Agonist- and depolarization-induced signals for myosin light chain phosphorylation and force generation of cultured vascular smooth muscle cells

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Summary

Phosphorylation of myosin light chain (MLC) and contraction of differentiated smooth muscle cells in vascular walls are regulated by Ca^{2+}-dependent activation of MLC kinase, and by Rho-kinase- or protein-kinases-C-dependent inhibition of MLC phosphatase (MLCP). We examined regulatory pathways for MLC kinase and MLCP in cultured vascular smooth muscle cells (VSMCs), and for isometric force generation of VSMCs reconstituted in collagen fibers. Protein levels of RhoA, Rho-kinase and MYPT1 (a regulatory subunit of MLCP) were upregulated in cultured VSMCs, whereas a MLCP inhibitor protein, CPI-17, was downregulated. Endothelin-1 evoked a steady rise in levels of Ca^{2+}, MLC phosphorylation and the contractile force of VSMCs, whereas angiotensin-II induced transient signals. Also, Thr853 phosphorylation of MYPT1 occurred in response to stimuli, but neither agonist induced phosphorylation of MYPT1 at Thr696. Unlike fresh aortic tissues, removal of Ca^{2+} or addition of voltage-dependent Ca^{2+}-channel blocker did not inhibit contractions of reconstituted VSMC fibers induced by agonists or even high concentrations of extracellular K^+ ions. Inhibitors of Ins(1,4,5)P_3-receptor and Rho-kinase antagonized agonist-induced or high-K^+-induced contraction in both reconstituted fibers and fresh tissues. These results indicate that both Ins(1,4,5)P_3-induced Ca^{2+} release and Rho-kinase-induced MYPT1 phosphorylation at Thr853 play pivotal roles in MLC phosphorylation of cultured VSMCs where either Ca^{2+}-influx or CPI-17-MLCP signaling is downregulated.

Key words: Calcium channel, Inositol 1,4,5-trisphosphate receptor, RhoA, CPI-17, Vascular smooth muscle, Arteriosclerosis

Introduction

Arterial vascular smooth muscle cells (VSMCs) play an important role in the function of many organ systems. Abnormality in the contractile and/or regulatory apparatus of smooth muscle is implicated in the pathogenesis of a variety of disease conditions such as hypertension, coronary and cerebral vasospasm, miscarriage, and erectile dysfunction. VSMCs in vivo show remarkable plasticity once they need to adapt to changes in environments, such as new development of vasculature and remodeling after vascular injury or during vascular diseases like arteriosclerosis (Owens, 1995). These arterial cells undergo rapid changes in shape and functional property from non-proliferative and contractile to proliferative and mobile phenotype.

Agonist stimulation of VSMCs induces phosphorylation of the 20 kDa regulatory light chain of myosin (MLC), which increases actin-activated myosin ATPase activity and contraction (Hartshorne, 1987; Somlyo and Somlyo, 2003). MLC phosphorylation is governed by the opposing actions of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). MLCK activity is regulated by intracellular Ca^{2+} levels through the binding of the $(Ca^{2+})_4$-calmodulin complex to MLCK (Kamm and Stull, 2001). Two major pathways leading to the Ca^{2+}-dependent activation of MLCK have been established in smooth muscle physiology (Somlyo and Somlyo, 1994). To increase Ca^{2+} influx, voltage-dependent L-type Ca^{2+}-channels can be opened directly by membrane depolarization through high concentrations of extracellular K^+ ions (high K^+) or indirectly through some agonists. Some agonists, through G-protein-coupled activation of PLCβ generate inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and thus open the Ins(1,4,5)P_3-channel in the sarcoplasmic reticulum (SR) to release Ca^{2+}. An increase in the intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$ and, thus, activation of the Ca^{2+}/calmodulin-dependent MLCK, has been directly demonstrated by Stull and co-workers using a fluorescent indicator for active MLCK during contraction (Isotani et al., 2004).

MLCP is a heterotrimeric enzyme comprised of a 36 kDa δ-isoform of the catalytic subunit of type-1 phosphatase (PP1Cδ), a 110-130 kDa myosin-targeting subunit (MYPT1) and an accessory 20-21 kDa subunit (Hartshorne et al., 1998). It has been well documented that MLCP activity in permeabilized smooth muscle tissues can be decreased (Kitazawa et al., 1991b; Kubota et al., 1992) or increased (Lee et al., 1997; Wu et al., 1998) by physiological stimuli even at constant Ca^{2+} concentrations, thereby decreasing or increasing the Ca^{2+} sensitivity (Ca^{2+}-desensitization or Ca^{2+}-sensitization, respectively) of contraction. This Ca^{2+}-independent MLCP regulation is also involved in the pathogenesis of abnormal

contraction of VSMCs in vascular diseases (Kandabashi et al., 2002). Two major pathways leading to inhibition of MLCP have been proposed. The first pathway involves phosphorylation of the MYPT1-targeting subunit (Trinkle-Mulcahy et al., 1995) that is mediated by the small GTPase RhoA, a member of the Ras super-family of monomeric G proteins (Hirata et al., 1992; Somlyo and Somlyo, 2003). A widely accepted downstream effector of RhoA in smooth muscles is Rho-kinase (Rho-activated kinase/ROKα/ROCK-II), which inhibits MLCP through MYPT1 phosphorylation at Thr696 (according to residue number of human MYPT1) (Kimura et al., 1996; Feng et al., 1999). In fact, an increase in the phosphorylation at the site was detected in smooth muscle tissues stimulated with agonists (Seko et al., 2003; Ito et al., 2004). On the contrary, no significant change in the phosphorylation level of Thr696 was found in smooth muscle tissues, such as artery, vein and vas deferens, during Ca^{2+}-sensitization and/or desensitization in response to agonists, GTPγS or Rho-kinase inhibitor (Kitazawa et al., 2003; Niiro et al., 2003; Wilson et al., 2005). Therefore, considerable controversy exists on the regulation of MLCP through the phosphorylation of MYPT1 at Thr696. MYPT1 Thr853 (according to residue number of human MYPT1), however, is exclusively phosphorylated by Rho-kinase (Feng et al., 1999), and phosphorylation at Thr853 is thereby used as an indicator of the in situ activity of Rho-kinase. Thr853 phosphorylation reduces the affinity of MYPT1 with myosin that causes a decrease in phosphatase activity in vitro (Velasco et al., 2002). The phosphorylation at Thr853 in response to agonists and Rho-kinase inhibitor increases and decreases, respectively, in smooth muscle tissues (Kitazawa et al., 2003; Niiro et al., 2003; Wilson et al., 2005). Therefore, phosphorylation of MYPT1 at Thr853 appears to be involved in agonist-induced inhibition of MLCP, whereas the role of phosphorylation of Thr696 is still controversial.

In addition to RhoA signals, PKC is involved in an increase in the Ca^{2+} sensitivity of MLC phosphorylation and contraction through inhibition of MLCP (Itoh et al., 1993; Masuo et al., 1994). PKC-induced Ca^{2+} sensitization in response to agonist stimulation is mediated by the smooth muscle-specific MLCP inhibitory protein CPI-17, of which a form phosphorylated at Thr38 directly binds to and inhibits the catalytic subunit (PP1Cδ) of MLCP (Eto et al., 1997; Li et al., 1998; Kitazawa et al., 2000; Eto et al., 2004). CPI-17 is also phosphorylated by several kinases such as Rho-kinase (Koyama et al., 2000; Pang et al., 2005). In fact, both Rho-kinase inhibitor and PKC inhibitor significantly inhibit agonist-induced CPI-17 phosphorylation as well as smooth muscle relaxation (Kitazawa et al., 2000; Kitazawa et al., 2003; Niiro et al., 2003). Expression levels of CPI-17 largely vary, depending on the type of smooth muscle (Woodsome et al., 2001) and the animal species (Kitazawa et al., 2004), and correlate with the degree of PKC-induced and GTPγS-induced Ca^{2+} sensitization of contraction and MLC phosphorylation. Thus, the Ca^{2+}-sensitizing signal transduction by phosphorylation of MYPT1 and CPI-17 depends on the agonist, tissue and cell type and is probably modulated in differentiation stages of VSMCs.

We evaluated the signaling pathways leading to MLC phosphorylation and contraction of VSMCs cultured from rat aorta and compared them with those in differentiated VSMCs in rat aorta tissues. Using site- and phosphorylation-specific antibodies, we examined agonist-induced changes in phosphorylation of MYPT1 at Thr696 and Thr853, and CPI-17 at Thr38, together with MLC phosphorylation and Ca^{2+} signals in cultured VSMCs and isometric force generation in reconstituted fibers and fresh tissues. Our results demonstrate dramatic changes in the expression profile of proteins controlling the Ca^{2+} signal and MLCP in proliferative VSMCs, which selectively use some but not all of the regulatory pathways for MLC phosphorylation seen in fresh tissues.

