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Essential Role for Class II Phosphoinositide 3-kinase α-Isoform in Ca^{2+}-Induced, Rho- and Rho Kinase-Dependent Regulation of Myosin Phosphatase and Contraction in Isolated Vascular Smooth Muscle Cells[S]

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ABSTRACT

The laser confocal fluorescent microscope-based observation of contractile responses in green fluorescent protein-expressing differentiated vascular smooth muscle cells, combined with the RNA interference-mediated gene-silencing technique, allowed us to determine the role of phosphoinositide 3-kinase (PI3K) class II α-isoform (PI3K-C2α) as a novel, Ca^{2+}-dependent regulator of myosin light-chain phosphatase (MLCP) and contraction. The Ca^{2+}-ionophore ionomycin induced a robust contractile response with an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). The PI3K-C2α-specific short interfering RNA (siRNA) induced a selective and marked reduction in PI3K-C2α protein expression. The siRNA-mediated knockdown of PI3K-C2α, but not class I PI3K p110α, suppressed ionomycin-induced contraction without altering Ca^{2+}-mobilization. PI3K-C2α is uniquely less sensitive to the PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) than the other PI3K members, including p110α. Ionomycin-induced contraction was inhibited only by a relatively high concentration of LY294002. Consistent with our previous observations showing that ionomycin and membrane depolarization induced Rho activation in vascular smooth muscle tissues in a Ca^{2+}-dependent manner, ionomycin-induced contraction was dependent on Rho and Rho-kinase. Ionomycin induced phosphorylation of the MLCP-regulatory subunit myosin targeting protein 1(MYPT1) at Thr^{850} and the 20-kDa myosin light chain (MLC) in a Rho kinase-dependent manner. Knockdown of PI3K-C2α suppressed phosphorylation of both MYPT1 and MLC. The receptor agonist noradrenaline, which induced a rapid increase in the $[Ca^{2+}]_i$ and Ca^{2+}-dependent contraction, stimulated phosphorylation of MYPT1 and MLC, which was also dependent on Ca^{2+}, PI3K-C2α, and Rho-kinase. These observations indicate that PI3K-C2α is necessary for Ca^{2+}-induced Rho- and Rho kinase-dependent negative regulation of MLCP and consequently MLC phosphorylation and contraction.

Membrane depolarization and excitatory receptor agonists, including noradrenaline, induce an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle, resulting in the activation of calmodulin-dependent myosin light-chain kinase (MLCK) and phosphorylation of the 20-kDa myosin light chain (MLC) (Morgan and Suematsu, 1990; Somlyo and Somlyo, 1994; Kamm and Stull, 2001). Excitatory receptor agonists also exert inhibitory regulation on the MLC dephosphorylating enzyme, myosin light-chain phosphatase (MLCP), which acts to potentiate Ca^{2+}-induced MLC phosphorylation and contraction (Pfitzer, 2001; Somlyo and Somlyo, 2003; Sward et al., 2003; Takuwa et al., 2005). Accumulating evidence implicates the small GTPase Rho and the Rho effector Rho-kinase in the negative regulation of MLCP by excitatory receptor agonists; excitatory receptor agonists trigger Rho activation (Sakurada et al., 2001), leading to MLCP inhibition through mechanisms involving Rho kinase-dependent phosphorylation of the 110-

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ABBREVIATIONS: MLCK, myosin light-chain kinase; MLCP, myosin light-chain 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 protein kinase C-potentiated inhibitory protein of PP1; GFP, enhanced green fluorescent protein; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*,*N*′-tetraacetic acid tetra (acetoxymethyl) ester; VSMC, vascular smooth muscle; PI3K, phosphoinositide 3-kinase; siRNA, short interfering RNA; C2α-siRNA, phosphoinositide 3-kinase-C2α-specific short interfering RNA; EGFP, enhanced green fluorescent protein; sc-siRNA, scrambled short interfering RNA; Y27632, *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride.

kDa myosin targeting subunit MYPT1/MBS of MLCP at Thr^{695} and/or Thr^{850} (numbering of chicken M133 isoform) (Noda et al., 1995; Kimura et al., 1996; Hartshorne et al., 2004) and of the smooth muscle-specific MLCP inhibitor protein CPI-17 (Kitazawa et al., 2000; Niiro et al., 2003). CPI-17 may also be phosphorylated by a protein kinase C-dependent mechanism (Eto et al., 2001). Thus, Rho acts as a switch molecule to negatively regulate MLCP in smooth muscle.

We and others have demonstrated that membrane depolarization and ionomycin induce Rho activation and MLCP inhibition in a Ca^{2+}-dependent manner in vascular smooth muscle (Mita et al., 2002; Sakamoto et al., 2003; Sakurada et al., 2003; Wang et al., 2006). Thus, it seems that an increase in the $[Ca^{2+}]_i$ not only activates MLCK but also inhibits MLCP in membrane depolarization- and ionomycin-stimulated muscle, like the case of excitatory receptor agonist stimulation. We also have shown that excitatory receptor agonist-induced Rho activation is Ca^{2+}-dependent (Wang et al., 2006), suggesting that the Ca^{2+}-dependent Rho activation mechanism, together with the receptor-coupled $G_{12/13}$-dependent mechanism (Somlyo and Somlyo, 2003), seems to operate in receptor agonist-stimulated smooth muscle. We demonstrated recently in vascular smooth muscle that phosphoinositide 3-kinase (PI3K) inhibitors suppress membrane depolarization- and receptor agonist noradrenaline-induced Rho activation and MYPT1 phosphorylation and MLC phosphorylation and contraction (Wang et al., 2006), suggesting that a PI3K plays a critical role in the activation of the Rho signaling pathway. We showed evidence that class II PI3K enzyme PI3K-C2α, which characteristically exhibits relatively lower sensitivities to PI3K inhibitors compared with other isoforms (Domin et al., 1997; Stein and Waterfield, 2000), is a PI3K isoform that is responsible for the receptor agonist noradrenaline-induced, PI3K inhibitor-sensitive Rho activation. However, it is not yet established whether PI3K-C2α is involved in Ca^{2+}-induced Rho activation and MLCP inhibition in vascular smooth muscle, although high concentrations of PI3K inhibitors inhibit membrane depolarization-induced Rho activation. In the present study, we addressed this question by taking advantage of RNA interference-mediated gene-silencing technique (Sharp, 2001) and differentiated vascular smooth muscle cells (VSMCs), which maintain contractile responses to various vasoactive substances (see the videos showing contractile responses in Supplementary Videos S1–S8).

