Role of Protein Kinase C ζ and Calcium Entry in KCl-Induced Vascular Smooth Muscle Calcium Sensitization and Feedback Control of Cellular Calcium Levels

Paul H. Ratz and Amy S. Miner

Departments of Biochemistry and Molecular Biology and Pediatrics, Virginia Commonwealth University, School of Medicine, Richmond, Virginia

Received June 16, 2008; accepted November 13, 2008

ABSTRACT

The degree of tonic force (F) maintenance induced in vascular smooth muscle upon K⁺ depolarization with 110 mM KCl can be greatly reduced by inhibition of rhoA kinase (ROCK). We explored the possibility that a protein kinase C (PKC) isotype may also play a role in causing KCl-induced Ca²⁺ sensitization. In isometric rings of rabbit artery, the PKC inhibitors, Go-6983 (3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione), GF-109203X (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide), and a cell-permeable (myristoylated) pseudosubstrate inhibitor of PKC ζ (PI_{PKC ζ}) inhibited KCl-induced tonic F. A myristoylated pseudosubstrate inhibitor of PKC α/β that inhibited phorbol dibutyrate-induced F slightly potentiated KCl-induced tonic F and attenuated 30 mM KCl-induced F. Although the ROCK inhibitor, H-1152 [(S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride], reduced basal phosphorylation of myosin light-chain phosphatase-

targeting subunit at Thr853 (MYPT1-pT853), 3 and 10 μ M GF-109203X inhibited only KCl-stimulated phosphorylation, not basal MYPT1-pT853. In fura-2-loaded tissues, GF-109203X and PI_{PKC ζ} elevated basal [Ca²⁺]_i (calcium) and potentiated KCl-induced tonic increases in calcium while reducing KCl-induced tonic increases in F. Blockade by nifedipine of Ca²⁺ entry through voltage-operated Ca²⁺ channels reduced KCl-induced Ca²⁺ sensitization and KCl-stimulated but not basal MYPT1-pT853. These data together support a model in which ROCK and PKC ζ are constitutively active and function in “resting” muscle to regulate the basal levels of MYPT1-pT853 and calcium, respectively. In this model, KCl-induced increases in calcium activate PKC ζ to feed forward and cause additional MYPT1-pT853 above that induced by constitutive ROCK, permitting Ca²⁺ sensitization and strong F maintenance. Active PKC ζ also feeds back to attenuate the degree of KCl-induced increases in calcium.

The primary mechanism causing smooth muscle cross-bridge activation leading to isometric force (F) development involves increases in calcium and MLCK activity, causing increased myosin light-chain phosphorylation (MLCP) (for review, see Kamm and Stull, 2001). Ca²⁺ sensitization is the term used for a control system identified in smooth muscle

nearly 2 decades ago (Nishimura et al., 1988; Kitazawa et al., 1989), in which Ca²⁺-dependent cross-bridge activation and, therefore, contractile F is potentiated independently of further increases in calcium by inhibition of MLCP (for review, see Karaki, 1989).

A currently accepted model of Ca²⁺ sensitization thought originally to absolutely require G protein-coupled receptor (GPCR) activation (for review, see Somlyo and Somlyo, 2003) involves inhibition of MLCP by ROCK, PKC α/β , and PKC δ (Eto et al., 2001). MLCP inhibition produces an elevation in MLCP and F above that induced by activation of Ca²⁺-cal-

This work was supported by the National Institutes of Health [Grant R01-HL61320] and by the American Heart Association.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.142422.

ABBREVIATIONS: F, isometric force; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphorylation; MLCP, myosin light-chain phosphatase; GPCR, G protein-coupled receptor; ROCK, rhoA kinase; PKC, protein kinase C; VOCC, voltage-operated calcium channel; GF-109203X, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide; Y-27632, *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride; HA-1077, 1-(5-isoquinolinesulfonyl)homopiperazine hydrochloride; H-1152, (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride; PSS, physiological saline solution; MOPS, 4-morpholinepropanesulfonic acid; L₀, optimal length for muscle contraction; F₀, maximal force at the optimal length for muscle contraction; MYPT1-pT853, myosin light-chain phosphatase-targeting subunit Thr853 phosphorylation; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Go-6983, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; Go-6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile; DMSO, dimethyl sulfoxide; PI_{PKC}, myristoylated pseudosubstrate inhibitor of PKC; PE, phenylephrine; PD98059, 2'-amino-3'-methoxyflavone; VSM, vascular smooth muscle; CPI-17, 17-kDa protein kinase C-dependent MLCP inhibitor.

modulin-dependent MLCK, causing Ca^{2+} sensitization. However, recent studies have shown that membrane depolarization induced by high concentrations of extracellular KCl also can cause Ca^{2+} sensitization in many smooth muscles (for review, see Ratz et al., 2005). This was originally a surprising finding because KCl, which has traditionally been used by smooth muscle biologists as a stimulus that bypasses GPCR, was thought to act primarily by elevating voltage-operated calcium channel (VOCC)-dependent Ca^{2+} entry and increasing MLCK activity (Brozovich, 2003). The implication is that any stimulus that causes membrane depolarization and elevates calcium may cause Ca^{2+} sensitization. Thus, GPCR may not be absolutely required to cause Ca^{2+} sensitization in many smooth muscle types. Moreover, the membrane depolarization and Ca^{2+} entry induced by GPCR activation would be expected to contribute to that known to be induced by activation of G_q and $\text{G}_{12/13}$.

Although both GPCR activation and stimulation with KCl can cause Ca^{2+} sensitization, GPCR-induced Ca^{2+} sensitization involves activation of some PKC isotypes to cause phosphorylation and activation of the 17-kDa protein kinase C-dependent MLCP inhibitor (CPI-17), whereas KCl does not cause an increase in phospholipase C activation (Ratz, 1990) nor phosphorylation of CPI-17 (Kitazawa et al., 2000; Dimopoulos et al., 2007). These data might suggest that KCl does not cause PKC activation. In support of this hypothesis, 3 to 6 μM of the PKC inhibitor, GF-109203X, does not inhibit a KCl-induced contraction in vascular and airway smooth muscles (Kitazawa et al., 2003; Liu et al., 2005). In addition, 3 μM GF-109203X does not inhibit basal CPI-17 phosphorylation in rabbit femoral artery (Dimopoulos et al., 2007). However, these studies cannot rule out the possibility that $\text{PKC}\zeta$, an atypical PKC isotype, plays a role because higher concentrations of GF-109203X are required to inhibit $\text{PKC}\zeta$ (Martiny-Baron et al., 1993). Moreover, $\text{PKC}\alpha/\beta$ is known to be constitutively active in smooth muscle and plays an integral role in maintaining basal Ca^{2+} entry (Navedo et al., 2005). Because KCl-induced Ca^{2+} sensitization is dependent on Ca^{2+} entry (Sakurada et al., 2003) and $\text{PKC}\alpha/\beta$ participates in regulation of Ca^{2+} entry, $\text{PKC}\alpha/\beta$ could theoretically play a role in Ca^{2+} -dependent Ca^{2+} sensitization. For these reasons, the present study was designed to determine whether KCl-induced Ca^{2+} sensitization and tonic F maintenance does or does not involve activation of PKC. This study also examined the concentration-dependent abilities of the selective VOCC blocker, nifedipine, and the three primary ROCK inhibitors, Y-27632, HA-1077, and H-1152, to temporally reduce calcium, F, and Ca^{2+} sensitization induced by KCl. Understanding the precise subcellular mechanisms controlling Ca^{2+} sensitization is relevant to gaining deeper insights not only into vascular disorders involving hypercontraction but also into how multiple types of smooth muscles found in hollow organ systems respond to contractile, relaxant, and therapeutic agents (Uehata et al., 1997; Peters et al., 2006; Shimokawa and Rashid, 2007).