Results

Expression of selected proteins in cultured aortic smooth muscle cells

To elucidate the signal transduction pathways in cultured VSMCs, we first examined, by immunoblotting, the expression levels of contractile and/or regulatory proteins for myosin phosphorylation in cultured rat aortic VSMCs and compared them with those of rat aortic tissues. As seen in Fig. 1A, the relative expression levels of proteins specific to smooth muscle, such as CPI-17, α-actin, h-caldesmon and h1-calponin, were all decreased in the cultured VSMCs, indicating a typical dedifferentiation of VSMCs during culturing process. Relative amounts of total actin and β-actin isoforms were also decreased. By contrast, RhoA, Rho-kinase (ROKα/ROCK-II) and MYPT1 were upregulated in cultured rat aorta VSMCs. Interestingly, similar changes in protein expression were found in cultured porcine coronary artery VSMCs, although Rho-kinase and PKCα were downregulated (Bi et al., 2005). We further examined the time course of relative expression of selected proteins with cell passages. The total actin content in cultured VSMCs rather abruptly decreased to 20-30% of that expressed in aortic tissues and was maintained at this level from the second to the tenth passage (Fig. 1B). The expression levels of smooth muscle-specific α-actin isoform (Fig. 1C) and CPI-17 (Fig. 1E) gradually decreased during passaging to 30% and 10%, respectively, at the tenth passage. The expression level of *h*-calponin was relatively promptly reduced to 25% (Fig. 1D). However, the levels of MYPT1 were increased several-fold, followed by a reduction to levels similar to those in VSMC tissue at passage ten (Fig. 1F). After the tenth passage, CPI-17 levels were decreased further below detection. We used cells from passages 4-10 in subsequent experiments. Furthermore, 24-hour serum starvation (supplemented with insulin and antibiotics only) before experimentation enhanced contractile protein expression of α-actin, CPI-17, *h*-calponin and MYPT1 by approximately 20-40% (n=3; data not shown).

Agonist-induced Ca^{2+} signals in cultured VSMCs

We measured changes in $[Ca^{2+}]_i$ in response to several agonists in Fluo-3-loaded cultured VSMCs. Fig. 2 illustrates representative Ca^{2+} images of the cultured aorta smooth muscle cells before and after the cells was stimulated with 0.1 μM endothelin-1 (ET-1). After recording the background fluorescent signal, the agonist was added 5 seconds before the first image was collected. A synchronized increase in the Ca^{2+}-indicator fluorescence intensity was seen in entire fields, and we obtain the fluorescent intensity in each image area that usually included 10-20 cells as intracellular $[Ca^{2+}]$. Fig. 3A shows a representative time course of change in the total intensity of fluorescent Ca^{2+} signal in response to 1 μM angiotensin II (ATII) and 0.1 μM ET-1. Fig. 3B,C show average time courses

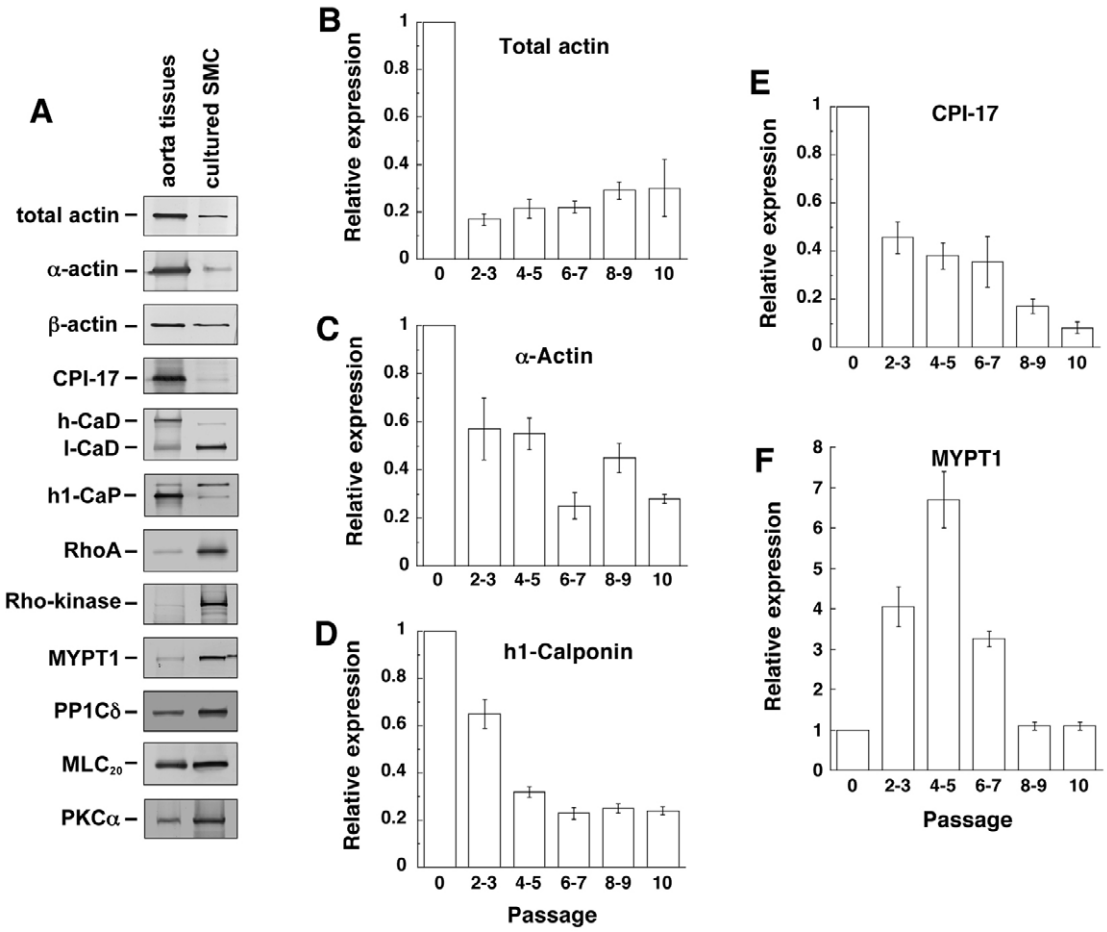

Fig. 1. Expression of contractile and/or regulatory proteins in primary cultured rat aorta VSMCs. The VSMCs from rat aorta were cultured up to passage ten. (A) Representative western blot images of various proteins in the cell lysates at passages 5-8. (B-F) Protein extracts were run on SDS gels and immunoblotted. For α-actin, caldesmon (CaD) and calponin (CaP) 2 μg of protein extract was used, all other protein extracts were used at 20 μg. Protein expression levels of (B) total actin, (C) smooth muscle α-actin isoform, (D) h-calponin, (E) CPI-17 and (F) MYPT1 were measured by immunoblotting and compared with the extract from intact rat aorta, which was normalized to one. *n*=3-10.

of ATII- and ET-1-induced $[Ca^{2+}]_i$ changes, respectively. Both agonists caused a transient increase in $[Ca^{2+}]_i$, which subsequently declined to a steady-state level that was similar to the resting $[Ca^{2+}]_i$ in the case of ATII (Fig. 3B) and significantly higher than the resting $[Ca^{2+}]_i$ for ET-1 (Fig. 3C). These responses did not appear to be dramatically affected by sequential addition of the agonists (Fig. 3B). However, typical α_1-agonists, PE (100 μM; Fig. 3D) and noradrenaline (10 μM; not shown) induced no increase and rather significant decrease in $[Ca^{2+}]_i$ in cells that were able to subsequently respond to ATII and ET-1. PDBu, a cell-permeable activator of PKC, did not alter Ca^{2+} levels at 1 μM (data not shown). For ET-1, the use of a Ca^{2+}-free, 2 mM EGTA-containing extracellular solution did not noticeably affect the peak Ca^{2+} transient, but the steady-

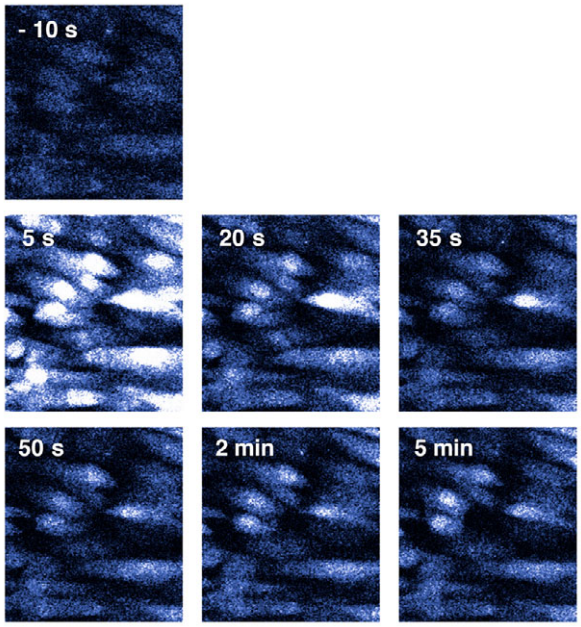

Fig. 2. Representative images of fluorescence of the Fluo-3 Ca^{2+}-indicator in cultured rat aortic smooth muscle cells. Images were captured every 15 seconds to avoid photo-bleaching. Baseline fluorescence was recorded before treatment with ET-1 (–10 seconds). ET-1 was added at 0 seconds and the first image was captured at 5 seconds. Although the cell boundary was not clear, a transient increase in fluorescence intensity was seen in almost all cells.

