Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase

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Depolarization of the sarcolemma of smooth muscle cells activates voltage-gated Ca^{2+} channels, influx of Ca^{2+} and activation of cross-bridge cycling by phosphorylation of myosin catalysed by Ca^{2+} /calmodulin-dependent myosin light-chain kinase (MLCK). Agonist stimulation of smooth muscle contraction often involves other kinases in addition to MLCK. In the present study, we address the hypothesis that membrane depolarization-induced contraction of rat caudal arterial smooth muscle may involve activation of Rho-associated kinase (ROK). Addition of 60 mM K^{+} to de-endothelialized muscle strips in the presence of prazosin and propranolol induced a contraction that peaked rapidly and then declined to a steady level of force corresponding to approx. 30% of the peak contraction. This contractile response was abolished by the Ca^{2+} -channel blocker nifedipine or the removal of extracellular Ca^{2+} . An MLCK inhibitor (ML-9) inhibited both the phasic and tonic components of K^{+} -induced contraction. On the other hand, the ROK inhibitors Y-27632 and HA-1077 abolished the tonic component of K^{+} -induced contraction, and

slightly reduced the phasic component. Phosphorylation levels of the 20-kDa light chain of myosin increased rapidly in response to 60 mM K^{+} and subsequently declined to a steady-state level significantly greater than the resting level. Y-27632 abolished the sustained and reduced the phasic elevation of the phosphorylation of the 20-kDa light chain of myosin, without affecting the K^{+} -induced elevation of cytosolic free Ca^{2+} concentration. These results indicate that ROK activation plays an important role in the sustained phase of K^{+} -induced contraction of rat caudal arterial smooth muscle, but has little involvement in the phasic component of K^{+} -induced contraction. Furthermore, these results are consistent with inhibition of myosin light-chain phosphatase by ROK, which would account for the sustained elevation of myosin phosphorylation and tension in response to membrane depolarization.

Key words: HA-1077, ML-9, myosin phosphorylation, myosin light-chain kinase, Y-27632.

INTRODUCTION

A rise in the concentration of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) is the major trigger for the contraction of smooth muscle [1]. Smooth muscle contraction is regulated by pharmacomechanical coupling mechanisms, whereby agonists induce contraction without depolarizing the membrane, and by electromechanical coupling mechanisms, which involve membrane depolarization [2]. Stimulation of vascular smooth muscle with various agonists leads to elevated $[\text{Ca}^{2+}]_i$ via Ca^{2+} influx from the extracellular space through Ca^{2+} -permeable non-specific cation channels [3] or voltage-dependent L-type Ca^{2+} channels, and Ca^{2+} release from the sarcoplasmic reticulum [4]. The elevation of $[\text{Ca}^{2+}]_i$ leads to calmodulin (CaM)-dependent activation of myosin light-chain kinase (MLCK) and hence phosphorylation of the 20-kDa light chains of myosin (LC_{20}) II at Ser¹⁹ [5,6]. This simple phosphorylation reaction triggers cycling of myosin cross-bridges along the actin filaments, which force development or shortening of the muscle. Relaxation generally occurs following a return of $[\text{Ca}^{2+}]_i$ to resting levels, resulting in dissociation of Ca^{2+} from CaM, inactivation of MLCK and dephosphorylation of myosin catalysed by myosin light-chain phosphatase (MLCP), a myosin-associated type-1 protein serine/threonine phosphatase

[7]. However, $[\text{Ca}^{2+}]_i$ does not always parallel the extent of LC_{20} phosphorylation and contraction [8–13]. During tonic force maintenance, for example, the relationship between force and LC_{20} -phosphorylation levels can be modified or even dissociated. Thus, secondary regulatory pathways are likely to be functionally important in the control of smooth muscle contractility. Additional important mechanisms of regulation that can modify the levels of LC_{20} phosphorylation and degree of contraction independent of $[\text{Ca}^{2+}]_i$ have been proposed, and are collectively referred to as Ca^{2+} -sensitization mechanisms [2]. Ca^{2+} -sensitizing agents include agonists that activate receptors coupled to heterotrimeric G-proteins, agents (GTP or the non-hydrolysable GTP analogue guanosine 5'-[γ -thio]triphosphate) that directly activate G-proteins [14–17], activators (phorbol esters) of conventional and novel protein kinase C (PKC) isoenzymes [14,18], and arachidonic acid [19–21]. Cellular signaling pathways involved in Ca^{2+} sensitization converge on an increase in LC_{20} phosphorylation, and analyses of kinase and phosphatase activities have indicated that LC_{20} dephosphorylation is selectively reduced in this process, i.e. Ca^{2+} sensitization is mediated by the inhibition of MLCP, rather than activation of MLCK [7,22,23].