Materials and Methods

Tissue Preparation and Isometric Force. Tissues were prepared and contractions measured as described previously (Ratz, 1993). In brief, femoral and renal arteries from adult New Zealand white rabbits were cleaned of adhering tissue, denuded of endothe-

lium, cut into 3- to 4-mm-wide rings, and stored for later analyses or immediately secured between wires in aerated myograph tissue chambers containing physiological salt solution (PSS) maintained at 37°C. To increase throughput and reduce the numbers of animals required, renal arteries were used for calcium measurements, and femoral arteries were used for all other studies. Renal and femoral arteries are approximately the same size and display approximately the same contraction profile to KCl. The PSS composition was 140 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.6 mM CaCl_2 , 1.2 mM NaH_2PO_4 , 2.0 mM MOPS (adjusted to pH 7.4), 0.02 mM EDTA (to chelate heavy metals), and 5.6 mM D-glucose. To produce K^+ depolarization, KCl (110 mM) was substituted isosmotically for NaCl. For each tissue preloaded to the optimal length for muscle contraction (L_0), the degree of KCl-induced steady-state F was equal to the optimal F for muscle contraction (F_0), and subsequent contractions were calculated as F/F_0 . Arteries contracted with KCl were incubated with 1 μM phentolamine to block potential α -adrenergic receptor activation caused by release of norepinephrine from periaarterial nerves.

$[\text{Ca}^{2+}]_i$. Calcium was measured in artery rings at L_0 as described previously (Han et al., 2006). Tissues were loaded for 2 h with 7.5 μM fura-2-PE3 (acetoxymethyl ester) and 0.01% (w/v) Pluronic F-127 (TEF Labs, Austin, TX) to enhance solubility. Fluorescence emission intensities at 510 nm collected by a photomultiplier tube were expressed as excitation ratios (340/380 nm, DeltaRam V; Photon Technology International, Lawrenceville, NJ) using Felix software (Photon Technology International). Background fluorescence, determined by incubating tissues in 4 mM MnCl_2 plus 20 μM ionomycin, was subtracted before calculating the fluorescence ratios. In the protocol used to assess the temporal affect of certain selective pharmacological agents on fura-2-loaded tissues in which F and calcium were simultaneously measured, and the degree of calcium sensitization (F/Ca) was calculated, tissues were contracted with KCl twice for 10 min each; the first F and calcium responses (F_1 and Ca_1) served as controls for the second F and calcium responses (F_2 and Ca_2), and the KCl used for the first contraction was washed from tissues for 15 min to allow complete relaxation before a pharmacological agent was equilibrated for 30 min (or 3 h for myristoylated pseudosubstrate inhibitors) before each tissue was again contracted by exposure to KCl the second time. For each tissue, F was reported as normalized (F_2 , normalized) by dividing F_2 by the 10-min control F_1 response or as F_2/F_1 . F_2 , normalized permitted comparisons of temporal contractile profiles, as in Fig. 4, A and B. F_2/F_1 essentially set control responses at any time point to approximately 1, and if a drug caused inhibition, then the F_2/F_1 value would be <1 , as in Fig. 5, A and D. Calcium responses were likewise converted to (Ca_2 , normalized) and Ca_2/Ca_1 , with "zero calcium" being the basal response produced just before the first KCl contraction. F/Ca was calculated as F_2/F_1 divided by Ca_2/Ca_1 . For the calcium response shown in Fig. 8, the minimal calcium value was calculated as that produced by an " R_{min} " solution consisting of PSS-absent CaCl_2 and with 110 mM KCl replacing NaCl plus 4 mM MnCl_2 and 20 μM ionomycin. This permitted an analysis of the ability of a Ca^{2+} -free solution (0Ca) and Ca^{2+} channel blockade (nifedipine) to reduce the basal level of calcium without introducing negative numbers.

Western Blot. MYPT1-pT853 was measured by Western blot analysis of artery ring homogenates as described previously (Porter et al., 2006). In brief, artery rings at L_0 were quick-frozen in an acetone-dry ice slurry, thawed, homogenized, heated, clarified by centrifugation, and stored or immediately assayed for protein concentration and loaded into gel wells. Proteins were separated by electrophoresis followed by Western blotting. MYPT1-pT853 was identified using anti-MYPT1-p853 antibody (Upstate, Billerica, MA), and total MYPT1 (BD Biosciences, San Jose, CA) was assessed to quantify loading accuracy. To compensate for gel-to-gel variability in efficiencies of Western blotting, antibody labeling, ECL reaction, and film development, a control basal sample was included in one lane of

each gel, and band intensities from other lanes were reported as the degree of change from control basal.

Fluorescence Immunohistochemistry and Confocal Microscopy. Tissues were prepared for fluorescence immunohistochemistry by methods described previously (Urban et al., 2003), with some modification. Arteries were quickly frozen in liquid nitrogen-cooled isopentane and stored at -80°C for later processing or immediately sectioned by cryostat to $8\text{ }\mu\text{m}$ and placed on a glass slide, fixed in 2% paraformaldehyde in PBS-0.1%/Tween 20 for 10 min, permeabilized in 0.5% Triton X-100 in PBS/Tween 20 for 30 min, and blocked in 10% BSA in PBS/Tween 20 for 1 h. Anti-PKC ζ antibody (Sigma-Aldrich, St. Louis, MO; rabbit polyclonal) was applied at 1:1000 overnight at 4°C , and the secondary antibody (Alexa Fluor 488 donkey anti-rabbit; Invitrogen, Carlsbad, CA) was applied at 1:500 for 1 h at room temperature the following day. Anti-vinculin antibody (Sigma-Aldrich; mouse monoclonal) was then be applied at 1:500 for 1 h at room temperature, and the secondary (Alexa Fluor 568 goat anti-mouse; Invitrogen) was applied at 1:500 for 1 h at room temperature. Cytosolic actin was counterstained with Phalloidin Alexa Fluor 647 (Invitrogen) at 1:40 in 0.1% BSA/PBS/Tween 20 for 10 min, and the nucleus was counterstained with $1\text{ }\mu\text{M}$ 4,6-diamidino-2-phenylindole (EMD Biosciences, San Diego, CA) in 0.1% BSA/PBS/Tween 20 for 1 min. VectaShield (Vector Laboratories, Burlingame, CA) was applied to reduce photobleaching. After immunolabeling, the sections were examined using a Leica TCS-SP2 (AOBS) confocal laser scanning microscope (Leica, Wetzlar, Germany), and 12-bit images were collected using a $63\times$, 1.4 numerical aperture Plan-Apo oil immersion lens.