Materials and Methods

Materials. LY294002, ionomycin, and BAPTA-AM were purchased from Merck-Calbiochem Biosciences (Darmstadt, Germany). Noradrenaline was bought from Sigma (St. Louis, MO). Insulin-like growth factor-I was bought from PeproTech (Rocky Hill, NJ). Laminin was bought from Asahi Techno Glass (Funabashi, Japan). Y27632 was donated by WelFide Corporation (Osaka, Japan). Fluo-4 acetoxymethyl ester was bought from Molecular Probes (Eugene, OR). Monoclonal antibody to PI3K-C2α (611046) was bought from BD Biosciences (San Jose, CA). Monoclonal antibodies to MLC (MY-21), smooth muscle α-actin (1A4), and MLCK (K36) were from Sigma. Rabbit polyclonal antibodies to phospho-MYPT1 (Thr^{850}) (36–003) and MYPT1 (PRB-457C) were bought from Upstate (Charlottesville, VA) and Covance Research Products (Berkeley, CA), respectively.

Differentiated VSMC Culture and Contraction Measurement. Rat aortic VSMCs were isolated from 5-week-old rat aortae by an enzyme-dispersion method essentially as described previously (Hayashi et al., 2001). In brief, aortae were dissected under sterile conditions and incubated at 37°C in 0.1% collagenase (type V; Sigma) and 0.05% elastase (type III, Sigma) for 30 min, followed by further incubation in the mixtures for 45 min after separating adventitia from aortic rings. Dispersed single cells were separated from undigested tissues by filtration and were collected by centrifugation. The cells thus obtained were cultured in the serum-free medium containing insulin-like growth factor-I (2 ng/ml) on laminin (20 μg/ml) in phosphate-buffered saline-coated glass-bottomed Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) for 3 days after isolation.

Ionomycin- and ligand-induced contractility of VSMCs was monitored as follows (Wang et al., 2006). To visualize VSMCs under the fluorescence microscope, the cells were transfected with either enhanced green fluorescent protein (EGFP) expression vector pEGFP-C1 (Clontech, Mountain View, CA), EGFP-tagged dominant-negative Rho mutant (N^{19}RhoA)-expression vector, which was kindly donated by Dr. Michael Way (Cancer Research Institute, London, UK), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were transferred into Leibovitz's L-15 medium (phenol-red free; Invitrogen) and then placed in a temperature-controlled incubator (Tokai Hit Co. Ltd., Shizuoka, Japan) to maintain the temperature at 37°C. Cell contractility of cultured VSMCs was observed at 37°C with an inverted microscope (Olympus IX70; Olympus, Tokyo, Japan)-coupled with CSU21 confocal unit (Yokogawa, Tokyo, Japan). The time-lapse images were acquired for 15 min at 6-s intervals using a cooled charge-coupled device camera (iXon EM-CCD; Andor, Belfast, UK) with IPLab image analysis software (Scanalytics, Fairfax, VA). To observe the effects of PI3K and Rho-kinase inhibition, cells were treated with LY294002 and Y27632 for 30 and 15 min, respectively, at indicated concentrations before time-lapse recording. In experiments to examine noradrenaline effects, propranolol (10 μM) was added to the media to block β-adrenergic receptors. Cell contractility was determined by measuring planar cell surface areas using ImageJ analysis software (http://rsb.info.nih.gov/ij/) and was expressed as the contraction index, $\Delta A/A_0$, in which a reduction of cell area ($\Delta A = A_0 - A_t$) at various time points after stimulation was normalized for the initial cell area at $t = 0$ (A_0). Data are given as mean ± S.E.M. and represent at least three independent experiments.

Determination of Fluo-4 Fluorescence. The VSMCs were seeded onto laminin-coated, glass-bottomed culture dishes (World Precision Instruments, Sarasota, FL) and used at 48 h after transfection. Cultures were incubated in the balanced salt solution (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES, pH 7.4) containing 2 μM fluo-4 acetoxymethyl ester/0.02% Pluronic F-127/2 mM probenecid for 45 min at 37°C. Cells were washed three times for 30 min using balanced salt solution containing 2 mM probenecid and viewed using the laser confocal microscope with excitation at 488 nm light and fluorescence detection at 510 nm, and images were captured every 500 ms with an EM-CCD cooled charge-coupled device camera (iXon; Andor). Pixel density was calculated from whole cell averages using the iXon iQ software (Andor). Temperature was maintained at 37°C for the duration of the experiments with the Olympus microscopic incubator system (Olympus, Tokyo, Japan).

Synthesis and Transfection of siRNA. Single-stranded rat PI3K-C2α-specific sense and antisense RNA oligonucleotides and control scrambled oligonucleotide were synthesized by in vitro transcription using the Silencer siRNA construction kit (Ambion, Austin, TX) and annealed to generate a RNA duplex, as described in detail previously (Usui et al., 2004). The target sequences of PI3K-C2α-specific siRNA 1, PI3K-C2α-specific siRNA 2, p110α-specific siRNA, and scrambled RNA duplex were 5′-AAGATATTGCTGGATGACAAT-3′, 5′-AATAGCAAGTACCTCAGAATT-3′, 5′-AACTGAGCAAGAGGCTCTGGA-3′, and 5′-AATCGACTGTGATACTACAAT-3′, respectively. The cells were transfected with short interfering RNA

(siRNA) (20 nM) using Lipofectamine 2000 with pEGF-C1 48 h before experiments. At least 60% of VSMCs were found to be transfected under our experimental condition, as evaluated by using fluorescent glyceraldehyde-3-phosphate dehydrogenase-specific siRNA (Ambion).

