Class II phosphoinositide 3-kinase α-isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle

Yu WANG*, Kazuaki YOSHIOKA*, Mohammed Ali AZAM*, Noriko TAKUWA*, Sotaro SAKURADA*, Yuji KAYABA†, Naotoshi SUGIMOTO*, Isao INOKI*, Takaharu KIMURA*, Tomoyuki KUWAKI† and Yoh TAKUWA*[1]

*Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, 920-8640, Japan, and †Department of Physiology, Chiba University Graduate School of Medical Sciences, 1-8-1 Inohana, Chiba, 260-8670, Japan

We demonstrated previously that membrane depolarization and excitatory receptor agonists such as noradrenaline induce Ca^{2+}-dependent Rho activation in VSM (vascular smooth muscle), resulting in MP (myosin phosphatase) inhibition through the mechanisms involving Rho kinase-mediated phosphorylation of its regulatory subunit MYPT1. In the present study, we show in de-endothelialized VSM strips that the PI3K (phosphoinositide 3-kinase) inhibitors LY294002 and wortmannin inhibited KCl membrane depolarization- and noradrenaline-induced Rho activation and MYPT1 phosphorylation, with concomitant inhibition of MLC (20-kDa myosin light chain) phosphorylation and contraction. LY294002 also augmented de-phosphorylation of MLC and resultantly relaxation in KCl-contracted VSM, whereas LY294002 was much less effective or ineffective under the conditions in which MP was inhibited by either a phosphatase inhibitor or a phorbol ester in Rho-independent manners. VSM express at least four PI3K isoforms, including the class I enzymes p110α and p110β and the class II enzymes PI3K-C2α and -C2β. The dose–response relationships of PI3K-inhibitor-induced inhibition of Rho, MLC phosphorylation and contraction were similar to that of PI3K-C2α inhibition, but not to that of the class I PI3K inhibition. Moreover, KCl and noradrenaline induced stimulation of PI3K-C2α in a Ca^{2+}-dependent manner, but not of p110α or p110β. Down-regulation of PI3K-C2α expression by siRNA (small interfering RNA) inhibited contraction and phosphorylation of MYPT1 and MLC in VSM cells. Finally, intravenous wortmannin infusion induced sustained hypotension in rats, with inhibition of PI3K-C2α activity, GTP-loading of Rho and MYPT1 phosphorylation in the artery. These results indicate the novel role of PI3K-C2α in Ca^{2+}-dependent Rho-mediated negative control of MP and thus VSM contraction.

Key words: class II phosphoinositide 3-kinase α (PI3K-C2α), myosin phosphatase, myosin light-chain phosphorylation, phosphoinositide 3-kinase (PI3K), Rho, smooth muscle.

INTRODUCTION

Most vasoconstrictors directly act on VSM (vascular smooth muscle) via cell-surface GPCRs (G-protein-coupled receptors) to induce phospholipase C stimulation as well as plasma membrane Ca^{2+} channel opening, leading to an increase in the $[Ca^{2+}]_i$ (cytoplasmic free Ca^{2+} concentration) and resultant activation of MLCK (myosin light-chain kinase) [1,2]. MLCK phosphorylates MLC (20-kDa myosin light chain), leading to the initiation of contraction. Constrictors also down-regulate MP (myosin phosphatase) activity by mechanisms involving the small GTPase Rho and the Rho effector Rho kinase, thus sensitizing MLC phosphorylation to Ca^{2+} [3–9]. Rho kinase phosphorylates the 110-kDa myosin targeting subunit MYPT1/MBS of MP at Thr^{695} and/or Thr^{850} (numbering of chicken M133 isoform) to inhibit MP activity [7,9]. In addition, Rho kinase, as well as protein kinase C, may phosphorylate and activate the smooth-muscle-specific MP inhibitor protein, CPI-17 [10]. Thus Rho serves a role as a switching molecule in the negative regulation of MP [3,5,9]. Many vasoconstrictors indeed stimulate Rho activity in VSM [11,12]. Available evidence suggests that receptor agonist-induced Rho activation is mediated by the $G_{12/13}$-dependent mechanism in VSM [13]. We [12] and others [14] have unveiled the novel, Ca^{2+}-dependent Rho activation mechanism in VSM; KCl-induced membrane depolarization stimulates Rho in a manner strictly dependent on Ca^{2+} entry, and excitatory receptor agonists employ the G_q/Ca^{2+} pathway for activating Rho and down-regulating MP, besides the $G_{12/13}$ pathway [12]. However, it remains to be delineated how Ca^{2+} up-regulates Rho activity in VSM.

Mammalian PI3Ks (phosphoinositide 3-kinases) are a ubiquitously expressed enzyme family that phosphorylates membrane inositol lipids [15,16]. PI3Ks are divided into three main classes, based on their structure and substrate preference. Class I enzymes are heterodimers of the catalytic subunits, including p110α, p110β, p110γ and p110δ, and the regulatory subunits, including p85 and p101. Class I enzymes are activated rapidly by a variety of receptor tyrosine kinases and/or GPCRs, and exert diverse biological activities such as cell proliferation, survival, motility and control of carbohydrate metabolism. Previous studies have identified Akt as an important downstream effector of class I PI3Ks. Class II enzymes include PI3K-C2α, -β and -γ isoforms; however, it is poorly understood how their activities are regulated and what physiological functions they serve. Class III enzymes, a homologue of yeast Vps34p, play an important role in vesicular trafficking. LY (LY294002) and WMN (wortmannin) inhibit these PI3K enzymes with similar potencies, except for PI3K-C2α, which is at least one order less sensitive to either of PI3K inhibitors [16,17].

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; EGFP, enhanced green fluorescent protein; GPCR, G-protein-coupled receptor; IGF-I, insulin-like growth factor-I; IHC, immunohistochemistry; IM, ionomycin; LY, LY294002; MAP, mean arterial blood pressure; MLC, 20-kDa myosin light chain; MLCK, myosin light-chain kinase; MP, myosin phosphatase; NA, noradrenaline; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PI(3)P, phosphoinositide 3-phosphate; sc-siRNA, scrambled RNA duplex; siRNA, small interfering RNA; VSM, vascular smooth muscle; VSMC, VSM cell; WMN, wortmannin.

[1] To whom correspondence should be addressed (email ytakuwa@med.kanazawa-u.ac.jp).

Blood vessels are known to express multiple PI3Ks, including at least p110α, p110β and p110δ [17,18]. PI3K inhibitors act on vascular endothelial cells to suppress nitric oxide production through Akt inhibition [19]. In VSM, WMN and LY were demonstrated to suppress both contraction and MLC phosphorylation induced by receptor agonist and KCl [20–23]. However, it is not fully understood how PI3K inhibitors inhibit MLC phosphorylation, although reported evidence implicated PI3K activity in the opening of plasma membrane Ca^{2+} channels [21] and inhibition of the cAMP signalling pathway [23]. It also remains unknown which PI3K isoform is a target of PI3K inhibitors in suppressing VSM contraction. We show here that PI3K inhibitors suppress receptor agonist- and KCl-induced Rho activation with reversal of MP inhibition, resulting in augmentation of MLC dephosphorylation in VSM. Furthermore, our results indicate that the target molecule of PI3K inhibitors for these actions in VSM is not the class I enzymes, but the class II enzyme PI3K-C2α.

EXPERIMENTAL

Materials

WMN and LY were purchased from Wako and Merck Biosciences respectively. Y27632 was donated by WelFide Corporation. NA (noradrenaline), collagenase (Type V) and elastase (Type III) were bought from Sigma. IGF-I (insulin-like growth factor-I) and laminin were bought from PeproTech and Asahi Techno Glass respectively. Antibodies against RhoA (26C4), PI3K-p110α (N-20), PI3K-p110β (S-19) and PI3K-p110γ (F-11) were bought from Santa Cruz Biotechnology. Monoclonal antibodies against PI3K-C2α (611046), PI3K-C2β (611342) and PI3K-p110δ (611014) were bought from BD Biosciences. Rabbit polyclonal antibody against PI3K-C2α was raised in our laboratory according to the method described previously [24]. Monoclonal antibodies against MLC (MY-21), smooth muscle α-actin (1A4) and MLCK (K36) were from Sigma. Rabbit polyclonal antibodies against phospho-MYPT1 (Thr^{850}) (36-003), phospho-MYPT1 (Thr^{695}) (07-251), phospho-CPI-17 (Thr^{38}) (36-006) and CPI-17 (07-344) were bought from Upstate. Anti-MYPT1 antibody (PRB-457C) was bought from Covance Research Products (Berkley, CA, USA). Antibodies against Akt (9272) and phospho-Akt (Ser^{473}) (9276) were from Cell Signaling Technology.

Use of WMN and LY

For most of the analyses of PI3K inhibitor effects on contraction, MLC phosphorylation, Rho and MYPT1 phosphorylation (see Figures 1–3), both LY and WMN were used. Since WMN, but not LY, is a non-reversible PI3K inhibitor and is more soluble in the vehicle than LY, WMN was employed in the experiments shown in Figures 6, 7 and 10. Because WMN has significant MLCK inhibitor activity [25], whereas LY has only a minimal MLCK inhibitor activity [22,26,27], LY was employed for the experiments to see PI3K inhibitor effects on MLC de-phosphorylation experiments in VSM (see Figure 4).

Tissue preparation and tension measurements

Japanese white rabbits were killed by overdoses of sodium pentobarbitone (60 mg/kg intravenous injection). The descending portion of a rabbit thoracic aorta was removed and dissected. The animals were maintained in compliance with the Guidelines of the Care and Use of Laboratory Animals in Kanazawa University (see [11]). De-endothelialized aortic rings were equilibrated in a modified Krebs–Henseleit buffer (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose) at 37 °C and gassed with 1:20 CO_2/O_2, and isometric tension was measured as described previously [11]. The tension during the measurement was expressed as the percentage of the maximum force during 60 mM KCl pre-contraction.