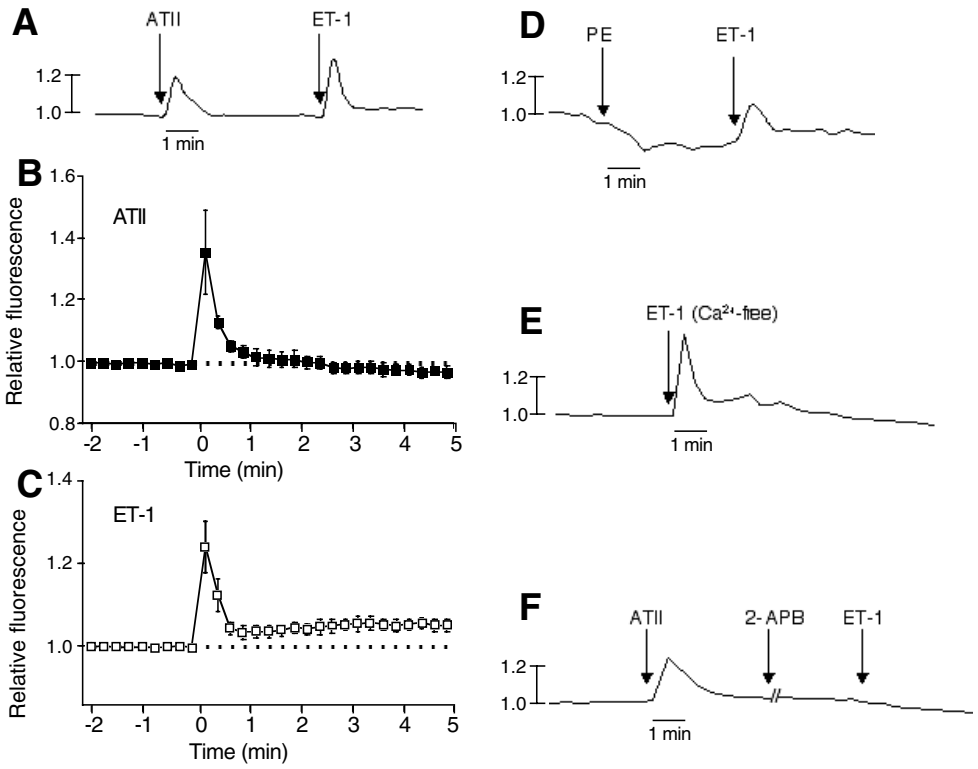

Fig. 3. Representative traces of fluorescence of the Ca^{2+}-indicator Fluo-3 in cultured VSMCs after various treatments. (A-F) Cells were serum-starved, loaded with Fluo-3 (see Materials and Methods for details), and then subjected to various agonists (1 μM ATII, 0.1 μM ET-1, or 100 μM phenylephrine (PE). (B,C) Average time courses of changes in $[Ca^{2+}]_i$ in cells stimulated with (B) ATII and (C) ET-1 (n=3). Dotted lines in A-C show the equivalent levels of $[Ca^{2+}]_i$ before stimulation. (D) PE-induced decrease in $[Ca^{2+}]_i$, although ET-1 still increased $[Ca^{2+}]_i$ in the presence of PE. (E,F) Cells treated with (E) Ca^{2+}-free solution or (F) of the Ins(1,4,5)P_3-receptor antagonist 2-APB m (90 μM) for 10 minutes. Traces are representative of three to four independent experiments for each condition.

state Ca^{2+} levels was below that in the presence of 2 mM Ca^{2+} (Fig. 3E). When 90 μM of the Ins(1,4,5)P_3-receptor antagonist 2-aminoethoxydiphenyl borate (2-APB) (Maruyama et al., 1997; Ascher-Landsberg et al., 1999) was added 10 minutes before ET-1 (Fig. 3F) or ATII stimulation (not shown), the rise in $[Ca^{2+}]_i$ was eliminated.

Contraction of reconstituted cultured rat aorta VSMC fibers and fresh rat aorta tissues

MLC phosphorylation triggers a contractile force in cultured cells that is required for cell locomotion, such as migration or cytokinesis (Fukata et al., 2001). We measured contractility of cultured VSMCs, using reconstituted VSMC fibers in a 3D-collagen gel (see Materials and Methods). We confirmed that, as reported by Oishi et al. and Song et al., expression levels of α-actin and, furthermore, CPI-17 and MYPT1 were not significantly changed whether fibers were cultured in the collagen gel or on plastic dishes (Oishi et al., 2000; Song et al., 2000) (data not shown). Fig. 4A shows a representative isometric contraction of the reconstituted VSMC fibers in response to high (124 mM) K^+, 200 μM ATP, 10 μM serotonin (5-HT), 1 μM ATII and 1 μM ET-1. When compared to the amplitude of high-K^+-induced contraction, ATP, 5-HT, and ET-1 produced an increase in contraction of 147±9% (n=4), 129±10% (n=8) and 241±14% (n=15), respectively. ATII evoked an contraction increase with a small transient peak (137±19%; n=8) followed by a decrease to a level that was still higher than basal. The ATII-induced contraction was reproducible after an extensive 30-minute wash (see Fig. 7B). Similar to the Ca^{2+} signals (Fig. 3D), neither the α_1-agonist PE nor noradrenaline (NA) evoked a contraction but rather reduced the 'resting tension' (n=6; Fig. 4B). This reduction was blocked by adding 30 μM of the β-receptor-blocker propranolol, suggesting a functional expression of β_2 receptors, which are stimulated by PE and NA and coupled to the downstream mechanisms through cAMP production for a decrease in $[Ca^{2+}]_i$ (Fig. 3D) and possibly contractile Ca^{2+} desensitization (Pfitzer et al., 1985). Neither histamine (30 μM) nor PDBu (1 μM; Fig. 4A) evoked significant contractions in the reconstituted VSMC fibers, similar to the non-increased Ca^{2+} signals (n=4).

Under the same conditions used for the reconstituted fibers, the average strength of contraction in aorta-tissue strips induced by ATP, 5-HT, ATII and ET-1 was 28±2 (n=8), 26±5 (n=8), 67±5 (n=14) and 243±9% (n=14), respectively, of high-K^+-induced contraction (Fig. 4C). The ATII-induced contractions were always transient and hardly reproduced, even after a 1-hour wash (data not shown). In contrast to the reconstituted fibers, PDBu (1 μM) effectively produced contractions equivalent to 193±13% (n=3, also see Fig. 8D). PE, a common and strong vascular agonist produced only small contractions (24±3% of high K^+; n=10) even at 100 μM in rat aorta tissues (Fig. 4D). The small PE-induced contractions were significantly enhanced by the presence of 30 μM propranolol by 3.4 times (n=3), basically similar to that of the reconstituted VSMC fibers (Fig. 4B). This enhancement of PE-induced contraction by propranolol was not seen in the femoral and mesenteric arteries of the same animals. Histamine did not evoke any contraction even in the presence of H_2 blocker tiotidine (Trzeciakowski and Levi, 1980) at 10 μM in the rat aorta, mesenteric and femoral arteries (n=3; not shown) similar to that of the reconstituted fibers.

Agonist-induced phosphorylation of MLC, CPI-17 and MYPT1

Phosphorylations of MLC, CPI-17 at Thr38, MYPT1 at

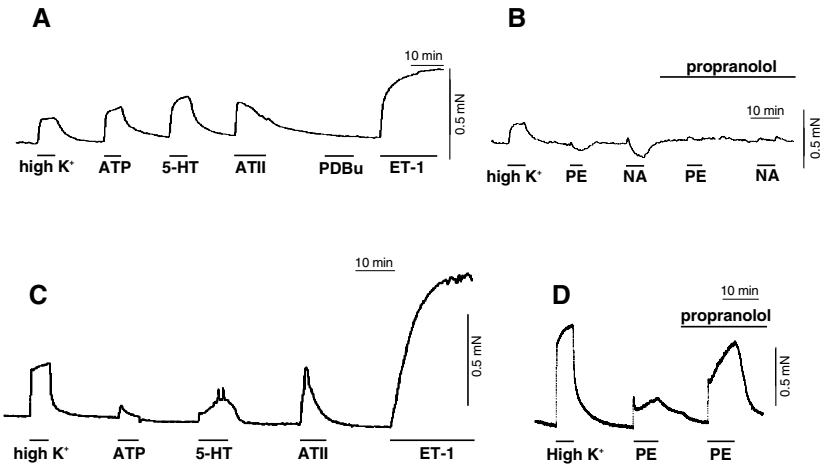

Fig. 4. Representative traces of contractile responses to various agonists in (A,B) reconstituted rat aorta VSMC fibers and (C,D) fresh tissues. Reconstituted VSMC fibers were cultured in collagen 3D-matrix in FBS-supplemented medium for 2 weeks and in the serum-free culture medium for an additional day (see Materials and Methods). (A) Contractions were induced with high-K^+ solution (124 mM), ATP (200 μM), serotonin (5-HT, 10 μM), ATII (1 μM), PDBu (1 μM) or ET-1 (1 μM). (B) Phenylephrine (PE, 100 μM) and noradrenaline (NA, 10 μM) both induced relaxation, which was blocked by 30 μM propranolol. (C) Contractions of the fresh rat aorta tissues were induced by high-K^+ solution, ATP, 5-HT, ATII, or ET-1 at the concentrations given for A. (D) A small contraction induced by 100 μM PE was enhanced by the presence of 30 μM propranolol. Force traces are representative of at least four (in A-C) and three (in D) similar experiments.