Determination of Phosphorylation of MLC and MYPT1. The VSMCs were quickly rinsed once with ice-cold Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline and fixed with ice-cold stop buffer containing 10% trichloroacetic acid, 150 mM NaCl, and 4 mM EGTA (Nagumo et al., 2000). The cells were scraped and centrifuged at 4°C at 15,000 rpm for 10 min. The resultant pellet was washed with ether two times and dissolved in Laemmli's SDS sample buffer. The samples were separated by 8% SDS-polyacrylamide gel electrophoresis followed by Western analysis using either anti-MLC antibody (MY21) or anti-mono- (Ser^{19})- and di- (Thr^{18} and Ser^{19}) phosphorylated MLC-specific antibodies (Sakurada et al., 1998) (gifts from Dr. M. Seto in Asahi Chemical Industry, Fuji, Japan), respectively. For quantitation of MLC monophosphorylation and diphosphorylation, densities of bands detected by antimonophosphorylated and diphosphorylated MLC antibodies were corrected by MLC protein amounts, and the results were expressed as multiples over a value in nontreated cells, which is expressed as 1.0. For the determination of MYPT1 phosphorylation, the VSMCs were treated as described for the determination of MLC monophosphorylation and diphosphorylation and analyzed by Western blotting using MYPT1-Thr^{850} phosphospecific antibody and an antibody that recognizes both phosphorylated and nonphosphorylated forms of MYPT1, as described previously (Wang et al., 2006). The amounts of phospho-MYPT1 quantitated by densitometry were normalized for total amount of MYPT1 in each sample, and the quantitative data of normalized amounts of the phosphoproteins were expressed as multiples over a value in unstimulated tissues, which is expressed as 1.0.

Statistics. All data are shown as mean ± S.E.M. One-way or two-way analysis of variance followed by Dunnett's test or unpaired *t* test were performed to determine the statistical significance of differences between mean values. For all statistical comparisons, $p < 0.05$ was considered significant.

Results

Knockdown of PI3K-C2α by siRNA Inhibits Ionomycin-Induced Contraction. Transfection of VSMCs with either PI3K-C2α-specific siRNA 1 (C2α-siRNA1) or PI3K-C2α-specific siRNA 2 (C2α-siRNA2) induced a marked reduction in the expression of PI3K-C2α protein (approximately 90% decrease) but not class I PI3K isoform p110α, MLCK, or smooth muscle-specific α-actin compared with the scrambled RNA counterpart (sc-siRNA) (Fig. 1A). On the other hand, transfection with PI3K p110α-specific siRNA (p110α-siRNA) strongly inhibited the p110α protein expression but not PI3K-C2α expression. Thus, the effects of C2α-siRNAs were specific.

We used the Ca^{2+} ionophore ionomycin to induce Ca^{2+}-dependent contraction in isolated VSMCs instead of high KCl membrane depolarization stimulus, because membrane depolarization-induced contraction was weak in VSMC cultures most likely because of down-regulation of voltage-dependent Ca^{2+}-channel expression (Ihara et al., 2002). Ionomycin (1 μM) induced a rapid increase in the $[Ca^{2+}]_i$ (Fig. 1B) with a robust contractile response (see Supplementary Video S1), as evaluated by using the fluorescent Ca^{2+} indicator fluo-4. Ionomycin-induced $[Ca^{2+}]_i$ response was not changed by PI3K-C2α knockdown (Fig. 1B). To evaluate ionomycin-induced contractile responses more quantitatively, the VSMCs were transfected with an EGFP expression vector and observed under a fluorescence laser confocal microscope, which allowed for the accurate determination of a contractile response as described under *Materials and Methods*. The addition of ionomycin (1 μM) induced a gradual decrease in the planar surface area of the VSMCs as a result of their contraction (Fig. 1C and Supplementary Video S2). Contraction was detected within 1 min and reached a nearly maximal extent at 10 min. Certain cells became much more quickly shortened because they were detached from the substrate at one end because of the generation of a strong tension. Quantitative analysis showed that ionomycin induced dose-dependent decreases in the planar surface area ($\Delta A/A_0$) with a maximal 50% decrease by 1 μM ionomycin in sc-siRNA-treated VSMCs (Fig. 1D). Knockdown of PI3K-C2α expression by either C2α-siRNA1 or C2α-siRNA2 substantially (approximately 35–45%) inhibited ionomycin-induced contraction (see Supplementary Video S3). In contrast, p110α-siRNA did not affect ionomycin-induced contraction (Supplementary Video S4). PI3K-C2α is less sensitive to the PI3K inhibitor LY294002 compared with the other PI3K isoforms (Domin et al., 1997; Wang et al., 2006). Consistent with the notion that PI3K-C2α is involved in Ca^{2+}-mediated contraction, a high (100 μM) but not low (10 μM) concentration of LY294002 inhibited ionomycin-induced contraction (Fig. 1E). These observations indicate that ionomycin-induced Ca^{2+}-mediated contraction is dependent on PI3K-C2α.

The Expression of a Dominant-Negative Rho Mutant and the Addition of a Rho-Kinase Inhibitor Suppress Ionomycin-Induced Contraction. In control VSMCs that had been transfected with EGFP, ionomycin induced a marked contractile response (Fig. 2, A and B, and Supplementary Video S5). The expression of an EGFP-tagged dominant-negative form of Rho, GFP-N^{19}RhoA, induced profound inhibition (approximately 80%) of ionomycin-induced contraction (Supplementary Video S6). Likewise, the Rho-kinase inhibitor Y27632 (10 μM) strongly inhibited ionomycin-induced contraction (Supplementary Video S7).

PI3K-C2α Knockdown and a Rho-Kinase Inhibitor Suppress Ionomycin-Induced Phosphorylation of MYPT1 and MLC. Ionomycin induced an increase in phosphorylation of the MLCP-regulatory subunit MYPT1 at Thr^{850} (Fig. 3A). Consistent with the inhibition of ionomycin-induced contraction by PI3K-C2α knockdown, either C2α-siRNA1 or C2α-siRNA2 abolished ionomycin-induced MYPT1 phosphorylation (Fig. 3A). Likewise, the addition of Y27632 abolished ionomycin-induced MYPT1 phosphorylation at Thr^{850} (Fig. 3B). Y27632 also reduced the basal level of MYPT1 phosphorylation. Ionomycin induced several-fold increases in mono- and diphosphorylation of MLC (Fig. 3C). The siRNA-mediated PI3K-C2α knockdown inhibited ionomycin-induced mono- and diphosphorylation of MLC. These observations suggest that PI3K-C2α participates in Ca^{2+}-induced contraction by regulating MLC phosphorylation through the mechanism involving Rho kinase-dependent phosphorylation of MLCP.

