amount in each sample and the quantitative data of normalized amounts of PI3P were expressed as multiples over a value in unstimulated tissues, which is expressed as 1.0.

**Statistics**

All data are shown as mean ± SEM. One-way or two-way ANOVA (analysis of variance) followed by Dunnet's test to determine the statistical significance of differences between mean values, except Fig. 1C and Fig. 4A where unpaired t-test were performed for the comparison between two groups. For all statistical comparisons, $p<0.05$ was considered significant.

## RESULTS

**Forskolin and dibutyryl cAMP inhibit membrane depolarization-induced contraction and MLC phosphorylation, but not an increase in the $[Ca^{2+}]_i$**

KCl membrane depolarization induces MLC phosphorylation and contraction strictly in

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a $Ca^{2+}$-dependent manner (Sakurada et al., 2003). The preincubation of de-endothelialized VSM with the adenylate cyclase activator FSK (30 μM) inhibited KCl (40 mM)-induced, $Ca^{2+}$-dependent contraction at both the initial and sustained phases by more than 70% (Fig. 1A). Concomitantly, FSK reduced KCl-induced increases in total MLC phosphorylation determined at 2, 5 and 10 min by 75-90%. Most (more than 95%) of the phosphorylated form of MLC was mono-phosphorylated MLC. Different from FSK, the inactive FSK analogue dd-FSK, which is incapable of stimulating adenylate cyclase (Seamon et al., 1984), failed to inhibit either contraction or MLC phosphorylation induced by KCl (Fig. 1A and B). The cell permeable cyclic AMP analogue, dbcAMP (5 mM), inhibited KCl-induced contraction and MLC phosphorylation as effectively as FSK.

We examined the effect of FSK on KCl-induced increases in the $[Ca^{2+}]_i$ by simultaneously monitoring fura-PE3 fluorescence and tension in VSM. KCl (40 mM) induced a rapid and sustained increase in the $[Ca^{2+}]_i$ as reported previously (Sato et al., 1988). Pretreatment with FSK (30 μM) inhibited KCl-induced contraction by approximately 70%, but not significantly affected KCl-induced rise in the $[Ca^{2+}]_i$ at either the peak value or the value in 10 min stimulation (Fig. 1C). The observations suggest that FSK induces inhibition of KCl-induced contraction and MLC phosphorylation largely through a $[Ca^{2+}]_i$-independent effect under the experimental condition.

**FSK inhibits membrane depolarization-induced activation of Rho and PI3K-C2α and phosphorylation of MYPT1 and CPI-17**

KCl (40 mM) induced a maximal 4-fold increase in the amount of the GTP-bound,

active form of Rho (GTP-Rho). FSK abolished KCl-induced increases in the amount of GTP-Rho at every time point examined (Fig. 2A). The addition of dbcAMP also abolished KCl-induced Rho activation (Fig. 2B). We recently showed that PI3K-C2α is involved in $Ca^{2+}$-mediated Rho activation in VSM (Wang et al., 2006). Therefore, we explored the possible inhibitory effect of FSK on KCl-induced PI3K-C2α stimulation. KCl induced stimulation (approximately 2.5-fold) of PI3K-C2α activity (Fig. 2C). FSK nearly abolished KCl-induced PI3K-C2α activation. These observations suggest that the site of the FSK action in inhibiting VSM contraction involves PI3K-C2α.

Consistent with the stimulatory effect of membrane depolarization on Rho activity (Fig. 2A), KCl induced several fold increases in $Thr^{850}$ phosphorylation of MYPT1 (numbering of chicken M133 isoform) at 5 and 10 min (Fig. 3A). FSK abolished KCl-induced MYPT1 phosphorylation at $Thr^{850}$. KCl membrane depolarization also stimulated MYPT1 phosphorylation at $Thr^{695}$ although slightly (approximately maximal 50% increase) (Fig. 3B). FSK partially inhibited MYPT1 phosphorylation at $Thr^{695}$. KCl membrane depolarization also induced 2-fold stimulation of CPI-17 phosphorylation at $Thr^{38}$ (Fig. 3C). FSK abolished KCl-induced stimulation of CPI-17 phosphorylation. The di-phosphorylation of MLC is stimulated when MLCK activity becomes very high or MLCP is inhibited (Somlyo and Somlyo, 2003). We previously showed that KCl induced an increase in di-phosphorylation of MLC (Wang et al., 2006). We analyzed the effect of FSK on KCl-induced MLC di-phosphorylation by Western blotting using anti-diphospho-specific MLC antibody because this method has a high sensitivity in the quantitative detection of MLC di-phosphorylation (Sakurada et al., 1998). FSK abolished

KCl-induced MLC di-phosphorylation (Fig. 3D), which was consistent with the FSK-induced suppression of Rho and phosphorylation of MYPT1 and CPI-17.

**Isoproterenol inhibits membrane depolarization-induced activation of Rho and PI3K-C2α and phosphorylation of MYPT1 and MLC**

Isoproterenol (ISO) induces an increase in cAMP content through acting via β-adrenergic receptor. ISO inhibited KCl-induced contraction by approximately 60% (Fig. 4A). ISO strongly suppressed KCl-induced increase in the amount of GTP-Rho and MYPT1 $Thr^{850}$ phosphorylation (Fig. 4 B and C). ISO also tended to inhibit KCl-induced PI3K-C2α stimulation ($p$=0.10) (Fig. 4D). Consistent with the prevention of KCl-induced Rho activation and MYPT1 phosphorylation by ISO, ISO markedly suppressed KCl-induced increase in MLC di-phosphorylation (Fig. 4E). Thus, ISO exerted similar inhibitory effects on $Ca^{2+}$- and PI3K-C2α-dependent Rho activation, MYPT1 phosphorylation and MLC di-phosphorylation.

**Membrane depolarization-induced phosphorylation of CPI-17 and MYPT1 are PI3K- and Rho kinase-dependent**

CPI-17 phosphorylation was previously shown to be mediated by both Rho kinase and protein kinase C in vascular and other smooth muscle types (Eto et al., 2001; Niiro et al., 2003), and membrane depolarization was suggested to stimulate phospholipase C, activating protein kinase C (Slish et al., 2002). KCl membrane depolarization-induced CPI-17 phosphorylation was not inhibited by the protein kinase C inhibitor GF109203X (5

μM) (Fig. 5A). However, GF109203X abolished PDBu-induced CPI-17 phosphorylation (Fig. 5B), confirming that GF109203X was effective in inhibiting protein kinase C-mediated CPI-17 phosphorylation. Instead, either the Rho kinase inhibitor Y27632 or the PI3K inhibitor LY294002 abolished KCl-induced CPI-17 phosphorylation (Fig. 5A). In contrast, PDBu-induced CPI-17 was insensitive to Y27632. These observations together indicate that membrane depolarization-induced CPI-17 phosphorylation is dependent on PI3K and Rho kinase, different from PDBu-induced CPI-17 phosphorylation. Similarly, either LY294002 or Y27632 but not GF109203X abolished KCl-induced MYPT1 phosphorylation at $Thr^{850}$ and Rho activation (Fig. 5 C and D). LY294002 and Y27632, but not GF109203X, markedly inhibited KCl-induced contraction (Fig. 5E). Furthermore, either LY294002 or Y27632, but not GF109203X, abolished KCl-induced di-phosphorylation of MLC (Fig. 3D). These observations suggest that the PI3K/Rho/Rho kinase-dependent pathway, but not protein kinase C, plays an essential role in membrane depolarization-induced CPI-17 and MYPT1 phosphorylation, consequent MLC phosphorylation and contraction.