Differentiated VSMC (VSM cell) culture and contraction measurement

Rat aortic VSMCs were isolated from 5-week-old rat aortae by an enzyme-dispersion method as essentially described in [28]. Briefly, aortae were dissected under sterile conditions, and incubated at 37 °C in 0.1 % collagenase (Type V) and 0.05 % elastase (Type III) 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 through Cell Strainers (BD Falcon), and were collected by centrifugation at 500 ***g*** for 5 min. The cells thus obtained were cultured in the medium containing IGF-I (2 ng/ml) on laminin (20 μg/ml in PBS)-coated glass-bottomed LabTek chamber slides (Nalge Nunc International) for 3 days after isolation.

Ligand-induced contractility of VSMCs was monitored as follows. To visualize VSMCs under the fluorescence microscope, the cells were transfected with EGFP (enhanced green fluorescent protein)-expression vector pEGFP-C1 (Clontech), using Lipofectamine™ 2000 (Invitrogen). At 24 h after transfection, the cells were transferred into Leibovitz's L-15 medium (Phenol Red-free; Invitrogen) and were then placed in a temperature-controlled incubator (Tokai Hit Co.) to maintain the temperature at 37 °C. Cell contractility of cultured rat aortic VSMCs was observed at 37 °C with an inverted microscope (Olympus IX70) coupled with a CSU21 confocal unit (Yokogawa). The time-lapse images were acquired for 15 min at 6-s intervals using a cooled CCD (charge-coupled device) camera (iXon EM-CCD; Andor) with IPLab image analysis software (Scanalytics). To observe the effects of PI3K and Rho kinase inhibition, cells were treated with LY and Y-27632 respectively for 30 min at the concentrations indicated before time-lapse recording. In experiments to examine NA effects, propranolol (10 μM) was added to the medium to block β-adrenergic receptors. Cell contractility was determined by measuring planar cell-surface areas using Image-J analysis software (National Institutes of Health), and was expressed as the contraction index, $\Delta A/A_0$, where 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 means $\pm$ S.E.M. and represent at least three independent experiments.

Determination of phosphorylation of MLC and MYPT1 and GTP-RhoA

Aortic strips mounted for isometric studies were frozen rapidly by immersion in solid CO_2/acetone/trichloroacetic acid as described in [12]. Myosin was extracted, and phosphorylation was analysed for determination of total MLC phosphorylation by urea/glycerol PAGE [5,8] and for determination of MLC di-phosphorylation by SDS/15 % PAGE, followed by Western blot analysis using either anti-MLC antibody (MY21) or anti-[di-phosphorylated (Thr^{18} and Ser^{19}) MLC] antibody [29], which was donated by Dr Seto (Asahi Chemical Industry, Fuji, Japan). For quantification of total MLC phosphorylation, densities of bands corresponding to MLC were quantified, and a ratio value of the sum of mono-phosphorylated and di-phosphorylated forms over total MLC were calculated as described previously [5]. For quantification

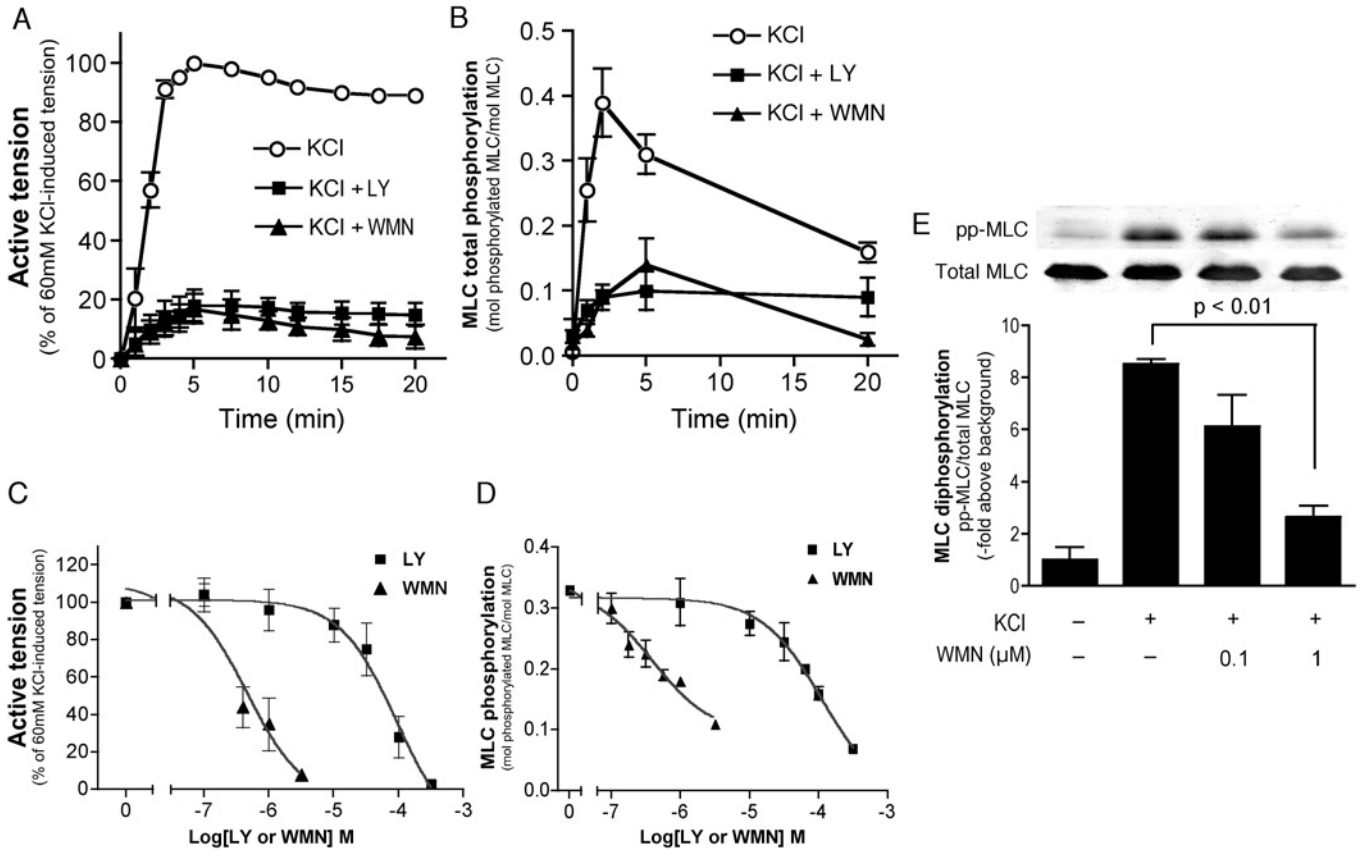

Figure 1 Inhibition of membrane depolarization-induced contraction and MLC phosphorylation by PI3K inhibitors

Time-dependent changes of KCl (60 mM)-induced contraction (**A**) and total MLC phosphorylation (**B**) in the presence and absence of WMN (1 μM) or LY (100 μM). Dose-dependent inhibition of KCl (60 mM)-induced contraction (**C**) and total MLC phosphorylation (**D**) at 5 min by PI3K inhibitors. (**E**) Inhibition of KCl-induced increase in the content of pp-MLC (di-phosphorylated form of MLC) by a high, but not a low, concentration of WMN. VSM was pretreated with the indicated concentrations of WMN and stimulated with 60 mM KCl for 5 min. The content of pp-MLC was analysed by using anti-pp-MLC-specific antibody. A portion of extracts was analysed for the content of total MLC by Western blot analysis using anti-MLC antibody (MY21). In all panels, PI3K inhibitors were added to VSM 30 min before KCl stimulation.

of MLC di-phosphorylation, a density of the band detected by anti-(di-phosphorylated MLC) antibody was corrected by an amount of MLC, and the results were expressed as multiples over a value for the non-treated samples. For determination of MYPT1 phosphorylation, VSM was homogenized and analysed by Western blotting using a phospho-specific antibody and an antibody that recognizes both phosphorylated and non-phosphorylated MYPT1, as described previously [12]. For quantification of MYPT1 phosphorylation, the values of phospho-specific antibody-reactive bands were corrected to amounts of total MYPT1, and the results were expressed as multiples over a value for the non-treated or sc-siRNA (scrambled RNA duplex)-treated control. Determination of a GTP-bound active form of RhoA in aortic VSM tissues was described previously [11]. Briefly, aortic rings that were contracted isometrically were frozen rapidly by immersing in liquid nitrogen. Frozen tissues were homogenized in a homogenizing buffer [50 mM Tris/HCl (pH 7.2), 500 mM NaCl, 10 mM $MgCl_2$, 1 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholic acid, 0.1 % (w/v) SDS, 20 mg/ml each of leupeptin and aprotinin, and 1 mM PMSF] and centrifuged at 18 000 ***g*** for 10 min. The resultant supernatants were subjected to pull-down assays using glutathione S-transferase–mouse rhotekin-(7–89) fusion protein immobilized on to glutathione–Sepharose 4B beads (Amersham Biosciences). RhoA bound to beads was analysed by Western blotting using a specific anti-RhoA antibody, and the results were expressed as described in [11].

For determination of phosphorylation of MLC and MYPT1 in VSMCs, the cells were rinsed rapidly once with ice-cold Ca^{2+}- and Mg^{2+}-free PBS (Dulbecco's PBS) and fixed with ice-cold stop buffer containing 10 % tricholoacetic acid, 150 mM NaCl and 4 mM EGTA [5,8,12]. The cells were scraped and centrifuged at 18 000 ***g*** for 10 min at 4 °C. The resultant pellet was washed twice with ether and dissolved in the urea sample buffer (20 mM Tris, pH 8.6, 23 mM glycine, 8 M urea and 2 mM dithiothreitol) for determination of total MLC phosphorylation or Laemmli's SDS sample buffer for determination of MYPT1 phosphorylation.

PI3K assay

Rings frozen by immersion in liquid nitrogen were homogenized in a lysis buffer containing 20 mM Tris/HCl, pH 7.5, 1 % Nonidet P40, 150 mM NaCl, 5 mM EDTA and 1 mM Na_3VO_4, and were centrifuged at 18 000 ***g*** for 10 min at 4 °C, as described in [30]. The resultant supernatants were subjected to immunoprecipitation using polyclonal anti-PI3K-C2α antibody and other PI3K isoform-specific antibodies. The anti-PI3K immunoprecipitates were incubated with 50 μl of the kinase assay buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 20 mM $MgCl_2$, 10 μM ATP, 1.6 MBq/ml of [γ-^{32}P]ATP and 200 μg/ml phosphatidylinositol) at 25 °C for 30 min. The reaction was terminated by adding 100 μl of methanol/chloroform/HCl (2:1:0.02 by vol.). Extracted lipid fraction was separated by

TLC on Silica gel 60 plates (Merck) and the solvent system of methanol/chloroform/25 % NH_4OH/water (43:38:5:7, by vol.). Radioactivity in the spot corresponding to PI(3)P (phosphoinositide 3-phosphate) was determined using a Fuji BAS Bioimage analyser 2000.