Thr696 and MYPT1 at Thr853 were simultaneously measured after stimulation with ET-1 or ATII. Basal MLC phosphorylation in the normal external growth-factor-free media for 1 day was already 34±4% (n=4) of total MLC. Continuous presence of growth factors in the media further increased MLC phosphorylation level to 44±3% (n=3). MLC phosphorylation was rapidly increased to high (70-80%) levels in response to both ET-1 and ATII (Fig. 5A). The

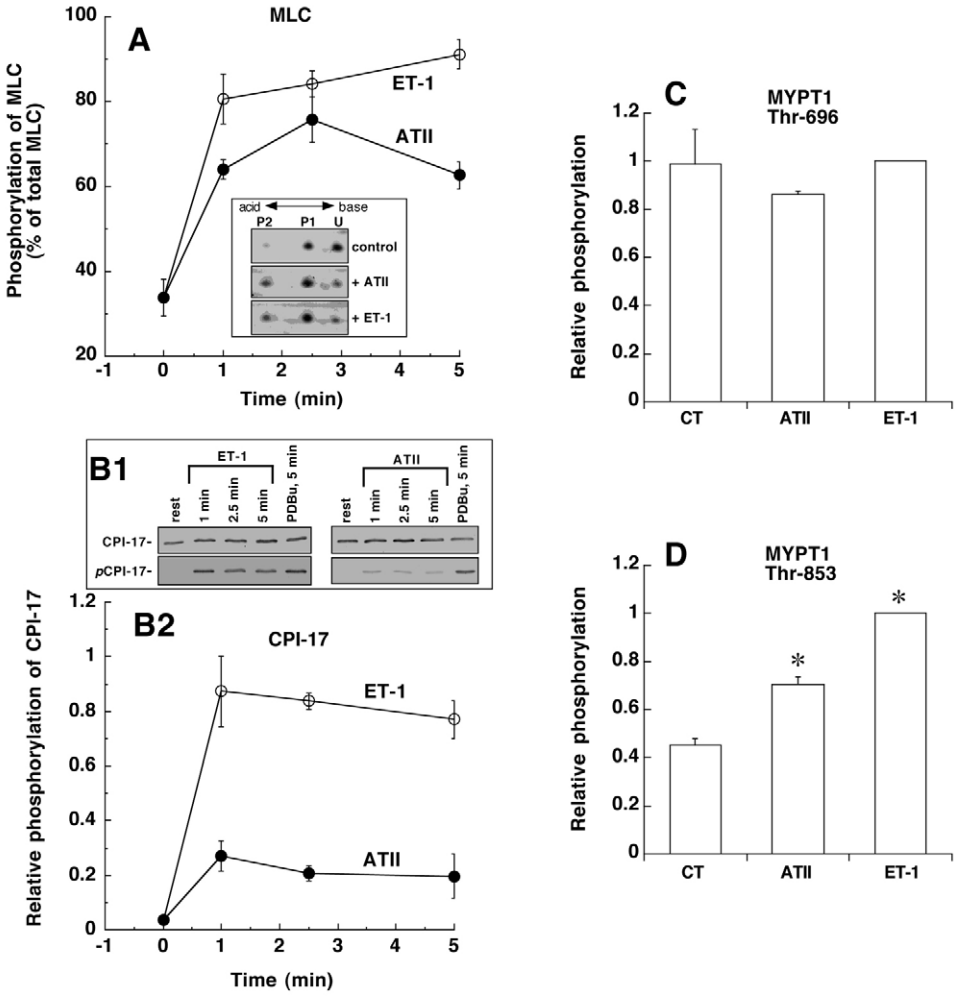

Fig. 5. Agonist-induced changes in phosphorylation of MLC, CPI-17 and MYPT1 in cultured VSMCs. (A) MLC phosphorylation is expressed as percent of total MLC (see Materials and Methods). Either ATII (1.0 μM) or ET-1 (0.1 μM) was added at 0 minutes. Inset shows representative 2D gel patterns of 20 kDa MLC of cultured VSMCs at rest (control), stimulated with ATII (+ATII) and ET-1 for 2.5 minutes (+ET-1). (B-D) Western blot results. Of each protein extract 20 μg were loaded onto two identical polyacrylamide gels and each was transferred onto nitrocellulose membrane. The total amount of (non-phosphorylated and phosphorylated) MYPT1 or CPI-17 was then determined with respective antibodies on one membrane (see Materials and Methods). We then compared the ratios of phosphorylated CPI-17 or MYPT1 to the total CPI-17or MYPT1 in the paired set of western blots and expressed relative phosphorylation levels as the percentage of control phosphorylation. (B1,B2) Western blots of total (CPI-17) and phosphorylated CPI-17 (pCPI-17) in cultured VSMCs at rest or VSMCs stimulated for 1, 2.5 and 5 minutes with ET-1 and for 5 minutes with the activator of PKC PDBu (B1). For average CPI-17 phosphorylation, the response to the 5-minute incubation with PDBu was normalized to 1 (B2). (C,D) Phosphorylation of MYPT1 at (C) Thr696 and (D) Thr853 after 2.5-minute incubation with ET-1 (0.1 μM) or ATII (1.0 μM). Responses in the presence of ET-1 were normalized to 1; *, significantly different to control (CT); n=3-5; $P \leq 0.05$.

increased MLC phosphorylation level was well maintained in response to ET-1, but declined in the presence of ATII after 5 minutes.

Although total the CPI-17 content was reduced in VSMCs (Fig. 1), the phosphorylation at Thr38 was increased by a membrane-permeable PKC activator PDBu (15-fold after 1 minute and over 20-fold after 2.5 and 5 minutes compared with the phosphorylation level at rest). Phosphorylation was also increased by ET-1 (Fig. 5B) and much less potently by ATII (Fig. 5B), similar to the results described for fresh rabbit arterial tissues (Kitazawa et al., 2000; Eto et al., 2001; Woodsome et al., 2001).

As shown in Fig. 5C, basal phosphorylation levels of MYPT1 at Thr696 were not significantly changed in response to stimulation with either ATII or ET-1 for 2.5 minutes. Stoichiometry of MYPT1 phosphorylation at Thr696 was 0.51±0.09 mol Pi/mol MYPT1 at rest and 0.58±0.07 mol Pi/mol MYPT1 at 2.5 minutes after ET-1 stimulation (n=3). The phosphorylation under resting condition was reduced by the non-selective kinase inhibitor staurosporine (Ruegg and Burgess, 1989) at 1 μM to 17±3% of control. Our previous data showed that phosphorylation at Thr696 was increased by calyculin A, the inhibitor of PP1 and PP2A phosphatases (Kitazawa et al., 2003). These data suggest that Thr696 phosphorylation is maintained by a high ratio of kinase to phosphatase activity, even under resting condition.

In contrast to Thr696, phosphorylation at Thr853 (Fig. 5D) significantly increased after treatment with ATII or ET-1, similar to the results obtained in fresh rabbit tissues (Kitazawa et al., 2003; Niiro et al., 2003). Furthermore, the ET-1-induced rise in MYPT1 Thr853 phosphorylation was 2.2 times greater than the effects of ATII. It is worthwhile to mention that the resting-phosphorylation level at Thr853 was already more than 40% of that stimulated by ET-1. The stoichiometry of MYPT1 phosphorylation at Thr853 was estimated to be 0.37±0.03 mol Pi/mol MYPT1 at rest and reached 0.84±0.10 mol Pi/mol MYPT1 after 2.5 minutes of ET-1 stimulation (n=3). Unlike Thr696, the phosphorylation at Thr853 is regulated in response to ET-1 stimulation.

Effects of kinase inhibitors on MYPT1 and CPI-17 phosphorylation

To investigate which kinases are involved in CPI-17 or MYPT1 phosphorylation, the cells were pretreated with the Rho-kinase inhibitor Y-27632 (Uehata et al., 1997) or the PKC inhibitor GF109203X (Toullec et al., 1991). A 30-minute pretreatment with 30 μM Y-27632 did not significantly change phosphorylation levels at Thr696 in the presence of either ET-1 or ATII (Fig. 6A). By contrast, the ATII-induced increase in MYPT1 phosphorylation at Thr853 was prevented by Y-27632 (Fig. 6B). The same concentration of Y-27632 attenuated the ET-1-induced MYPT1 phosphorylation at Thr853 by 60%, but phosphorylation levels still remained greater than resting levels.

Pretreatment with 10 μM of GF109203X for 30 minutes completely blocked any increase in CPI-17 phosphorylation at Thr38 in response to ET-1 (Fig. 6C). By contrast, treatment with 30 μM Y-27632 for 30 minutes had no effect on CPI-17 phosphorylation, suggesting that ET-1-induced phosphorylation of CPI-17 at Thr38 is mediated through PKC but not Rho-kinase.