## DISCUSSION

An increase in the intracellular cAMP or cGMP in response to various physiological stimuli including hormones, neurotransmitters and autacoids induces relaxation in VSM contracted by excitatory receptor agonists and membrane depolarization. The relaxing actions of both cAMP and cGMP involve stimulation or de-inhibition of MLCP (Somlyo and Somlyo 2003), leading to $Ca^{2+}$-desensitization of the contractile proteins. Rho and Rho

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kinase, the major signaling pathway to negatively regulate MLCP, is activated by the receptor-coupled, $G_{12/13}$-mediated mechanism as well as the recently discovered $Ca^{2+}$-mediated, PI3K-C2α-dependent one (Pfitzer, 2001; Somlyo and Somlyo, 2003; Hartshorne et al., 2004; Takuwa et al., 2005). Receptor-coupled Rho signaling pathway is effectively inhibited by both cAMP and cGMP. On the other hand, whether and how the latter $Ca^{2+}$-mediated Rho activation and MLCP inhibition are regulated by cAMP remained unknown. The present results, together with the recent observations by Porter et al. (2006) that FSK inhibited KCl-induced MYPT1 phosphorylation and promoted relaxation of $Ca^{2+}$-induced contraction, demonstrate that cAMP can inhibit the $Ca^{2+}$-mediated Rho activation and consequent MLCP suppression without reducing the $[Ca^{2+}]_i$ in intact VSM, that the cAMP inhibition is likely mediated through prevention of Rho kinase-dependent phosphorylation of MYPT1 and CPI-17, and that the site of the cAMP inhibition involves the Rho regulator PI3K-C2α (Fig. 6).

We previously demonstrated that $Ca^{2+}$ mediated Rho activation in VSM stimulated by membrane depolarization and the $Ca^{2+}$ ionophore ionomycin (Sakurada et al., 2003). In the present study, in order to explore a possible, $Ca^{2+}$ lowering-independent effect of cAMP on $Ca^{2+}$-mediated Rho activation, we chose the concentrations of extracellular $K^+$ and FSK that do not confer effective inhibition of KCl-induced $[Ca^{2+}]_i$ increase (Fig. 1C) but strongly suppress MLC phosphorylation and contraction (Fig. 1 A and B). Under this condition, forskolin and dbcAMP abolished membrane depolarization-induced Rho activation (Fig. 2 A and B). The cAMP-elevating receptor agonist ISO also inhibited KCl-induced Rho activation (Fig. 4B). These observations indicate that cAMP exerts an

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inhibitory effect on Rho without lowering the $[Ca^{2+}]_i$. Because our recent study (Wang et al., 2006) suggested that PI3K-C2α mediates $Ca^{2+}$-induced Rho activation in VSM, we next examined the effect of FSK on KCl-induced PI3K-C2α activation. FSK nearly abolished KCl-induced PI3K-C2α activation (Fig. 2C). Thus, these observations suggest that cAMP inhibits the upstream Rho regulator PI3K-C2α, resulting in inhibition of $Ca^{2+}$-mediated Rho activation.

We showed that the PI3K inhibitor LY294002 as well as wortmannin suppressed KCl-induced, $Ca^{2+}$-dependent stimulation of PI3K-C2α, Rho, MYPT1 and MLC phosphorylation and contraction (Wang et al., 2006, and the present study). In differentiated vascular smooth muscle cells, selective PI3K-C2α downregulation by a specific short interfering RNA duplex suppressed $Ca^{2+}$-dependent phosphorylation of MYPT1and MLC, and abolished further inhibition of these responses by LY294002. These observations suggested PI3K-C2α as a target for LY294002 in vascular smooth muscle. On the other hand, it was previously reported that LY294002 but not wortmannin inhibited $Ca^{2+}$ influx through both voltage-dependent and receptor-coupled $Ca^{2+}$ channels in smooth muscle and non-muscle cells overexpressing a $Ca^{2+}$ channel (Takayama et al., 1996; Tolloczko et al., 2004; Welling et al., 2005). We observed that LY294002 at its lower concentration (10 μM) that was previously shown to inhibit voltage-dependent $Ca^{2+}$ channel (Welling et al., 2005) failed to inhibit KCl-induced $Ca^{2+}$ entry in rabbit aortic smooth muscle (Sato K et al., unpublished observation). These observations may suggest that the inhibitory effect of LY on $Ca^{2+}$ channels could be different among smooth muscle types and animal species probably because of differences in $Ca^{2+}$ channel isotypes and accessory proteins. However,

it was not possible to determine the effect of LY294002 at higher concentrations on KCl-induced $[Ca^{2+}]_i$ increase in the present study because LY294002 highly interfered with fura3 fluorescence. We cannot exclude that inhibition of $Ca^{2+}$ entry by LY contributed to LY inhibition of PI3K-C2α and Rho particularly at its higher concentrations.

A recent study by Huang et al. (2006) demonstrated that stimulation of isolated intestinal smooth muscle cells by $G_i$-coupled receptor agonists induced activation of integrin-linked kinase (ILK) through a PI3K-dependent mechanism and that ILK mediated phosphorylation of CPI-17 and MLC. The study showed that a relatively low concentration (10 μM) of LY294002 nearly abolished agonist-induced phosphorylation of CPI-17 and MLC although the responsible PI3K isoform was not identified. On the other hand, we observed that this low concentration of LY294002 failed to inhibit membrane depolarization-induced Rho activation, phosphorylation of MYPT1, CPI-17 and MLC, and contraction in intact VSM, but a higher concentration of LY294002 inhibited these $Ca^{2+}$-dependent responses (Wang et al., 2006). Our observations are quite consistent with the notion that PI3K-C2α, which is clearly less sensitive to LY294002 than the other PI3K isoforms, is responsible for the mediation of these $Ca^{2+}$-dependent responses. In addition, ILK activation is not dependent on $Ca^{2+}$, whereas activation of both PI3K-C2α and Rho by membrane depolarization is dependent on $Ca^{2+}$. The observations by Huang et al. and us may suggest that more than a single PI3K isoforms participate in MLCP regulation through different mechanisms in smooth muscle type- and stimulus-specific manners.