Several different mechanisms have been proposed to account for this inhibition of MLCP: (i) the small GTPase RhoA, a

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; CaM, calmodulin; DTT, dithiothreitol; H-T solution, Hepes/Tyrode solution; LC_{20} , 20-kDa light chain of myosin; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; NPY, neuropeptide Y; PKC, protein kinase C; ROK, Rho-associated kinase.

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member of the Rho subfamily of the Ras superfamily of monomeric GTPases, activates Rho-associated kinase (ROK), which in turn phosphorylates the 130-kDa myosin-binding subunit of MLCP, inhibiting phosphatase activity [24–28]; (ii) PKC [29] phosphorylates the protein kinase C-potentiated inhibitory protein for protein phosphatase-1 of 17-kDa ('CPI-17') [30–32], which thereby becomes a potent inhibitor of MLCP; (iii) arachidonic acid inhibits MLCP activity either directly [19], or via activation of an atypical PKC isoenzyme [21] or ROK [33]. Of these signal transduction pathways, the RhoA–ROK pathway appears to play a major role in agonist-induced Ca^{2+} sensitization of smooth muscle contraction (reviewed in [34,35]). Receptor stimulation by agonists can thus activate multiple cellular signaling mechanisms that can change the level of force, without necessarily changing membrane potential.

Contraction of smooth muscle is regulated by electromechanical, in addition to pharmacomechanical coupling mechanisms. Electromechanical coupling operates through changes in surface membrane potential, which affect $[\text{Ca}^{2+}]_i$. Stimulation by K^+ induces depolarization of the cell membrane, which opens voltage-gated Ca^{2+} channels causing Ca^{2+} influx to increase $[\text{Ca}^{2+}]_i$ [36]. Therefore, K^+ -induced contraction may depend entirely on the influx of extracellular Ca^{2+} and is not mediated by receptors. We reported previously [37,38] α_1 -adrenoceptor-mediated contraction and phosphorylation of myosin in de-endothelialized rat caudal arterial smooth muscle. During investigations into the role of the RhoA–ROK pathway in α_1 -adrenoceptor-mediated contraction in this tissue, we observed inhibition of K^+ -induced contraction by ROK inhibitors. However, little is known about the possible role of ROK in K^+ -induced contraction. The goal of this study, therefore, was to investigate the potential role of ROK in K^+ -induced contraction of rat caudal arterial smooth muscle. Specifically, we assessed the contributions of MLCK and ROK to K^+ -induced contraction of de-endothelialized rat caudal arterial smooth muscle using well-characterized inhibitors. We conclude that membrane depolarization with the influx of extracellular Ca^{2+} activates ROK, in addition to MLCK, and ROK activation is required for the sustained, but not the phasic, contractile response to depolarization.

MATERIALS AND METHODS

Materials

Prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furan-ylcarbonyl)-piperazine], DL-propranolol [1-(isopropylamino)-3-(1-naphthoxy)-2-propanol] and nicardipine hydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.). ML-9 [1-(5-chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine] was from Seikagaku (Tokyo, Japan). Y-27632 [(*R*)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] was generously given by the Welfide Corporation (Osaka, Japan). HA-1077 was from ICN Biomedicals (Aurora, OH, U.S.A.). Dithiothreitol (DTT) was from Wako Pure Chemical Industries (Osaka, Japan). Hepes and fura 2 acetoxymethyl ester (fura 2/AM) were from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade. Stock solutions were prepared in water for prazosin, propranolol, Y-27632 and HA-1077, and in DMSO for ML-9.

Preparation of muscle strips

Male Sprague–Dawley rats (300–400 g) were killed by a blow on the neck and exsanguination, as approved by the Institutional Ethics Committee for Animal Research (Meiji Pharmaceutical

University). Caudal arterial smooth muscle strips were prepared by the method of Mita and Walsh [37]. The endothelial layer and adventitial tissue were removed mechanically. Helical strips [0.5 mm × (6–7) mm] were cut and stored in Hepes/Tyrode (H-T) solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose and 10 mM Hepes, pH 7.4).

