The importance of Rho-associated kinase-induced Ca^{2+} sensitization as a component of electromechanical and pharmacomechanical coupling in rat ureteric smooth muscle

Lyudmyla Borysova^a, S. Shabir^{a,1}, Michael P. Walsh^b, and Theodor Burdyga^{a,*}

^aDepartment of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Crown Street, Liverpool L69 3BX, UK

^bSmooth Muscle Research Group, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1

Abstract

Ureteric peristalsis, which occurs via alternating contraction and relaxation of ureteric smooth muscle, ensures the unidirectional flow of urine from the kidney to the bladder. Understanding of the molecular mechanisms underlying ureteric excitation–contraction coupling, however, is limited. To address these knowledge deficits, and in particular to test the hypothesis that Ca^{2+} sensitization via activation of the RhoA/Rho-associated kinase (ROK) pathway plays an important role in ureteric smooth muscle contraction, we carried out a thorough characterization of the electrical activity, Ca^{2+} signaling, MYPT1 (myosin targeting subunit of myosin light chain phosphatase, MLCP) and myosin regulatory light chain (LC_{20}) phosphorylation, and force responses to membrane depolarization induced by KCl (electromechanical coupling) and carbachol (CCh) (pharmacomechanical coupling). The effects of ROK inhibition on these parameters were investigated. We conclude that the tonic, but not the phasic component of KCl- or CCh-induced ureteric smooth muscle contraction is highly dependent on ROK-catalyzed phosphorylation of MYPT1 at T855, leading to inhibition of MLCP and increased LC_{20} phosphorylation.

Keywords

Rho-kinase; Ureter smooth muscle; Ca^{2+} sensitization

1. Introduction

Ureteric peristalsis ensures the unidirectional flow of urine from the kidney to the bladder and is regulated by myogenic mechanisms and neurogenic factors [1,2]. Urolithiasis (the formation of kidney stones) is a common disease [3] and stones are frequently located in the ureter where they compromise ureteric function [4]. In order to improve the treatment of

*Corresponding author. Tel.: +44 151 794 4971; fax: +44 151 794 5321. burdyga@liverpool.ac.uk (T. Burdyga).

¹Present address: Jack Birch Unit of Molecular Carcinogenesis, Department of Biology, University of York YO10 5YW, UK.

Conflict of interest statement

The authors confirm that they do not have any conflict of interest.

urolithiasis and facilitate stone passage, greater understanding of the signal transduction pathways involved in regulation of ureteric smooth muscle contraction and relaxation is essential.

Ca^{2+} is the primary activator of smooth muscle contraction [5]. An increase in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) can be achieved by depolarization of the plasma membrane leading to Ca^{2+} entry via voltage-gated L-type Ca^{2+} channels, Ca^{2+} entry via receptor- or store-operated channels, Ca^{2+} release from intracellular stores, or a combination of these mechanisms. A global rise in $[\text{Ca}^{2+}]_i$ activates Ca^{2+} /calmodulin (CaM)-dependent myosin light chain kinase (MLCK), which phosphorylates the 20-kDa regulatory light chains of myosin (LC_{20}), thereby triggering cross-bridge cycling and contraction [6]. The type of Ca^{2+} signaling varies depending on the particular smooth muscle and the stimulus. In phasic smooth muscles, such as the uterus, ureter, bladder and gastrointestinal tract, Ca^{2+} signaling is controlled by action potentials. In ureteric smooth muscle, the amplitude of phasic contraction depends mainly on the duration of the Ca^{2+} transient, controlled by the duration of the plateau component of the action potential [7]. This tissue exhibits a non-linear relationship between LC_{20} phosphorylation and force, and force lags significantly behind LC_{20} phosphorylation [8], suggesting that the rate-limiting step in force production is not LC_{20} phosphorylation but rather formation of the force-generating state of cross-bridges. Normally, when $[\text{Ca}^{2+}]_i$ is restored to resting levels following termination of the action potential discharge, the muscle relaxes in a manner that is dependent on the rate of LC_{20} dephosphorylation by myosin light chain phosphatase (MLCP) [9].

It is well established that a decrease or increase in MLCP activity at a constant level of MLCK activity, at steady-state $[\text{Ca}^{2+}]_i$, can sensitize or desensitize smooth muscle, which results in an increase or decrease, respectively, in LC_{20} phosphorylation and force [10,11]. Thus the extent of LC_{20} phosphorylation and force results from a balance between MLCK and MLCP activities. Some early studies performed on intact tissue showed that contractile agonists acting via G protein-coupled receptors can generate more force for a given increase in $[\text{Ca}^{2+}]_i$ than KCl, which activates Ca^{2+} entry via L-type voltage-gated Ca^{2+} channels [12–14].

Ca^{2+} sensitization of smooth muscle contraction involves primarily inhibition of MLCP activity by ligand occupancy of receptors that are coupled to guanine nucleotide exchange factors via heterotrimeric G proteins of the $\text{G}_{12/13}$ family [11]. MLCP is a heterotrimeric enzyme composed of a 38 kDa type-1 phosphatase catalytic subunit (PP1c δ), a 110–130 kDa myosin-targeting subunit (MYPT1) and a 20 kDa subunit that is not required for activity or regulation [9]. ROK phosphorylates MYPT1 at T697 and/or T855 (rat numbering), leading to inhibition of MLCP activity [15–17]. There is abundant evidence that phosphorylation of T855 catalyzed by ROK occurs in a variety of smooth muscle tissues in response to diverse stimuli; however, the physiological importance of T697 phosphorylation by ROK remains uncertain. Thus, while there are several examples of T697 phosphorylation occurring in some types of smooth muscles [18–30], there are many examples of T855 phosphorylation occurring without T697 phosphorylation in smooth muscle tissues in response to a variety of contractile stimuli [30–39]. MLCP activity can also be inhibited via the $\text{G}_{q/11}$ -protein kinase C (PKC) pathway (for review see [10]) leading to phosphorylation

at T38 of the 17-kDa cytosolic phosphatase inhibitor protein, CPI-17, which, in the phosphorylated state, binds to the catalytic subunit of MLCP, inhibiting the phosphatase activity and resulting in an increase in LC₂₀ phosphorylation and force [40–42].

Several groups have demonstrated that KCl can also increase the Ca²⁺ sensitivity of contraction in some types of smooth muscles via activation of RhoA and ROK [43–49].