Accumulating evidence shows that cAMP and cGMP exert stimulating or de-inhibiting effects on MLCP by acting at more than a single sites (Somlyo and Somlyo, 2003). Protein

kinase A (PKA) and protein kinase G (PKG) were shown to phosphorylate RhoA at $Ser^{188}$ and to inhibit RhoA activity through enhancement of RhoA and GDI (GDP-dissociation inhibitor) interaction in various cell types including smooth muscle (Sauzeau et al., 2000; Murthy et al., 2003; Bonnevier et al., 2004). Antagonism of Rho and Rho kinase by cyclic nucleotides may also be caused by PKA- and PKG-mediated $G\alpha_{13}$ phosphorylation that induces inhibition of $G\alpha_{13}$-mediated Rho activation (Manganello et al., 2003). PKG was also suggested to inhibit geranyl-geranylation of Rho, a maturation process of Rho that is required for the Rho translocation to the plasma membrane (Begum et al., 2002). The $Ca^{2+}$-mediated Rho activation mechanism, together with $G\alpha_{12/13}$–coupled mechanism, contributes to excitatory receptor agonist-induced Rho activation in VSM (Sakurada et al., 2003; Takuwa et al., 2005). Therefore, the cAMP-induced PI3K-C2α inhibition may at least partially contribute to cAMP-induced Rho inhibition in receptor agonist-stimulated VSM. Both cAMP and cGMP also appear to stimulate MLCP independently of inhibiting Rho and Rho kinase because the cyclic nucleotides inhibit the activity of MLCP in Triton X100-permeabilized smooth muscle in which Rho- and Rho kinase-signaling is disrupted (Ruegg et al., 1981). The Rho mechanism-independent stimulation of MLCP by the cyclic nucleotides may involve MLCP stimulation by direct PKG binding to MLCP (Surks et al., 1999), MYPT1 phosphorylation by PKG and PKA (Wooldridge et al., 2004), and telokin phosphorylation by these kinases (Walker et al., 2001). Telokin expression is more abundant in phasic type of smooth muscle and relatively lower in tonic smooth muscle. We have shown the operation of $Ca^{2+}$- and PI3K-C2α-mediated Rho activation in tonic VSM (Wang et al., 2006). However, it is yet unknown how this $Ca^{2+}$- and PI3K-C2α-mediated

Rho activation mechanism contributes to Rho regulation in other smooth muscle types. It is likely that mechanisms by which cAMP induces MLCP stimulation might be different among various smooth muscle types.

Cyclic AMP inhibited KCl-induced, $Ca^{2+}$-induced stimulation of PI3K-C2α. It is unknown how $Ca^{2+}$ exactly induces PI3K-C2α activation and how stimulation of PI3K-C2α leads to Rho activation. Because $Ca^{2+}$ is not required for the activity of PI3K-C2α despite of the presence of C2 domain in PI3K-C2α molecule (Arcaro et al., 2000), it is unlikely that $Ca^{2+}$ directly activates PI3K-C2α. How cAMP brings about inhibition of $Ca^{2+}$-induced PI3K-C2α stimulation in a $Ca^{2+}$-independent manner also remains to be clarified.

Membrane depolarization induced a several fold increase in $Thr^{850}$ phosphorylation and a much smaller increase in $Thr^{695}$ phosphorylation of MYPT1 (Fig. 3 A and B). Membrane depolarization also induced an increase in CPI-17 phosphorylation at $Thr^{38}$ (Fig. 3C). FSK inhibited all of phosphorylation of these MLCP-regulatory proteins although FSK inhibition of MYPT1 $Thr^{695}$ phosphorylation was partial. These results suggest that the mechanism for cAMP-induced MLCP inhibition involves inhibition of phosphorylation of both MYPT1 and CPI-17. Previous studies (Eto et al., 2001) showed that excitatory receptor agonist-induced CPI-17 phosphorylation was dependent on both Rho kinase and protein kinase C. In the present study, membrane depolarization-induced phosphorylation of CPI-17 as well as MYPT1 was abolished by the Rho kinase inhibitor Y27632 but not the non-selective protein kinase C inhibitor GF109203X (Fig. 5 A and C). These results are similar to that observed previously in GTPγS-stimulated permeabilized VSM (Niiro et al.,

2003). In contrast, phorbol ester-induced CPI-17 phosphorylation was abolished by GF109203X but not Y27632 (Fig. 5B). These observations suggest that membrane depolarization-induced MLCP inhibition is caused by Rho kinase-mediated phosphorylation of both MYPT1 and CPI-17. The signaling mechanism to induce CPI-17 phosphorylation at $Thr^{38}$ appears to be different, depending on contractile stimuli.

It is known that inhibition of MLCP is associated with elevation of di-phosphorylation level of MLC (Noda et al., 1995; Somlyo and Somlyo, 2003; Hartshorne et al., 2004). We observed that membrane depolarization induced an increase in MLC di-phosphorylation (Fig. 3D), consistent with the notion that membrane depolarization induces MLCP inhibition. FSK and ISO abolished membrane depolarization-induced MLC di-phosphorylation as well as total phosphorylation, which was well in agreement with the facts that FSK and ISO suppressed Rho activation and phosphorylation of MYPT1 and CPI-17.

In conclusion, the present study demonstrates that cAMP exerts inhibitory effects on $Ca^{2+}$-mediated Rho activation and resultant MLCP suppression in a manner independent of the $Ca^{2+}$-lowering effect of cAMP. The actions of cAMP on Rho and MLCP appear to involve inhibition of the Rho regulator PI3K-C2$\alpha$. These results indicate the novel mechanism of cAMP-induced inhibition of contraction.

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## REFERENCES

Arcaro A, Zvelebil MJ, Wallasch C, Ullrich A, Waterfield MD and Domin J (2000) Class II phosphoinositide 3-kinase are downstream targets of activated polypeptide growth factor receptors. *Mol Cell Biol* **20**:3817-3830.

Begum N, Sandu OA & Duddy N (2002) Negative regulation of rho signaling by insulin and its impact on actin cytoskeleton organization in vascular smooth muscle cells: role of nitric oxide and cyclic guanosine monophosphate signaling pathways. *Diabetes* 51: 2256-2263.

Bonnevier J, Fassler R, Somlyo AP, Somlyo AV & Arner A (2004) Modulation of $Ca^{2+}$ sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *J Biol Chem* 279: 5146-5151.

Eto M, Ohomori T, Suzuki M, Furuya K and Morita F (1995) A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. *J Biochem* 118: 1104-1107.

Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y and Brautigan DL (2001) Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein of myosin phosphatase by protein kinase C alpha and delta isoforms. *J Biol Chem* **276**:29072-29078.

Etter EF, Eto M, Wardle RL, Brautigan DL & Murphy RA (2001) Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. *J Biol Chem* **276:**34681-34685.

Gudi T, Chen JC, Casteel DE, Seasholtz TM, Boss GR & Pilz RB (2002) cGMP-dependent

protein kinase inhibits serum-response element-dependent transcription by inhibiting rho activation and functions. *J Biol Chem* 277:37382-37393.

Hartshorne DJ, Ito M and Erdodi F (2004) Role of protein phosphatase type I in contractile functions: myosin phosphatase. *J Biol Chem* **279**:37211-37214.