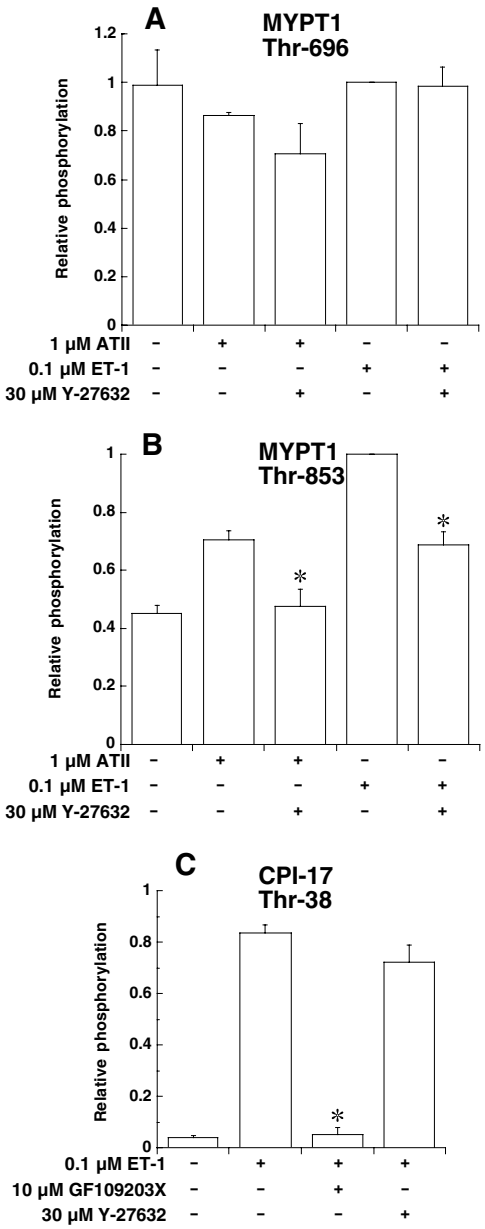

Fig. 6. Effect on phosphorylation of MYPT1 at (A) Thr696 and (B) Thr853, and of (C) CPI-17 at Thr38 after pretreatment with inhibitors. Cells were pretreated for 30 minutes with Y-27632 (30 μM) before addition of either ATII (1 μM) or ET-1 (0.1 μM) for 2.5 minutes. ET-1-treated samples were normalized to 1 for (A) MYPT1 Thr696, (B) MYPT1 Thr853 and (C) CPI-17 Thr38. *, significantly different to normal agonist treatments (n=3-4).

Effects of inhibitors on various contractions in reconstituted VSMC fibers and aortic tissue strips

High-K^+-induced contraction in reconstituted VSMC fibers was, in marked contrast to fresh aorta tissues (see below), not significantly inhibited by the Ca^{2+}-free extracellular solution that contained 2 mM EGTA (108±4% of control; n=8; Fig. 7A), or by 1 μM of the L-type Ca^{2+}-channel blocker nicardipine (data not shown). This extracellular Ca^{2+}-independent, high-K^+-induced contraction of cultured VSMCs was almost completely relaxed by the inhibition of Rho-kinase with 10 μM

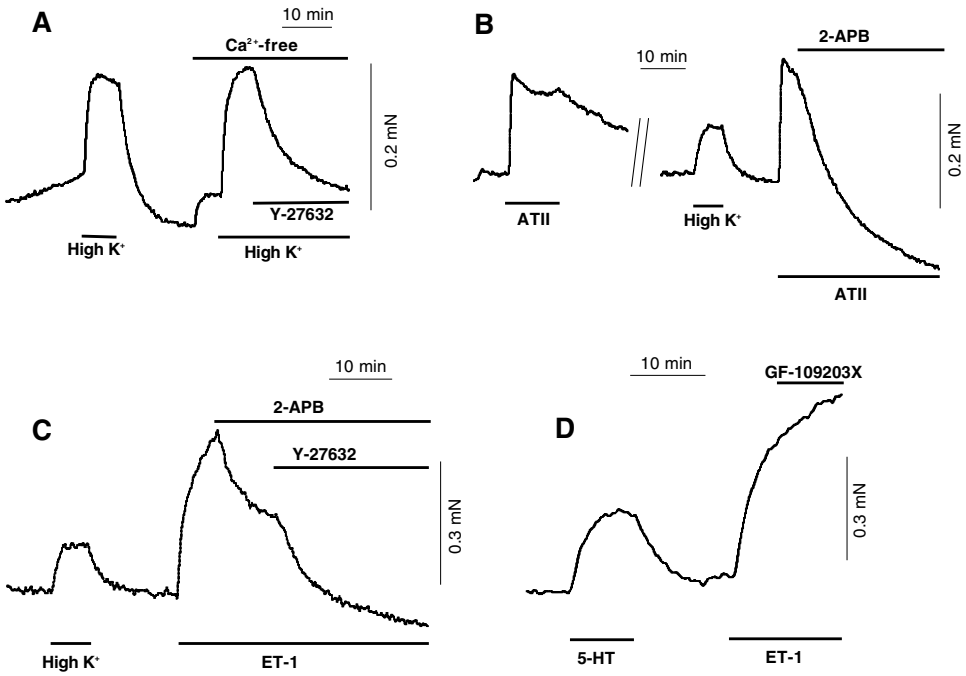

Fig. 7. Effect of 2-APB, GF-109203X and Y-27632 on agonist-induced contraction in reconstituted rat aortic VSMC fibers. (A) High levels of K^+ induced a contraction in the Ca^{2+}-free [EGTA-containing (2 mM)] solution similar to that of the control solution containing 2 mM Ca^{2+}. Y-27632 (10 μM) was added at the peak of contraction in response to the addition of high-K^+ solution to the Ca^{2+}-free solution. (B) The first contraction induced by 1 μM ATII was used as the control for the reconstituted VSMC fibers. After a 45-minute wash, the second addition of ATII resulted in a contraction to a level similar to the one after the first addition of ATII. Addition of 2-APB (30 μM) caused a faster relaxation in the presence of ATII than that by simple removal of ATII. (C) ET-1-induced contraction in the reconstituted VSMC fibers was partially inhibited by 2-APB (30 μM) alone and subsequently completely inhibited by the mixture of 2-APB and Y-27632 (10 μM). (D) ET-1-induced contraction was not affected by addition of GF-109203X (3 μM). The figures are representative of 3-5 similar experiments.

Y-27632 (Fig. 7A) or the inhibition of the intracellular Ca^{2+}-release with 30 μM 2-APB (not shown). ATII-induced contraction was also reduced by 30 μM 2-APB to a level below the base line (n=3; Fig. 7B). Pretreatment of the fibers with 2-APB also significantly prevented the development of ATII-induced contraction by 75±4% of control (n=3). The maintained tonic contraction induced by ET-1 was reduced partially by 30 μM 2-APB and strongly by further addition of 10 μM Y-27632 (n=4; Fig. 7C). The resultant level of relaxation by the mixture of inhibitors was always below the base line. Addition of single dose of 30-90 μM 2-APB, 10 μM Y-27632 or 100 μM ML-9 also produced a relaxation of the tonic phase of ET-1-induced contraction below the base line to, respectively, –3±10%, –9±9% or –15±10% of control (n=3-4). Pretreatment of the VSMC fibers with 30 μM 2-APB inhibited the development of ET-1-induced contraction by 64±12% of control (n=3). However, the pretreatment with Y-27632 alone had a tendency, but did not significantly (by only 15±11% of control, n=4), suppress the development of ET1-induced contraction. Either 1 μM nicardipine or 3 μM GF-109203X did not inhibit the development of ET-1-induced contraction or induced a significant relaxation of the tonic contraction (n=4; Fig. 7D). Removal of Ca^{2+} and addition of 2 mM EGTA did not prevent the development of contraction induced by agonists, such as ET-1, ATII and 10 μM 5-HT (not shown).

In fresh rat aorta tissues, in contrast to reconstituted VSMC fibers, the development of high-K^+-induced contraction was almost completely blocked by removal of Ca^{2+} and addition of 2 mM EGTA (by 91±1% of control; n=4; Fig. 8A), by inhibition of Ca^{2+} channels with 1 μM nicardipine (by 100±0%; n=3) or inhibition of MLC kinase with 100 μM ML-9 (by 95±1%; n=3). It was and partially inhibited by 10 μM Y-27632 (by 33±8% at initial phasic contraction and by 71±4% at tonic phase of contraction; n=3) or by 30 μM 2-APB (by 50±11% at phasic contraction and 69±10% at tonic phase of contraction; n=4). Ca^{2+}-free solution containing 2 mM EGTA and the presence of 30 μM 2-APB (Fig. 8B) strongly inhibited the development of transient ATII-induced contraction by 88±10% and 75±5%, respectively, and 1 μM nicardipine, 3 μM GF-109203X and 10 μM Y-27632 partially inhibited the development of transient ATII-induced contraction by 44±3%, 38±14% and 20±6%, respectively (n=3-9). The tonic phase of ET-1-induced contraction in aorta strips was effectively reduced by all three inhibitors (2-APB, Y-27632 and GF-109203X) (Fig. 8C). The development of contraction by ET-1 was significantly suppressed by the Ca^{2+}-free solution and the presence of 2-APB or nicardipine by, respectively, 89±3% and 40±1% or 36±3% of control (n=4-7). Pretreatment with Y-27632 or GF-109203X, however, did not significantly prevent the development of ET-1-induced contraction [14±12% (n=9) or 5±5% (n=4) of control, respectively]. The PDBu-induced contraction was reduced by 3 μM GF-109203X to near base line (Fig. 8D).