Drugs. Go-6983, nifedipine, and phentolamine were from Sigma-Aldrich. Ionomycin, Go-6976, GF-109203X, Y-27632, HA-1077, H-1152, and cell-permeable (myristoylated) pseudosubstrate peptide inhibitors of PKC ζ and PKC α/β were from EMD Biosciences. Fura-2-PE3 (acetoxymethyl ester) and Pluronic F-127 were from TEF Labs. Nifedipine and ionomycin were dissolved in ethanol; Go-6976, Go-6983, and GF-109203X were dissolved in dimethyl sulfoxide (DMSO), and all other compounds were dissolved in distilled water. Ethanol and DMSO were added at a final concentration no greater than 0.1%, a concentration that had no effect on KCl-induced contraction.

Statistics. The null hypothesis was examined using Student's *t* test (when two groups were compared) or using a one-way analysis of variance. To determine differences between groups after analysis of variance, the Student-Newman-Keuls post hoc test was used. In all cases, the null hypothesis was rejected at $P < 0.05$. For each study described, the *n* value was equal to the number of rabbits from which arteries were taken.

Results

Effect of Inhibitors of PKC on KCl-Induced Contraction. To determine whether KCl-induced contractions are dependent on PKC activity, arteries were contracted with KCl in the absence (control) and presence of the bisindolylmaleimide PKC inhibitors, Go-6976, Go-6983, and GF-109203X (Martiny-Baron et al., 1993; Gschwendt et al., 1996; Eto et al., 2001; Pang et al., 2005). Go-6976 inhibits conventional PKC isotypes, and the IC_{50} for inhibition of PKC α/β is 2 to 6 nM. Go-6983 and GF-109203X can inhibit several PKC isotypes in a concentration-dependent manner. The IC_{50} for inhibition of PKC α/β , PKC γ , and PKC δ by Go-6983 is 6 to 10 nM and for inhibition of the atypical PKC isotype, PKC ζ , is 60 nM, whereas the IC_{50} for inhibition of PKC μ is 20 μM . The IC_{50} values for inhibition of PKC α/β , PKC δ , and PKC ζ by GF-109203X are, respectively, 8 to 18 nM, 200 nM, and 5 μM . At 0.1 μM , the conventional PKC inhibitor, Go 6976, had no effect on a KCl-induced contraction (Fig. 1A). However, 0.1 μM Go-6983 and 1, 3, and 10 μM

Fig. 1. KCl-induced contractions in the absence (Control) and presence of the bisindolylmaleimide PKC inhibitors, Go-6976 and Go-6983 (A) and GF-109203X (B) and statistical comparisons from A and B of the effects of PKC inhibitors on the tonic phase (10 min) of KCl-induced F (C). Data in are means (solid lines and bars) $\pm$ S.E. (dotted lines in A and B), $n = 3$ to 5. *, $P < 0.05$ compared with control (Con).

GF-109203X inhibited the KCl-induced tonic more than the early, phasic phase of contraction (Fig. 1, B and C). Based on the known IC_{50} values for these inhibitors, these data suggest that PKC participates in a KCl-induced contraction of rabbit artery.

Effect of Myristoylated PKC α/β and PKC ζ Pseudosubstrate Inhibitors on KCl-Induced Contraction. GF-109203X inhibits multiple PKC isotypes and may also inhibit other kinases at the concentrations used in this study (Davies et al., 2000). Therefore, we examined the ability of the cell-permeable (myristoylated) form (Eichholtz et al., 1993) of the pseudosubstrate peptide inhibitor of PKC ζ ($\text{PI}_{\text{PKC}\zeta}$) to attenuate a KCl-induced contraction. The data obtained were compared with the effects of $\text{PI}_{\text{PKC}\alpha/\beta}$ (Eichholtz et al., 1993). It is interesting that when tissues were incubated with peptide inhibitors for 3 h, 10 μM $\text{PI}_{\text{PKC}\zeta}$ produced a very weak (approximately 1% F_0) but significant increase in basal ("passive") F (Fig. 2A, solid circles), and 10 μM $\text{PI}_{\text{PKC}\alpha/\beta}$ produced a very weak (approximately 2% F_0) but significant reduction in passive F (Fig. 2A, solid squares). As expected, $\text{PI}_{\text{PKC}\alpha/\beta}$ strongly inhibited a contraction induced by phorbol dibutyrate (Fig. 2B), an allosteric activator of PKC α/β . Like GF-109203X, $\text{PI}_{\text{PKC}\zeta}$ significantly inhibited a KCl-induced contraction (Fig. 2C). It is notable that the tonic phase of a KCl-induced contraction (Fig. 2C, 5 and 10 min) was inhibited more than the early, phasic phase (Fig. 2C, 16 s). To our surprise, $\text{PI}_{\text{PKC}\alpha/\beta}$ caused a small but significant potentiation of the tonic phase of a KCl-induced contraction (Fig. 2C). Moreover, 5 μM GF-109203X and 10 μM $\text{PI}_{\text{PKC}\zeta}$ significantly inhibited KCl-induced contractions at lower KCl stimulation levels (Fig. 2D, 30–60 mM). It is interesting that 10 μM $\text{PI}_{\text{PKC}\alpha/\beta}$ produced inhibition at 30 mM KCl but not at lower and higher KCl concentrations (Fig. 2D; *, all of the PKC inhibitors significantly attenuated contraction; ψ , $\text{PI}_{\text{PKC}\zeta}$ and GF-109203X but not $\text{PI}_{\text{PKC}\alpha/\beta}$ attenuated contraction). These data together suggest that 1) a fraction of passive force in rabbit muscular arteries is "actively" and oppositely regulated by constitutively active PKC α/β and PKC ζ ; 2) during maximal KCl-induced contraction, PKC α/β attenuates, whereas PKC ζ potentiates tonic F maintenance; and 3) PKC α/β and PKC ζ participate in force production induced by submaximal KCl concentrations. The α -adrenergic receptor

Fig. 2. Effects of the cell-permeable PI_{PKCα/β} (10 μM) and PI_{PKCζ} (10 μM) on passive (A) and KCl-activated (C and D) F. The effect of PI_{PKCα/β} on phorbol dibutyrate-induced contraction was also examined (B). PI_{PKCα/β} potentiated the tonic phase of a 110 mM KCl-induced contraction [C, 5 (5') and 10 (10') min] and did not affect the peak of the early, phasic phase of contraction [D, 16 s (16'')]. PI_{PKCζ} inhibited both the early and tonic phases of a 110 mM KCl-induced contraction (C). GF-109203X (5 μM; PKC inhibitor), and PI_{PKCζ} inhibited contractions induced by low and high KCl concentrations (D), whereas PI_{PKCα/β} caused inhibition at a moderate (30 mM) KCl concentration. Data are means ± S.E. (vertical lines with caps in A, C, and D; dotted lines in B), $n = 3$ to 5. *, $P < 0.05$ compared with dashed line (zero, A) and control (B and C). D, *, all three drugs caused inhibition at $P < 0.05$ compared with the vehicle control (DMSO); ψ , only PI_{PKCζ} and GF-109203X, but not PI_{PKCα/β}, caused inhibition at $P < 0.05$ compared with the vehicle control (DMSO).

agonist, phenylephrine (PE), causes contraction in part by elevating $[Ca^{2+}]_i$. We showed previously that both GF-109203X and Y-27632 can inhibit the tonic contraction induced by 1 μM PE (Ratz et al., 2005). Thus, we determined whether PI_{PKCζ} can also inhibit a 1 μM PE-induced tonic (10 min) contraction. Compared with control tissues (0.94 ± 0.04 F/F₀), those incubated for 3 h with 10 μM PI_{PKCζ} then stimulated with 1 μM PE produced significantly less tonic (10 min) force (0.68 ± 0.12 F/F₀, $n = 3$, $P < 0.05$).