Lee MR, Li L & Kitazawa T (1997) Cyclic GMP causes Ca2+ desensitization in vascular smooth muscle by activating the myosin light chain phosphatase. *J Biol Chem* 272:5063-5068

Manganello JM, Huang JS, Kozasa T, Voyno-Yasenetskaya TA & Le Breton GC (2003) Protein kinase A-mediated phosphorylation of the G13 switch I region alters the G13-G protein-coupled receptor complex and inhibits Rho activation. *J Biol Chem* 278:124-130.

Mita M, Yanagihara H, Hishinuma S, Saito M and Walsh MP (2002) Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem J* **364**:431-440.

Murthy KS, Zhou H, Grider JR & Makhlouf GM (2003) Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA. *Am J Physiol* 284:G1006-G1016.

Nagumo H, Sasaki Y, Ono Y, Okamoto H, Seto M and Takuwa Y (2000) Rho kinase inhibitor HA1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. *Am J Physiol* **278**:C57-C65.

Niiro N, Koga Y and Ikebe M (2003) Agonist-induced changes in the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase and CPI-17, two

regulator factors of myosin light chain phosphatase, in smooth muscle. *Biochem J* **369**:117-128.

Nishimura J & van Breemen C (1989) Direct regulation of smooth muscle contractile elements by second messengers. *Biochem Biophys Res Commun* 163:929-935

Noda M, Yasuda–Fukazawa C, Moriishi K, Kato T, Okuda T, Kurokawa K and Takuwa Y (1995) Involvement of Rho in GTPγS-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. *FEBS Lett* **367**:246-250.

Pfitzer G (2001) Regulation of myosin phosphorylation in smooth muscle. *J Appl Physiol* 91:497-503.

Porter M, Evans MC, Miner AS, Berg KM, Ward KR & Ratz PH (2006) Convergence of $Ca^{2+}$-desensitizing mechanisms activated by forskolin and phenylephrine pretreatment, but not 8-bromo-cGMP. *Am J Physiol* 290:C1552-C1559.

Ruegg JC, Sparrow MP & Mrwa U (1981) Cyclic-AMP mediated relaxation of chemically skinned fibers of smooth muscle. *Pflugers Arch* 390:198-201.

Sakurada K, Seto M and SasakiY (1998) Dynamics of myosin light chain phosphorylation at Ser19 and Thr18/Ser19 in smooth muscle cells in culture. *Am J Physiol* **274**:C1563-1C72.

Sakurada S, Okamoto H, Takuwa N, Sugimoto N and Takuwa Y (2001) Rho activation in excitatory agonist-stimulated vascular smooth muscle. *Am J Physiol* **281**:C571-C578.

Sakurada S, Takuwa N, Sgimoto N, Wang Y, Seto M, Sasaki Y and Takuwa Y (2003) $Ca^{2+}$ -dependent activation of Rho and Rho kinase in membrane depolarization-induced

and receptor stimulation-induced vascular smooth muscle contraction. *Circ Res* **93**:548-556.

Sato K, Ozaki H, Karaki H (1988) Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. *J Pharmacol Exp Ther* 246:294-300.

Sato K, Satake N, Shibata S, Adachi Y, Karaki H (2000) Mechanisms of vasoinhibitory effect of cardioprotective agent, CP-060S, in rat aorta. *Eur J Pharmacol* 21:225-230.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P & Loirand G (2000) Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle. *J Biol Chem* 275:21722-21729.

Seamon KB, Vaillancourt R, Edwards M & Daly JW. (1984) Binding of [3H]forskolin to rat brain membranes. *Proc Natl Acad Sci U.S.A.* 81:5081-5085.

Slish DF, Welsh DG & Brayden JE (2002) Diacylglycerol and protein kinase C activate cation channels involved in myogenic tone. *Am J Physiol* 283:H2196-H2201.

Somlyo AP and Somlyo AV (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**:231-236.

Somlyo AP and Somlyo AV (2003) $Ca^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* **83**:1325-1358.

Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM & Mendelsohn ME (1999) Regulation of myosin phosphatase by a specific

interaction with cGMP-dependent protein kinase Ialpha. *Science* 286:1583-1587.

Stein RC and Waterfield MD (2000) PI3-kinase inhibition: a target for drug development? *Mol Med Today* **6**:347-357.

Takayama M, Ozaki H, Karaki H (1996) Effects of a myosin light chain kinase inhibitor, wortmannin, on cytoplasmic Ca2+ levels, myosin light chain phosphorylation and force in vascular smooth muscle *Naunyn Schmiedebergs Arch Pharmacol* 354:120-127.

Takuwa Y and Rasmussen H (1987) Measurement of cytoplasmic free Ca2+ concentration in rabbit aorta using the photoprotein, aequorin. Effect of atrial natriuretic peptide on agonist-induced $Ca^{2+}$ signal generation. *J Clin Invest* **80**:248-257.

Takuwa Y, Yoshioka K, Takuwa N, Wang Y, Azam MA and Sugimoto N (2005) Calcium-dependent regulation of Rho and myosin phosphatase in vascular smooth muscle. *Biomed Res* **16**:13-21.

Tolloczko B, Turkewitsch P, Al-Chalabi M, Martin JG (2004) LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] affects calcium signaling in airway smooth muscle cells independently of phosphoinositide 3-kinase inhibition. *J Pharmacol Exp Ther* 311:787-793.

Wang Y, Yoshioka K, Azam MA, Takuwa N, Sakurada S, Kayaba Y, Sugimoto N, Inoki I, Kimura T, Kuwaki T and Takuwa Y (2006) Class II phosphoinositide 3-kinase α-isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle. *Biochem J* **394**:581-592.

Welling A, Hofmann F, Wegener JW (2005) Inhibition of L-type Cav1.2 Ca2+ channels by

2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and 2-[1-(3-dimethyl-aminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Go6983). *Mol Pharmacol* 67:541-544.

Walker LA, MacDonald JA, Liu X, Nakamoto RK, Haystead TA, Somlyo AV & Somlyo AP (2001) Site-specific phosphorylation and point mutations of telokin modulate its Ca2+-desensitizing effect in smooth muscle. *J Biol Chem* 276:24519-24524.

Wooldridge AA, MacDonald JA, Erdodi F, Ma C, Borman MA, Hartshorne DJ & Haystead TA (2004) Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of serine 695 in response to cyclic nucleotides. *J Biol Chem* 279:34496-34504.

Wu X, Somlyo AV & Somlyo AP (1996) Cyclic GMP-dependent stimulation reverses G-protein-coupled inhibition of smooth muscle myosin light chain phosphatase. *Biochem Biophys Res Commun* 220:658-663.