Discussion

This study provides a view of multiple signaling pathways that increase MLC phosphorylation in response to agonists in cultured proliferative vascular smooth muscle cells. Accumulated evidence, by using strips of vascular smooth muscle tissues (Somlyo and Somlyo, 1994; Somlyo and Somlyo, 2003) (Fig. 9), demonstrates that agonists mainly increase MLC phosphorylation and contraction through an increase in the activity ratio of MLCK to MLCP. This is achieved by pathways such as, (1) Ca^{2+} channel→Ca^{2+}→CaM→MLCK, (2) Ins(1,4,5)P_3→SR→Ca^{2+}→CaM→

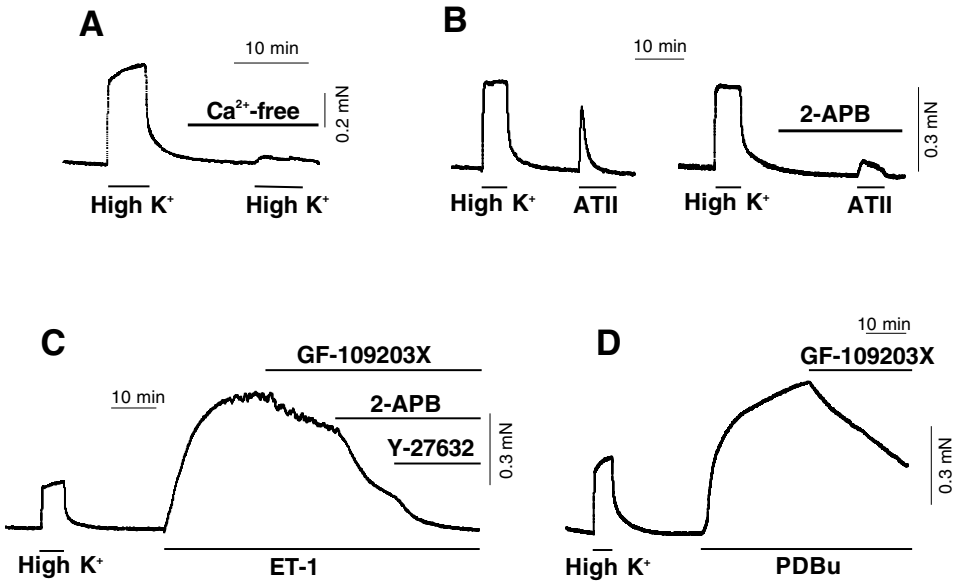

Fig. 8. Effect of various inhibitors on high-K^+- and agonist-induced contractions in fresh rat aorta tissues. (A) Ca^{2+}-free, EGTA-containing solution inhibited the development of contractions induced by high K^+. (B) The first force trace shows a control contraction, transient and practically irreversible, induced by 1 μM ATII after a contraction induced by high K^+. The second trace (from another tissue strip) shows that the development of transient ATII-induced contraction is prevented by in presence of 30 μM 2-APB. (C) ET-1 (1 μM) produced a large sustained contraction, which was relaxed to the base line by 3 μM GF-109203X, 30 μM 2-APB and 10 μM Y-27632. (D) PDBu (1 μM) generated a large contraction, which was reduced by 3 μM GF-109203X. All figures are representative of 3-5 similar experiments.

MLCK. Moreover, by Ca^{2+}-independent inhibitory pathways such as (3) PKC→CPI-17(Thr38-*P*)→PP1Cδ, (4) RhoA→Rho-kinase→CPI-17(Thr38-*P*)→PP1Cδ, and (5) RhoA→Rho-kinase→MYPT1(Thr853-*P*)→PP1Cδ. We have described here that, in cultured VSMCs agonists and even high-K^+ (membrane depolarization) produce a contraction via an Ins(1,4,5)P_3-dependent Ca^{2+}-release from the SR (pathway 2) as the main Ca^{2+} source for Ca^{2+}-dependent mechanism and via RhoA→Rho-kinase→MYPT1(Thr853-*P*)→PP1Cδ (pathway 5) as a main mechanism for Ca^{2+}-independent MLCP inhibition (see also Bi et al., 2005). In contrast to the aortic tissue strips, PKC inhibitor suppressed agonist-induced CPI-17 phosphorylation, but did not affect agonist-induced contraction, consistent with the downregulation of CPI-17 expression (Bi et al., 2005) (this study). Likewise, removal of extracellular Ca^{2+} or addition of voltage-dependent Ca^{2+}-channel blocker did not inhibit agonist- and membrane-depolarization-induced contractions. These provide evidence that Ca^{2+} influx and CPI-17 signaling are mostly downregulated and are minimally involved in an increase of MLC phosphorylation and contraction in cultured VSMCs.

Both ET-1 and ATII significantly increased $[Ca^{2+}]_i$, phosphorylation of MYPT1 at Thr853 (but not at Thr696), CPI-17 at Thr38 and MLC, and contraction in cultured VSMCs. However, several quantitative differences in the response to the two agonists were found here. First, although the initial Ca^{2+} transients induced by ET-1 and ATII were similar, and consisted of a rapid rise of $[Ca^{2+}]_i$ followed by a decline, the level of maintained $[Ca^{2+}]_i$ after the peak was different. In the continuous presence of ET1, maintained $[Ca^{2+}]_i$ was significantly higher than the basal level after a peak. However, in the case of ATII the Ca^{2+} signal was transiently returned to the basal level. The higher level of steady-state Ca^{2+} in the presence of ET-1 must result in a higher activity of Ca^{2+}-dependent MLCK during sustained contraction than that of ATII. Second, activation of the Ca^{2+}-independent signaling pathways was also significantly different between the two agonists. Phosphorylation of MYPT1 at Thr853 was

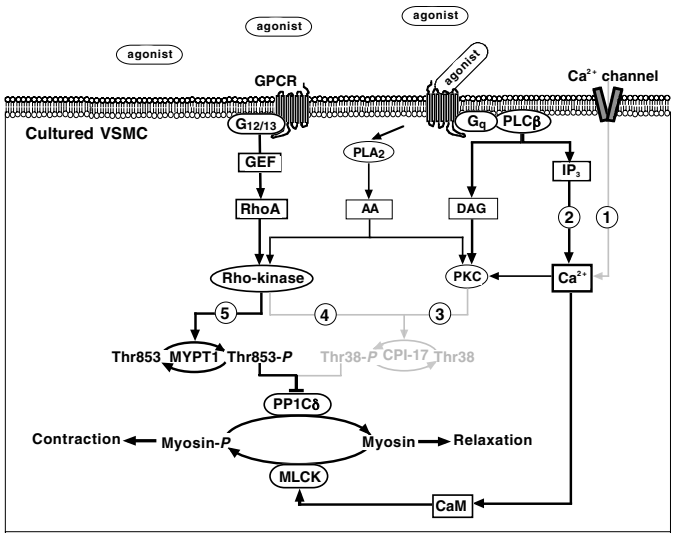

Fig. 9. Schematic diagram of signal transduction pathways towards phosphorylation of myosin and contraction in cultured VSMCs. In smooth muscle tissues, both pathways of signal transduction (black and gray) are significant in development of contraction. However, only signaling pathways indicated in black are active in cultured VSMCs. GPCR, G protein-coupled receptor; PLCβ, phospholipase Cβ; PLA_2, phospholipase A_2; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphatase; DAG, diacylglycerol; AA, arachidonic acid; GEF, guanine nucleotide exchange factor; PKC, protein kinase C; CPI-17, protein kinase C-potentiated myosin phosphatase inhibitor protein 17 kDa; MYPT1, myosin targeting subunit of myosin phosphatase; PP1Cδ, δ-isoform of type 1 protein phosphatase catalytic subunit; MLCK, myosin light chain kinase; CaM, calmodulin. All pathways represent signaling pathways leading to an increase in myosin-*P* and contraction. Pathway1 through voltage-dependent Ca^{2+} channels, pathway 2 through Ins(1,4,5)P_3-induced Ca^{2+} release from the SR, pathway 3 through phosphorylation of CPI-17 at Thr38 by PKC, pathway 4 through phosphorylation of CPI-17 by Rho-kinase and pathway-5 through phosphorylation of MYPT1 at Thr853 by Rho-kinase.