Noradrenaline-Induced Contraction and Phosphorylation of MYPT1 and MLC Are Dependent on Ca^{2+} and PI3K-C2α. The receptor agonist noradrenaline (10 μM) induced a rapid and transient increase in the $[Ca^{2+}]_i$ followed by a lower sustained increase with a robust contractile response (Fig. 4A and Supplementary Video S8). Depletion of the intracellular Ca^{2+} with the cell-permeable Ca^{2+} chelator

BAPTA-AM induced an approximately 60% inhibition of contraction. Noradrenaline induced an increase in MYPT1 phosphorylation at Thr^{850} (Fig. 4C). The Ca^{2+} depletion with BAPTA-AM treatment abolished noradrenaline-induced increase in MYPT1 phosphorylation at Thr^{850}, indicating that noradrenaline-induced MYPT1 phosphorylation and contraction are Ca^{2+}-dependent to the substantial degrees. However, Ca^{2+} depletion with BAPTA-AM did not affect the basal, nonstimulated level of MYPT1 phosphorylation, suggesting that the basal MYPT1 phosphorylation was Ca^{2+}-independent, different from noradrenaline-induced stimulation of MYPT1 phosphorylation. Y27632 reduced the basal level of MYPT1 phosphorylation and totally abrogated noradrenaline-induced stimulation of MYPT1 phosphorylation, indicating that MYPT1 phosphorylation under both the basal and stimulated conditions was dependent on Rho-kinase (Fig. 5A). Noradrenaline also induced an increase in MLC diphosphorylation (Fig. 5B), which was consistent with the observation that noradrenaline induced MYPT1 phosphorylation and MLCP inhibition. The siRNA-mediated PI3K-C2α knockdown inhibited noradrenaline-induced MLC diphosphorylation. Similar to ionomycin-induced contraction, LY294002 only at a high concentration (100 μM) inhibited noradrenaline-induced MLC diphosphorylation. PI3K-C2α knockdown by C2α-siRNA markedly reduced further inhibition of MLC diphosphorylation by LY294002, supporting the notion that PI3K-C2α is a target of LY294002 in inhibition of MLC phosphorylation.

Discussion

Ca^{2+} ion plays a central role in vascular smooth muscle contraction (Somlyo and Somlyo, 1994). The critical target molecule of Ca^{2+} in the regulation of smooth muscle contraction is the calmodulin-dependent enzyme MLCK. An increase in the $[Ca^{2+}]_i$ elicited by receptor activation and membrane depolarization activates MLCK (Morgan and Suematsu, 1990; Kamm and Stull, 2001), leading to stimulation of MLC phosphorylation. In contrast to the well-defined molecular mechanism of the Ca^{2+}-triggered MLC phosphorylation pro-