## Footnotes

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## Legends for Figures

**Figure 1. Inhibition of KCl-induced contraction and MLC phosphorylation, but not $[Ca^{2+}]_i$ increase by forskolin and dibutyryl cyclic AMP.**

De-endothelialized rabbit aortic rings were pre-treated with either 30 μM forskolin (FSK), or 30 μM 1,9-dideoxy-forskolin (dd-FSK) or 5 mM of the cell permeable cAMP analogue N6, 2'-O-dibutyryl cyclic AMP (dbcAMP) for 20 min, and stimulated with 40 mM KCl. (A) Isometric tension, (B) MLC total phosphorylation, and (C) $[Ca^{2+}]_i$ were determined. In (A) the tension was expressed as a percentage of 60mM KCl-induced maximal tension. Total MLC phosphorylation (the ratio of the sum of mono-phosphorylated and di-phosphorylated MLC over total MLC) was determined as described in "Materials and Methods". In (C) the $[Ca^{2+}]_i$ and tension were simultaneously monitored in fura-PE3-loaded aortic VSM strips and expressed as a percentage of the control response as described in detail in "Materials and Methods". Values are means ± SE of 4-8 determinations. The symbol ★★ denotes statistical significance at the level of $p<0.01$ compared with the group of "None".

**Figure 2. Inhibition of KCl-induced Rho activation and PI3K-C2α by forskolin**

Aortic rings were pre-treated with 30 μM FSK or 5 mM dbcAMP for 20 min or unpretreated, and then stimulated with 40 mM KCl for indicated time periods (A) or for 10 min (B and C). Aortic rings were rapidly frozen and analyzed for amounts of GTP-Rho (A and B) and PI3K-C2α activity (C) by the pull-down assay and the immunoprecipitation

kinase assay, respectively, as described in "Materials and Methods". Values are means ± SE of 4-6 determinations. The symbols ★★ and †† denote statistical significance at the level of $p<0.01$ compared with "None" and "KCl", respectively.

**Figure 3. Inhibition of KCl-induced phosphorylation MYPT1 and CPI-17 by forskolin**

Aortic rings were pre-treated with 30 μM FSK for 20 min or unpretreated, and then stimulated with 40 mM KCl for indicated time periods. Aortic rings were rapidly frozen and analyzed for phosphorylation of MYPT1 and CPI-17 by western blotting using respective anti-phosphoprotein-specific antibodies. (A) MYPT1 phosphorylation at $Thr^{850}$, (B) MYPT1 phosphorylation at $Thr^{695}$, and (C) CPI-17 phosphorylation at $Thr^{38}$. In (D), rings were pretreated with either of 5 μM GF109203X (GFX), 30 μM FSK, 100 μM LY 294002 (LY) and 10 μM Y27632 (Y) or non-pretreated for 30 min, and then stimulated with 40mM KCl for 10 min. MLC di-phosphorylation at $Ser^{18}$ and $Thr^{19}$ was determined by western blot analysis using anti-diphosphorylated MLC-specific antibody. Values are means ± SE of 4-6 determinations. The symbols ★ and ★★ denote statistical significance at the levels of $p < 0.05$ and $p < 0.01$, respectively, compared with the value in the non-stimulated rings, and the symbols † and †† denote statistical significance at the level of $p < 0.05$ and $p < 0.01$, respectively, compared with "KCl".

**Figure 4. Inhibition of KCl-induced contraction, Rho activation, MYPT1 phosphorylation, PI3K-C2α and MLC di-phosphorylation by isoproterenol**

Aortic rings were pre-treated with 100 μM ISO in the presence of 30 μM phentolamine for 10 min or unpretreated, and then stimulated with 40 mM KCl for 5 min. The isometric tension was recorded, and aortic rings were rapidly frozen and analyzed for amounts of GTP-Rho (B), MYPT1 phosphorylation at $Thr^{850}$(C), PI3K-C2α activity (D) and MLC di-phosphorylation, as described in Figs. 2 and 3. Values are means ± SE of 4 determinations. The symbols ★ and ★★ denote statistical significance at the level of $p<0.05$ and $p<0.01$, respectively, compared with "None". The symbols † and †† denote statistical significance at the level of $p<0.05$ and $p<0.01$, respectively, compared with "KCl".

**Figure 5. Inhibition of KCl-induced phosphorylation of CPI-17 and MYPT1, Rho stimulation and contraction by a PI3K inhibitor and a Rho kinase inhibitor but not a protein kinase C inhibitor**

Aortic rings were pretreated with either 5 μM GF109203X, 100 μM LY 294002 or 10 μM Y27632 for 30 min, and then stimulated with 40mM KCl for 10 min or indicated time periods, or with 1 μM PDBu for 60 min. Aortic rings were rapidly frozen and analyzed for CPI-17 $Thr^{38}$ phosphorylation (A) and (B), MYPT1 $Thr^{850}$ phosphorylation (C), and amounts of GTP-Rho (D) by western blotting using respective anti-phosphoprotein-specific antibodies or the pull-down assay. (E) Tension. Values are means ± SE of 4-6 determinations. The symbol ★★ denotes statistical significance at the level of $p < 0.01$, compared with the value in the non-stimulated rings, and the symbol †† denotes statistical

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significance at the level of $p < 0.01$, compared with "KCl" or "PDBu".

**Figure 6. The model depicting the sites of cAMP actions in the $Ca^{2+}$-induced, PI3K-C2α-dependent Rho signaling.**

KCl membrane depolarization induces stimulation of $Ca^{2+}$ influx across the plasma membrane through the voltage-dependent $Ca^{2+}$ channels (VDCC). Stimulation of $Ca^{2+}$ influx induces PI3K-C2α stimulation, which leads to Rho activation. Stimulation of $Ca^{2+}$ influx results in MLCP inhibition ($Ca^{2+}$-induced $Ca^{2+}$-sensitization) through mechanisms involving Rho- and Rho kinase-dependent phosphorylation of MYPT1 and CPI-17. $Ca^{2+}$-induced MLCK activation and MLCP inhibition synergistically induces efficient MLC phosphorylation and contraction. The adenylate cyclase (AC) activator forskolin (FSK) and the β-adrenergic agonist isoproterenol (ISO) stimulates cAMP production, which exerts inhibitory effects on $Ca^{2+}$-induced PI3K-C2α stimulation, which at least in part contributes to cAMP-induced Rho inhibition and MLCP de-inhibition. In addition to cAMP inhibition of PI3K-C2α, previous studies showed that cAMP also inhibits Rho by phosphorylating it at $Ser^{188}$, and de-inhibits MLCP by phosphorylating MYPT1 (please see "Discussion"). PM, plasma membrane; AC, adenylate cyclase; MLC-P, phosphorylated MLC; MYPT1-P, phosphorylated MYPT1; CPI17-P, phosphorylated CPI-17.