Force measurements

De-endothelialized muscle strips were mounted horizontally between two hooks and immersed in a pool of solution (750 μl), which was rounded by surface tension on a rotation plate of 4-cm diameter. One hook was connected to a force displacement transducer (TB-612T; Nihon Kohden, Tokyo, Japan) that was mounted on a micromanipulator, and the other to a second micromanipulator. Solution changes were effected rapidly by rotation of the plate. A resting tension of 75 mg was determined experimentally to give a maximal contractile response to 40 mM KCl and was used for all experiments. Once mounted, de-endothelialized muscle strips were held at this resting tension in H-T solution at 24 °C for 45 min, and then transferred to H-T solution in the presence of 1 μM prazosin and 0.1 μM propranolol for 10 min, before transfer to 60 mM K^+ solution in the presence of 1 μM prazosin and 0.1 μM propranolol. After stable 60 mM K^+ -induced contractions were achieved in the presence of 1 μM prazosin and 0.1 μM propranolol, the strips were transferred to H-T solution in the presence of 1 μM prazosin and 0.1 μM propranolol for 45 min before transfer to the test solutions (containing 1 μM prazosin and 0.1 μM propranolol), as indicated below. All buffers were at 24 °C and those used for intact strips were pre-oxygenated with 100% O_2 . K^+ solution (60 mM) was prepared by replacing the NaCl in H-T solution with equimolar KCl. K^+ (60 mM) induced a sub-maximal contraction (approx. 78.1% of the maximal contraction) of rat caudal arterial smooth muscle.

Measurement of changes in intracellular Ca^{2+} concentration

Muscle strips were incubated with H-T solution containing 16 μM fura 2/AM for 4 h in the dark at 24 °C. Fura 2-loaded muscle strips were washed with H-T solution containing 1 μM prazosin and 0.1 μM propranolol for 30 min. The fluorescence of the mounted muscle strips was recorded at an emission wavelength of 500 nm, with excitation at 340 nm, and 380 nm, using a CAF-100 spectrofluorimeter (Jasco, Tokyo, Japan). The fluorescence ratio (F_{340}/F_{380}) was monitored before (resting state) and during stimulation with 60 mM K^+ for 20 min in the absence and presence of 3 μM Y-27632.

Quantification of LC_{20} phosphorylation

Quantification of LC_{20} phosphorylation was achieved by the method of Mita and Walsh [37]. Tissue samples were frozen at selected times, following 60 mM K^+ stimulation by immersion in 10% trichloroacetic acid/10 mM DTT in solid CO_2 /acetone for 10 min. Frozen muscle strips were then washed with 10 mM DTT in acetone at 24 °C. The tissues were freeze-dried overnight and stored at –80 °C until LC_{20} extraction was carried out. Proteins were extracted from tissue strips in 30 μl of extraction buffer (6 M deionized urea, 20.1 mM Tris, 22.2 mM glycine, 10 mM DTT, 10 mM EGTA, 1 mM EDTA, 1 mM PMSF, 0.6 M KI and 0.15 mM Bromophenol Blue) by constant rotation in a microcentrifuge tube for 90 min at 24 °C. After filtration and centrifugation, phosphorylated and unphosphorylated LC_{20} were separated by urea/glycerol gel electrophoresis, and were trans-

Figure 1 Effect of ML-9 on K⁺-induced contraction of rat caudal arterial smooth muscle

(A) Effect of ML-9 on K⁺-induced sustained contraction of rat caudal arterial smooth muscle. ML-9 was added at the indicated concentrations to muscle strips that exhibited steady 60 mM K⁺-induced tonic contraction, and the time-course of relaxation was followed. Force is expressed as a percentage of the force of the tonic contraction developed in response to K⁺ before the addition of ML-9. The inset shows that treatment with vehicle (DMSO) alone [up to 1% (the maximal concentration used)] had no effect on K⁺-induced contraction. Results are expressed as the means $\pm$ S.E.M. ($n = 8$). (B) A typical trace showing the effect of pre-treatment with ML-9 on K⁺-induced contraction of rat caudal arterial smooth muscle. After stable 60 mM K⁺-induced contractions were achieved, strips were relaxed and incubated for 20 min with 30 μM ML-9 prior to K⁺ depolarization. The trace is representative of four experiments. (C) Effect of pre-treatment with ML-9 on K⁺-induced contraction of rat caudal arterial smooth muscle. After stable 60 mM K⁺-induced contractions were achieved, strips were relaxed and incubated for 20 min with ML-9 prior to K⁺ depolarization. Upper panels show the time-courses of K⁺-induced contraction in the absence of ML-9 (○) or presence of the indicated concentrations of ML-9 (●). Lower panels show the effects of ML-9 on the phasic and tonic components of K⁺-induced contraction. Force development is expressed as a percentage of the maximal force of the phasic contraction developed in response to 60 mM K⁺ without ML-9. The force of the tonic component was measured 30 min after K⁺ addition. Filled bars indicate the phasic contraction in response to K⁺ and open bars the tonic contraction. Results are expressed as the means $\pm$ S.E.M. ($n = 4$). *Significantly different from the force without ML-9 ($P < 0.005$, Student's t test).