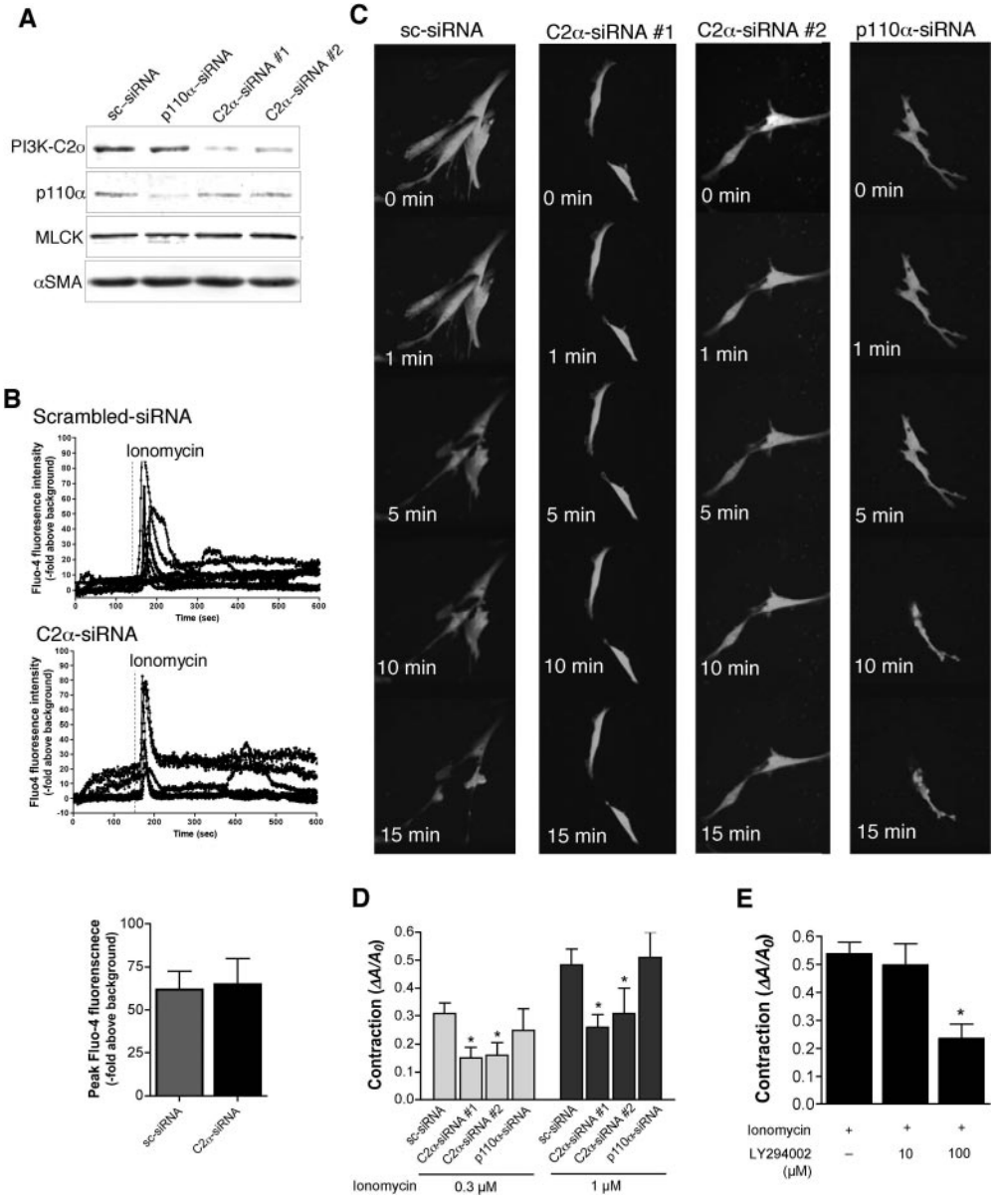


Fig. 1. Selective knockdown of PI3K-C2α protein expression by siRNA suppresses ionomycin-induced contraction. The VSMCs were transfected with either PI3K-C2α-specific siRNAs (C2α-siRNA1 and -2), PI3K p110α-specific siRNA, or scrambled RNA duplex (sc-siRNA). In C, D, and E, the cells were cotransfected with the GFP expression vector pEGFP-C1, and cell contraction was observed by using a laser confocal microscope. A, analysis of PI3K-C2α, p110α, and MLCK protein expression in the siRNA-treated VSMCs by Western blotting. B, no difference in the $[Ca^{2+}]_i$ response to ionomycin between sc-siRNA- and C2α-siRNA1-treated, fluo-4-loaded VSMCs. Ionomycin (1 μM)-induced changes in fluo-4 fluorescence in representative eight different VSMCs (top and middle), and quantified data (bottom) are shown. C, representative GFP fluorescence contraction images in the VSMCs treated with either sc-siRNA, C2α-siRNA1, or p110α-specific siRNA. The cells were stimulated with ionomycin (1 μM), and changes in the planar cell surface area were continuously monitored for 15 min. D, quantified results of ionomycin (0.3 and 1 μM)-induced contraction at 15 min. E, inhibition of ionomycin-induced contraction by LY294002. The cells were pretreated with low (10 μM) or high (100 μM) concentrations of LY294002 or nonpretreated, and ionomycin (1 μM)-induced contraction was determined at 15 min. Each datum of cell contraction in D and E is a mean ± S.E. of values from 15 to 45 cells. *, $p < 0.05$ compared with sc-siRNA-treated cells in (D) and LY294002-nontreated cells in (E).

cess, very little was known about possible Ca^{2+} regulation of MLC dephosphorylation process catalyzed by MLCP. We demonstrated previously in vascular smooth muscle that Ca^{2+} exerts an inhibitory effect on MLCP through inducing Rho activation (Sakurada et al., 2003). Based on the experimental results obtained largely by using pharmacological PI3K inhibition, we suggested that a PI3K is involved in Ca^{2+}-dependent Rho stimulation and MLCP inhibition (Wang et al., 2006). We showed by taking advantage of siRNA-mediated gene-silencing that PI3K-C2α isoform plays a thus-far-unrecognized role in the receptor agonist noradrenaline-induced contraction. However, the involvement of PI3K-C2α in Ca^{2+}-induced contraction and regulation of MLCP and MLC was not yet examined directly. The present study shows that PI3K-C2α plays an indispensable role in Ca^{2+}-induced Rho- and Rho-kinase-dependent MLCP inhibition, MLC phosphorylation, and contraction.

The physiological function of class II member PI3K-C2α has not been well understood. PI3K-C2α uniquely exhibits lower sensitivities to two structurally different PI3K inhibitors, LY294002 and wortmannin, compared with the seven other PI3K isoforms (Domin et al., 1997; Stein and Waterfield, 2000). Our observations (Wang et al., 2006) that membrane depolarization-induced Ca^{2+}-dependent Rho activation, phosphorylation of MLC and MYPT1, and contraction were all relatively less sensitive to the PI3K inhibitors than Akt phosphorylation, which is a well-known downstream signaling event of class I PI3K enzymes (Franke et al., 1995), led us to the hypothesis that PI3K-C2α might be involved in Ca^{2+}-induced Rho activation and contraction. In the present study, PI3K-C2α knockdown by two different specific siRNAs suppressed Ca^{2+}-induced contraction and phosphorylation of MLC and MYPT1, and these Ca^{2+}-induced responses were all Rho kinase-dependent. The siRNA effect on contraction was specific for PI3K-C2α, because class I p110α-specific siRNA was ineffective. In agreement with the present data obtained by using siRNA-mediated PI3K-C2α and also our previous results in vascular smooth muscle tissues (Wang et al., 2006), a lower concentration (10 μM) of LY294002, which can effectively inhibit various effects mediated by PI3K isoforms other than PI3K-C2α (including Akt phosphorylation, cell migration, and cell survival and proliferation) (Franke et al., 1995; King et al., 1997), failed to inhibit the Ca^{2+}-induced responses in VSMCs.

Ca^{2+}-induced MLCK activation occurs via the binding of the Ca^{2+}-calmodulin complex to MLCK (Somlyo and Somlyo, 1994; Kamm and Stull, 2001). Ca^{2+}-induced MLCP inhibition is mediated through Rho kinase-dependent phosphory-