In a previous study, we provided evidence for an important role of ROK in action potential-induced phasic contractions of rat ureteric smooth muscle [8]. Specifically, we demonstrated that ROK inhibition significantly decreased the amplitude of electric field stimulation-induced phasic contractions and Ca²⁺ transients, and increased the rate of relaxation and LC₂₀ dephosphorylation. Other studies support a role for ROK in sheep, human and rabbit ureteric contractility [50–52], but not in guinea-pig [8]. Rabbits with unilateral ureteric obstruction exhibited increased expression of both isoforms of ROK (ROKI or ROK β and ROKII or ROK α) as well as enhanced contractile responses to electric field stimulation, KCl, phenylephrine and CCh, all of which were attenuated by the ROK inhibitor Y-27632 [52]. ROK inhibitors, therefore, represent a promising novel therapy for the prevention of renal colic-associated pain and kidney damage, and have the potential to facilitate stone passage.

In the present study, we examined the effects of ROK inhibition on Ca²⁺ signaling, LC₂₀ phosphorylation, MYPT1 phosphorylation (at T697 and T855) and force activated by two stimuli that evoke contractile responses via distinct mechanisms – high [K⁺]-induced depolarization (electromechanical coupling) and the muscarinic agonist carbachol (pharmacomechanical coupling) – in order to better understand the role and mechanism of action of ROK in phasic ureteric smooth muscle.

2. Methods

2.1. Materials

Commercial antibodies were purchased from the following sources: mouse monoclonal anti-LC₂₀ (Sigma) was used at 1:1000 dilution; rabbit polyclonal anti-pT697-MYPT1 (Upstate) raised against a synthetic phosphopeptide corresponding to the region around pT697 of the human protein was used at 1:1000 dilution; rabbit polyclonal anti-pT855-MYPT1 (Upstate) raised against a synthetic phosphopeptide corresponding to the region around pT855 of the human protein was used at 1:1000 dilution; rabbit polyclonal anti-actin (Cytoskeleton) raised against a synthetic peptide corresponding to the C-terminal 11 residues of human actin (SGPSIVHRKCF) was used at 1:500 dilution; rabbit polyclonal anti-caldesmon was raised in-house against full-length chicken gizzard *h*-caldesmon, purified as previously described [53] and used at 1:10,000 dilution.

2.2. Tissue preparation

Rats (~200 g) were humanely killed using CO₂ anaesthesia followed by cervical dislocation, in accordance with UK legislation. The ureters were dissected, carefully cleaned of connective and paraureteric adipose tissue using fine curved scissors, keeping the sharp edges away from the tissue to avoid physical damage of the ureteric bundles.

2.3. Fluo 4 loading

To load the tissue with fluo 4, strips of ureter were placed in a plastic cuvette containing 1 ml of HEPES-buffered Krebs solution containing 15 μ M-fluo 4-AM dissolved in dimethyl sulphoxide premixed with Pluronic F127 (final concentration of 0.01%). Loading was performed at room temperature for 3 h with the cuvettes wrapped in black tape and rotated at 30 rpm. Tissue samples were then removed from the loading medium and placed in normal Krebs solution for at least 30 min to allow cleavage of fluo 4-AM to fluo 4 by intracellular esterases.

2.4. Confocal imaging

We used fast Nipkow disc-based confocal imaging attached to a high sensitivity (iXon Andor) CCD camera, which allowed acquisition of images at 60–200 fps and thereby accurate measurement of temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells and bundles of intact ureteric strips. To measure temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells, a 60 $\times$ water objective (NA 1.2) was used, while low-power dry objectives (4 $\times$, NA 0.13; 20 $\times$, NA 0.7) were used to measure temporal and spatial characteristics of Ca^{2+} signaling in whole ureteric muscle bundles. Data acquisition was performed using Andor iQ software.

For simultaneous recording of Ca^{2+} signaling and force ureters were cut into small segments (3–4 mm in length), clipped at both ends using aluminium foil clips (Laser Services, USA) and attached to a force transducer (WPI) at one end and a stainless steel hook fixed to the bottom of the experimental chamber at the other end. The force transducer was attached to a 3-d manipulator (Narashige, Japan), allowing movement of the strip in the *X–Y–Z* directions to position it in the focal plane of the objective and apply an optimal stretch, which did not exceed 40% of active maximal force induced by high $[\text{K}^+]$ depolarization. For simultaneous force, Ca^{2+} signaling and electrical activity measurements, a modified tissue bath was used, as detailed elsewhere [54,55]. Briefly the bath became a sucrose-gap chamber with a cover slip at its base, to enable the optical measurements to be made.

2.5. Measurement of LC_{20} phosphorylation and force

Tissue homogenization, urea-gel electrophoresis and Western blotting with anti- LC_{20} were carried out as previously described [56]. LC_{20} phosphorylation was quantified by scanning densitometry. Data are expressed as percentage phosphorylated LC_{20} , determined as the ratio of the signal intensity of the phosphorylated LC_{20} band to the sum of the signal intensities of the phosphorylated and unphosphorylated bands. Because it was not technically feasible to rapidly freeze smooth muscles while mounted on the confocal system, LC_{20} phosphorylation and tension were measured in separate strips using another experimental set-up, which allowed quick freezing of ureteric tissues at different times of force development by rapidly submerging the muscle strips in an acetone-dry ice slurry [8]. The moment of freezing was detected on the tension trace, which was used to correlate LC_{20} phosphorylation and force during the development of high $[\text{K}^+]$ - or CCh-induced contractions. Force transients induced by KCl or CCh and recorded on the confocal rig at a fast flow rate, and those induced by quickly submerging in high $[\text{K}^+]$ or CCh solutions using the freezing rig were identical. By superimposing force transients obtained in the two