blotted on to 0.2-μm nitrocellulose membrane (BioRad, Mississauga, Ontario, Canada) in 10 mM sodium cyclohexylamino-propanesulphonic acid, pH 11, in a Mini Transblot Cell (BioRad) at 27 V and 5 °C for 16 h. LC₂₀ was detected using a polyclonal antibody raised in rabbits against purified chicken gizzard LC₂₀ [37,38]. The antibody was purified from antiserum using HiTrap Protein A (Pharmacia, Baie d'Urfe, Québec, Canada) and was used at a dilution of 1:1000 with the BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Phosphorylated and unphosphorylated LC₂₀ bands were quantified by densitometric scanning using a Pharmacia Image Master Desktop Scanning

System. LC₂₀ phosphorylation levels were calculated by dividing the chemiluminescence signal (absorbance $\times$ area of the band) of the phosphorylated LC₂₀ peak by the total chemiluminescence signal of the phosphorylated and unphosphorylated LC₂₀ peaks. We observed no diphosphorylated LC₂₀ in any experiment.

Statistical analysis

All statistical analyses were performed using StatView-J5.0 (SAS Institute, Cary, NC, U.S.A.). Student's paired or unpaired t tests were performed and $P < 0.05$ was considered significant. Results

are expressed as the means $\pm$ S.E.M. Values of n indicate the numbers of strips.

RESULTS

Characterization of K⁺-induced contraction of rat caudal arterial smooth muscle

All experiments were carried out in the presence of 1 μ M prazosin and 0.1 μ M propranolol in order to block the α_1 - and β -adrenergic effects of noradrenaline, which is released from nerve terminals by depolarization [39]. The addition of 60 mM K⁺ to denuded helical strips of rat caudal arterial smooth muscle induced a contraction that peaked rapidly and then declined to a steady level corresponding to approx. 30% of the peak contraction (Figures 1B and 1C). The forces of the phasic and tonic components of contraction evoked by 60 mM K⁺ were 247.8 ± 13.4 mg and 76.9 ± 8.6 mg ($n = 4$) respectively.

Tetrodotoxin (0.1 μ M) or the P₂ purinoceptor antagonist suramine (10 μ M) did not affect 60 mM K⁺-induced contraction, indicating that release of ATP or neuropeptide Y, which are co-stored and co-released with noradrenaline from sympathetic nerve terminals [40–43], is not involved in K⁺-induced contraction (results not shown). Furthermore, the cyclo-oxygenase inhibitor indomethacin (3 μ M) had no effect on K⁺-induced contraction, indicating that prostaglandins also do not play a role in the contractile response to membrane depolarization (results not shown).

Both the phasic and tonic components of 60 mM K⁺-induced contraction were completely abolished by the Ca²⁺-channel blocker nifedipine or brief treatment with Ca²⁺-free solution containing 2 mM EGTA for 1 min to remove extracellular Ca²⁺ (results not shown). The K⁺-induced contraction was fully restored following replacement of extracellular Ca²⁺.

Figure 2 Effects of Y-27632 and HA-1077 on K⁺-induced sustained contraction of rat caudal arterial smooth muscle

Y-27632 (○) or HA-1077 (△) was added at the indicated concentrations to muscle strips exhibiting steady 60 mM K⁺-induced tonic contraction, and the time-course of relaxation was followed. Force is expressed as a percentage of the force of the tonic contraction developed in response to K⁺ before the addition of Y-27632 or HA-1077. Results are expressed as the means $\pm$ S.E.M. ($n = 4$).

Effects of ML-9 on K⁺-induced contraction

An MLCK inhibitor, ML-9, potently inhibited 60 mM K⁺-induced sustained contraction, causing a concentration-dependent relaxation to below the basal level of force with an IC₅₀ value of 2.39 ± 0.32 μ M (Figure 1A). Treatment with vehicle (DMSO) alone had no effect on K⁺-induced contraction (Figure 1A, inset). Pre-treatment of muscle strips with ML-9 inhibited both the phasic and tonic components of K⁺-induced contraction in a concentration-dependent manner (Figures 1B and 1C).