Identification of PKCζ in VSM of Rabbit Artery. To determine whether PKCζ was expressed by rabbit artery, and where within the VSM cell PKCζ was located, unstimulated tissues were frozen and prepared for fluorescence immunohistochemistry and confocal microscopy. Rabbit VSM expressed abundant PKCζ (Figs. 3C, green) localized primarily within perinuclear sites (Fig. 3E, green located outside of the blue nucleus) that did not seem to stain with phalloidin (Fig. 3E, green seems to not colocalize with purple, see arrows) and also diffusely within the cytosol and in the cell periphery (Fig. 3, C and F, green; see arrowheads in Fig. 3E). Peripheral PKCζ seemed to be localized as punctate aggre-

Fig. 3. A 63× confocal image taken of a cross-section of a rabbit artery showing expression and prominent perinuclear (C and E, green) and peripheral punctate (C and F, green) locations of PKCζ. A to D, actin (purple, phalloidin), nuclei (blue, 4,6-diamidino-2-phenylindole), PI_{PKCζ} (green, anti-PI_{PKCζ} antibody), and focal contacts (red, anti-vinculin antibody), respectively. Two sections (bounded by the white boxes labeled e and f) were zoomed 3.3-fold, and composites are shown in E (actin, nuclei, and PI_{PKCζ}) and F (vinculin and PI_{PKCζ}), respectively. Example of an $n = 3$.

gates alternating with vinculin (Fig. 3F, green and red, see arrowheads), which is a marker for dense bodies.

Effect of ROCK and PKC Inhibitors on KCl-Induced F, Calcium, and the F/Ca Ratio. The isoquinolinesulfonamide ROCK inhibitors, HA-1077 and H-1152 (Hidaka and Kobayashi, 1992; Sasaki et al., 2002), effectively inhibited a KCl-induced contraction when used at, respectively, 10 and 0.1 μM (Fig. 4A). The early (approximately 20 s) peak F (Fig. 4A, "phasic phase") was not inhibited, but the tonic phase contraction was strongly inhibited. It is interesting that H-1152 also produced a small but significant reduction in F at times preceding the peak. Although F was reduced by H-1152 and HA-1077, the KCl-induced increase in calcium was not (Fig. 4B). Y-27632, a nonisoquinolinesulfonamide ROCK inhibitor (Uehata et al., 1997), reduced the phasic phase increases in both F (Fig. 4A) and calcium (Fig. 4B) induced by KCl and strongly attenuated the tonic phase F (Fig. 4A, 2–10 min) without reducing the tonic phase calcium (Fig. 4B, 2–10 min). The reductions in KCl-induced tonic increases in F without changes in KCl-induced tonic increases in calcium by the ROCK inhibitors resulted in reductions in the tonic F/Ca ratio (i.e., they caused Ca^{2+} desensitization) that mirrored the reductions in F (Fig. 5, compare C with A). Thus, at the concentrations used, all of the ROCK

Fig. 4. KCl-induced phasic phase (up to approximately 20 s) and tonic phase (after approximately 20 s) increases in F (A and C) and calcium (B and D) produced in the absence (control) and presence of the isoquinolinesulfonamide ROCK inhibitors, H-1152 (H-11) and HA-1077 (HA-10) and in the nonisoquinolinesulfonamide ROCK inhibitor, Y-27632 (Y-27), and in the absence (control) and presence of the PKC inhibitors, GF-109203X and the cell-permeable pseudosubstrate PKC ζ inhibitor, PI_{PKC ζ} . Data are means $\pm$ S.E., $n = 3$ to 4. *, $P < 0.05$ compared with control. ψ , $P < 0.05$ comparing 2- and 10-min control values.

inhibitors caused Ca^{2+} desensitization of tonic contraction, implying that ROCK played a significant role in KCl-induced Ca^{2+} sensitization and tonic F maintenance.

At 3 μ M, the PKC inhibitor GF-109203X acted in a manner similar to the isoquinolinesulfonamide ROCK inhibitors in that it inhibited KCl-induced increases in F during the tonic but not phasic phase (Fig. 4C) and had no apparent effect on calcium (Fig. 4D). However, although the level of calcium decreased significantly from 2 to 10 min in control tissues, this was not the case in tissues treated with 3 μ M GF-109203X. When normalized internally by dividing the second by the first response, the KCl-induced increase in calcium at 10 min in the presence of 3 μ M GF-109203X was significantly elevated above the control value (Fig. 5E, cross-hatched bar labeled "3GF109").

At 10 μ M, GF-109203X inhibited the KCl-induced increases in F at all time points measured beyond 5 s, although tonic F was inhibited comparatively more than the early, phasic phase F (Fig. 4C). GF-109203X at 10 μ M significantly increased basal calcium without causing a concomitant increase in basal F [Fig. 4, D and C, respectively; compare data points (square symbol) at time = 0 and potentiated the KCl-induced increase in calcium (Fig. 4D)]. PI_{PKC ζ} acted similarly to 10 μ M GF-109203X. A notable difference was that although 10 μ M GF-109203X elevated basal calcium and not

Fig. 5. Effect of ROCK inhibitors (A–C) and PKC inhibitors (D–F) on KCl-induced tonic (10-min) increases in F (A and D) and calcium (B and E) expressed as the ratios of the second over the first responses (F_2/F_1 and Ca_2/Ca_1) and on KCl-induced Ca^{2+} sensitization (C and F) expressed as the ratio of the second F response over the second calcium response (F/Ca). Control responses were produced by KCl in the absence of inhibitors. Data are means $\pm$ S.E., $n = 3$ to 12. *, $P < 0.05$ compared with control.

basal F , PI_{PKC ζ} elevated both [Fig. 4, C and D, compare data points (inverted triangle) at time = 0]. This difference may have been because of the well documented PKC isotype non-selectivity of GF-109203X (i.e., 10 μ M GF-109203X will inhibit PKC α/β and PKC ζ , whereas PI_{PKC ζ} should selectively inhibit PKC ζ). The extent of increase in basal F induced by PI_{PKC ζ} was greater in tissues that were loaded with fura-2 (approximately 20% F_0 ; see Fig. 4C, inverted triangle at $t = 0$) than in tissues not loaded (approximately 1% F_0 ; see Fig. 2A, circles). The potentiation of the tonic phase of a KCl-induced increase in calcium when PKC was inhibited by PI_{PKC ζ} and by 10 μ M GF-109203X was greater than could be accounted for by the increase in basal calcium. Thus, PI_{PKC ζ} and GF-109203X reduced KCl-induced tonic F (Fig. 5D) and potentiated KCl-induced tonic calcium (Fig. 5E), causing substantial Ca^{2+} desensitization (Fig. 5F) equivalent to that induced by the ROCK inhibitors (compare Fig. 5, F and C). These data together support the hypothesis that PKC ζ is constitutively active, that basal activity participates in inhibition of calcium entry, and that the further activation PKC ζ above the basal level by KCl participates in causing KCl-induced Ca^{2+} sensitization and tonic force maintenance. However, unlike the Ca^{2+} sensitization induced by ROCK activation, that induced by PKC ζ activation involved a dual effect of increasing F while reducing calcium.