systems, we could correlate $[Ca^{2+}]_i$, LC₂₀ phosphorylation and force induced by high $[K^+]$ depolarization and CCh (Figs. 4, 7, 9 and 10). It was essential to perform all experiments at the same temperature, which was achieved by working in an air-conditioned experimental room. The temperature of the bath was 22–23 °C. Tissue samples were secured with aluminium foil clips (Laser Services, USA), which allowed easy transfer between recording chambers. We considered the possibility that the temporal characteristics of the Ca^{2+} signal recorded from muscle strips could be affected by two factors: (1) asynchronous activation of muscle bundles in the whole strip due to diffusion of KCl or CCh in the bath and (2) recording of Ca^{2+} images at low frame rate. We have carried out additional sets of experiments to determine the conditions under which the Ca^{2+} signal induced by high $[K^+]$ depolarization or CCh is well synchronized in the whole strip; this was important to avoid inaccuracies in correlating Ca^{2+} signaling with LC₂₀ phosphorylation and force. To define the optimal perfusion rate, we compared the Ca^{2+} transient induced by high $[K^+]$ and CCh measured from the whole preparation (average signal) with that from a small region at different perfusion rate (Supplementary Fig. 1S and Video 1). The threshold of activation of the fast-propagating action potential in ureteric bundles exposed to high $[K^+]$ was reached within 150 ms (Fig. 1A). This resulted in the rapid (2–4 cm/s) spread of an intercellular Ca^{2+} wave causing a homogeneous rise of $[Ca^{2+}]_i$ throughout the strip within 300–500 ms, even at a normal flow rate of the bath solution (2.5 ml/min) (Fig. 1S and Video 1). In contrast, the speed of propagation of the Ca^{2+} transient induced by CCh applied in Ca^{2+} -free solution (Fig. 1S and Video 1) or in the presence of nifedipine was limited by the speed of diffusion of the agonist in the chamber (data not shown). At the standard flow rate (2.5 ml/min), CCh diffused in the bath at a speed of ~300 μ m/s, so that the regional changes of the Ca^{2+} transient were significantly higher than the average signal (Fig. 1S and Video 1). In an attempt to activate the tissue more rapidly, we increased the flow rate to 25 ml/min, which allowed rapid exchange of the bath solution and achievement of a synchronous rise in $[Ca^{2+}]_i$ even during CCh stimulation in Ca^{2+} -free solution (Figs. 1, 3, 4, 8, 9 and 10). By acquiring images at 33–126 fps, we found that recording of Ca^{2+} images at 33 fps was sufficient to accurately measure the temporal and spatial characteristics of Ca^{2+} transients induced by KCl or CCh with good signal-to-noise ratio.

2.6. SDS-PAGE and Western blotting

SDS-PAGE was carried out in 1.5-mm thick mini-gels (12.5% acrylamide in the running gel with a 5% acrylamide stacking gel) at 200 V for 45 min in a Mini Protean 3 Cell (Bio-Rad). Following electrophoresis, gels were equilibrated at room temperature for 1 h with Transblot Buffer (25 mM Tris-HCl, pH 7.5, 192 mM glycine, 20% methanol, 0.1% SDS for MYPT1 and caldesmon, or 25 mM Tris-HCl, pH 7.5, 192 mM glycine, 20% methanol for actin) prior to Western blotting. Proteins separated by SDS-PAGE were transblotted to nitrocellulose (0.2 μ m) at 100 V for 1.5 h (for MYPT1 and caldesmon) or 1 h (for actin) at 4 °C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Nitrocellulose membranes were washed (3 $\times$ 5 min) in phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and proteins fixed in 0.5% glutaraldehyde in PBS for 15 min. After washing (3 $\times$ 5 min) in Tris-buffered saline containing Tween-20 (TBST: 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 3 mM KCl, 0.05% Tween-20), membranes were blocked with 5% non-fat dried milk in TBST for 1 h at room temperature,

and incubated with shaking in 1% milk in TBST containing the appropriate primary antibody overnight at 4 °C. Membranes were washed (4× 5 min) in TBST, incubated with anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution in 1% milk in TBST; Chemicon) for 1 h and washed (4× 5 min) with TBST and (1× 5 min) with TBS before chemiluminescence signal detection using the Super-Signal West Femto reagent (Pierce). The emitted light was detected and quantified with a chemiluminescence imaging analyzer (LAS3000mini; Fujifilm) and images were analyzed with Multi Gauge v3.0 software (Fujifilm). Molecular weight markers were purchased from Fermentas. Samples of tissue extracts in SDS-gel sample buffer (40 µl each) were subjected to SDS-PAGE and Western blotting with antibodies to pT697-MYPT1, pT855-MYPT1, caldesmon or actin. pMYPT1 signals were normalized to caldesmon and actin. Representative Western blots are shown in the appropriate figures with cumulative data presented in the form of histograms. Values represent the mean ± SEM.

2.7. Solutions

The standard physiological solution was a HEPES-buffered modified Krebs solution containing (in mM): NaCl, 120.4; KCl, 5.9; CaCl₂, 2; MgSO₄, 1.2; HEPES, 11.6; and glucose, 11; pH 7.4 adjusted with NaOH. Solutions with increased [K⁺] were obtained by replacing Na⁺ by equimolar K⁺. The Ca²⁺-free solutions contained 2 mM EGTA.

2.8. Statistics

Values are given as means ± SEM and *n* is the number of animals and *N* the number of tissue preparations. Differences were considered significant for *P* < 0.05 using the appropriate Student's *t* test or ANOVA.

3. Results

3.1. Effects of high [K⁺]-induced depolarization and carbachol on electrical activity, Ca²⁺ signaling and force

In order to correlate electrical activity and force with the spatio-temporal characteristics of the Ca²⁺ transient induced by high [K⁺] or CCh, combined confocal imaging and the double sucrose gap method were used [57]. Two protocols were used in this series of experiments. In the first protocol, high [K⁺] (120 mM) or CCh (100 µM) was applied locally from a 100-µm pipette delivery tip for 1 s. The diffusion of K⁺ and CCh through the preparation was limited by rapid washout and therefore effects were limited to the part of the ureteric wall located in close proximity to the delivery tip. These experimental conditions enabled study of the effects of local application of both high [K⁺] and CCh on electrical activity, Ca²⁺ signaling and force in intact ureteric strips. In region 1 (close to the point of application), smooth muscles cells were directly exposed to high [K⁺] or CCh, while in region 2 (distal to the point of application), only the Ca²⁺ transient controlled by propagating action potentials could be recorded (Videos 2 and 3). Brief local application of high [K⁺] in the presence of extracellular Ca²⁺ induced a gradual rise in [Ca²⁺]_i in the muscle bundles in region 1, associated with gradual membrane depolarization, which, upon reaching a threshold, fired a rapidly propagating plateau-type action potential that triggered a fast-propagating intercellular Ca²⁺ transient that was detected in both regions 1 and 2. This led to the

generation of a brief phasic contraction (Fig. 1Aii and iii, C panel 1 and Video 2). When ureteric strips were pre-incubated in Ca^{2+} -free solution containing 2 mM EGTA for 2 min, the action potential, Ca^{2+} transient and force induced by brief application of high $[\text{K}^+]$ were completely abolished (Fig. 1Aiv and C panel 3).