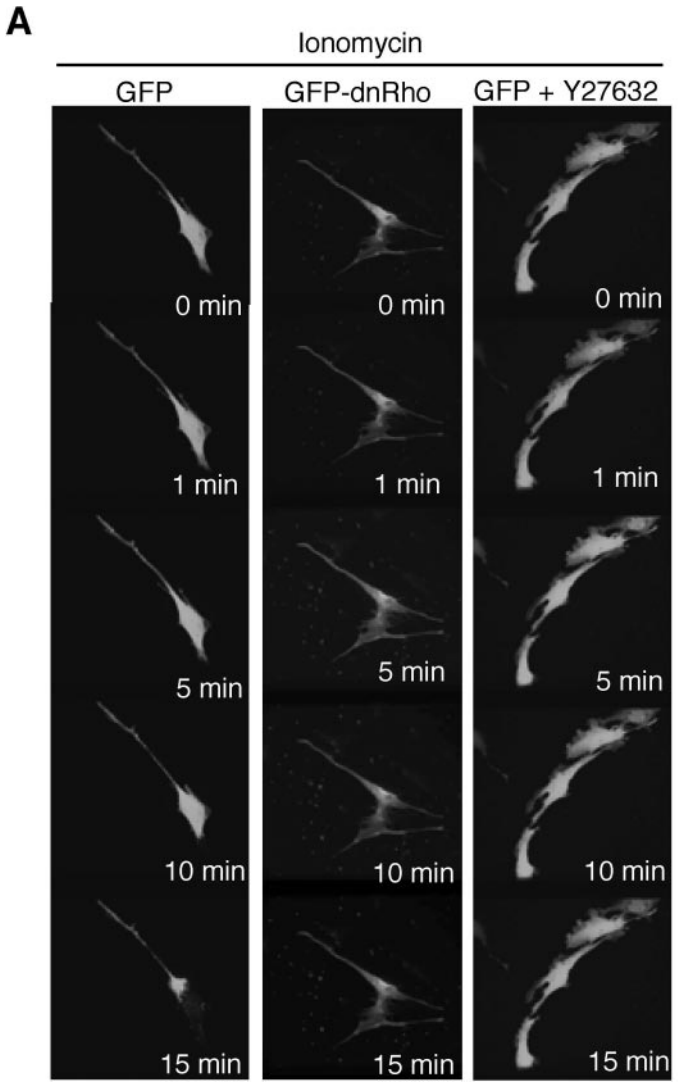


Fig. 2. The expression of a dominant-negative Rho mutant and a Rho-kinase inhibitor suppress ionomycin-induced contraction. A, representative GFP-fluorescence contraction images in the VSMCs transfected with GFP- or the dominant-negative GFP-N^{19}RhoA-expression vectors or treated with the Rho-kinase inhibitor Y27632. B, quantified results of inhibition of ionomycin-induced contraction at 15 min by the dominant-negative Rho mutant and Y27632. The VSMCs were transfected with either GFP or GFP-N^{19}Rho (GFP-dnRho) expression vectors 2 days before experiments or pretreated with Y27632 (10 μM) for 30 min immediately before the experiment and stimulated with ionomycin (1 μM) for up to 15 min. Contraction was analyzed as in Fig. 1. **, $p < 0.01$ compared with GFP-transfected, ionomycin-stimulated control. Each datum is a mean ± S.E. of values from 13 to 32 cells.

lation of the MLCP regulatory proteins MYPT1 and CPI-17 in vascular smooth muscle tissues (Sakurada et al., 2003; Wang et al., 2006). Because membrane depolarization-induced activation of PI3K-C2α and Rho, but not Rho-kinase activation itself, is dependent on Ca^{2+}, and PI3K-C2α is located upstream of Rho, the step of PI3K-C2α stimulation seems to be critically Ca^{2+}-dependent. Because PI3K-C2α by itself does not require Ca^{2+} for its activity (Arcaro et al., 2000), a regulatory molecule necessary for PI3K-C2α activation at the cell membrane might be sensitive to Ca^{2+}. Further investigations are necessary to delineate how Ca^{2+} induces PI3K-C2α stimulation and how PI3K-C2α stimulation leads to Rho activation.

The present results indicated that PI3K-C2α and Rho induce inhibition of MLCP, leading to potentiation of Ca^{2+}-induced MLC phosphorylation. However, it could also be possible that the PI3K-C2α and Rho pathway might positively regulate MLC phosphorylating enzymes including MLCK, potentiating MLC phosphorylation, and contraction. We (Noda et al., 1995) and others (Kitazawa et al., 1991) showed previously in permeabilized vascular smooth muscle preparations that guanosine 5′-3-*O*-(thio)triphosphate stimulation of Rho did not increase MLC kinase activity, suggesting that Rho enhanced MLC phosphorylation probably by inhibiting MLCP. Marked inhibition of Ca^{2+}-induced MLC phosphorylation and contraction by either PI3K inhibitor, a dominant-negative Rho mutant or a Rho-kinase inhibitor (Figs. 2 and 3), might be explained by a relatively high MLCP activity compared with MLC kinase activity in the aortic vascular tissue.

In addition to PI3K-C2α, vascular smooth muscle expresses at least three other PI3K members: class I enzymes p110α and p110β, and class II enzyme PI3K-C2β (Wang et al., 2006). The roles of the latter three PI3K isoforms in vascular smooth muscle contraction may not be significant, because relatively lower concentrations of PI3K inhibitors do not inhibit contractions induced by either membrane depolarization or receptor agonists, despite that PI3K inhibitors suppress these PI3K isoforms at the concentrations used. However, class I p110α, p110β, and PI3Kγ are expressed in vascular endothelial cells and have a stimulatory role in the regulation of the endothelial nitric-oxide synthase (Fulton et al., 1999), thus indirectly regulating vascular smooth muscle tone through the control of nitric oxide production. In addition, class I PI3K p110δ and PI3Kγ were suggested to be involved in enhanced spontaneous tone and reactive oxygen species-mediated, Akt-dependent stimulation of Ca^{2+} entry, respectively, in some blood vessels from animals (Northcott