Effect of ROCK and PKC Inhibitors on Basal and KCl-Induced MYPT1-pT853. H-1152 (0.3 μ M) produced a strong inhibition of basal MYPT1-pT853 by nearly 75% (Fig. 6A, compare the basal control level, "B," with the basal level produced in the presence of 0.3 μ M H-1152, "0.3H"). KCl produced an approximate 1.75- to 2-fold increase in MYPT1-pT853 in the presence and absence of H-1152 (Fig. 6A, compare B with "C, 5' KCl" and compare 0.3H with "0.3H, 5' KCl"). That is, 0.3 μ M H-1152, a concentration 3-fold greater than that used to inhibit the KCl-induced tonic contraction shown in Fig. 3A, did not prevent KCl from producing an approximately 2-fold increase in MYPT1-pT853. However, because of the dramatic diminution in basal MYPT1-pT853 induced by H-1152, the increase above the basal level produced by KCl in the presence of H-1152 was insufficient to elevate MYPT1-pT853 even back to the control, basal level (Fig. 6A, 0.3H, 5' KCl was significantly below the dotted line). In comparison, when used at 3 and 10 μ M, the PKC inhibitor GF-109203X, did not inhibit basal MYPT1-pT853 but did abolish the ability of KCl to increase MYPT1-pT853 above the basal level (Fig. 6A). Likewise, $PI_{PKC\zeta}$ did not reduce the basal level of MYPT1-pT853 (0.99- $\pm$ 0.13-fold basal, $n = 6$, not significantly different from 1.0). These data together suggest that constitutive ROCK activity supports

basal MYPT1-pT853, and KCl-stimulated $PKC\zeta$ activity elevates MYPT1-pT853 above the basal level. The drugs used did not affect total MYPT1 expression (Fig. 6B).

Effect of Nifedipine on KCl-Induced F, Calcium, and F/Ca. Nifedipine differentially inhibited the phasic [data taken at 15 s (15'')] and tonic [data taken at 10 min (10')] phases of KCl-induced increases in F and calcium. This was best visualized by plotting the data as nifedipine concentration-response curves (Fig. 7, A and B). The concentration-dependent effect of nifedipine on Ca^{2+} sensitization was shown by plotting the F/Ca ratio (Fig. 7C). Low nifedipine concentrations ($<10^{-8}$ M) tended to increase the F/Ca ratio during both phasic (15'') and tonic (10') phases of contraction (Fig. 7C) by inhibiting calcium (Fig. 7B) more so than F (Fig. 7A). Higher nifedipine concentrations (10^{-8} and 10^{-7} M) inhibited F (Fig. 7A) more than calcium (Fig. 7B) only during the tonic phase of contraction, causing a large reduction in the F/Ca ratio (Fig. 7C). At 10^{-8} M, nifedipine significantly elevated the F/Ca ratio produced during the phasic phase of contraction (Fig. 7C, diamond) and significantly reduced the F/Ca ratio produced during the tonic phase of contraction (Fig. 7C, inverted triangle). These data support the hypothesis that calcium entry through VOCCs participates in causing Ca^{2+} sensitization during the tonic phase of contraction. In addition, the data suggest that low levels of calcium support Ca^{2+} sensitization. For example, at 10 min of a KCl-

Fig. 6. Effect of 3 μ M of the ROCK inhibitor, H-1152 (0.3H), and 3 and 10 μ M of the PKC inhibitor, GF-109203X (3GF and 10GF, respectively) on basal (B) and the KCl-stimulated tonic (5' KCl) increase in MYPT1-pT853 (A). Total MYPT1 was unaffected (B). C, control level produced by a 5' stimulation with KCl. Data are means $\pm$ S.E., $n = 3$ to 7. *, $P < 0.05$, less than and greater than the basal value of 1. ψ , $P < 0.05$ comparing KCl-stimulated 0.3H and basal 0.3H.

Fig. 7. Nifedipine (Nif) concentration-response curves comparing the relative ability of nifedipine to inhibit KCl-induced increases in F (A), calcium (B), and the F/Ca ratio (C). Open symbols demarcated by a vertical dotted line are control values. D, KCl-induced F tracings produced in the absence (control) and presence of 1 μ M nifedipine (Nif). E, tissues treated as in D were frozen at 0 (Basal), 30 s (K 30''), 2 min (K 2'), and 5 min (K 5') of a KCl-induced contraction and processed to measure the MYPT1-pT853. Data are means $\pm$ S.E., $n = 3$ to 5. *, $P < 0.05$ compared with control.

induced contraction, tonic F and calcium were reduced by 10^{-7} M nifedipine, to approximately 15 and 40%, respectively. Reducing the degree of calcium channel blockade by using 10^{-8} rather than 10^{-7} M nifedipine did not cause a measurable increase calcium (Fig. 7B) but did increase F (Fig. 7A).

Effect of Nifedipine on the KCl-Induced Increase in MYPT1-pT853. To test the hypothesis that nifedipine at a concentration that inhibited Ca^{2+} sensitization could also inhibit ROCK activity, tissues treated with 1 μM nifedipine and contracted with KCl were frozen and processed to measure ROCK substrate phosphorylation (MYPT1-pT853). KCl induced an approximate 1.75- to 2-fold increase above the basal level in MYPT1-pT853 at 30 s and 2 and 5 min (Fig. 7E, K30", 2', and 5', open bars). One micromolar nifedipine nearly abolished the tonic phase of a KCl-induced contraction (Fig. 7D) and abolished the ability of KCl to increase MYPT1-pT853 but had no effect on basal MYPT1-pT853 (Fig. 7E, cross-hatched bars). Thus, although H-1152 inhibited basal MYPT1-pT853 (see Fig. 6A), nifedipine (Fig. 7E), GF-109203X (see Fig. 6A), and $\text{PI}_{\text{PKC}\zeta}$ did not. Moreover, although H-1152 did not prevent a near doubling of MYPT1-pT853 by KCl (see Fig. 6A), both GF-109203X (see Fig. 6A) and nifedipine (Fig. 7E) did. These data together suggest that Ca^{2+} entry induced by KCl activates PKC ζ rather than ROCK.

Effect of Reduced Ca^{2+} Entry on GF-109203X- and $\text{PI}_{\text{PKC}\zeta}$ -Induced Changes in Basal Calcium and KCl-Induced Increases in Calcium. Tissues incubated for approximately 30 min in a nominally Ca^{2+} -free solution or with 10^{-7} M nifedipine were exposed to 10 μM GF-109203X for approximately 30 min, and temporal changes in calcium and F were assessed. The Ca^{2+} -free solution (Fig. 8, B and E, black horizontal bar, 0Ca) was PSS minus CaCl_2 , to which EGTA was not added. Calcium was normalized so that the minimal value was equal to that produced by exposure of tissues to ionomycin and MnCl_2 in a Ca^{2+} -free solution containing 1 mM EGTA (Fig. 8, R_{min}) so that the relative reduction in calcium induced by exposure to 0Ca and nifedipine could be evaluated. As shown also in Fig. 4, GF-109203X induced an increase in basal calcium (Fig. 8, A and G) without causing an increase in basal F (Fig. 8D). Incubation of tissues in 0Ca reduced basal calcium by approximately 10% and abolished the ability of GF-109203X to cause an increase in calcium (Fig. 8, B and G). Nifedipine had no effect on basal calcium and did not inhibit the increase in basal calcium induced by GF-109203X (Fig. 8, C and G). $\text{PI}_{\text{PKC}\zeta}$ elevated basal calcium to a level similar to that induced by GF-109203X (Fig. 8G), and nifedipine did not seem to abolish this increase (data not shown, $n = 1$). These data together support the hypothesis that constitutive PKC ζ activity helps to maintain a low basal calcium through a mechanism distinct from inhibition of L-type VOCCs.