A 1-s application of CCh through the same delivery tip induced a large Ca^{2+} transient in region 1 associated with slow depolarization and force development, which, upon reaching a threshold, also triggered an action potential, causing a fast-propagating Ca^{2+} transient, detected in both regions 1 and 2, with similar spatio-temporal characteristics to that induced by high $[\text{K}^+]$ (Fig. 1Bii and iii, C panel 2 and Video 2). In region 1, an action potential-mediated global Ca^{2+} transient was superimposed on a local Ca^{2+} transient induced by direct action of CCh on the smooth muscle cells. When ureteric strips were pre-incubated in Ca^{2+} -free solution containing 2 mM EGTA for 2 min, a Ca^{2+} transient was recorded only in region 1 where smooth muscle cells were exposed directly to CCh, while the action potential, Ca^{2+} transient in region 2 and phasic contraction were completely abolished (Fig. 1Biv, C panel 4 and Video 3).

In the second protocol, depolarization of the cell membrane induced by exposure to high $[\text{K}^+]$ for a longer time (1 min) triggered one or two action potentials at the onset of depolarization when the threshold was reached (Fig. 2, top trace), which quickly propagated through the tissue (Videos 1 and 2), resulting in generation of a rapid rise in $[\text{Ca}^{2+}]_i$ (Fig. 2, middle trace) associated with development of the phasic component of the high $[\text{K}^+]$ -induced contracture (Fig. 2, bottom trace). High $[\text{K}^+]$ -induced depolarization caused rapid inactivation of the action potentials, resulting in a decrease in the amplitude of the phasic component of the Ca^{2+} transient and force (Fig. 2). As depolarization progressed, it caused Ca^{2+} influx, seen as the sustained component of the Ca^{2+} transient (Fig. 2, middle trace). This biphasic Ca^{2+} transient caused a contractile response following an approximately 300 ms delay, which also consisted of a phasic followed by a tonic component (Fig. 2, bottom trace).

In contrast, prolonged application of CCh (10 μM) in the presence of extracellular Ca^{2+} induced a small sustained depolarization, which resulted in activation of several action potentials (Fig. 2, top trace). The Ca^{2+} transient induced by CCh was complex and consisted of a slow component, which was not associated with action potentials, on which were superimposed Ca^{2+} oscillations associated with action potentials. This complex Ca^{2+} signaling resulted in a strong CCh-induced contraction, on which were superimposed mechanical oscillations associated with the action potentials (Fig. 2, bottom trace).

3.2. Effects of CCh following high $[\text{K}^+]$ -induced depolarization

In the next series of experiments, we studied the effects of CCh on ureteric strips depolarized by high $[\text{K}^+]$ to determine if CCh could produce additional effects on $[\text{Ca}^{2+}]_i$ and force. Tissue samples were perfused at a faster flow rate (25 ml/min compared to 2.5 ml/min in the previous experiments) to achieve a synchronous rise in $[\text{Ca}^{2+}]_i$ in all muscle bundles, and CCh was used at 100 μM to produce maximal stimulation (as in Fig. 1). Fig. 3A shows that when CCh was applied in Ca^{2+} -free solution, following incubation of the ureteric strips in Ca^{2+} -free solution for 1 min, it produced a brief Ca^{2+} transient associated

with a short phasic contraction, the amplitude of which was comparable to that of the phasic contraction induced by high $[K^+]$ depolarization. Increasing the pre-incubation period in Ca^{2+} -free solution to 5 min resulted in a significant decrease in the amplitude of both the Ca^{2+} transient (30–40% of peak KCl) and force (25–40% of peak KCl), suggesting that the intracellular stores quickly lost their Ca^{2+} content due to passive Ca^{2+} leak and extrusion of Ca^{2+} from the cell ($n = 5$, data not shown). Application of CCh at a time when $[Ca^{2+}]_i$ and force induced by high $[K^+]$ depolarization were at steady state produced a transient Ca^{2+} spike similar to that seen in Ca^{2+} -free solution, which quickly returned to the level of the high $[K^+]$ -induced sustained component (Fig. 3A). Unlike the Ca^{2+} transient, which was greater than that induced by high $[K^+]$ depolarization, force increased only to the level observed in response to high $[K^+]$ and then declined to a steady-state level greater than the sustained force in response to high $[K^+]$ alone (Fig. 3A). The amplitude of tonic contraction induced by high $[K^+]$ depolarization (measured at 40 s in the absence of CCh) was $78.9 \pm 5.5\%$ and in the presence of CCh $96.3\% \pm 1.5\%$ of the peak phasic contraction induced by high $[K^+]$ ($n = 7$, $N = 12$); these values are statistically significantly different. The half-time of relaxation of the Ca^{2+} transient in the absence and presence of CCh was 22.3 ± 0.4 and 23.5 ± 0.9 s, respectively ($n = 5$, $N = 9$), i.e. there was no significant effect of CCh on the rate of decline of the Ca^{2+} signal. In contrast to the Ca^{2+} transient, the rate of relaxation of force in the presence of CCh was significantly slowed (Fig. 3A). The half-time of relaxation of the tonic contraction induced by removal of external Ca^{2+} and repolarization of the plasma membrane was 25.3 ± 1.5 and 48.0 ± 2.3 s in the absence and presence of CCh, respectively ($n = 5$, $N = 11$). This suggests that there was a small but statistically significant Ca^{2+} -independent effect of CCh on the amplitude of the tonic contraction induced by sustained high $[K^+]$ depolarization and on the rate of relaxation of the tonic contraction induced by removal of external Ca^{2+} and repolarization of the plasma membrane (Fig. 3A).