increased by ET-1 from 37% at the basal level to 85% of total MYPT1. This was twice as much as that of ATII, suggesting much higher activity of Rho-kinase in response to ET-1 than ATII. This phosphorylation is possibly responsible for the inhibition of the cellular MLCP activity (Velasco et al., 2002) and the increase in the Ca^{2+} sensitivity of MLC phosphorylation in cultured VSMCs. It should also be noticed that the phosphorylation of MYPT1 at Thr696 was 50-60% of total MYPT1 regardless of agonist stimulation. Therefore, what role does the inactive form of MLCP have in its function and localization, and which, if any, mechanism(s) are responsible for the reactivation of inactive MLCP. Active Rho-kinase, in response to ET1, might directly phosphorylate more MLC (Amano et al., 1996) than ATII in cultured VSMCs. Such a direct phosphorylation of MLC at Ser19 by Rho-kinase rather than MLCK is very unlikely to occur in fresh arterial tissues, because the rate of MLC phosphorylation is not affected by agonists and GTPγS when MLCP is inhibited (Kitazawa et al., 1991b; Masuo et al., 1994). Nonetheless, it is still possible that Rho-kinase directly phosphorylates MLC in cultured VSMCs, in which RhoA and Rho-kinase are extremely upregulated. Together, these results suggest that ET-1 can efficiently activate both Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways, and thus maintain higher level of MLC phosphorylation and contraction as compared with those of ATII. Indeed, ATII-induced contraction was strongly abolished by 2-APB alone, inhibiting Ins(1,4,5)P_3-induced Ca^{2+} release, whereas Y-27632 together with 2-APB is required for the strong inhibition of ET-1-induced contraction. This, again, confirms that ATII primarily activates a transient Ca^{2+}-dependent signaling pathway that includes MLCK, and thus increases MLC phosphorylation and contraction in a transient manner. This is consistent with the observation that ATII receptor activation is not coupled to RhoA activation that activates Rho-kinase in rabbit aorta tissues (Sakurada et al., 2001).

Considering fresh smooth muscle tissues, there is no doubt that high K^+ causes membrane depolarization of smooth muscle cells (and also nerve cells remaining in some tissues) (Kitazawa et al., 2003), followed by the opening of Ca^{2+} channels and Ca^{2+} influx (Fig. 9). The resultant increase in $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin-dependent MLCK, which increases MLC phosphorylation and contraction. Indeed, Ca^{2+}-channel blocker, removal of extracellular Ca^{2+} and the MLCK inhibitor ML-9 all prevented the high-K^+-induced contraction in the aorta tissues. In the reconstituted VSMC fibers, however, the high-K^+-induced contraction was not inhibited by the Ca-channel blocker or a Ca^{2+}-chelator (2 mM EGTA), suggesting that the depolarization-induced contraction does not rely on Ca^{2+} influx across the plasma membrane. Since the MLCK inhibitor ML-9 relaxed contractions, the high-K^+-induced contraction appears to require activation of MLCK. The Ins(1,4,5)P_3-receptor antagonist 2-APB also inhibited the development of high K^+-induced contraction, suggesting that high K^+ increases the concentration of Ins(1,4,5)P_3 to induce Ca^{2+} release from the intracellular Ca^{2+} stores and evoke a contraction. Together, these results suggest that Ca^{2+} from intracellular stores but not from across the plasma membrane triggers the development of depolarization-induced contraction of the reconstituted VSMC fibers (Fig. 9). The lack of Ca^{2+} entry due to membrane depolarization may be ascribed at least in part to the following: (1) The resting membrane potential in cultured smooth muscle cells is already at considerably depolarized state (Platoshyn et al., 2000), so that most of voltage-dependent Ca^{2+} channels are supposedly in inactivated state even under 'resting conditions' and cannot be opened by membrane depolarization, and/or (2) the expression of L-type Ca^{2+} channels is downregulated (Gollasch et al., 1998) similar to that of smooth-muscle-specific α-actin and CPI-17 (this study). Interestingly, Y-27632 strongly relaxed the high-K^+-induced contraction and also prevented the development of contraction by high K^+ in the reconstituted fibers. Initially, the Rho-kinase inhibitor Y-27632 was thought to have no effect on the high-K^+-induced contraction in smooth muscle tissues (Uehata et al., 1997). However, more recent studies show that the late plateau-phase (but not the initial phase) of high K^+-induced contraction in smooth muscle tissues is very sensitive to the Rho-kinase inhibitors, not only to Y-27632 but also to the structurally different HA1077 (Mita et al., 2002; Urban et al., 2003; Janssen et al., 2004). Since Y-27632 does not block an initial Ca^{2+} transient induced by high K^+, the initial development of contraction is not prevented by the presence of the inhibitor. These results suggest that Rho-kinase has a significant role in the maintenance of the plateau-phase of high-K^+-induced contraction in smooth muscle cells. Sakurada et al. demonstrated that, the active form of RhoA was indeed increased by high K^+ and ionomycin, and inhibited by the removal of extracellular Ca^{2+} (Sakurada et al., 2003). However, it is still not clear whether Ca^{2+} alone, Ca^{2+} and membrane depolarization together or the resultant increase in second messenger(s) besides Ca^{2+}, such as arachidonic acid, have a role in activation of RhoA and/or Rho-kinase. Interestingly, Murata et al. identified the existence of a non-channel protein having both voltage-sensor- and phosphatase-domains, by which the membrane potential directly regulates the phosphoinositide-turnover rate (Murata et al., 2005). Although further studies are needed to clarify the mechanism(s), the reconstituted VSMC fibers appear to be a very useful model for depolarization-induced and Y-27632-sensitive contraction because the other Ca^{2+}-sensitizing pathway towards MLCP inhibition, i.e. CPI-17, is downregulated.

The basal level of MLC phosphorylation was significantly increased (34% of total MLC) in cultured VSMCs in spite of high MLCP expression. Would this happens in the fresh tissues, they would produce 20-50% of maximum contraction at pCa ($-\log_{10}[Ca^{2+}]$) 5, dependent on the fiber type (Kitazawa et al., 1991a). Indeed, inhibitors Y-27632, 2-APB and ML-9, but not GF-109203X, all decreased the isometric tension below the basal level, suggesting that Rho-kinase, Ins(1,4,5)P_3-dependent Ca^{2+} and MLCK, but not PKC–CPI-17, play a significant role in the high basal activity of cultured VSMCs without stimulation (Fig. 9). The high MLC phosphorylation and high basal tone may be relevant to high proliferation rate and high plasticity of the cultured VSMCs, which are always prepared to proliferate and move. The basal MYPT1 phosphorylation at Thr853 was also very high (37% of total MYPT1) in spite of high expression levels of MYPT1. These results suggest that kinases, including MLCK and Rho-kinase, in cultured cells are so active that they can considerably overcome the activity of phosphatases, even under basal conditions.

In conclusion, multiple pathways lead to MLC

phosphorylation, activation of myosin and contraction in the agonist-specific and phenotype-dependent manner. In the cultured rat aortic VSMCs where Ca^{2+}-influx and CPI-17–MLCP signaling is downregulated, ET-1 stimulation causes potent activation of mainly two parallel pathways Ins(1,4,5)P_3→Ca^{2+}→MLCK (pathway 2) and RhoA→Rho-kinase→MLCP (pathway 5) to evoke a large monotonic contraction. By contrast, ATII appears to act mainly on the Ins(1,4,5)P_3→Ca^{2+}→MLCK pathway, with a weak activation of the Ca^{2+}-independent MLCP-inhibition pathways to produce smaller and more transient contractions than those of ET-1. Thus, this study provides valuable information on signaling pathways underlying the regulation of contractile machinery in fresh and cultured smooth muscle cells, and contributes to further understanding of smooth muscle pathophysiology.

Materials and Methods

Standard cell culture

Primary culture cells from adult male Sprague-Dawley rat aortas were grown according to the protocol provided by Biowhittaker (Cambrex; East Rutherford, NJ). Briefly, the growth medium SmBM (modified MCDB131 medium; Cambrex) was supplemented with 0.5 ng/ml human recombinant epidermal growth factor (hEGF), 5 μg/ml insulin, 1 ng/ml human recombinant fibroblast growth factor (hFGF), 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 5% fetal bovine serum (FBS). Cells were grown at 37°C in 5% CO_2 and the medium was changed regularly (every 3 days) until 2 or 3 days after the cells reached confluence. Cells at 80-90% confluence were either propagated through trypsin treatment or grown to 100% confluence. For subculturing, a hemocytometer was used to seed at a density of 3500-4000 cells/cm^2. Confluent cells were further incubated in serum-free medium (supplemented with insulin and antibiotics only) for 24 hours before experiments.