IHC (immunohistochemistry)

Paraformaldehyde (4 %)-fixed paraffin-embedded tissues were de-paraffinized, re-hydrated in graded alcohols, and stained using the immunoperoxidase method using the Dako Envision™ kit, according to the manufacturer's instructions. Endogenous peroxidase was blocked using 0.3 % H_2O_2 in methanol. Tissue sections were incubated overnight at 4 °C with the primary antibodies which were diluted in Dulbecco's PBS containing 0.1 % BSA at 1:200. Reactivity was visualized using 3′-diaminobenzidine, which produces a brown reaction product, and tissues were then lightly counterstained with haematoxylin. Control slides, where the primary antibody was replaced with PBS or diluted non-immune mouse IgG, were checked for non-specific reactivity before assessment of the staining intensity. To confirm the specificity of the immunoreactive signal detected by IHC, the PI3K-C2α antigen peptide fragment was pre-incubated with the antibody for 2 h at room temperature (25 °C) before use for immunostaining.

Synthesis and transfection of siRNA (small interfering RNA)

Single-stranded, rat PI3K-C2α- and p110α-specific sense and antisense RNA oligonucleotides were synthesized by *in vitro* transcription using a Silencer siRNA construction kit (Ambion) and annealed to generate an RNA duplex [17]. The targeted sequences of rat PI3K-C2α and p110α were 5′-AAGATATTG-CTGGATGACAAT-3′ and 5′-AACTGAGCAAGAGGCTCTG-GA-3′. The specificity of these sequences in PI3K-C2α and p110α mRNAs was confirmed by a BLAST search in the NCBI database. The sequence of control scrambled oligonucleotide was 5′-AATCGACTGTGATACTACAAT-3′. The cells were transfected with siRNA (20 nM) using Lipofectamine™ 2000 with pEGFP-C1 48 h before the experiments. At least 60 % of VSMCs were found to be transfected under our experimental conditions, as evaluated by using fluorescent GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific siRNA (Ambion).

In vivo measurement of arterial blood pressure

Male Wistar rats (9–10-week-old) were anaesthetized with isoflurane and ventilated with oxygen-enriched room air through a face mask. The right femoral artery and vein were cannulated with polyethylene tubing for blood pressure measurement and drug delivery respectively. The other ends of the cannulae were externalized at the back of the neck through a subcutaneous tunnel [31]. After the rats were allowed to recover from surgery for at least 2 h, pulsatile blood pressure and heart rate were recorded with the rats conscious and unrestrained. Each animal was injected with 0, 1, 3, 5 or 7 mg of WMN/kg of body mass (each group contained five rats) dissolved in 20 % DMSO-containing saline.

Statistics

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

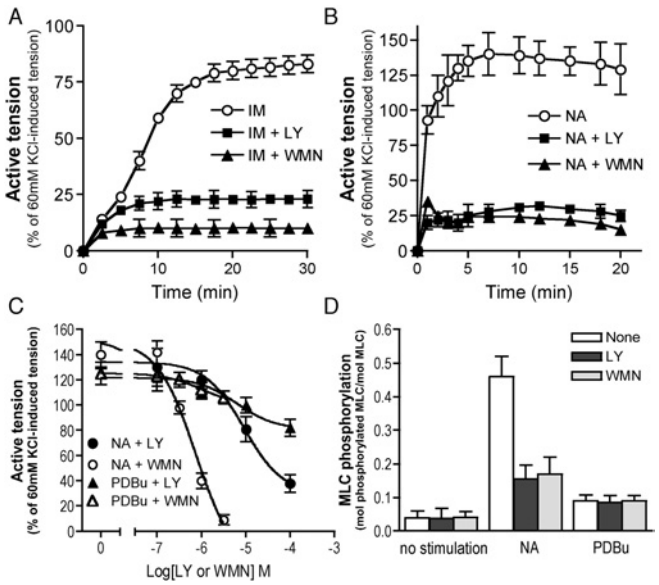

Figure 2 Inhibition of IM- and NA-induced contraction and MLC phosphorylation by PI3K inhibitors

(**A**) Time-dependent changes of IM (5 μM)-induced contraction of VSM in the presence and absence of WMN and LY as in Figure 1(A). (**B**) Time-dependent changes of NA (3 μM)-induced contraction in the presence and absence of WMN and LY as in (**A**). (**C**) Dose-dependent inhibition of NA- and phorbol-ester-induced contraction by PI3K inhibitors. VSM was stimulated with NA (3 μM) for 5 min or by phorbol 12,13-dibutyrate (PDBu) (1 μM) for 60 min in the presence of various concentrations of WMN or LY. (**D**) Inhibition of NA (3 μM)- and PDBu (1 μM)-induced total MLC phosphorylation by WMN (1 μM) and LY (100 μM). VSM was treated as in (**C**). In all panels, PI3K inhibitors were added to VSM 30 min before KCl stimulation.

RESULTS

Inhibition by PI3K inhibitors of membrane depolarization- and receptor agonist-induced Ca^{2+}-dependent contraction and MLC phosphorylation

KCl membrane depolarization induces a sustained contraction, which is totally dependent on extracellular Ca^{2+} [12]. Pretreatment with WMN (1 μM) markedly inhibited KCl-induced contraction in de-endothelialized rabbit aortic VSM (Figure 1A). The structurally different PI3K inhibitor LY (100 μM) also suppressed KCl-induced contraction. WMN was previously shown to inhibit MLCK [25]. On the other hand, LY exhibits a much weaker or minimal, if any, inhibitory activity on MLCK [22,26,27]. LY, as well as WMN, reduced KCl-induced MLC phosphorylation (Figure 1B). Inhibition of contraction and MLC phosphorylation by each of these PI3K inhibitors was dose-dependent with quite good agreement of the IC_{50} values for both responses (approx. 0.5 and 70 μM for WMN and LY respectively) (Figures 1C and 1D), suggesting that inhibition of MLC phosphorylation is a primary mechanism for WMN- and LY-induced contraction inhibition. It is also of note that these IC_{50} values are higher than those reported previously for inhibition of PI3K-mediated effects including Akt stimulation and cell survival [16,19,32]. The di-phosphorylation of MLC is stimulated when MLCK activity becomes very high or MP is inhibited [2]. KCl also induced an increase in di-phosphorylation of MLC, which was markedly inhibited by a high (1 μM), but not a low (0.1 μM), concentration of WMN (Figure 1E).

The Ca^{2+} ionophore ionomycin (IM) and excitatory receptor agonist NA induced sustained contractile responses (Figures 2A–2C), which are also strongly dependent on extracellular Ca^{2+} [12]. WMN and LY suppressed contractions by IM and NA.

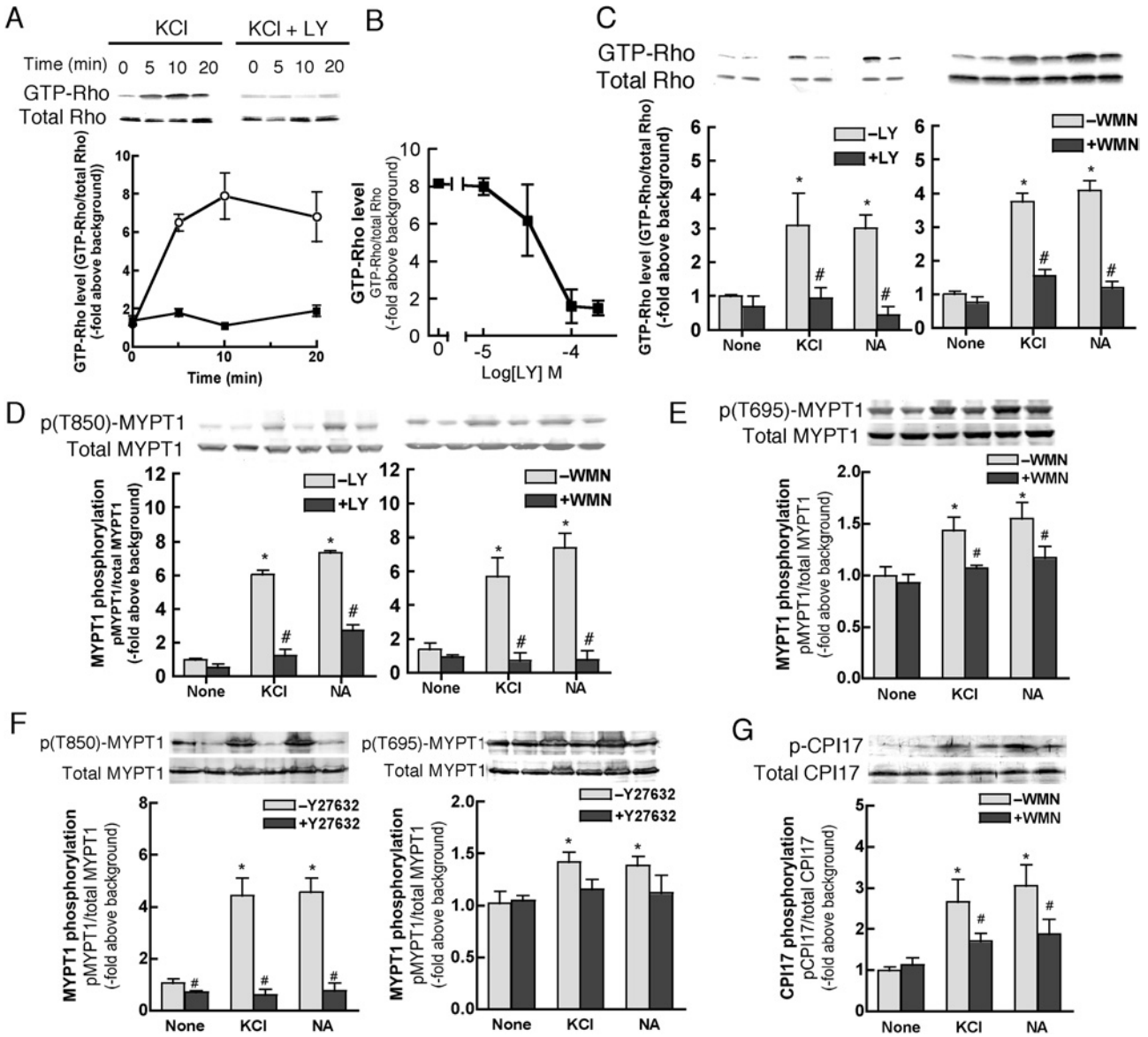

Figure 3 Inhibition by PI3K inhibitors of KCl- and NA-induced activation of Rho and phosphorylation of MYPT1 and CPI-17