Effects of Y-27632 and HA-1077 on K⁺-induced contraction

The ROK inhibitors, Y-27632 and HA-1077, potently inhibited K⁺-induced sustained contraction, causing a concentration-dependent relaxation to the basal force with IC₅₀ values of 0.41 ± 0.02 μ M and 0.49 ± 0.06 μ M respectively (Figure 2). Force was well maintained in the vehicle (water) alone over the same number of solution changes for the same duration (results not shown). Pre-treatment of muscle strips with Y-27632 (Figure 3) or HA-1077 (Figure 4) potently inhibited the tonic component of K⁺-induced contraction with a small, but significant, decrease in the phasic component. Furthermore, pre-treatment with the ROK inhibitors increased the rate of relaxation after the peak response (Figures 3 and 4).

Effect of Y-27632 on [Ca²⁺]_i

Stimulation of fura 2/AM-loaded rat caudal arterial smooth muscle strips with 60 mM K⁺ led to a transient increase in [Ca²⁺]_i, which settled at a sustained level significantly above resting [Ca²⁺]_i (Figure 5A, a). Y-27632 (3 μ M) had no effect on the [Ca²⁺]_i response to 60 mM K⁺ (Figure 5A, b). Figure 5(B) shows the summarized results of the effect of 3 μ M Y-27632 on the F_{340}/F_{380} ratio in response to 60 mM K⁺. The values of F_{340}/F_{380} after K⁺ addition in the absence of Y-27632 were 0.483 ± 0.012 in the resting state, 0.671 ± 0.019 at the peak of the response and 0.612 ± 0.014 during the sustained response. On the other hand, in the presence of Y-27632, the values were 0.448 ± 0.028 , 0.631 ± 0.025 and 0.563 ± 0.022 respectively, i.e. not significantly different from those in the absence of Y-27632.

Effect of Y-27632 on LC₂₀ phosphorylation

Under control conditions, LC₂₀ phosphorylation levels paralleled force, increasing rapidly in response to 60 mM K⁺ from a resting level of 0.12 ± 0.04 mol of P_i/mol of LC₂₀ to a peak of 0.54 ± 0.03 mol of P_i/mol of LC₂₀ at 15 s after 60 mM K⁺ addition (Figure 6). LC₂₀ phosphorylation then declined to a steady level of 0.35 ± 0.08 mol of P_i/mol of LC₂₀ at 15 min after K⁺ addition. In the presence of 3 μ M Y-27632, LC₂₀ phosphorylation levels increased rapidly in response to K⁺ from a resting level of 0.18 ± 0.03 mol of P_i/mol of LC₂₀ to a peak of 0.34 ± 0.06 mol of P_i/mol of LC₂₀ at 15 s after 60 mM K⁺ addition and then declined rapidly to resting levels (Figure 6). Thus, Y-27632 reduced the transient increase in LC₂₀ phosphorylation, but completely abolished the sustained elevation of LC₂₀ phosphorylation (0.14 ± 0.05 mol of P_i/mol of LC₂₀ at 15 min after 60 mM K⁺ addition, which is indistinguishable from the resting value).

DISCUSSION

Electromechanical coupling describes the signaling pathways whereby depolarization of the sarcolemma leads to muscle

Figure 3 Effect of pre-treatment with Y-27632 on K⁺-induced contraction of rat caudal arterial smooth muscle

After stable 60 mM K⁺-induced contractions were achieved, strips were relaxed and incubated for 20 min with Y-27632 prior to K⁺ depolarization. (A) A typical trace showing the effect of pre-treatment with 3 μM Y-27632 on K⁺-induced contraction of rat caudal arterial smooth muscle. The trace is representative of four experiments. (B) Upper panels show the time courses of K⁺-induced contraction in the absence of Y-27632 (○) or presence of the indicated concentrations of Y-27632 (●). Lower panels show the effects of Y-27632 on the phasic and tonic components of K⁺-induced contraction. Force development is expressed as a percentage of the maximal force of the phasic contraction developed in response to K⁺ without Y-27632. The force of the tonic component was measured 30 min after K⁺ addition. Filled bars indicate the phasic contraction in response to K⁺ and open bars the tonic contraction. Results are expressed as the means ± S.E.M. (*n* = 4 for 1 μM and 3 μM Y-27632; *n* = 6 for 10 μM Y-27632). *Significantly different from the force without Y-27632 (*P* < 0.005, Student's *t* test).