These results are interpreted as follows. High $[K^+]$ elicits rapid action potentials, which trigger entry of Ca^{2+} via voltage-gated Ca^{2+} channels and contraction. High $[K^+]$ also induces a slow depolarization of the cell membrane, which maintains Ca^{2+} entry and steady-state force development (Figs. 2 and 3A). CCh triggers the rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR) that causes the initial spike of $[Ca^{2+}]_i$ and contraction, followed by a series of action potentials that give rise to Ca^{2+} oscillations that correlate with oscillations in force (Fig. 2). The initial Ca^{2+} spike and transient contraction are retained in the absence of extracellular Ca^{2+} (Fig. 3A), supporting the conclusion that they are due to Ca^{2+} release from the SR. Finally, the observation that steady-state force in the presence of CCh and high $[K^+]$ is greater than that in the presence of high $[K^+]$ alone, and that relaxation induced by removal of external Ca^{2+} in the presence of CCh proceeds much more slowly than in the absence of CCh (Fig. 3A), although $[Ca^{2+}]_i$ declines at the same rate, indicates that CCh also induces Ca^{2+} sensitization.

There is a significant delay between the rise in $[Ca^{2+}]_i$ and the development of force in response to high $[K^+]$ in the presence of extracellular Ca^{2+} (Fig. 3B) or CCh in the absence of extracellular Ca^{2+} (Fig. 3C). This delay is also evident in Fig. 3D and Video 4, which show that a marked increase in $[Ca^{2+}]_i$ in response to a brief (1 s) application of high $[K^+]$ occurs much earlier than muscle shortening. Fig. 3E shows that $[Ca^{2+}]_i$ is close to its peak

value before shortening is observed, and $[Ca^{2+}]_i$ has declined to ~25% of its peak value by the time force reaches its peak.

3.3. Temporal relationship between $[Ca^{2+}]_i$, LC₂₀ phosphorylation and force induced by high $[K^+]$ depolarization or carbachol

Fig. 4A demonstrates a close correlation between $[Ca^{2+}]_i$, LC₂₀ phosphorylation and force in response to high $[K^+]$. The initial Ca^{2+} spike induced a rapid increase in LC₂₀ phosphorylation that peaked at ~70–75% pLC₂₀ (0.70–0.75 mol P/mol LC₂₀) within 1 s and corresponded to 80–90% of the peak of the Ca^{2+} transient (Fig. 4B). The Ca^{2+} transient peaked within 2–2.5 s while force lagged significantly behind both $[Ca^{2+}]_i$ and LC₂₀ phosphorylation, reaching its peak level within 5–7 s. After reaching its peak level, the Ca^{2+} transient declined to a new steady-state level, which was $67.9 \pm 7.1\%$ of the peak value (Fig. 4A). This significant decline in $[Ca^{2+}]_i$ produced a fall in LC₂₀ phosphorylation and force. Finally, removal of the depolarizing stimulus resulted in a rapid return of $[Ca^{2+}]_i$ to baseline, but a slower decline in LC₂₀ phosphorylation and force.

The Ca^{2+} spike induced by CCh in Ca^{2+} -free solution also triggered an increase in LC₂₀ phosphorylation to ~0.6–0.7 mol P/mol LC₂₀ (Fig. 4C and D). After reaching its peak, $[Ca^{2+}]_i$ rapidly declined to its resting level with a half-time of 3.8 ± 0.8 s. $[Ca^{2+}]_i$ returned to baseline in the continued presence of CCh in Ca^{2+} -free solution, presumably due to depletion of the SR Ca^{2+} store and leakage of Ca^{2+} from the cytosol to the extracellular space. This rapid decline in $[Ca^{2+}]_i$ correlated with a much slower rate of dephosphorylation of LC₂₀ (Fig. 4C). The half-time of dephosphorylation of LC₂₀ was 11.4 ± 1.2 s, i.e. ~3 times higher than that of relaxation of the Ca^{2+} transient. During the rising phase, force induced by CCh significantly lagged behind both the Ca^{2+} transient and LC₂₀ phosphorylation (Fig. 4C) and peaked at a time when $[Ca^{2+}]_i$ had decreased to ~50% and LC₂₀ phosphorylation to ~70% of their peak levels, respectively. After reaching its peak, force spontaneously relaxed to the baseline and the relaxation correlated well with the slow rate of dephosphorylation of LC₂₀ (Fig. 4B). The half-time of relaxation of force was 14.8 ± 2.1 s, which did not differ significantly from that of LC₂₀ dephosphorylation.

3.4. Effects of ROK inhibition

Our observation that CCh induced Ca^{2+} sensitization of contraction in rat ureteric strips prompted us to investigate the underlying mechanism. We therefore addressed the hypothesis that CCh-induced Ca^{2+} sensitization involves activation of ROK and phosphorylation of MYPT1. It has been demonstrated in other smooth muscle tissues that K^+ -induced membrane depolarization also induces ROK activation [44,47,49] and therefore we investigated this possibility in ureteric smooth muscle. Firstly, we demonstrated that both ROK isoforms, α and β , are expressed in rat ureteric smooth muscle (Fig. 5).

The involvement of ROK in K^+ - and CCh-induced contraction of the rat ureter was investigated using the selective ROK inhibitor, H-1152 [58]. Pre-incubation with H-1152 had two effects on the high $[K^+]$ response: (i) the amplitude of the plateau component of the action potential was reduced (Fig. 6B), which attenuated the phasic component of the Ca^{2+} transient and force (Fig. 6A) and (ii) the tonic component of the contractile response was

markedly inhibited, while steady-state $[Ca^{2+}]_i$ was decreased by only $18.0 \pm 0.5\%$ (Fig. 6A) with no change in the time course of Em changes (Fig. 6B).

Fig. 7A shows that pre-treatment with H-1152, despite some decrease in the amplitude of the phasic component of the Ca^{2+} transient caused by partial inhibition of the action potential, had no significant effect on the rapid increase in LC₂₀ phosphorylation, although the contractile response was markedly decreased. The sustained component of both LC₂₀ phosphorylation and force was abolished. Western blotting with phosphospecific antibodies revealed a basal level of phosphorylation of MYPT1 at both T697 and T855 in the unstimulated tissue (Fig. 7B and C). High $[K^+]$ -induced depolarization increased MYPT1 phosphorylation at T855, but not at T697, during steady-state contraction. H-1152 prevented this increase and reduced the basal level of phosphorylation at both sites (Fig. 7B and C).