(**A**) Time-dependent changes of the amounts of GTP-RhoA in KCl-stimulated VSM in the presence of absence of LY. VSM was treated as in Figures 1(A) and 1(B). A portion (1/27) of tissue extracts was subjected to Western blot analysis for evaluating amounts of total RhoA in each sample. (**B**) Dose-dependent inhibition of KCl-induced RhoA activation by LY. VSM was treated as in Figure 1(C). (**C**) Inhibition of KCl (60 mM)- and NA (3 μM)-induced Rho activation at 5 min by LY (100 μM) and WMN (1 μM). (**D**) Inhibition of KCl- and NA-induced Thr^{850} MYPT1 phosphorylation by LY and WMN. (**E**) Inhibition of KCl- and NA-induced Thr^{695} MYPT1 phosphorylation by WMN. In (**C**) and (**D**), VSM was treated as in (**C**), and was analysed for Thr^{850} and Thr^{695} phosphorylation of MYPT1 by Western blotting using phosphorylation site-specific anti-phospho-MYPT1 antibodies. (**F**) Effects of Y-27632 on KCl- and NA-induced Thr^{850} and Thr^{695} MYPT1 phosphorylation. VSM was pretreated with 10 μM Y-27632 for 30 min and then stimulated as in (**C**). (**G**) Inhibition of KCl- and NA-induced Thr^{38} CPI-17 phosphorylation by WMN. VSM was treated as in (**C**). * and #, $P < 0.05$ compared with non-treated control and non-inhibitor-treated stimulation respectively.

Consistently, LY and WMN inhibited NA-induced MLC phosphorylation (Figure 2D). In contrast, the inhibitory effects of WMN and LY on phorbol-ester-induced contraction and MLC phosphorylation was much weaker or was absent (Figures 2C and 2D).

Suppression of Rho activation and MYPT1 phosphorylation by PI3K inhibitors

KCl (Figure 3A), NA (Figure 3C) and IM (results not shown) induced severalfold sustained increases in the amount of a GTP-bound active form of RhoA (GTP-Rho) in Ca^{2+}-dependent manners, consistent with our previous observations [12]. Either LY (100 μM) or WMN (1 μM) nearly totally suppressed KCl-induced Rho stimulation (Figures 3A and 3C). LY inhibition of KCl-induced Rho stimulation was dose-dependent (Figure 3B) with an IC_{50} value of approx. 60 μM, which is similar to those for contraction and MLC phosphorylation (Figures 1C and 1D). LY, as well as WMN, also inhibited NA-induced RhoA activation (Figure 3C). Consistent with the stimulatory effects on Rho, KCl and NA both stimulated Thr^{850} phosphorylation of MYPT1 (Figure 3D). Either LY or WMN inhibited KCl- and NA-stimulated Thr^{850} MYPT1 phosphorylation and tended to inhibit the basal Thr^{850} MYPT1 phosphorylation. The Rho kinase inhibitor Y-27632 inhibited KCl- and NA-induced Thr^{850}

phosphorylation of MYPT1 as well as the basal phosphorylation (Figure 3F), suggesting that MYPT1 phosphorylation at Thr^{850} was dependent on Rho kinase. KCl and NA only slightly (by approx. 40–50 %) stimulated Thr^{695} phosphorylation of MYPT1. WMN inhibited KCl- and NA-induced stimulated Thr^{695} phosphorylation of MYPT1, but not the basal phosphorylation (Figure 3E). Y-27632 tended to inhibit KCl- and NA-stimulated Thr^{695} MYPT1 phosphorylation, but not the basal phosphorylation (Figure 3F). KCl and NA also stimulated phosphorylation of CPI-17 at Thr^{38}, which was inhibited by WMN (Figure 3G). We showed previously that Y-27632 inhibited MLC phosphorylation and contraction induced by KCl and NA [12]. The pretreatment of VSM with Y-27632 substantially attenuated LY-induced inhibition of contraction. Thus these observations indicate that KCl induces Rho activation and, consequently, MP inhibition through the mechanisms involving MYPT1 and CPI-17 phosphorylation, as well as NA, and suggest that PI3K inhibitors reverse Rho-mediated MP inhibition.

Acceleration of relaxation and MLC de-phosphorylation by a PI3K inhibitor

We examined whether LY could affect MLC de-phosphorylation. The addition of the MLCK inhibitor ML9 (30 μM) induced relaxation of KCl-contracted VSM (Figure 4A). This concentration of ML9 appeared to be sufficient for MLCK inhibition because pretreatment of VSM with the same concentration of ML9 nearly abolished contraction elicited by KCl. The simultaneous addition of LY (100 μM) and ML9 substantially augmented the relaxation. Concomitant with this, LY augmented ML9-induced reduction in MLC phosphorylation (Figure 4B). In contrast, under the conditions where MP was inhibited by the addition of a phosphatase inhibitor, calyculin A, LY only a little or hardly attenuated slowly developing contraction and MLC phosphorylation (Figures 4C and 4D).

Expression and regulation of PI3K isoforms, and sensitivities to a PI3K inhibitor in VSM

Rabbit and rat aortic VSM detectably express at least the class I PI3Ks p110α and p110β, and the class II enzymes PI3K-C2α and -C2β among six isoforms examined, as evaluated with Western blot analysis (Figure 5A). Cultured VSMCs also express these four isoforms. The immunohistochemical staining with anti-PI3K-C2α antibody, but not antigen peptide-pre-absorbed anti-PI3K-C2α antibody, showed that PI3K-C2α was distributed in endothelial cells and the medial smooth muscle cell layer of rabbit aorta (Figure 5B, middle and bottom panels).

Previous investigations have shown that the PI3K-C2α isoform is clearly less sensitive to PI3K inhibitors compared with other PI3K isoforms [16,17]. This raises the possibility that PI3K-C2α may be a PI3K isoform involved in contraction, because higher concentrations of PI3K inhibitors were required for inhibiting Rho, MLC phosphorylation and contraction, compared with other PI3K-mediated responses, including Akt stimulation and cell survival (Figures 1C and 1D) [16,19,32]. We first examined the sensitivity of PI3Ks to a PI3K inhibitor. We immunoprecipitated each PI3K isoform from VSM by using isoform-specific antibodies and determined the *in vitro* activities of PI3K isoforms in the presence of various concentrations of WMN. The PI3K-C2α activity was not inhibited *in vitro* by 0.1 μM WMN and was 50 % inhibited by 1 μM WMN, whereas p110α was 80–90 % inhibited by 0.01 and 0.1 μM WMN (Figure 6A). p110β exhibited intermediate sensitivity to WMN. WMN covalently binds to PI3Ks and irreversibly inhibits PI3Ks [25]. By taking advantage of this property of WMN, we determined the *in situ* WMN sensitivity of PI3Ks in intact VSM. VSM was pretreated with various concentrations of WMN, and then PI3K isoforms were immunoprecipitated from WMN-treated VSM using isoform-specific

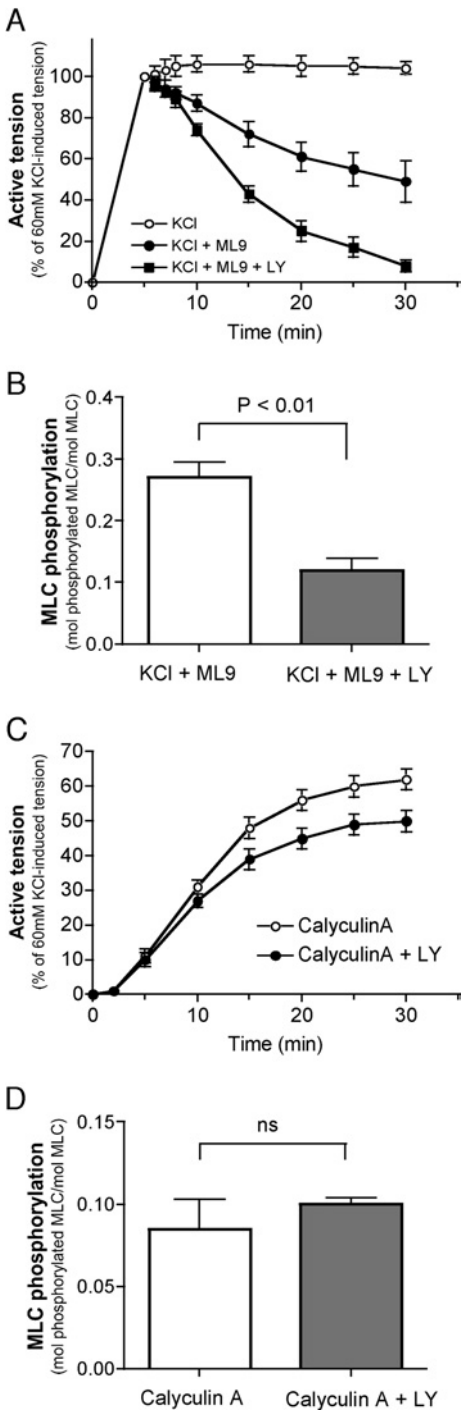

Figure 4 Augmentation by a PI3K inhibitor of MLC de-phosphorylation and relaxation

(**A**) Augmentation by LY of the MLCK inhibitor ML9-induced relaxation of KCl-contracted VSM. At 5 min after KCl (60 mM) stimulation, relaxation was induced by adding ML9 (30 μM) with or without LY (100 μM). (**B**) Augmentation of ML9-induced MLC de-phosphorylation by LY. VSM was treated as in (**A**). MLC phosphorylation levels were determined at 25 min after the addition of either ML9 (KCl + ML9) or ML9 plus LY (KCl + ML9 + LY). (**C**) Slight inhibition of the phosphatase inhibitor calyculin A-induced contraction by LY. LY (100 μM) was added 30 min before calyculin A addition. (**D**) No inhibition of calyculin A-induced MLC phosphorylation by LY. VSM was treated as in (**C**). ns, no statistical significance.