In the absence of extracellular Ca^{2+} (0Ca) and the presence of 10 μM GF-109203X, KCl failed to cause an increase in calcium (Fig. 8, B and H) and F (Fig. 8, E and I). A surprising finding was that the washout solution that included CaCl_2 caused a strong increase in calcium (Fig. 8B, second "W") but not F (Fig. 8E, second W). The absence of an increase in F was not because of an irreversible down-regulation of contractile proteins because exposure of tissues to KCl, ionomycin, and CaCl_2 subsequent to wash in the R_{min} solution induced a strong contraction [1.21 ± 0.07 F (normalized),

Fig. 8. Effect of a nominally Ca^{2+} -free solution (0Ca) and 100 nM nifedipine (Nif) on the basal increase in calcium induced by 10 μM GF-109203X (GF) and on KCl-induced tonic (10 min) increases in calcium and F. A to F, examples of calcium (A–C) and F (D–F) tracings, and data in G to I are means $\pm$ S.E., $n = 3$ to 12. Calcium data in A to C and G are normalized so that 1 is the 10-min KCl-induced contraction and zero is the response produced by exposure to the R_{min} solution. Data for control (Con), GF, and $\text{PI}_{\text{PKC}\zeta}$ (PI-PKC ζ) groups in H and I are from Fig. 5. *, $P < 0.05$ compared with GF.

data not shown, $n = 3$). These data suggest that a portion of the fura-2-sensitive calcium resided in a compartment distinct from that directly responsible for contractile protein activation.

$\text{PI}_{\text{PKC}\zeta}$, like GF-109203X, potentiated the KCl-induced increase in calcium (Fig. 8H) and attenuated the KCl-induced increase in force (Fig. 8I). Although not affecting the increase in basal calcium induced by 10 μM GF-109203X, nifedipine did abolish the potentiation by 10 μM GF-109203X of the KCl-induced tonic increase in calcium (Fig. 8, C and H) and nearly abolished KCl-induced tonic F (Fig. 8, F and I). A similar result was obtained in a single tissue exposed to both 10^{-7} M nifedipine and $\text{PI}_{\text{PKC}\zeta}$ (data not shown, $n = 1$).

Discussion

$\text{PI}_{\text{PKC}\zeta}$ and 10 μM GF-109203X potentiated the KCl-induced tonic increase in calcium while causing a concomitant inhibition of KCl-induced tonic F, resulting in a decrease in Ca^{2+} sensitization (i.e., in the F/Ca ratio). To our knowledge, these are the first data that support the hypothesis that PKC ζ plays a role in causing KCl-induced Ca^{2+} sensitization and F maintenance. KCl caused a nearly 2-fold increase above the basal level in MYPT1-pT853, a signaling molecule downstream of ROCK that causes MLCP inhibition resulting

in Ca^{2+} sensitization (Murányi et al., 2005; Dimopoulos et al., 2007). Although the selective ROCK inhibitor, H-1152, reduced basal MYPT1-pT853 by approximately 75%, KCl could still induce a nearly 2-fold increase in MYPT1-pT853 above the basal level. This increase, however, did not elevate MYPT1-pT853 above the control basal level. In contrast, nifedipine, GF-109203X, and $\text{PI}_{\text{PKC}\zeta}$ had no effect on basal MYPT1-pT853, but nifedipine and GF-109203X abolished the ability of KCl to cause an increase in MYPT1-pT853 above the basal level. Finally, $\text{PI}_{\text{PKC}\zeta}$ and GF-109203X caused an increase in both basal and KCl-stimulated calcium, suggesting that $\text{PKC}\zeta$ regulates Ca^{2+} homeostasis basally and during muscle stimulation. These data together support the hypotheses that 1) ROCK and $\text{PKC}\zeta$ are basally (i.e., constitutively) active, 2) constitutive ROCK regulates basal MYPT1-pT853, 3) constitutive $\text{PKC}\zeta$ regulates basal calcium, and 4) the KCl-dependent increase in Ca^{2+} entry through VOCCs leads to elevated $\text{PKC}\zeta$ activity, which in turn causes a reduction in calcium and a concomitant elevation in MYPT1-pT853, causing Ca^{2+} sensitization (Fig. 9). Although it is generally agreed that pseudosubstrate peptide inhibitors are highly selective agents, it remains possible that the very closely related atypical PKC isotype, $\text{PKC}\lambda$, may have played a role, or that the effects were because of an unrecognized action of $\text{PI}_{\text{PKC}\zeta}$. Moreover, among the many PKC isotypes, we tested only for the effects of $\text{PKC}\alpha$ and $\text{PKC}\zeta$. Thus, these data do not rule out the participation of other PKC isotypes present in rabbit arteries in KCl-induced Ca^{2+} sensitization.

Fig. 9. Working model of KCl-induced Ca^{2+} sensitization in VSM. We propose that: 1) $\text{PKC}\zeta$ and ROCK are constitutively active as reflected by basal inhibition of Ca^{2+} entry and basal MYPT1-pT853, respectively; 2) the increases in $[\text{Ca}^{2+}]_i$ (Ca^{2+}) caused by KCl-induced membrane depolarization feeds forward to cause 2a) further activation of $\text{PKC}\zeta$ above the basal level (arrow from Ca^{2+} to $\text{PKC}\zeta$), adding to the degree of MYPT1-pT853 induced by constitutive (basal) ROCK activity (arrows joining $\text{PKC}\zeta$ and ROCK) and 2b) activation of MLCK; 3) the increased MYPT1-pT853 further “turns off” MLCP activity so that in conjunction with MLCK activation, MLCP “builds up” above approximately 15%, causing contraction [the threshold MLCP level for F development is approximately 15% (Rembold et al., 2004)]; 4) activation of $\text{PKC}\zeta$ also feeds back to attenuate the stimulus-induced increase in calcium (dotted line with ball at end indicating inhibition), as evidenced by the fall in KCl-induced increases in Ca^{2+} over time after the initial peak response. Inhibition of MLCP permits F maintenance in the face of falling Ca^{2+} , and this model proposes that $\text{PKC}\zeta$ is the signaling molecule that causes this Ca^{2+} sensitization by inhibiting MLCP and Ca^{2+} entry. Dashed lines connect inhibitors used in this study with their proposed sites of action.