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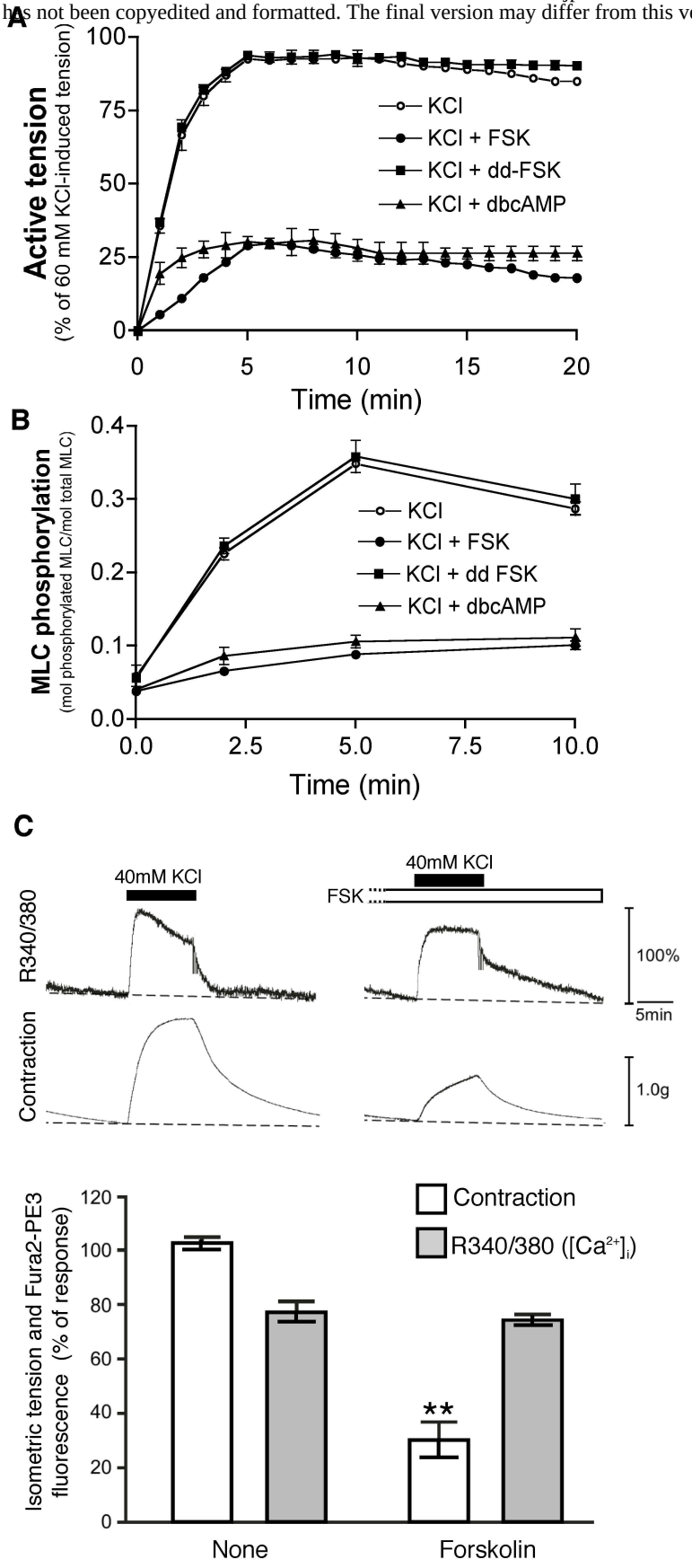


Fig.1

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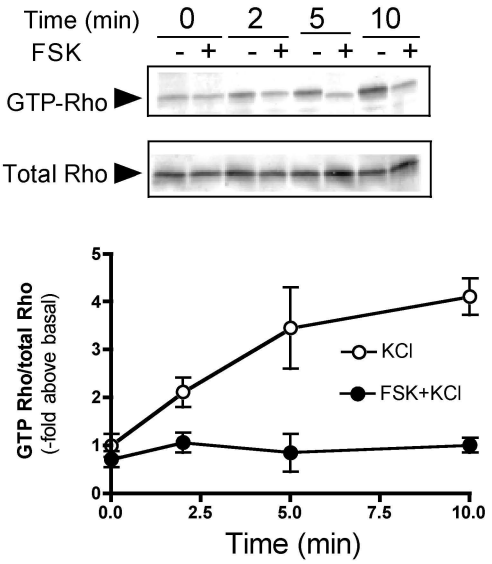


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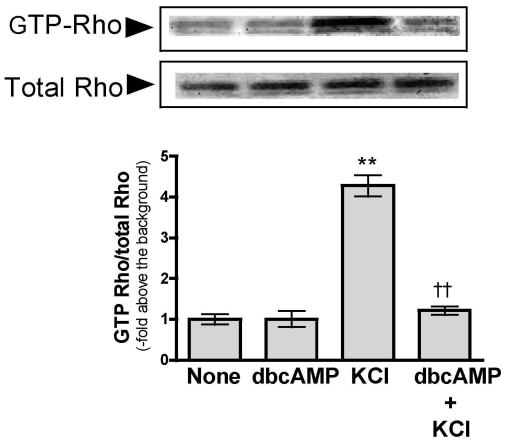


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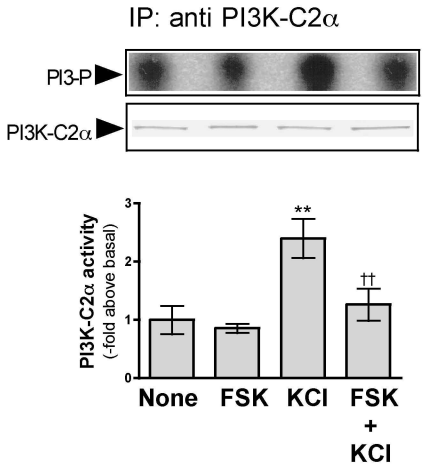