contraction. In the context of smooth muscle contraction, it is well established that membrane depolarization opens voltage-gated Ca²⁺ channels allowing Ca²⁺ entry to the cytosol [4]. The increase in [Ca²⁺]_i results in binding of Ca²⁺ to CaM, which activates MLCK. The active kinase then catalyses phosphorylation of myosin, which triggers cross-bridge cycling [2]. During the course of studies of pharmacomechanical coupling mechanisms [44], which trigger contraction without a change in membrane potential, we obtained evidence from the control experiments that depolarization of the smooth muscle sarcolemma by K⁺ results in activation of ROK, which could play a role in the contractile response. This prompted us to conduct a detailed analysis of the activation of this kinase by membrane depolarization and its potential involvement in this contractile response. Therefore, in the present study, we examined the effects

of two well-characterized selective ROK inhibitors on the contractile properties of endothelium-free smooth muscle helical strips of the rat caudal artery. In order to eliminate effects of noradrenaline released from nerve terminals, prazosin and propranolol were used to block the α₁- and β-adrenergic effects of this neurotransmitter. It was found unnecessary to antagonize other neurotransmitters, such as ATP and neuropeptide Y or prostaglandins.

The contractile response to K⁺-induced membrane depolarization was found to be absolutely dependent on the influx of Ca²⁺ through voltage-gated Ca²⁺ channels, since it could be blocked by a Ca²⁺-channel blocker or by removal of extracellular Ca²⁺. This contrasts with α₁-adrenoceptor-induced contraction of the rat caudal artery, in which the phasic component involves the release of Ca²⁺ from the sarcoplasmic reticulum, whereas the

Figure 4 Effect of pre-treatment with HA-1077 on K⁺-induced contraction of rat caudal arterial smooth muscle

After stable 60 mM K⁺-induced contractions were achieved, strips were relaxed and incubated for 20 min with HA-1077 prior to K⁺ depolarization. (A) A typical trace showing the effect of pre-treatment with 3 μM HA-1077 on K⁺-induced contraction of rat caudal arterial smooth muscle. The trace is representative of four experiments. (B) Upper panels show the time-courses of K⁺-induced contraction in the absence of HA-1077 (○) or presence of the indicated concentrations of HA-1077 (●). Lower panels show the effects of HA-1077 on the phasic and tonic components of K⁺-induced contraction. Force development is expressed as a percentage of the maximal force of the phasic contraction developed in response to K⁺ without HA-1077. The force of the tonic component was measured 30 min after K⁺ addition. Filled bars indicate the phasic contraction in response to K⁺ and open bars the tonic contraction. Results are expressed as the means ± S.E.M. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.005; significantly different from the value of the force without HA-1077 with the Student's *t* test.

tonic component involves influx of Ca²⁺ from the extracellular milieu [37].

K⁺-induced contraction of rat caudal arterial smooth muscle strips was inhibited by ML-9, a highly selective inhibitor of MLCK with a *K_i* value of 3.8 μM [45,46], similar to the IC₅₀ value for inhibition of K⁺-induced contraction obtained in the present study (2.39 ± 0.32 μM). It is noticeable that ML-9-induced relaxation to levels of force well below resting levels (Figure 1A), suggesting that resting tone is due to the low basal level of LC₂₀ phosphorylation (0.12 mol of P_i/mol of LC₂₀; Figure 6) and that MLCK is responsible for this basal phosphorylation. Pre-treatment of arterial strips with ML-9 inhibited both the phasic and tonic components of K⁺-induced contraction in a concentration-dependent manner (Figures 1B and 1C).

Ca²⁺ sensitization of smooth muscle contraction involves the small GTPase RhoA. Agonists activate RhoA via receptors that are coupled to the G_{12/13} family of heterotrimeric G-proteins [47],

and the activated RhoA interacts with and activates ROK [34]. Activated ROK subsequently phosphorylates the 130-kDa myosin-binding subunit of MLCP [27], inhibiting the activity of MLCP [28,48]. Y-27632 [49] and HA-1077 [50] are selective ROK inhibitors, and Ca²⁺ sensitization induced by receptor stimulation or the activation of G-proteins is inhibited by these compounds. In the present study, the K⁺-induced sustained contraction of smooth muscle strips was inhibited by Y-27632 and HA-1077 in a concentration-dependent manner with IC₅₀ values of 0.41 ± 0.02 μM and 0.49 ± 0.06 μM respectively (Figure 2). These IC₅₀ values are similar to previously reported values for agonist-induced contractions and to the *K_i* values reported using purified ROK (0.14 μM for Y-27632 and 0.40 μM for HA-1077) [49,50]. We observed that pre-treatment of arterial strips with Y-27632 or HA-1077 abolished the tonic component of K⁺-induced contraction, with a relatively minor effect on the phasic component (Figures 3 and 4). On the other hand, the ROK inhibitors