Although muscle cells are generally considered to be “off” until “turned on” by a contractile stimulus, there is now ample evidence that several signaling systems that participate in regulation of contraction are at least partially “on” when smooth muscle cells are in the basal, resting state. Results from this study support the hypothesis that ROCK and $\text{PKC}\zeta$ are constitutively active in rabbit artery stretched to L_0 . Extracellular signal-regulated kinase mitogen-activated protein kinase seems also to be constitutively active in rabbit artery because the mitogen-activated protein kinase inhibitor, PD98059, reduces basal diphosphorylation of extracellular signal-regulated kinase mitogen-activated protein kinase (Ratz, 2001). A recent study by Guo et al. (2003), using rabbit venous smooth muscle, revealed the importance of constitutive Ca^{2+} -independent phospholipase A_2 activity in α -adrenergic receptor activation-induced Ca^{2+} sensitization in VSM. A recent study by Rembold et al. (2004) supports the notion that basal MLCp must be at least approximately 15% before significant active F can be generated. There is a high, basal Ca^{2+} entry in VSM that involves multiple Ca^{2+} entry pathways (Poburko et al., 2004; Navedo et al., 2005), and a significant portion of this basal Ca^{2+} entry is taken up by the peripheral SR acting as a superficial buffer barrier (for review, see van Breemen et al., 1995). Constitutively active $\text{PKC}\alpha$ is responsible for the constitutive activity of VOCCs responsible, in part, for basal calcium entry in VSM (Navedo et al., 2005). In support of this concept, we found that passive force was very slightly but significantly reduced by a $\text{PI}_{\text{PKC}\alpha/\beta}$. The present study provides evidence that constitutively active $\text{PKC}\zeta$ also participates in regulation of VSM Ca^{2+} homeostasis. However, rather than elevating calcium, our data suggest that $\text{PKC}\zeta$ reduced global Ca^{2+} levels. Moreover, our results support the hypothesis that $\text{PKC}\zeta$ also moderated the increase in KCl-stimulated calcium while reducing the dependence of contractile proteins on Ca^{2+} by elevating MYPT1-pT853 and causing Ca^{2+} sensitization. In short, results from this study support the notion that constitutive and activated ROCK and $\text{PKC}\zeta$ activities couple the regulation of Ca^{2+} homeostasis with the regulation of Ca^{2+} sensitization and the level of sustained contraction.

VOCCs and at least two additional channel types participate in causing basal Ca^{2+} entry in smooth muscle (Poburko et al., 2004). In the present study, the inhibitors of $\text{PKC}\zeta$, $\text{PI}_{\text{PKC}\zeta}$ and 10 μM GF-109203X, increased basal calcium, and this increase was abolished by the removal of CaCl_2 from the bathing solution but not by 10^{-7} M nifedipine. However, nifedipine did inhibit the potentiation by 10 μM GF-109203X and $\text{PI}_{\text{PKC}\zeta}$ of KCl-stimulated increases in calcium. Moreover, 1 μM nifedipine abolished the KCl-induced increase in MYPT1-pT853 but did not reduce basal MYPT1-pT853. These data together suggest that constitutive $\text{PKC}\zeta$ activity exerted basal inhibition of non-VOCC-mediated calcium entry and that stimulated $\text{PKC}\zeta$ activity attenuated KCl-stimulated VOCCs. Although speculative, this differential activity may be because of differential subcellular compartmentation of ion channels and $\text{PKC}\zeta$. Although we determined that $\text{PKC}\zeta$ was localized in a punctate pattern at the cell periphery, whether $\text{PKC}\zeta$ colocalized with some but not all populations of VOCCs was not determined.

Inhibition of $\text{PKC}\alpha/\beta$ by $\text{PI}_{\text{PKC}\alpha/\beta}$ reduced passive F, inhibited a contraction induced by 30 mM KCl, and potentiated the tonic phase of a 110 mM KCl-induced contraction. This

latter effect was a surprising finding because PKC α/β participates in causing Ca²⁺ sensitization of contractions induced by GPCR agonists by increasing the phosphorylation of CPI-17, which inhibits MLCP (Eto et al., 2001; Dimopoulos et al., 2007). However, KCl does not induce CPI-17 phosphorylation of rabbit artery (Dimopoulos et al., 2007), and PKC α/β has been shown to cause inhibition of contraction by phosphorylating myosin light chain at Ser1 and 2, which reduces the ability of MLCK to phosphorylate and activate cross-bridges (Nishikawa et al., 1984; Obara et al., 2006). Thus, the contribution to the contractile state of PKC α/β seems quite complex. Whether the modest potentiation of the KCl-induced tonic contraction by PI_{PKC α/β} was caused by relief of PKC α/β -induced partial inhibition of cross-bridges was not further explored in this study.

Inhibition of PKC ζ by PI_{PKC ζ} had the reverse effect, elevating passive F and inhibiting KCl-induced tonic contraction. The elevation of passive F induced by PI_{PKC ζ} was qualitatively the same but quantitatively greater in tissues loaded with fura-2 than in tissues not loaded with this Ca²⁺ indicator. As an added weak intracellular calcium buffer (Neher, 1995), fura-2 could potentially slightly modify the Ca²⁺ signaling network. Thus, although speculative, the quantitative difference between fura-2-loaded and -non-loaded tissues may have been because of a difference in the local Ca²⁺-buffering action at or near the plasma membrane.

In most studies, the ROCK inhibitor Y-27632 has been shown to inhibit KCl-induced F without inhibiting KCl-induced increases in calcium (for review, see Ratz et al., 2005). However, 1 μ M Y-27632 dramatically inhibits VOCC activity in the phasic rat ureter (Shabir et al., 2004), and Y-27632 can inhibit nonselective cation channels that are distinct from VOCCs (Ghisal et al., 2003). Our data showed that Y-27632 caused inhibition of the early phase of the KCl-induced increase in calcium, but HA-1077 and H-1152, ROCK inhibitors structurally distinct from Y-27632 (Hidaka and Kobayashi, 1992; Uehata et al., 1997; Sasaki et al., 2002), did not. These data suggest that Y-27632 may have exerted some direct calcium channel blockade. However, this seems unlikely because this inhibitory effect was not evident during the tonic phase of the KCl-induced increase in calcium. Alternatively, this activity by Y-27632 may have been because of inhibition of another kinase that participates in regulating calcium during the early but not tonic phase of a KCl-induced contraction. Although Y-27632 is highly selective for ROCK (Uehata et al., 1997; Davies et al., 2000), 3 μ M Y-27632 can inhibit PKC δ (Eto et al., 2001). Thus, although speculative, these data suggest that PKC δ , which is known to participate in regulation of GPCR-induced smooth muscle contraction (Eto et al., 2001), also participates in regulation of KCl-induced increases in calcium and contraction.

In summary, we and others have proposed that KCl-induced tonic F maintenance is dependent on ROCK activity (for review, see Ratz et al., 2005). The present study confirms this hypothesis, and supports a working model (Fig. 9) in which both ROCK and PKC ζ converge as activators of MYPT1-pT853 and Ca²⁺ sensitization, and PKC ζ also feeds back to buffer increases in Ca²⁺. This constitutive and calcium-dependent subcellular signaling network may represent a common mechanism that probably participates in regulating calcium and Ca²⁺ sensitization induced by any natural stimulus that elevates calcium. Because calcium plays a cen-

tral role in the action of many contractile and relaxant agents, an accurate understanding of KCl-induced Ca²⁺ sensitization should facilitate comparative studies of other VSM stimuli and other smooth muscles and provide a molecular foundation for efforts to design therapeutic agents that selectively target disorders involving smooth muscle hypercontraction (Uehata et al., 1997; Peters et al., 2006; Shimokawa and Rashid, 2007).