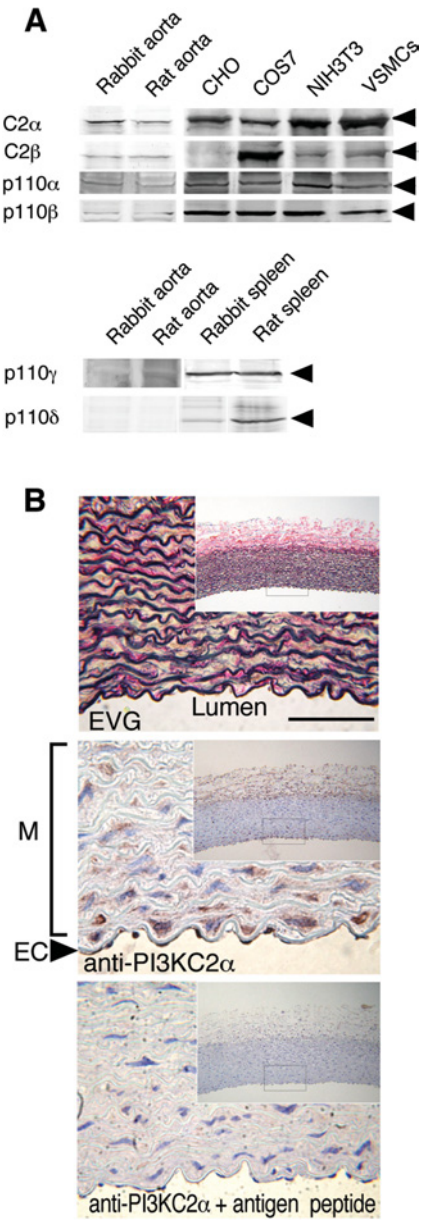

Figure 5 Expression of PI3K-C2α in the aortae and cultured cells, and its regulation and sensitivity to a PI3K inhibitor

(**A**) Expression of PI3K isoforms in the aorta and cultured cells. Homogenates of rabbit and rat aortae and spleens (100 μg of protein each) and several different cultured cell types (30 μg of protein each), including rat aortic VSMCs, were analysed by immunoblotting analysis using isoform-specific antibodies. (**B**) Immunohistochemical detection of PI3K-C2α in VSM and endothelial cells in the rabbit aortic tissue. The aortic tissue sections were stained using the elastica van Gieson (EVG) method (top panel), antigen peptide-non-pre-absorbed (middle panel) and pre-absorbed (bottom panel) anti-PI3K-C2α antibodies. The dotted box areas in the insets were magnified. M, media; EC, endothelium. Scale bar, 20 μm.

antibodies, followed by *in vitro* PI3K assay. The PI3K-C2α activity from 0.1 μM WMN-treated muscle showed only 15 % inhibition and that from 3 μM WMN-treated muscle showed 60 % inhibition (Figure 6B). In contrast, the p110α activity was 80 % inhibited by 0.1 μM WMN (Figure 6C). Likewise, PDGF (platelet-derived growth factor)-induced phosphorylation of Akt, a downstream effector of the class I PI3K [32], was also nearly completely abolished by 0.1 μM WMN (Figure 6D). Thus the WMN sensitivities of MLC phosphorylation and contraction

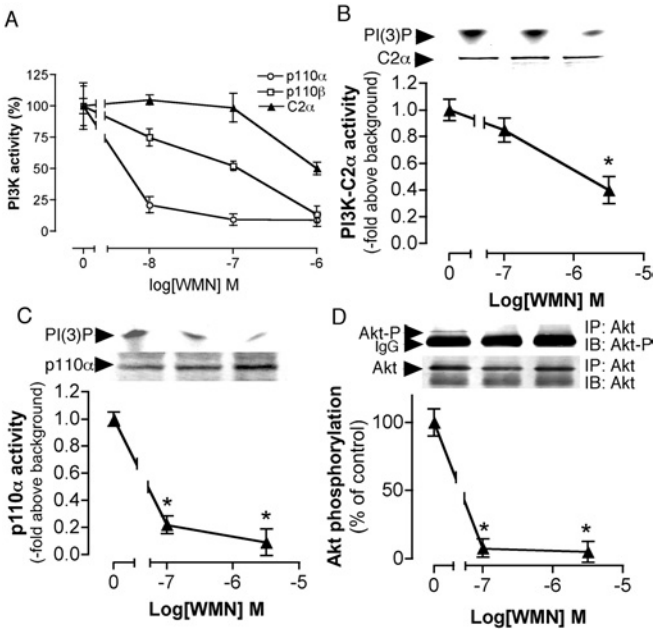

Figure 6 The sensitivity of PI3K isoforms and Akt to a PI3K inhibitor

(**A**) Different *in vitro* sensitivities of p110α, p110β and PI3K-C2α to WMN. The PI3K isoforms were immunoprecipitated from rabbit aortae, followed by *in vitro* PI3K assay in the presence of the indicated concentrations of WMN. (**B**) Lower sensitivity of PI3K-C2α to WMN *in vivo* in VSM. Intact VSM was treated with the indicated concentrations of WMN for 30 min, and then PI3K-C2α was immunoprecipitated from WMN-treated VSM followed by *in vitro* PI3K assay. Above the histograms are autoradiographs of the PI(3)P band and protein bands of PI3K-C2α in portions of immunoprecipitates. Sensitivity of (**C**) p110α and (**D**) Akt phosphorylation to WMN *in vivo* in VSM. In (**C**) and (**D**), VSM was pretreated with WMN as in (**B**). In (**D**), VSM was stimulated with PDGF-B chain (30 ng/ml) for 10 min at 20 min after WMN addition.*, $P < 0.01$ compared with WMN non-treated control.

(Figures 1C and 1D) appeared to be close to the WMN sensitivity of PI3K-C2α rather than those of class I PI3Ks in intact VSM.

Stimulation with either KCl, NA or IM induced an increase in the PI3K-C2α activity immunoprecipitated by a subtype-specific antibody (Figure 7A). The extent of PI3K-C2α stimulation by these stimuli was relatively small (30–80 %), but was significant and consistent in the repeated experiments. NA-induced PI3K-C2α stimulation was abolished by extracellular Ca^{2+} depletion by incubation in a Ca^{2+}-free EGTA-containing buffer (Figure 7B), indicating that NA-induced PI3K-C2α stimulation was Ca^{2+}-dependent. In contrast, the activities of p110α and p110β showed decreases in response to either KCl or NA (Figures 7C and 7D).

Inhibition of contraction, MLC phosphorylation and MYPT1 phosphorylation by siRNA-mediated PI3K-C2α down-regulation

To test directly the possibility that PI3K-C2α might mediate contraction, we examined the effect of siRNA-mediated PI3K-C2α down-regulation on contraction in VSMCs. VSMCs, which are enzymatically dispersed from the aortic medium and cultured in serum-free chemically defined medium [28], maintain contractile responses to various vasoactive substances, including NA. The addition of siRNAs specific to either PI3K-C2α or p110α markedly inhibited the expression of PI3K-C2α and p110α proteins (approx. 60 % decreases) respectively, but not MLCK or α-smooth muscle actin, compared with control cells treated with sc-siRNA (Figure 8). The siRNA-mediated PI3K-C2α down-regulation by approx. 70 % inhibited NA-induced contraction (Figures 9A–9C, and see Supplementary Videos 1 and 2 at http://www.BiochemJ.org/bj/394/bj3940581add.htm).

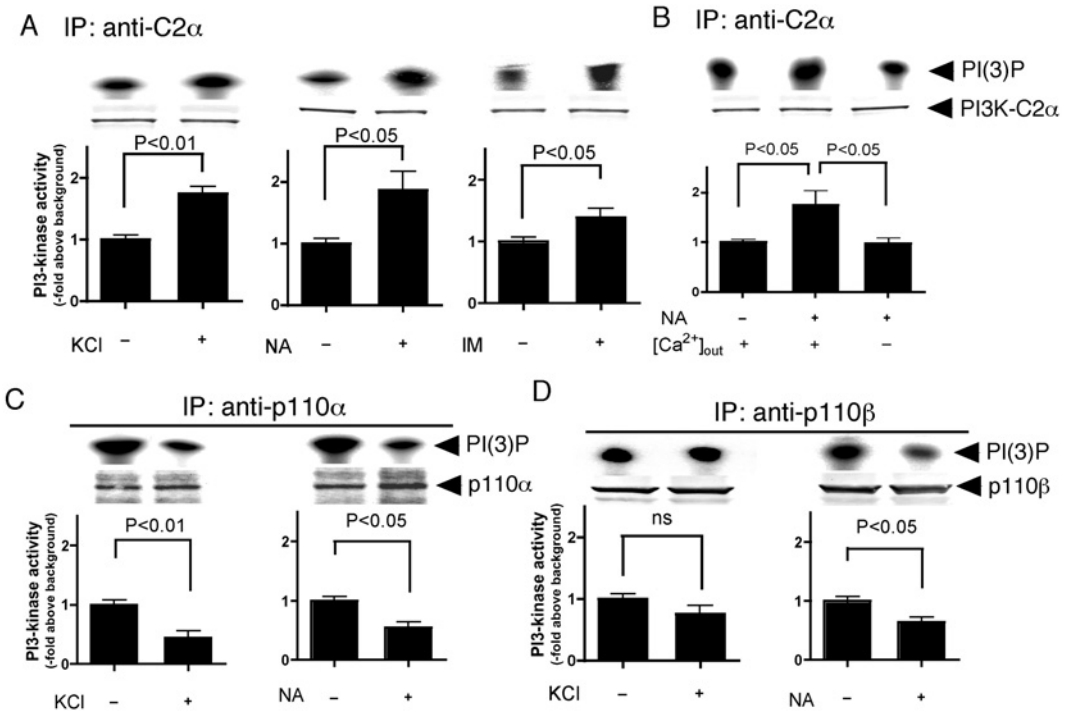

Figure 7 Regulation of the activities of PI3K isoforms by KCl and NA

(**A**) Stimulation of PI3K-C2α by KCl and NA. VSM was stimulated by either KCl (60 mM), NA (3 μM) or IM (5 μM) for 5 min. (**B**) Dependence of NA-induced PI3K-C2α activation on extracellular Ca^{2+}. VSM was incubated in either normal Ca^{2+}-containing or Ca^{2+}-free EGTA (1 mM)-containing buffer for 5 min, and stimulated with NA (3 μM) for 5 min. PI3K-C2α was immunoprecipitated with a specific antibody followed by *in vitro* PI3K assay. (**C**) and (**D**) The effects of KCl and NA respectively on the activities of p110α and p110β. VSM was stimulated with either KCl or NA for 5 min and analysed for PI3K activity as in (**A**). ns, no statistical significance.