Similar effects of pre-incubation with H-1152 were observed when CCh was used to elicit a contractile response in the presence of extracellular Ca^{2+} . Thus, H-1152: (i) reduced the duration of the plateau component of the CCh-induced action potential (Fig. 8B), resulting in a decrease in the amplitude of the phasic component of the Ca^{2+} transient and force (Fig. 8A) and (ii) abolished the tonic component of the CCh-induced contraction without affecting steady-state Em or $[Ca^{2+}]_i$ (Fig. 8A). The ROK inhibitor Y-27632 had similar effects on the CCh-induced action potential and Ca^{2+} transient [8]. Pre-treatment with H-1152 had no effect on the Ca^{2+} transient induced by CCh in Ca^{2+} -free solution (Fig. 9A, top panel), nor did it affect the rapid increase in LC₂₀ phosphorylation (Fig. 9A, middle panel) or the initial increase in force (Fig. 9A, bottom panel). However, LC₂₀ dephosphorylation and relaxation were more rapid in the presence of H-1152 (Fig. 9A and C), and force peaked earlier and was reduced in size.

The L-type Ca^{2+} channel blocker nifedipine (1–10 μ M) completely blocked action potentials, Ca^{2+} transients and force induced by high $[K^+]$ (data not shown). CCh, in the presence of nifedipine, elicited a rapid rise in $[Ca^{2+}]_i$ (Fig. 10A, top panel), LC₂₀ phosphorylation (middle panel) and force (bottom panel). $[Ca^{2+}]_i$ then declined to a steady-state level that preceded a decrease in LC₂₀ phosphorylation and force, which also reached steady-state levels. Steady-state levels of $[Ca^{2+}]_i$, LC₂₀ phosphorylation and force induced by CCh in the presence of nifedipine were two to three times smaller than those induced by high- $[K^+]$ depolarization (compare Figs. 10 and 7). Pre-treatment with H-1152 had no effect on the Ca^{2+} transient or the phasic component of LC₂₀ phosphorylation, but it reduced the phasic component of the contractile response, and abolished the tonic component of both LC₂₀ phosphorylation and force. MYPT1 phosphorylation at T855, but not T697, increased during the sustained phase of the contractile response (Fig. 10C). This increase was blocked by H-1152, which actually decreased the resting level of MYPT1 phosphorylation at both sites.

4. Discussion

In a previous study [8], we showed that ROK plays a key role in control of electromechanical coupling in phasic rat ureteric smooth muscle via both Ca^{2+} -dependent and Ca^{2+} -independent mechanisms involving modulation of Ca^{2+} influx through L-type

voltage-gated Ca^{2+} channels and modulation of MLCP activity, respectively. However, the mechanism of Ca^{2+} sensitization was not investigated. In the present study, we examined the effects of the ROK inhibitor H-1152 on the electrical activity, Ca^{2+} signaling, LC_{20} and MYPT1 phosphorylation, and force activated by high $[\text{K}^+]$ -induced depolarization or carbachol in order to study the role of ROK in electro- and pharmacomechanical coupling in phasic ureteric smooth muscle. Firstly, however, the responses of the tissue to brief and prolonged application of KCl or CCh were characterized in detail.

A brief (1 s) localized application of KCl (120 mM) in the presence of extracellular Ca^{2+} induced gradual membrane depolarization and increased $[\text{Ca}^{2+}]_i$ in proximity to the stimulus. Firing of a single action potential occurred once a threshold was reached, and this evoked a Ca^{2+} wave, which triggered a transient contraction (Fig. 1A and C panel 1). In the absence of extracellular Ca^{2+} , high $[\text{K}^+]$ failed to elicit any increase in $[\text{Ca}^{2+}]_i$ (Fig. 1A and C panel 3). Similar application of CCh (100 μM) in the presence of extracellular Ca^{2+} initially elicited a large increase in $[\text{Ca}^{2+}]_i$ in proximity to the stimulus, which was not associated with membrane depolarization, and subsequently evoked an action potential that was associated with a Ca^{2+} wave that triggered a transient contraction (Fig. 1B and C panel 2). In the absence of extracellular Ca^{2+} , CCh evoked a rapid, localized Ca^{2+} transient that failed to elicit a contractile response (Fig. 1B and C panel 4). This Ca^{2+} transient corresponded to the initial Ca^{2+} transient induced by CCh in the presence of extracellular Ca^{2+} , and can be ascribed to the localized release of Ca^{2+} from the SR.

Prolonged depolarization induced by exposure to KCl (120 mM) for 1 min evoked several action potentials, which rapidly inactivated as depolarization occurred (Fig. 2). These action potentials gave rise to a very rapid increase in $[\text{Ca}^{2+}]_i$ (seen as fast-propagating intercellular Ca^{2+} waves), which peaked within 500 ms. A 300 ms delay occurred between the onset of the Ca^{2+} transient and the initiation of force generation, similar to previous reports on different smooth muscles [5,13,59–62]. This contraction was preceded by a rapid increase in LC_{20} phosphorylation, which reached its maximal level at a time when the Ca^{2+} transient was ~80–90% of its peak level (Fig. 4B). It should be noted that we were not able to measure LC_{20} phosphorylation during the 300 ms latency period as no force was generated at this time, making it impossible to define precisely the point of freezing. The Ca^{2+} transient induced by CCh in Ca^{2+} -free solution did not differ significantly in amplitude from that induced by high $[\text{K}^+]$ depolarization (Fig. 3A), and the temporal relationship between the Ca^{2+} transient and LC_{20} phosphorylation during the rising phase of the Ca^{2+} transient was similar to that of the rising phase of the high $[\text{K}^+]$ -induced response (Fig. 4D). These data emphasize two important points: (1) there is a rapid rise in LC_{20} phosphorylation, which reaches its maximal level (0.70–0.75 mol P/mol LC_{20}) at approximately the same $[\text{Ca}^{2+}]_i$ in response to either the high $[\text{K}^+]$ -induced Ca^{2+} transient caused by Ca^{2+} influx or CCh-induced Ca^{2+} release from the SR and (2) there is a significant delay between LC_{20} phosphorylation and force development in response to both stimuli. We conclude that the maximal level of LC_{20} phosphorylation occurs at submaximal $[\text{Ca}^{2+}]_i$. Evidence has been presented that a specific “contractile” pool of CaM is tightly bound to the contractile machinery [63]. Once this pool has been saturated with Ca^{2+} , leading to maximal activation of MLCK, a further increase in $[\text{Ca}^{2+}]_i$ would have no further effect on MLCK activity and, therefore, LC_{20} phosphorylation. In fact, a further increase in $[\text{Ca}^{2+}]_i$ could result in

decreased MLCK activity through phosphorylation of the kinase by Ca^{2+} /CaM-dependent kinase II, which reduces the affinity of MLCK for Ca^{2+} /CaM [64,65].