References

- Brozovich FV (2003) Rho signaling: agonist stimulation and depolarization come together. *Circ Res* **93**:481–483.
- Davies SP, 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.
- Dimopoulos GJ, Semba S, Kitazawa K, Eto M, and Kitazawa T (2007) Ca²⁺-dependent rapid Ca²⁺ sensitization of contraction in arterial smooth muscle. *Circ Res* **100**:121–129.
- Eichholtz T, de Bont DB, de Widt J, Liskamp RM, and Ploegh HL (1993) A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. *J Biol Chem* **268**:1982–1986.
- Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y, and Brautigan DL (2001) Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. *J Biol Chem* **276**:29072–29078.
- Ghisal P, Vandenberg G, and Morel N (2003) Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. *J Physiol* **551**:855–867.
- Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, and Johannes FJ (1996) Inhibition of protein kinase C mu by various inhibitors: differentiation from protein kinase c isoenzymes. *FEBS Lett* **392**:77–80.
- Guo Z, Su W, Ma Z, Smith GM, and Gong MC (2003) Ca²⁺-independent phospholipase A2 is required for agonist-induced Ca²⁺ sensitization of contraction in vascular smooth muscle. *J Biol Chem* **278**:1856–1863.
- Han S, Speich JE, Eddinger TJ, Berg KM, Miner AS, Call C, and Ratz PH (2006) Evidence for absence of latch-bridge formation in muscular saphenous arteries. *Am J Physiol Heart Circ Physiol* **291**:H138–H146.
- Hidaka H and Kobayashi R (1992) Pharmacology of protein kinase inhibitors. *Annu Rev Pharmacol Toxicol* **32**:377–397.
- Kamm KE and Stull JT (2001) Dedicated myosin light chain kinases with diverse cellular functions. *J Biol Chem* **276**:4527–4530.
- Karaki H (1989) Ca²⁺ localization and sensitivity in vascular smooth muscle. *Trends Pharmacol Sci* **10**:320–325.
- Kitazawa T, Eto M, Woodsome TP, and Brautigan DL (2000) Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J Biol Chem* **275**:9897–9900.
- Kitazawa T, Eto M, Woodsome TP, and Khalequzzaman M (2003) Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca²⁺ sensitization in rabbit smooth muscle. *J Physiol* **546**:879–889.
- Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV, and Somlyo AP (1989) Receptor-coupled, permeabilized smooth muscle: role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca²⁺. *J Biol Chem* **264**:5339–5342.
- Liu C, Zuo J, Pertens E, Helli PB, and Janssen LJ (2005) Regulation of Rho/ROCK signaling in airway smooth muscle by membrane potential and [Ca²⁺]_i. *Am J Physiol Lung Cell Mol Physiol* **289**:L574–L582.
- Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marmé D, and Schächtele C (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268**:9194–9197.
- Murányi A, Derkach D, Erdodi F, Kiss A, Ito M, and Hartshorne DJ (2005) Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett* **579**:6611–6615.
- Navedo MF, Amberg GC, Votaw VS, and Santana LF (2005) Constitutively active L-type Ca²⁺ channels. *Proc Natl Acad Sci U S A* **102**:11112–11117.
- Neher E (1995) The use of fura-2 for estimating Ca buffers and Ca fluxes. *Neuropharmacology* **34**:1423–1442.
- Nishikawa M, Sellers JR, Adelstein RS, and Hidaka H (1984) Protein kinase C modulates in vitro phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. *J Biol Chem* **259**:8808–8814.
- Nishimura J, Kolber M, and van Breemen C (1988) Norepinephrine and GTP-g-S increase myofilament Ca²⁺ sensitivity in a-toxin permeabilized arterial muscle. *Biochem Biophys Res Commun* **157**:677–683.
- Obara K, Uchino M, Koide M, Yamanaka A, and Nakayama K (2006) Stretch-induced triphosphorylation of myosin light chain and myogenic tone in canine basilar artery. *Eur J Pharmacol* **534**:141–151.
- Pang H, Guo Z, Su W, Xie Z, Eto M, and Gong MC (2005) RhoA-Rho kinase pathway mediates thrombin- and U-46619-induced phosphorylation of a myosin phosphatase inhibitor, CPI-17, in vascular smooth muscle cells. *Am J Physiol Cell Physiol* **289**:C352–C360.
- Peters SL, Schmidt M, and Michel MC (2006) Rho kinase: a target for treating urinary bladder dysfunction? *Trends Pharmacol Sci* **27**:492–497.
- Poburko D, Lhote P, Szado T, Behra T, Rahimian R, McManus B, van Breemen C, and Ruegg UT (2004) Basal calcium entry in vascular smooth muscle. *Eur J Pharmacol* **505**:19–29.
- Porter M, Evans MC, Miner AS, Berg KM, Ward KR, and Ratz PH (2006) Convergence of calcium desensitizing mechanisms activated by forskolin and phenyleph-

- rine pretreatment, but not 8-bromo-cGMP. *Am J Physiol Cell Physiol* **290**:C1552–C1559.
- Ratz PH (1990) Effect of the kinase inhibitor, H-7, on stress, crossbridge phosphorylation, muscle shortening and inositol phosphate production in rabbit arteries. *J Pharmacol Exp Ther* **252**:253–259.
- Ratz PH (1993) High α_1 -adrenergic receptor occupancy decreases relaxing potency of nifedipine by increasing myosin light chain phosphorylation. *Circ Res* **72**:1308–1316.
- Ratz PH (2001) Regulation of ERK phosphorylation in differentiated arterial muscle of the rabbit. *Am J Physiol Heart Circ Physiol* **281**:H114–H123.
- Ratz PH, Berg KM, Urban NH, and Miner AS (2005) Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol Cell Physiol* **288**:C769–C783.
- Rembold CM, Wardle RL, Wingard CJ, Batts TW, Etter EF, and Murphy RA (2004) Cooperative attachment of cross bridges predicts regulation of smooth muscle force by myosin phosphorylation. *Am J Physiol Cell Physiol* **287**:C594–C602.
- Sakurada S, Takuwa N, Sugimoto 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.
- Sasaki Y, Suzuki M, and Hidaka H (2002) The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Ther* **93**:225–232.
- Shabir S, Borisova L, Wray S, and Burdyga T (2004) Rho-kinase inhibition and electromechanical coupling in phasic smooth muscle: Ca^{2+} -dependent and independent mechanisms. *J Physiol* **560**:839–855.
- Shimokawa H and Rashid M (2007) Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci* **28**:296–302.
- Somlyo AP and Somlyo AV (2003) Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* **83**:1325–1358.
- Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, et al. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**:990–994.
- Urban NH, Berg KM, and Ratz PH (2003) K^+ depolarization induces RhoA kinase translocation to caveolae and Ca^{2+} sensitization of arterial muscle. *Am J Physiol Cell Physiol* **285**:C1377–C1385.
- van Breemen C, Chen Q, and Laher I (1995) Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. *Trends Pharmacol Sci* **16**:98–105.

Address correspondence to: Dr. Paul H. Ratz, Departments of Biochemistry and Molecular Biology and Pediatrics, Virginia Commonwealth University, School of Medicine, P.O. Box 980614, 1101 East Marshall Street, Richmond, VA 23298-0614. E-mail: phratz@vcu.edu