Fig. 2

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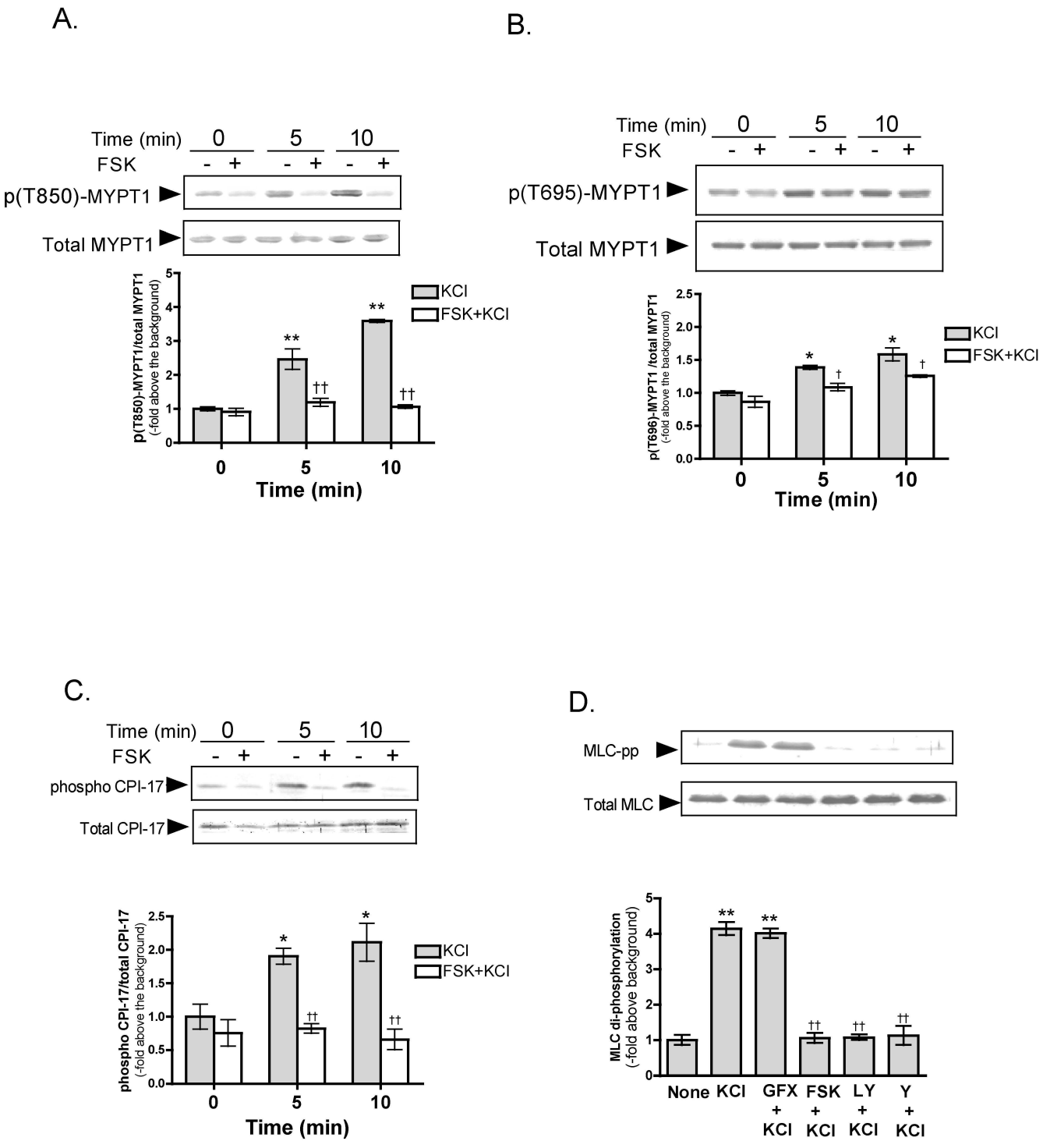


Fig. 3

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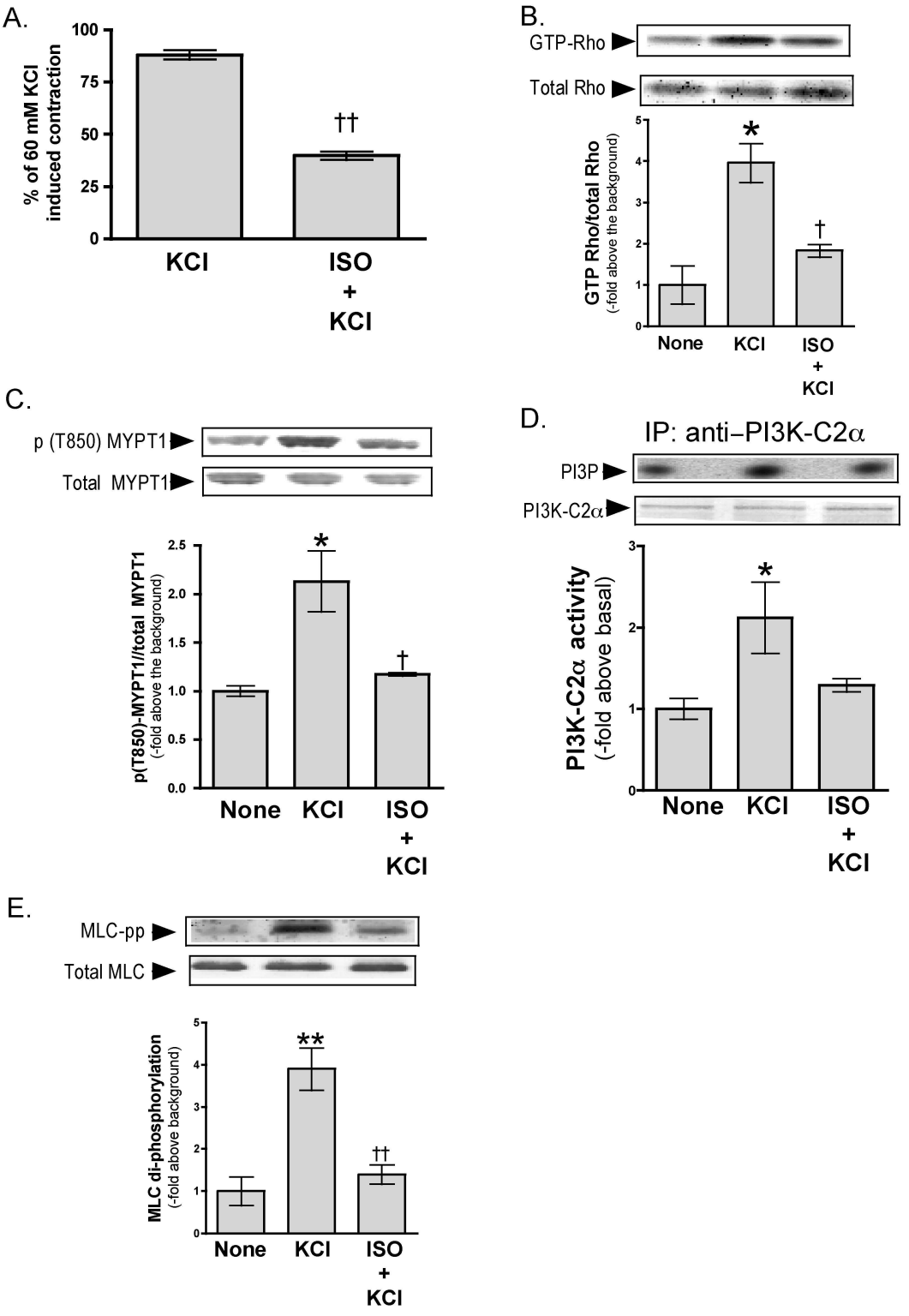