The fact that force lags significantly behind LC_{20} phosphorylation during the rising phase of both high $[\text{K}^+]$ - and CCh-induced Ca^{2+} transients, suggests that the rate-limiting step in force generation is not LC_{20} phosphorylation but lies downstream, perhaps at the level of cross-bridges. Numerous studies have shown that the dependence of force on LC_{20} phosphorylation is steep and curvilinear and that the increase in force is normally preceded by a rapid increase in $[\text{Ca}^{2+}]_i$ and LC_{20} phosphorylation [5,13,59,60,62]. Interestingly, in tonic and phasic smooth muscles, the rate of LC_{20} phosphorylation has even been found to be greater than the rate of increase in $[\text{Ca}^{2+}]_i$ and to reach its peak value well before the Ca^{2+} transient and force [66].

Inhibition of ROK by H-1152 shortened the duration of the action potential induced by prolonged treatment with high $[\text{K}^+]$ (Fig. 6B). This reduced the amplitude of the phasic component of the Ca^{2+} transient and the force response (Fig. 6A), but the mechanism is unknown. Furthermore, the peak of LC_{20} phosphorylation was unaffected by H-1152 (Fig. 7A), again consistent with maximal activation of MLCK at submaximal $[\text{Ca}^{2+}]_i$ and delayed activation of ROK (~5.5 s), which was suggested to be caused by rate-limiting steps including the dissociation of RhoA-GTP from GDI, translocation of RhoA-GTP to the membrane, activation of ROK, and inhibition of MLCP [10]. The most dramatic effect of ROK inhibition was observed on the tonic phase of the response to high $[\text{K}^+]$: LC_{20} was completely dephosphorylated and the muscle relaxed with a modest ($18.0 \pm 0.5\%$) decrease in $[\text{Ca}^{2+}]_i$ (Figs. 6A and 7A).

It is becoming increasingly clear that force maintenance in various smooth muscle tissues involves Ca^{2+} sensitization via ROK-catalyzed phosphorylation of MYPT1 and consequent inhibition of MLCP activity [10,67]. We found that MYPT1 is partially phosphorylated at both T697 and T855 (the ROK sites) in resting ureteric smooth muscle (Fig. 7B and C), suggesting partial inhibition of MLCP activity by constitutively active ROK under these conditions. High $[\text{K}^+]$ increased MYPT1 phosphorylation at T855, but not at T697, which would further reduce MLCP activity and thereby explain K^+ -induced Ca^{2+} sensitization. ROK inhibition prevented the K^+ -induced increase in MYPT1 phosphorylation at T855 and, in fact, reduced the basal level of phosphorylation at both ROK sites. This would result in an increase in MLCP activity, which would account for the rapid dephosphorylation of LC_{20} and relaxation of the muscle in the presence of H-1152 and high $[\text{K}^+]$ (Fig. 7A). We can also conclude from these experiments that the basal levels of MYPT1 phosphorylation at T855 and T697 are due, in part, to ROK that is partially active under these conditions.

ROK inhibition had no effect on the Ca^{2+} transient elicited by CCh in the absence of extracellular Ca^{2+} (Fig. 9A). Inhibition of ROK also had no effect on the initial phases of LC_{20} phosphorylation and force development in response to CCh stimulation, suggesting that, during the rising phase of the Ca^{2+} transient, MLCK is maximally activated by Ca^{2+} /CaM, and MLCP inhibition does not contribute to this initial phase of LC_{20} phosphorylation and force. If Ca^{2+} entry through voltage-gated L-type Ca^{2+} channels was prevented by pre-treatment with nifedipine, an initial rapid increase in $[\text{Ca}^{2+}]_i$ in response to CCh was

followed by a sustained elevation of $[Ca^{2+}]_i$ above resting levels (Fig. 10A). $[Ca^{2+}]_i$ returned to resting levels upon removal of the stimulus. The phasic rise in $[Ca^{2+}]_i$ was accompanied by a rapid increase in LC₂₀ phosphorylation, followed by force. As $[Ca^{2+}]_i$ declined to a steady-state level in the continued presence of CCh, LC₂₀ phosphorylation and force also declined to steady-state levels. However, when $[Ca^{2+}]_i$ is at steady state, or declines, inhibition of ROK has a dramatic effect on LC₂₀ phosphorylation and force, i.e. there is a significant contribution of MLCP inhibition to LC₂₀ phosphorylation and force when $[Ca^{2+}]_i$ reaches steady state or begins to decline. Inhibition of ROK has a dramatic effect on the balance between MLCK and MLCP activities during this period so that an increase in MLCP activity, caused by dephosphorylation of MYPT1 at T697 and T855, results in complete dephosphorylation of LC₂₀ and relaxation of tonic force induced by CCh with no change in $[Ca^{2+}]_i$.

In summary, these findings shed significant new light on the importance of ROK in regulation of ureteric smooth muscle contraction by both electromechanical and pharmacomechanical coupling mechanisms, and support the exploitation of ROK inhibitors for the treatment of ureteric stones via their ability to induce relaxation of ureteric smooth muscle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from The British Heart Foundation, The Medical Research Council, The Physiological Society (to TB) and The Canadian Institutes of Health Research (to MPW). MPW is an Alberta Heritage Foundation for Medical Research Scientist and recipient of a Canada Research Chair (Tier 1) in Vascular Smooth Muscle Research.