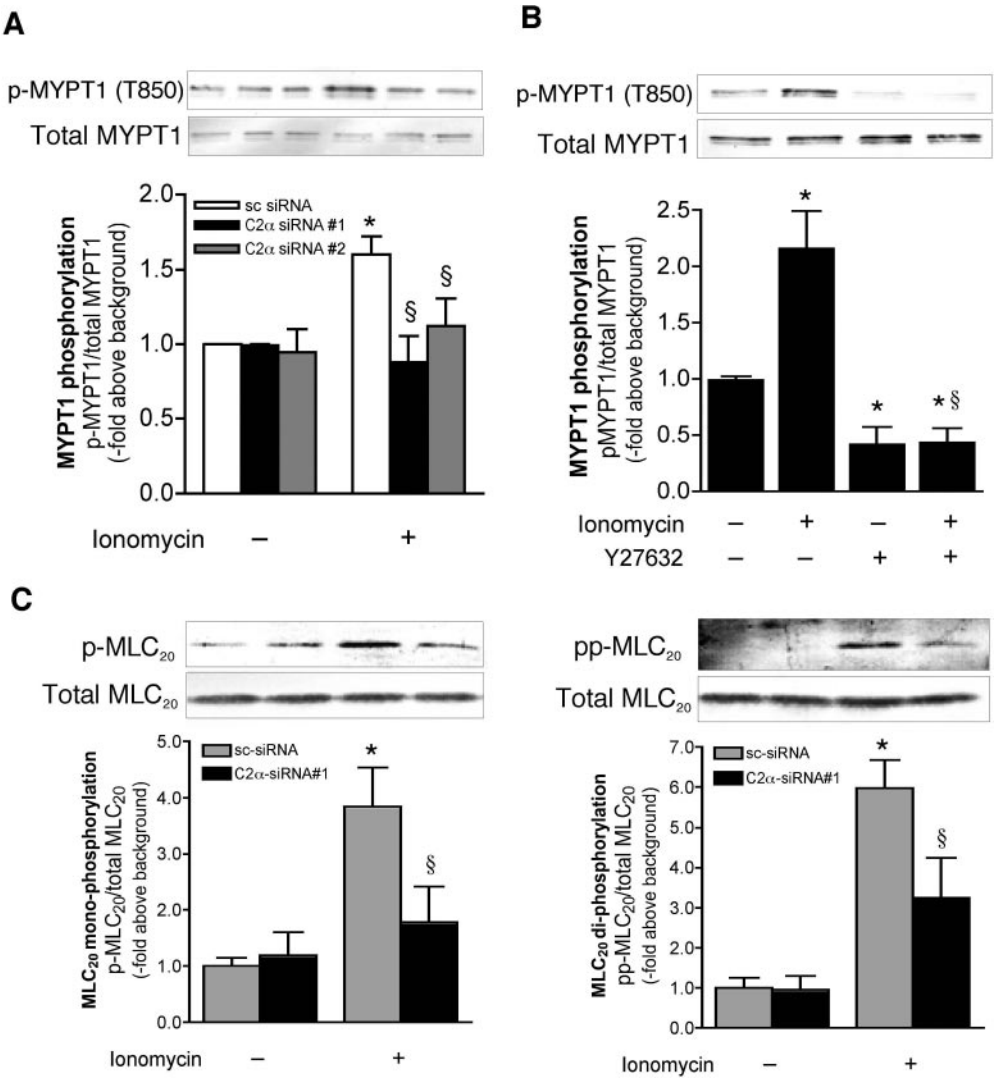
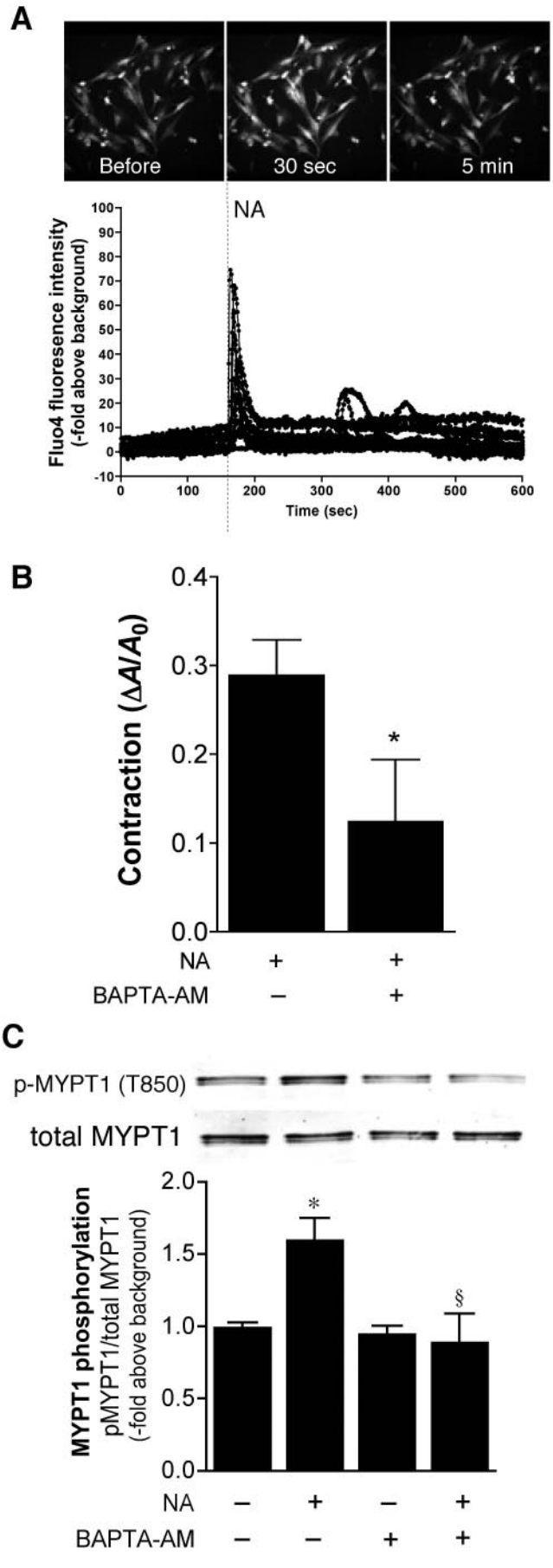


Fig. 3. PI3K-C2α knockdown by siRNA and a Rho-kinase inhibitor suppress ionomycin-induced phosphorylation of MLC and MYPT1. A, inhibition of ionomycin-induced MYPT1 phosphorylation at Thr^{850} by PI3K-C2α knockdown. B, inhibition of ionomycin-induced MYPT1 phosphorylation at Thr^{850} by the Rho-kinase inhibitor Y27632. C, inhibition of ionomycin-induced MLC phosphorylation by PI3K-C2α knockdown. p-MLC and pp-MLC, mono- and diphosphorylated forms of MLC, respectively. The VSMCs were transfected with either C2α-siRNA1, C2α-siRNA2, or sc-siRNA, stimulated with ionomycin (1 μM) for 10 min, and analyzed for Thr^{850} phosphorylation of MYPT1 and mono- (Ser^{19})- and di- (Thr^{18} and Ser^{19}) phosphorylation of MLC and by Western blotting using respective antiphosphospecific antibodies. In B and C, portions of cell extracts were analyzed for contents of total MYPT1 and MLC, respectively, by Western blotting using anti-MYPT1 and anti-MLC antibodies. *, $p < 0.05$ compared with sc-siRNA-treated or nontreated ionomycin-nonstimulated control. §, $p < 0.05$, compared with ionomycin-stimulated cells.



et al., 2002; Vecchione et al., 2005). Unlike PI3K-C2α, both PI3K p110δ and PI3Kγ are well-sensitive to relatively lower concentrations of PI3K inhibitors (Stein and Waterfield, 2000), and indeed low concentrations of PI3K inhibitors suppressed these vascular effects mediated by p110δ and PI3Kγ. Therefore, it is possible that more than a single PI3K isoform could participate in vascular smooth muscle contraction through different mechanisms and that there might be a species-dependent difference in the PI3K-dependent mechanisms.