Figure 5 Effect of Y-27632 on K⁺-induced changes in [Ca²⁺]_i in rat caudal arterial smooth muscle

Intact rat caudal arterial smooth muscle strips were loaded with fura 2/AM. The F_{340}/F_{380} ratio was recorded following stimulation with 60 mM K⁺ in the absence or presence of 3 μM Y-27632. Y-27632 was present 20 min prior to, and during, the 60 mM K⁺-induced contraction. (A) Representative traces showing the change in the F_{340}/F_{380} ratio induced by 60 mM K⁺ in the absence (a) and presence (b) of 3 μM Y-27632. (B) The F_{340}/F_{380} ratio for resting, peak and sustained components in response to 60 mM K⁺ in the absence (open bars) and presence (filled bars) of 3 μM Y-27632. The fluorescence intensity of the sustained component was measured 20 min after K⁺ addition. Results are expressed as the means ± S.E.M. ($n = 8$). F_{340}/F_{380} values with Y-27632 were not significantly different from those without Y-27632 with the Student's *t* test.

had no effect on K⁺-induced changes in [Ca²⁺]_i (Figure 5). Comparison of the upper panels in Figures 3(B) and 4(B) indicates that Y-27632 and HA-1077, but not ML-9 (Figure 1C), enhance the rate of relaxation following the peak of the phasic component of K⁺-induced contraction, which is consistent with an increase in MLCP activity and faster LC₂₀ dephosphorylation. We conclude, therefore, that the tonic component of K⁺-induced contraction involves activation of ROK with resultant inhibition of MLCP. Similar results were obtained by receptor stimulation of guinea-pig ileum [28]. Pre-treatment with HA-1077 abolished the tonic component of carbachol-induced contraction and the sustained elevation of LC₂₀ phosphorylation, but had no effect on the transient or sustained increase in [Ca²⁺]_i. These results indicate that physiological elevations of [Ca²⁺]_i and the consequent MLCK activation are not sufficient to cause a full-force response, unless GTPase-dependent pathways leading to MLCP inhibition are simultaneously activated. It therefore seems likely that maintained contraction in response to K⁺, as well as agonists, depends on both MLCK and ROK activities.

Figure 6 Effect of Y-27632 on K⁺-induced LC₂₀ phosphorylation in rat caudal arterial smooth muscle

After stable 60 mM K⁺-induced contractions were achieved, strips were relaxed and incubated for 20 min in the absence or presence of 3 μM Y-27632 prior to K⁺ depolarization. Tissues were frozen at the indicated times and LC₂₀ phosphorylation quantified, as described in the Materials and methods section. (A) Representative Western blots showing separation of phosphorylated and unphosphorylated LC₂₀ by urea/glycerol gel electrophoresis. Lane 1: resting conditions without (a) or with (b) Y-27632. Lanes 2–8: 15 s, 30 s, 1 min, 2 min, 5 min, 15 min and 30 min respectively, following addition of 60 mM K⁺ in the absence (a) or presence (b) of Y-27632. (B) Quantitative results pooled from several experiments [$n = 5$ for the results in the absence of Y-27632 (○), and $n = 6$ for the results in the presence of Y-27632 (●)]. Results are expressed as the means ± S.E.M.

In contrast with our present observations with rat caudal artery, Uehata et al. [49] reported that Y-27632 caused a small concentration-dependent inhibition of contraction induced by high [K⁺] in rabbit aortic strips. These two vascular smooth muscles exhibit major differences in their contractile responses to K⁺: in the rat caudal artery, the initial phasic force transient is followed by a decline in tension (Figures 1, 3 and 4), whereas in the rabbit aorta there is a continuous increase in tension during prolonged depolarization [51]. This difference is likely due to regional and species differences in the action potentials between these two smooth muscle cell types. It is generally assumed that vascular smooth muscle cells in capacitative vessels, similar to the aorta, are electrically quiescent (reviewed in [52]). It seems, therefore, that the smooth muscle of caudal artery may exhibit qualitatively different electrical properties from those of the aorta, and there may be some divergence between the generation of electrical events and contractile signal transduction. Smooth muscle can be classified into two major subtypes: phasic and tonic [53]. Smooth muscles respond to K⁺ depolarization with a relatively rapid contraction that, in phasic smooth muscles, declines within minutes to an intermediate level, whereas, in tonic smooth muscles, it is followed by sustained increases in force for up to 30 min [54]. It has been suggested that the Ca²⁺ sensitivity of the contractile elements might differ between phasic and tonic