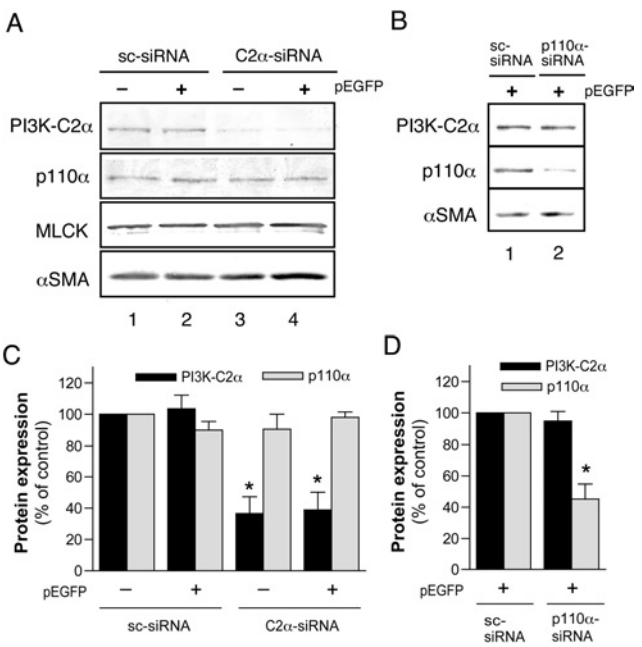

Figure 8 Selective inhibition of PI3K-C2α protein expression by siRNA specific to PI3K-C2α

(**A**) Effects of C2α-siRNA (PI3K-C2α-specific siRNA) on protein expression. (**B**) Effects of p110α-siRNA (p110α-specific siRNA) on protein expression. In (**A**) and (**B**), the VSMCs were transfected with C2α-siRNA, p110α-siRNA or sc-siRNA with or without the EGFP expression vector pEGFP, and were analysed for PI3K-C2α, p110α, smooth muscle α-actin (αSMA) and MLCK protein expression by Western blotting. (**C**) and (**D**) Quantitative data of the expression of PI3K-C2α and p110α proteins. *, $P < 0.01$ compared with sc-siRNA-treated control.

In contrast, p110α down-regulation did not inhibit NA-induced contraction (Figure 9C). NA-induced contraction was inhibited by Y-27632 (Figure 9D). Inhibition of NA-induced contraction by PI3K-C2α down-regulation was accompanied by inhibition of MLC phosphorylation (Figure 9E). NA induced MYPT1 phosphorylation at both Thr^{850} and Thr^{695}, although stimulation of Thr^{695} MYPT1 phosphorylation was slight (Figures 9F and 9G). PI3K-C2α down-regulation inhibited NA-induced Thr^{850} MYPT1 phosphorylation and tended to inhibit Thr^{695} MYPT1 phosphorylation. A high (100 μM), but not a low (1 or 10 μM), concentration of LY strongly inhibited NA-induced contraction and MYPT1 phosphorylation (Figures 9B and 9F). The effects of the high concentration of LY were markedly diminished in PI3K-C2α-down-regulated VSMCs. These observations suggest that NA-induced phosphorylation of MYPT1 and MLC and contraction are dependent on PI3K-C2α and that the molecular target of LY for inhibiting these responses is PI3K-C2α.

WMN infusion induces hypotension and inhibition of vascular PI3K-C2α, Rho and MYPT1 phosphorylation

Finally, we examined whether a PI3K inhibitor exerted inhibitory effects on Rho activity and MYPT1 phosphorylation in the blood vessel *in vivo*. The bolus intravenous infusion of WMN lowered the MAP (mean arterial blood pressure) of rats in a dose-dependent manner, except the lowest dose (1 mg/kg of body mass), where the MAP was increased transiently by a maximal 15 mmHg (Figure 10A). At doses of 3 and 5 mg/kg, the MAP reached the nadir at 10 min after the injections, and then gradually recovered. At a dose of 7 mg/kg, the MAP decrease was sustained throughout the observation period. The heart rate was reciprocally increased in rats that received doses of 3

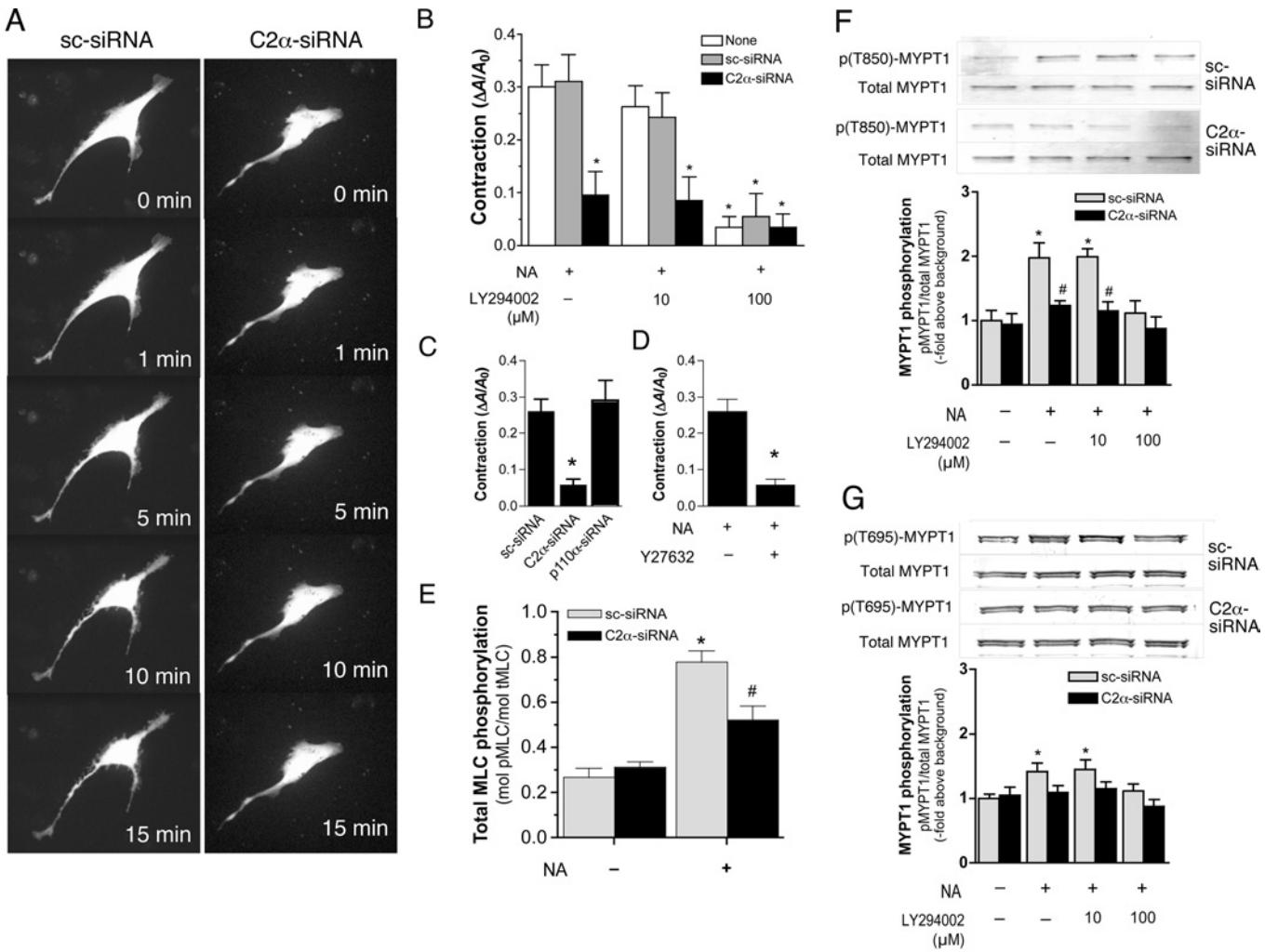

Figure 9 Inhibition of contraction and phosphorylation of MLC and MYPT1 by siRNA-mediated PI3K-C2α silencing, a PI3K inhibitor and a Rho kinase inhibitor

(**A**) Inhibition of NA-induced contraction by PI3K-C2α silencing. The VSMCs that had been transfected with either C2α-siRNA or sc-siRNA were stimulated with NA (10 μM), and changes in the planar cell surface area were monitored continuously for 15 min. (**B**) Quantified results of NA-induced contraction of VSMCs transfected with siRNAs and/or treated with LY at the concentrations indicated. The VSMCs were pretreated with LY or left unpretreated for 30 min, and stimulated with NA (10 μM) for up to 15 min. *, $P < 0.01$ compared with sc-siRNA-treated NA-stimulated control without LY. (**C**) Ineffectiveness of p110α silencing in inhibiting NA-induced contraction. (**D**) Inhibition of NA-induced contraction by the Rho kinase inhibitor Y-27632. The VSMCs were pretreated with Y27632 (10 μM) or left unpretreated for 30 min, and stimulated with NA (10 μM) for up to 15 min. *, $P < 0.01$ compared with NA-stimulated Y27632-unpretreated control. (**E**) Inhibition of NA-induced total MLC phosphorylation. The VSMCs that had been transfected with either C2α-siRNA or sc-siRNA were stimulated with NA (10 μM) for 10 min. *, $P < 0.01$ compared with sc-siRNA-treated non-stimulated control. #, $P < 0.05$ compared with sc-siRNA-treated NA-stimulated control. (**F**) Inhibition of NA-induced Thr^{850} MYPT1 phosphorylation by siRNA-mediated PI3K-C2α silencing and/or LY. (**G**) Inhibition on NA-induced Thr^{695} MYPT1 phosphorylation of siRNA-mediated PI3K-C2α silencing and/or LY. In (**F**) and (**G**), the VSMCs that had been transfected with either C2α-siRNA or sc-siRNA were with LY at the concentrations indicated or left unpretreated for 30 min, stimulated with NA (10 μM) for 10 min, and analysed for Thr^{850} and Thr^{695} phosphorylation of MYPT1 by Western blotting using phosphorylation site-specific anti-phospho-MYPT1 antibodies. *, $P < 0.05$ compared with sc-siRNA-treated non-stimulation without LY. #, $P < 0.05$ compared with sc-siRNA-treated, NA-stimulated control. Results are means ± S.E.M. of values from 15–45 cells.