Reconstituted VSMC fibers

To examine contractile activity of cultured cells, reconstituted VSMC fibers in the 3D-collagen matrix were prepared using the method that was similar to those of Kolodney and Wysolmerski, and Oishi et al. (Kolodney and Wysolmerski, 1992; Oishi et al., 2000). The collagen-gel solution was prepared from sterile pepsin-solubilized bovine dermal collagen solution (95-98% was type I and the remainder type III; Cohesion Technologies in Angiotech Biomaterials, Palo Alto, CA) according to the manufacturer's instruction. All solutions used to prepare the reconstituted VSMC fibers in the collagen matrix were kept on ice to decelerate the collagen polymerization. The suspended rat aortic VSMCs (final concentration ~5×10^5 cells/ml) were gently mixed with the neutralized collagen solution (pH 7.4) containing 1.2 mg/ml of collagen in phosphate-buffered saline solution. The resultant collagen solution was cast in a rectangular trough (17 mm long, 5.5 mm wide, 1.7 mm deep) with a 1.6-mm-diameter Teflon pole near each end. The trough was made of silicone elastomer (Sylgard 184; Dow Corning, Midland, MI) in 5-cm-diameter culture dishes. After the collagen solution containing VSMCs became firm at 37°C within 1 hour, the supplemented culture medium SmGM described above was added to the dishes and the culture was continued for 7-14 days. Fresh culture medium was applied every 2 days. The cells in the 3D-collagen matrix appeared to be spindle-shaped and quiescent in terms of cell proliferation. The collagen gels with cells started to shrink within a day and formed a rod-shape (~400 μm in diameter) supported by a Teflon pole at each end (see Fig. 1 of Oishi et al., 2000). The collagen gel fibers were kept for one or two additional days in the serum-free culture media. Oishi et al showed that cultured smooth muscle cells in reconstituted fibers exhibited elongated bipolar shapes and were oriented parallel to the longitudinal axis of the fibers (Oishi et al., 2000; Oishi et al., 2002). The rod-shaped fibers were cut into 4-mm-long strips. One end of the segment was tied to a force transducer and the other to a micromanipulator with a monofilament silk thread to monitor isometric contraction as was the fresh arterial tissue strips. The fibers were stretched to 1.2 times of slack length and equilibrated in the normal external solution for at least 1 hour before experiments. To avoid lack of cellular glucose and oxygen during contraction, cells were kept at temperature of 30°C instead of 37°C throughout the experiments to reduce the consumption of energy and oxygen (because of high Q_{10}) with little effect on speed of diffusion (low Q_{10}). The compositions of the normal external and high (124 mM) K^+ solutions have been described previously (Woodsome et al., 2001). The force was monitored using a force transducer (AE801; SensoNor, Horten, Norway) as previously described (Masuo et al., 1994).

Tissue preparations

All animal procedures were approved by the Animal Care and Use Committee of the Boston Biomedical Research Institute. Adult male Sprague-Dawley rats (250-300 g) were euthanized with CO_2. Smooth-muscle-tissue strips (750 μm wide and 2.5 mm long with natural wall thickness) were dissected from thoracic aorta and cleaned from endothelial cells and fluffy connective tissues. Force levels were monitored at 30°C as described previously (Masuo et al., 1994). Prior to experimentation, the strips were stimulated several times with a high-K^+ (124 mM) solution until a steady maximal response was obtained.

Antibodies and chemicals

Polyclonal anti-CPI-17, anti-phosphorylated(Thr38)-CPI-17, and anti-phosphorylated(Thr696)-MYPT1 antibodies were prepared as described previously (Kitazawa et al., 2000; Kitazawa et al., 2003). Polyclonal anti-MYPT1 and anti-phosphorylated(Thr853)-MYPT1 antibodies was purchased from BabCO (Richmond, CA) and Upstate Biotechnology, respectively. We used these antibodies to monitor the phosphorylation levels of Thr38 of CPI-17, and Thr696 and Thr853 of MYPT1 extracted from rat aorta VSMCs as described previously (Kitazawa et al., 2000; Kitazawa et al., 2003). Polyclonal anti-PP1Cδ antibody was prepared and affinity-purified (Eto et al., 1999). Polyclonal anti-h-caldesmon and anti-h-calponin antibodies were provided by K. Mabuchi (Mabuchi et al., 1996). Monoclonal anti-α-smooth muscle actin, anti-β-actin and anti-MLC_{20} antibodies, and polyclonal anti-actin(20-33) and anti-PKCα antibodies were from Sigma (St Louis, MO), monoclonal anti-RhoA antibody from SantaCruz Biotech (Santa Cruz, CA), and monoclonal anti-Rho-kinase (ROKα) from Transduction Laboratories (Lexington, KY).

Alkaline-phosphatase-conjugated secondary antibody against IgY was purchased from Promega (Madison, WI), anti-rabbit and mouse secondary antibodies were from Chemicon (Temecula, CA), and anti-sheep antibody was from Sigma. GF-109203X was from BioMol (Plymouth Meeting, PA), and 2-aminoethoxy-diphenylborate (2-APB) from Calbiochem (San Diego, CA). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries (Iruma, Saitama, Japan).

Standard immunoblotting

The protocol for agonist treatment was as follows: cells were first washed three times with prewarmed (37°C) extracellular solution (150 mM NaCl, 4 mM KCl, 2 mM Ca^{2+} methanesulphonate, 2 mM Mg^{2+} methanesulphonate, 5.6 mM glucose, and 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid). Then, cells were either subjected to the same buffer (control) or treated with buffer containing selected agonists for various periods of time. The solutions were then aspirated from the flasks and the cell populations immediately fixed in cold 10% trichloroacetic acid (TCA) in H_2O. After fixation, the cells were removed with a scraper and the suspension was transferred to a 1.5 ml centrifuge tube. The samples were washed with acetone, dried and homogenized in Laemmli sample buffer as described previously (Woodsome et al., 2001).

Immunoblotting has been described in detail previously (Kitazawa et al., 2000; Woodsome et al., 2001). Briefly, the homogenized samples were heated at 95°C for 5 minutes and centrifuged. Protein concentration of the supernatants was adjusted to 2 mg/ml

Ca^{2+} signaling

To determine the presence of functional agonist receptors, intracellular Ca^{2+} was measured as described by Wang et al. (Wang et al., 2000). Cells were grown to confluence on number 1.5 coverglass chambers (Nunc; Rochester, NY) as described above and then incubated for an additional 24 hours in growth-factor-free media prior to Ca^{2+} measurement. The cells were then incubated in the extracellular solution supplemented with 0.55 μM Fluo-3 AM (Molecular Probes). After rinsing out the indicator dye, Ca^{2+} fluorescence was recorded using a Noran Odyssey XL laser scanning confocal microscope (Noran Instruments, Madison, WI) with a Zeiss 40× water-immersion objective lens. The excitation wavelength of the argon ion laser was set at 488 nm and fluorescence light greater than 510 nm was detected. Images were obtained every 15 seconds (to prevent photobleaching) and recorded with Intervision software on a Silicon Graphics workstation. For some experiments, Ca^{2+}-free extracellular solution (150 mM NaCl, 4 mM KCl, 2 mM $Mg(Ms)_2$, 5 mM HEPES, 11.5 mM Glucose, 2 mM EGTA, pH 7.4) was used to determine the source of increase in intracellular Ca^{2+}. The cell-permeable Ins(1,4,5)P_3-receptor antagonist 2-APB was also used to evaluate intracellular Ca^{2+} signaling.

Measurement of MLC phosphorylation

Cultured cells were fixed with cold 10% TCA 0, 1, 2.5, and 5 minutes after agonist stimulation. The fixed samples were collected into a centrifuge tube, thoroughly washed with acetone and dried under vacuum at room temperature. The dried cells were homogenized in a buffer containing 0.1% SDS, 20 mM DTT, 10% glycerol, and 0.1 mg/ml BSA. The samples were then subjected to 2D-electrophoresis to identify the phosphorylation states of MLC as described previously (Kitazawa et al., 1991a). Since di-phosphorylation of MLC at Ser19 and Thr18 has no addition effect on the in vitro motility of myosin mono-phosphorylated at Ser19 of MLC (Umemoto et al., 1989; Bresnick et al., 1995), we assume that effect of the di-phosphorylated myosin on isometric contraction is equivalent to that of mono-phosphorylated myosin. For evaluation of MLC phosphorylation, therefore, the equation used was percent phosphorylation=100×(P1+P2)/(U1+P1+P2) where

U1=unphosphorylated, P1=monophosphorylated, and P2=diphosphorylated MLC. If non-muscle cells had been present, a spot at P2 was been seen at control conditions (Kitazawa et al., 1991a). However, P2-staining under control conditions was barely visible, which suggests minimal contamination by other cell types.

Quantitative immunoblotting

Quantification of phosphorylation of MYPT1 and CPI-17 using immunoblotting (Kitazawa et al., 2000; Kitazawa et al., 2003) is as follows: Acid-fixed and dried samples were thoroughly homogenized in Laemmli sample buffer as described above. Immunoblotting experiments for measurements of protein phosphorylation were always carried out in duplicate. Equal amounts (20 μg) of each protein extract were loaded onto two identical polyacrylamide gels composed of 15% acrylamide at the bottom (for CPI-17) and 8% in the middle (for MYPT1) with a stacking gel on top. Separated proteins were transferred to the same nitrocellulose membranes. The membranes were blocked in a Tris-buffered saline solution containing 0.05% Tween-20, 5% non-fat milk and 1% BSA. The membranes were then incubated with a primary antibody followed by an alkaline-phosphatase-conjugated secondary antibody. The immunoblots were developed with an alkaline phosphatase substrate solution (Sigma) to visualize immunoreactive proteins. The bands of alkaline phosphatase products were digitized and analyzed with an image processing software (Signal Analytics Co., Vienna, VA). We compared the ratios of phosphorylated to total protein (CPI-17 and MYPT1) in the paired set of western blots and expressed relative phosphorylation levels as the percentage of control phosphorylation.

Statistics

Results are expressed as the means ± s.e.m. of *n* experiments. Statistical significance was evaluated with one-way ANOVA; $P<0.05$ was considered statistically significant.

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