The receptor agonist noradrenaline induces Rho activation in vascular smooth muscle (Sakurada et al., 2001). The α_1 adrenergic receptor for noradrenaline is a major adrenergic receptor subtype expressed in vascular smooth muscle. Noradrenaline induces a robust increase in the $[Ca^{2+}]_i$ via the α_1 receptor coupling to G_q in vascular smooth muscle (Takuwa and Rasmussen, 1987). In the present study, Ca^{2+} depletion with BAPTA-AM suppressed noradrenaline-induced, Rho kinase-dependent MYPT1 phosphorylation and contraction (Fig. 4). Moreover, noradrenaline-induced MLC and MYPT1 phosphorylation was suppressed by PI3K-C2α knockdown (Fig. 5). We also found previously that the PI3K inhibitors efficiently suppressed noradrenaline-induced Rho activation, phosphorylation of MYPT1 and MLC, and contraction in isolated arterial smooth muscle tissues (Wang et al., 2006). Taken together, these observations suggest that noradrenaline induces Rho activation and MYPT1 phosphorylation in a Ca^{2+}- and PI3K-C2α-dependent manner, although the $G_{12/13}$-dependent mechanism was also suggested to contribute to adrenergic receptor-mediated Rho stimulation (Gohla et al., 2000; Maruyama et al., 2002). Thus, the Ca^{2+}/PI3K-C2α pathway mediates not only membrane depolarization-induced but also excitatory receptor agonist-induced regulation of the Rho/Rho-kinase/MLCP.

In the present study, we used the VSMC culture on the laminin-coated substrate in the serum-free, chemically defined medium to evaluate contractile responses and their sensitivity to inhibitors. In general, the culture of VSMCs in the presence of bovine serum after their isolation from blood vessels induces cell proliferation, which is accompanied by dedifferentiation of VSMCs, including down-regulation of expression levels of contractile proteins and cell surface receptors, resulting in loss of contractility (Campbell and Campbell, 1993). The VSMCs used in the present study maintains high levels of protein expression of smooth muscle-specific α-actin and MLCK and contractility (Hayashi et al., 2001). This VSMC culture is also sensitive to gene transduction to a reasonable extent (see *Materials and Methods*). Cotransfection of VSMCs with an EGFP expression vector in combination with the observation under a fluorescence laser confocal

Fig. 4. Ca^{2+} depletion inhibits noradrenaline-induced contraction and MYPT1 phosphorylation. A, the $[Ca^{2+}]_i$ response to noradrenaline (NA). The cells were loaded with Fluo-4 and changes in the $[Ca^{2+}]_i$ in response to 10 μM noradrenaline were monitored as in Fig. 1B. Ionomycin-induced changes in Fluo-4 fluorescence in representative, 10 different VSMCs are shown. B, inhibition of noradrenaline-induced contraction by Ca^{2+} depletion. C, inhibition of noradrenaline-induced MYPT1 phosphorylation by Ca^{2+} depletion. The VSMCs were preincubated with BAPTA-AM (50 μM) for 15 min and stimulated with noradrenaline (10 μM) for 10 min. Contraction and MYPT1 phosphorylation at Thr^{850} were analyzed as in Figs. 1 and 3. *, $p < 0.05$ compared with noradrenaline-nonstimulated control in the absence of BAPTA-AM. §, $p < 0.05$ compared with noradrenaline-stimulated cells.

microscope equipped with a CCD camera permits accurate determination of single-cell surface areas and thus quantitative analysis of contractile responses (Fig. 1, D and E, and Supplementary Videos S2–S7). Loading the VSMCs with a fluorescent Ca^{2+} indicator and observation with a fluorescence microscope enabled us to simultaneously monitor the $[Ca^{2+}]_i$ change and a contractile response (Supplementary Videos S1 and S8). The differentiated VSMC culture in combination with gene manipulation techniques, including forced gene expression and siRNA-mediated gene silencing, would be a useful tool for analyzing molecular mechanisms of muscle contraction regulation.

In conclusion, we identified the class II PI3K isoform PI3K-C2α as a novel regulator of Ca^{2+}-induced contraction in vascular smooth muscle. PI3K-C2α participates in Ca^{2+}-induced MLC phosphorylation by inhibiting MLCP through mechanisms involving Rho kinase-dependent phosphorylation of its regulatory subunit MYPT1. The findings, together with our recent results (Wang et al., 2006), support the notion that PI3K-C2α is involved in Ca^{2+}-dependent Rho activation and its downstream signaling events.

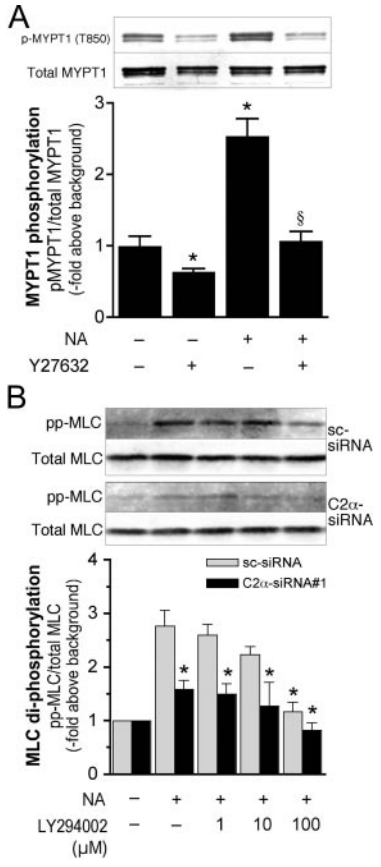


Fig. 5. PI3K-C2α knockdown by siRNA and a Rho-kinase inhibitor suppress noradrenaline-induced MYPT1 and MLC phosphorylation. A, inhibition of noradrenaline-induced MYPT1 phosphorylation at Thr^{850} by Y27632. The VSMCs were pretreated or nonpretreated with Y27632 (10 μM) for 15 min and stimulated with noradrenaline (10 μM) for 10 min. MYPT1 phosphorylation at Thr^{850} was analyzed as in Fig. 3. *, $p < 0.05$ compared with nonstimulated control in the absence of Y27632. §, $p < 0.05$ compared with noradrenaline-stimulated cells. B, inhibition of noradrenaline-induced di-phosphorylation. The VSMCs that had been transfected with either C2α-siRNA1 or sc-siRNA were pretreated with indicated concentrations of LY294002 or left nonpretreated for 30 min and stimulated with noradrenaline (10 μM) for 10 min, followed by analysis of di- (Thr^{18} and Ser^{19}) phosphorylation of MLC by Western, as in Fig. 3C. pp-MLC, diphosphorylated form of MLC. *, $p < 0.05$ compared with sc-siRNA-treated LY294002-nontreated noradrenaline-stimulated cells.

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