and 5 mg/kg, which probably reflected the baroreceptor reflex, but was decreased in rats given 7 mg/kg, which would be most likely to be due to suppression of the vasomotor centre in the brain as a result of hypotension. PI3K-C2α was immunoprecipitated from aortae of rats given either WMN (5 mg/kg) or vehicle, followed by PI3K assay *in vitro*. WMN infusion reduced PI3K-C2α activity in the aorta by 45% compared with control rats (Figure 10B), with concomitant inhibition of Rho activity and MYPT1 phosphorylation in the aorta (Figure 10C and 10D).

DISCUSSION

The best characterized link of PI3Ks to the vascular function is that the class I PI3K enzymes are involved in activation of the nitric-oxide-synthesizing enzyme, eNOS (endothelial nitric oxide synthase), in endothelial cells [19]. Our data demonstrate for the first time that PI3K inhibitors suppress membrane depolarization- and receptor agonist-induced activation of Rho that serves as a molecular switch to negatively control MP in VSM. Furthermore, we found that the class II PI3K-C2α, but not the class I PI3Ks, is the molecular target for these effects of PI3K inhibitors. Thus the present study indicates the novel role of a PI3K in smooth muscle signalling to regulate MP and contraction.

We showed in the present study that the PI3K inhibitor LY suppressed Ca^{2+}-dependent Rho activation with concomitant suppression of Rho kinase-dependent MYPT1 phosphorylation (Figure 3), stimulation of MLC de-phosphorylation and relaxation (Figure 4). In addition, the dose–response relationship of LY-induced Rho inhibition (Figure 3B) was similar to LY inhibition of

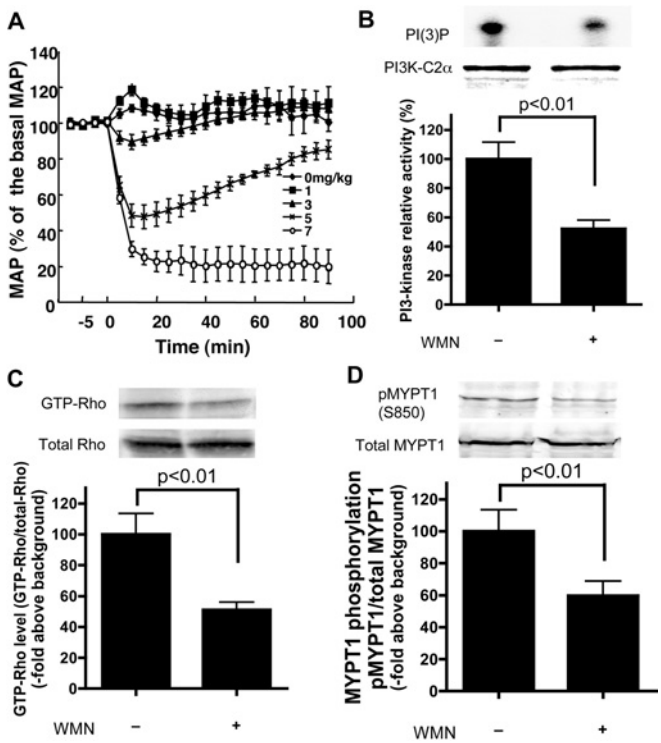

Figure 10 Depressor response and inhibition of PI3K-C2α, Rho and MYPT1 phosphorylation induced by WMN infusion in rats

(**A**) Changes in MAP induced by WMN infusion in rats. At zero time, varying doses of WMN were intravenously bolus-infused and the MAP was monitored continuously through a catheter inserted into the femoral artery. (**B**) Inhibition of aortic PI3K-C2α activity by WMN infusion. Rats were killed 10 min after WMN (5 mg/kg of body mass) infusion, and the aortae were removed. PI3K-C2α was immunoprecipitated and analysed for *in vitro* PI3K activity. (**C**) Inhibition of aortic Rho activity by WMN infusion. Rats were treated, and the aortae were removed in as in (**B**). Amounts of GTP-RhoA in the aortae were determined as in Figures 3(A)–3(C). (**D**) Inhibition of aortic MYPT1 Thr^{850} phosphorylation by WMN infusion. Rats were treated, and the aortae were removed in as in (**B**). Phosphorylation of MYPT1 in the aorta was determined as in Figure 3(D).

MLC phosphorylation and contraction (Figures 1C and 1D). All of these data suggest that LY-induced reversal of Rho-mediated MP inhibition was largely responsible for LY-induced inhibition of MLC phosphorylation and contraction. The structurally different PI3K inhibitor WMN inhibited KCl- and NA-induced Rho activation and MYPT1 phosphorylation, as well as LY (Figures 3C and 3D). Previous investigations demonstrated a MLCK inhibitor activity of WMN [25,27]. However, the present observations showing the effectiveness of LY rather suggest that inhibition by WMN of MLC phosphorylation in intact VSM might be due to inhibition of the Rho signalling pathway. In WMN inhibition of MLC phosphorylation and contraction, the exact contribution of Rho inhibition remains to be determined.

In agreement with the known lower sensitivity of PI3K-C2α to the PI3K inhibitors compared with the other PI3K members, including p110α, p110β and PI3K-C2β [15–17,33], PI3K-C2α in VSM was indeed at least one order less sensitive to WMN than p110α and p110β, whether either immunoprecipitated PI3Ks (Figure 6A) or intact VSM were treated with WMN (Figure 6B–6D) for the determination of WMN sensitivity. Consistent with a high sensitivity of class I p110α, WMN also inhibited Akt, a downstream effector of class I PI3Ks, in VSM with a similar high potency (Figure 6D). Moreover, the dose–response relationships of WMN- and LY-induced inhibition of Rho, MLC phosphorylation and contraction (Figures 1C, 1D and 3B) were roughly similar to inhibition of PI3K-C2α rather than class I enzymes p110α and p110β. The class I PI3Ks and class II PI3K-C2α were also different in their regulation by membrane depolarization and NA; either KCl or NA stimulated PI3K-C2α activity, but inhibited the activities of p110α and p110β (Figures 7C and 7D), although the mechanisms of the PI3K isoform-specific regulation are still unknown. Moreover, PI3K-C2α activation was totally dependent on extracellular Ca^{2+}. All of these observations suggested that PI3K-C2α might be a PI3K isoform involved in the regulation of Rho and MP, and also the target of the PI3K inhibitors for the regulation of contraction in VSM. By adopting siRNA-mediated gene silencing, we found that PI3K-C2α is involved in inhibitory regulation of MP, and thereby MLC phosphorylation and contraction (Figures 8 and 9).

Recently, Class I PI3K p110δ was suggested to be involved in enhanced spontaneous tone in hypertensive rat aorta [18]. They observed up-regulation of p110δ protein expression and activity in the hypertensive rat aorta compared with the normotensive control rat aorta, and inhibition of spontaneous tone by a p110δ-specific inhibitor. However, they did not show whether p110δ was involved in receptor agonist- and membrane depolarization-induced active tension generation. The expression of p110δ was very low or undetectable in the aorta of a rabbit as well as a normotensive rat, although we readily detected it in rabbit and rat spleens (Figure 5A). Moreover, unlike PI3K-C2α, p110δ is as sensitive to lower concentrations of WMN and LY as p110α [34]. The role of p110δ in Rho and MP regulation awaits further investigation. Another group showed that class I PI3Kγ is involved in angiotensin II-, but not the α-adrenergic agonist phenylephrine-induced vasoconstriction through reactive oxygen species-mediated Akt-dependent stimulation of Ca^{2+} entry in mice [35]. Different from the present study, a low concentration of WMN was effective in inhibiting angiotensin II-induced contraction in that study [35]. Additionally, in the present study, PI3K inhibitors suppressed contraction induced by the Ca^{2+} ionophore IM, as well as by KCl and NA, suggesting that the sites of action of PI3K inhibitors are unlikely to involve Ca^{2+} channels. Also, WMN was shown not to affect membrane depolarization-induced $[Ca^{2+}]_i$ increase in rat aortae [20]. These observations may suggest that more than a single PI3K isoform could participate in VSM contraction through different mechanisms and that there could be a species-dependent difference in the PI3K-dependent mechanisms.

Previous studies demonstrated that receptor agonists and non-hydrolysable GTP analogue stimulated phosphorylation of MYPT1 at Thr^{850}, but not at Thr^{695}, and of CPI-17 in intact and membrane-permeabilized smooth muscle [36,37]. In agreement with these studies, we observed in the present study that KCl as well as NA stimulated phosphorylation of MYPT1 at Thr^{850} severalfold in a Rho kinase-dependent manner, which was consistent with the observation that KCl and NA stimulated Rho. In contrast with Thr^{850} MYPT1 phosphorylation, an increase in the extent of Thr^{695} MYPT1 phosphorylation in KCl- and NA-stimulated VSM was only modest. We also observed KCl- and NA-induced CPI-17 phosphorylation, which was sensitive to a PI3K inhibitor. Thus the PI3K-C2α-dependent signalling pathway negatively regulates MP most likely through phosphorylation of both MYPT1 and CPI-17.