Fig. 4

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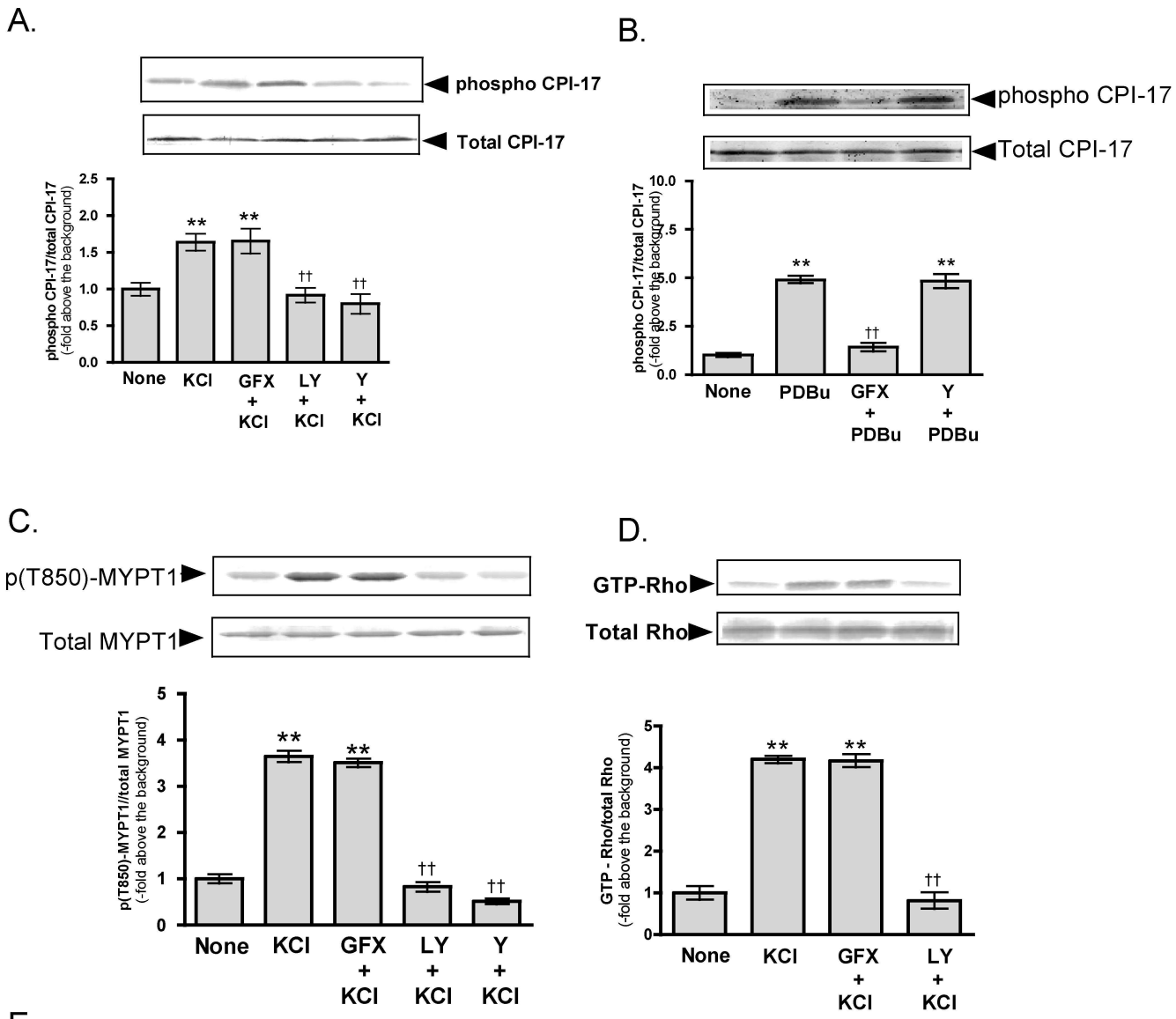


Fig. 5

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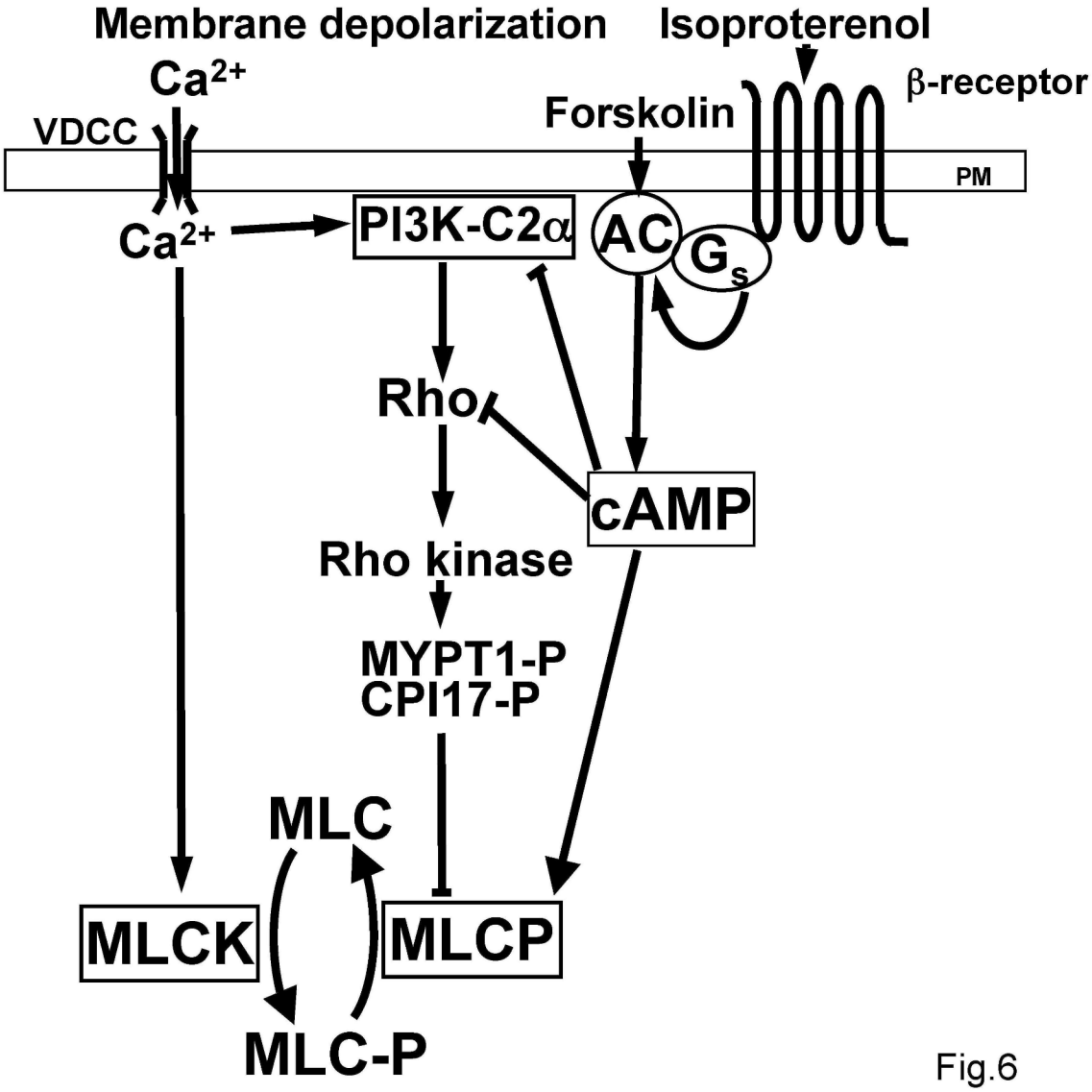


Fig.6