Figure 7 Proposed signal transduction pathways involved in K^+ -induced contraction of rat caudal arterial smooth muscle

smooth muscles [55]. In addition, MLCP and MLCK activities vary among smooth muscles: MLCP and MLCK activities are 2.0- and 1.9-fold lower respectively in tonic compared with phasic smooth muscles [56]. Smooth muscle-specific differences between the inhibitory effects of Y-27632 on K^+ -induced contractions of different smooth muscles may, therefore, be due to differences in MLCP activity in different smooth muscles.

Phosphorylation levels of LC_{20} increased rapidly in response to 60 mM K^+ and declined to a steady-state level that was significantly higher than the resting level (Figure 6). This phosphorylation of LC_{20} closely matched the time-course of contraction. Y-27632 (3 μ M) had a modest inhibitory effect on the transient increase in LC_{20} phosphorylation (Figure 6). This effect was maximal since, in the presence of a higher concentration of Y-27632 (10 μ M), LC_{20} phosphorylation levels also increased rapidly in response to K^+ to the same level (0.34 ± 0.03 mol of P_i /mol of LC_{20} 15 s after 60 mM K^+ addition) (results not shown). On the other hand, Y-27632 (3 μ M) abolished the sustained elevation of LC_{20} phosphorylation (Figure 6). The time courses of LC_{20} phosphorylation, therefore, correlate well with the force responses to 60 mM K^+ in the absence and presence of Y-27632. We conclude that the tonic phase of K^+ -induced contraction is mediated via inhibition of MLCP activity by ROK activation, and requires MLCK activity. Swärd et al. [28] reported a similar finding for carbachol-induced contraction of guinea-pig ileum: pre-treatment of intact ileal strips with HA-1077 abolished the tonic component of carbachol-induced contraction and the sustained elevation of LC_{20} phosphorylation, but had little effect on the phasic contraction or the transient increase in LC_{20} phosphorylation induced by carbachol, suggesting that the ROK-mediated increase in LC_{20} phosphorylation is due to MLCP inhibition, not MLCK activation.

In fura 2-loaded rat caudal arterial strips, the time course of the change in $[Ca^{2+}]_i$ stimulated by K^+ closely matched the time courses of contraction and LC_{20} phosphorylation (Figures 3–6). Moreover, Y-27632 had no significant effect on K^+ -induced changes in $[Ca^{2+}]_i$ (Figure 5). HA-1077 also had no effect on the K^+ -induced Ca^{2+} transient (results not shown). ROK inhibitors,

therefore, caused complete reduction of sustained contraction without associated changes in $[Ca^{2+}]_i$. It was reported that in guinea-pig trachealis Y-27632 inhibited methacholine-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner with an IC_{50} value of 524.1 ± 31.0 μ M [57]. However, at the concentrations of Y-27632 used in our experiments (1–10 μ M), methacholine-induced contraction was inhibited with little change in $[Ca^{2+}]_i$. We suggest, therefore, that during the phasic component of K^+ -induced contraction, $[Ca^{2+}]_i$ is high enough to activate MLCK to a level that is much greater than that of MLCP. However, the lower $[Ca^{2+}]_i$ during the sustained contraction may not be high enough to activate MLCK to a level sufficient to overcome the phosphatase. In this case, inhibition of MLCP by ROK is required for the activity ratio of MLCK to MLCP to shift far enough in the direction of the kinase to maintain LC_{20} phosphorylation elevated. These results suggest that RhoA/ROK activation plays an important role in the tonic component of K^+ -induced contraction and LC_{20} phosphorylation in rat caudal arterial smooth muscle, but has no significant role in the phasic component.

In conclusion, our results indicate that vascular smooth muscle contraction elicited by membrane depolarization comprises two phases, both of which are dependent on influx of extracellular Ca^{2+} (Figure 7): (i) a rapid phasic component that involves phosphorylation of myosin by Ca^{2+} /CaM-dependent MLCK and (ii) a sustained tonic component that involves these mechanisms, as well as activation of ROK and Ca^{2+} sensitization.

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