The infusion of WMN induced substantial decreases in the blood pressure in rats (Figure 10A). We did not determine directly the effect of the PI3K inhibitor on vascular resistance in the present study; however, the depressor response was accompanied by reciprocal increases in the heart rate except at the highest WMN dose, suggesting that the primary site of WMN action included

the resistance vessels. We observed inhibition of PI3K-C2α, Rho and MYPT1 phosphorylation in VSM together with the depressor response (Figure 8B–8D). Thus WMN at the dose administered was effective in inhibiting PI3K-C2α and its downstream pathway in VSM *in vivo*. These observations are consistent with the hypothesis that PI3K-C2α is involved in maintaining the arterial basal tone.

We [12] and others [13] demonstrated previously in VSM that excitatory receptor agonist-induced Rho activation occurs through both Ca^{2+}-dependent and $G_{12/13}$-dependent mechanisms. We suggested that the former mechanism was G_q-dependent [12]. The relative contribution of these two pathways for Rho activation appears to be different among receptor agonists: NA-induced Rho activation was totally abolished by Ca^{2+}-depletion, whereas the U46619-induced one was only partially inhibited by Ca^{2+}-depletion [12]. Thus the extent of Ca^{2+}-dependence of receptor agonist-induced Rho activation as well as contraction appears to be different, depending upon agonists. NA-induced PI3K-C2α stimulation was dependent on extracellular Ca^{2+} (Figure 2D), like Rho activation. Hence, the contribution of Ca^{2+}-dependent PI3K-C2α activation to agonist-induced contraction may be different in an agonist-dependent manner. The exact contribution of this Ca^{2+}-dependent mechanism remains to be determined for each agonist. A previous study demonstrated that Ca^{2+} does not activate PI3K-C2α activity directly [38]. The molecular mechanism for Ca^{2+}-dependent PI3K-C2α activation remains unknown at present. In arteries from hypertensive animals, increased Rho activity and MYPT1 phosphorylation was shown [39]. It is an interesting possibility that a PI3K is involved in dysregulation of Rho activity in diseased blood vessels. The PI3K isoform in VSM may be a useful new target for the development of therapeutics to treat abnormalities of vascular contraction.

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Japan Society for the Promotion of Science, the Space Utilization Program by Japan Space Forum, Mitsubishi Pharma Research Foundation and Novartis Pharma. We thank Ms Chiemi Hirose and Yumiko Ohta for assistance in the experiments and in preparing the manuscript.

REFERENCES

1 Somlyo, A. P. and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. Nature (London) **372**, 231–236
2 Hartshorne, D. J., Ito, M. and Ikebe, M. (1989) Myosin and contractile activity in smooth muscle. Adv. Exp. Med. Biol. **255**, 269–277
3 Somlyo, A. P. and Somlyo, A. V. (2003) Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. **83**, 1325–1358
4 Kitazawa, T., Gaylinn, B. D., Denney, G. H. and Somlyo, A. P. (1991) G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. J. Biol. Chem. **266**, 1708–1715
5 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
6 Lucius, C., Anders, A., Steusloff, A., Troschka, M., Hofman, F., Aktories, K. and Pfitzer, G. (1998) *Clostridium difficile* toxin B inhibits carbachol-induced force and myosin light chain phosphorylation in guinea-pig smooth muscle: role of Rho proteins. J. Physiol. (Cambridge, U.K.) **506**, 83–93
7 Feng, J., Ito, M., Ichikawa, K., Nishikawa, M., Hartshorne, D. J. and Nakano, T. (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. J. Biol. Chem. **274**, 37385–37390
8 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. Cell Physiol. **278**, C57–C65
9 Hartshorne, D. J., Ito, M. and Erdodi, F. (2004) Role of protein phosphatase type I in contractile functions: myosin phosphatase. J. Biol. Chem. **279**, 37211–37214
10 Kitazawa, T., Eto, M., Woodsome, T. P. and Brautigan, D. L. (2000) Agonist trigger G protein-mediated activation of the CPI-17 inhibitor phopsphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. J. Biol. Chem. **275**, 9897–9900
11 Sakurada, S., Okamoto, H., Takuwa, N., Sugimoto, N. and Takuwa, Y. (2001) Rho activation in excitatory agonist-stimulated vascular smooth muscle. Am. J. Physiol. Cell Physiol. **281**, C571–C578
12 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
13 Gohla, A., Schlutz, G. and Offermanns, S. (2000) Role for $G_{12/13}$ in agonist-induced vascular smooth muscle cell contraction. Circ. Res. **87**, 221–227
14 Mita, M., Yanagihara, H., Hishinuma, S., Saito, M. and Walsh, M. P. (2002) Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. Biochem. J. **364**, 431–440
15 Rameh, L. E. and Cantley, L. C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. J. Biol. Chem. **274**, 8347–8350
16 Stein, R. C. and Waterfield, M. D. (2000) PI3-kinase inhibition: a target for drug development? Mol. Med. Today **6**, 347–357
17 Domin, J., Pages, F., Volinia, S., Rittenhouse, S. E., Zvelebil, M. J., Stein, R. C. and Waterfield, M. D. (1997) Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem. J. **326**, 139–147
18 Northcott, C. A., Hayflick, J. S. and Watts, S. W. (2004) PI3-kinase upregulation and involvement in spontaneous tone in arteries from DOCA-salt rats: is p110δ the culprit? Hypertension **43**, 885–890
19 Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature (London) **399**, 597–601
20 Takayama, M., Ozaki, H. and Karaki, H. (1996) Effects of a myosin light chain kinase inhibitor, wortmannin, on cytoplasmic Ca^{2+} levels, myosin light chain phosphorylation and force in vascular smooth muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. **364**, 120–127
21 Northcott, C. A., Poy, M. N., Najjar, S. M. and Watts, S. W. (2002) Phosphoinositide 3-kinase mediates enhanced spontaneous and agonist-induced contraction in aorta of deoxycorticosterone acetate-salt hypertensive rats. Circ. Res. **91**, 360–369
22 Su, X., Smolock, E. M., Marcel, K. N. and Moreland, R. S. (2004) Phosphatidylinositol 3-kinase modulates vascular smooth muscle contraction by calcium and myosin light chain phosphorylation-independent and -dependent pathways. Am. J. Physiol. Heart Circ. Physiol. **286**, H657–H666
23 Komalavilas, P., Mehta, S., Wingard, C. J., Dransfield, D. T., Bhalla, J., Woodrum, J. E., Molinaro, J. R. and Brophy, C. M. (2001) PI3-kinase/Akt modulates vascular smooth muscle tone via cAMP signaling pathways. J. Appl. Physiol. **91**, 1819–1827
24 Turner, S. J., Domin, J., Waterfield, M. D., Ward, S. G. and Westwick, J. (1998) The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2α. J. Biol. Chem. **273**, 25987–25995
25 Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H. and Matsuda, Y. (1992) Wortmannin, a microbial product inhibitor of myosin light chain kinase. J. Biol. Chem. **267**, 2157–2163
26 Yano, H., Agatsuma, T., Nakanishi, S., Saitoh, Y., Fukui, Y., Nonomura, Y. and Matsuda, Y. (1995) Biochemical and pharmacological studies with KT7692 and LY294002 on the role of phosphatidylinositol 3-kinase in FcεRI-mediated signal transduction. Biochem. J. **312**, 145–150
27 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**, 95–105
28 Hayashi, K., Takahashi, M., Nishida, W., Yoshida, K., Ohkawa, Y., Kitabatake, A., Aoki, J., Arai, H. and Sobue, K. (2001) Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids. Circ. Res. **89**, 251–258
29 Sakurada, K., Seto, M. and Sasaki, Y. (1998) Dynamics of myosin light chain phosphorylation at Ser^{19} and Thr^{18}/Ser^{19} in smooth muscle cells in culture. Am. J. Physiol. **274**, C1563–C1572
30 Okamoto, H., Takuwa, N., Yokomizo, Y., Sugimoto, N., Sakurada, S., Shigematsu, H. and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol. Cell. Biol. **20**, 9247–9261
31 Kayaba, Y., Nakamura, A., Kasuya, Y., Ohuchi, T., Yanagisawa, M., Komuro, I., Fukuda, Y. and Kuwaki, T. (2003) Attenuated defense response and low basal blood pressure in orexin knockout mice. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **285**, R581–R593

32 Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81**, 727–736
33 Arcaro, A., Volinia, S., Zvelebil, M. J., Stein, R., Watton, S. J., Layton, M. J., Gout, I., Ahmadi, K., Downward, J. and Waterfield, M. D. (1998) Human phosphoinositide 3-kinase C2β, the role of calcium and the C2 domain in enzyme activity. J. Biol. Chem. **273**, 33082–33090
34 Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M. D. (1997) P110δ, a novel phosphoinositide 3-kinase in leukocytes. Proc. Natl. Acad. Sci. U.S.A. **94**, 4330–4335
35 Vecchione, C., Patrucco, E., Marino, G., Barberis, L., Poulet, R., Aretini, A., Maffei, A., Gentile, M. T., Storto, M., Azzolino, O. et al. (2005) Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3Kγ. Nat. Med. **201**, 1217–1228
36 Kitazawa, T., Eto, M., Woodsome, T. P. and Khalequzzaman, M. (2003) Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca^{2+} sensitization in rabbit smooth muscle. J. Physiol. (Cambridge, U.K.) **546**, 879–889
37 Niiro, N., Koga, Y. and Ikebe, M. (2003) Agonist-induced changes in the phosphorylation of the myosin-targeting subunit of myosin light chain phosphatase and CPI17, two regulatory factors of myosin light chain phosphatase, in smooth muscle. Biochem. J. **369**, 117–128
38 Arcaro, A., Zvelebil, M. J., Wallasch, C., Ullrich, A., Waterfield, M. D. and Domin, J. (2000) Class II phosphoinositide 3-kinase are downstream targets of activated polypeptide growth factor receptors. Mol. Cell. Biol. **20**, 3817–3830
39 Seko, T., Ito, M., Kureishi, Y., Okamoto, R., Moriki, N., Onishi, K., Isaka, N., Hartshorne, D. J. and Nakano, T. (2003) Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. Circ. Res. **92**, 411–418

Received 6 September 2005/30 November 2005; accepted 9 December 2005
Published as BJ Immediate Publication 9 December 2005, doi:10.1042/BJ20051471