References

1. Santicioli P, Maggi CA. Myogenic and neurogenic factors in the control of pyeloureteral motility and ureteral peristalsis. *Pharmacol Rev.* 1998; 50:683–721. [PubMed: 9860807]
2. Canda AE, Turna B, Cinar GM, Nazli O. Physiology and pharmacology of the human ureter: basis for current and future treatments. *Urol Int.* 2007; 78:289–298. [PubMed: 17495484]
3. Pak CYC. Kidney stones. *Lancet.* 1998; 51:1797–1801.
4. Porpiglia F, Destefanis P, Fiori C, Fontana D. Effectiveness of nifedipine and deflazacort in the management of distal ureteral stones. *Urology.* 2000; 56:579–582. [PubMed: 11018608]
5. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature.* 1994; 372:231–236. [PubMed: 7969467]
6. Allen BG, Walsh MP. The biochemical basis of the regulation of smooth-muscle contraction. *Trends Biochem Sci.* 1994; 19:362–368. [PubMed: 7985229]
7. Burdya TV, Wray S. The relationship between the action potential, intracellular calcium and force in intact phasic, guinea-pig ureteric smooth muscle. *J Physiol.* 1999; 520:867–883. [PubMed: 10545150]
8. Shabir S, Borisova L, Wray S, Burdya T. Rho-kinase inhibition and electromechanical coupling in rat and guinea-pig ureter smooth muscle: Ca^{2+} -dependent and -independent mechanisms. *J Physiol.* 2004; 560:839–855. [PubMed: 15331677]
9. Hartshorne DJ, Ito M, Erdodi F. Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J Biol Chem.* 2004; 279:37211–37214. [PubMed: 15136561]

10. Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and non-muscle myosin. II: Modulated by G proteins, kinases and myosin phosphatase. *Physiol Rev.* 2003; 83:1325–1358. [PubMed: 14506307]
11. Somlyo AP, Somlyo AV. Signal transduction through the RhoA/Rho-kinase pathway in smooth muscle. *J Muscle Res Cell Motil.* 2004; 25:613–615. [PubMed: 15750846]
12. Bradley AB, Morgan KG. Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. *J Physiol.* 1987; 385:437–448. [PubMed: 3477639]
13. Himpens B, Matthijs G, Somlyo AV, Butler TM, Somlyo AP. Cytoplasmic free calcium, myosin light chain phosphorylation, and force in phasic and tonic smooth muscle. *J Gen Physiol.* 1988; 92:713–729. [PubMed: 3216188]
14. Himpens B, Casteels R. Different effects of depolarization and muscarinic stimulation on the Ca^{2+} /force relationship during the contraction-relaxation cycle in the guinea pig ileum. *Pflüg Arch.* 1990; 416:28–35.
15. Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, Nakano T. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem.* 1999; 274:37385–37390. [PubMed: 10601309]
16. Velasco G, Armstrong C, Morrice N, Frame S, Cohen P. Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1 M at Thr850 induces its dissociation from myosin. *FEBS Lett.* 2002; 527:101–104. [PubMed: 12220642]
17. Murányi A, Derkach D, Erdodi F, Kiss E, Ito M, Hartshorne DJ. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett.* 2002; 579:6611–6615.
18. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guérin P, Sagan C, Pacaud P, Loirand G. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol.* 2005; 146:1010–1018. [PubMed: 16205723]
19. Wardle RL, Gu M, Ishida Y, Paul RJ. Ca^{2+} -desensitizing hypoxic vasorelaxation: pivotal role for the myosin binding subunit of myosin phosphatase (MYPT1) in porcine coronary artery. *J Physiol.* 2006; 572:259–267. [PubMed: 16439434]
20. Azam MA, Yoshioka K, Ohkura S, Takuwa N, Sugimoto N, Sato K, Takuwa Y. Ca^{2+} -independent, inhibitory effects of cyclic adenosine 5'-monophosphate on Ca^{2+} regulation of phosphoinositide 3-kinase C2alpha, Rho, and myosin phosphatase in vascular smooth muscle. *J Pharmacol Exp Ther.* 2007; 320:907–916. [PubMed: 17110524]
21. Kume H, Takeda N, Oguma T, Ito S, Kondo M, Ito Y, Shimokata K. Sphingosine 1-phosphate causes airway hyper-reactivity by Rho-mediated myosin phosphatase inactivation. *J Pharmacol Exp Ther.* 2007; 320:766–773. [PubMed: 17105828]
22. Gao Y, Portugal AD, Liu J, Negash S, Zhou W, Tian J, Xiang R, Longo LD, Raj JU. Preservation of cGMP-induced relaxation of pulmonary veins of fetal lambs exposed to chronic high altitude hypoxia: role of PKG and Rho kinase. *Am J Physiol.* 2008; 295:L889–L896.
23. Mizuno Y, Isotani E, Huang J, Ding H, Stull JT, Kamm KE. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. *Am J Physiol.* 2008; 295:C358–C364.
24. Choi SK, Ahn DS, Lee YH. Comparison of contractile mechanisms of sphingosylphosphorylcholine and sphingosine-1-phosphate in rabbit coronary artery. *Cardiovasc Res.* 2009; 82:324–332. [PubMed: 19218288]
25. Ding HL, Ryder JW, Stull JT, Kamm KE. Signaling processes for initiating smooth muscle contraction upon neural stimulation. *J Biol Chem.* 2009; 284:15541–15548. [PubMed: 19349274]
26. Kitazawa T, Semba S, Huh YH, Kitazawa K, Eto M. Nitric oxide-induced biphasic mechanism of vascular relaxation via dephosphorylation of CPI-17 and MYPT1. *J Physiol.* 2009; 587:3587–3603. [PubMed: 19470783]
27. Lee JH, Palaia T, Ragolia L. Impaired insulin-mediated vasorelaxation in diabetic Goto-Kakizaki rats is caused by impaired Akt phosphorylation. *Am J Physiol.* 2009; 296:C327–C338.
28. Nepl RL, Lubomirov LT, Momotani K, Pfitzer G, Eto M, Somlyo AV. Thromboxane A2-induced bi-directional regulation of cerebral arterial tone. *J Biol Chem.* 2009; 284:6348–6360. [PubMed: 19095646]

29. Qi F, Ogawa K, Tokinaga Y, Uematsu N, Minonishi T, Hatano Y. Volatile anesthetics inhibit angiotensin II-induced vascular contraction by modulating myosin light chain phosphatase inhibiting protein, CPI-17 and regulatory subunit MYPT1 phosphorylation. *Anesth Analg*. 2009; 109:412–417. [PubMed: 19608811]
30. El-Yazbi AF, Johnson RP, Walsh EJ, Takeya Y, Walsh MP, Cole WC. Pressure-dependent contribution of Rho kinase-mediated calcium sensitization in serotonin-evoked vasoconstriction of rat cerebral arteries. *J Physiol*. 2010; 588:1747–1762. [PubMed: 20351047]
31. Kitazawa T, Eto M, Woodsome TP, Khalequzzaman M. Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca^{2+} sensitization in rabbit smooth muscle. *J Physiol*. 2003; 546:879–889. [PubMed: 12563012]
32. Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. Thromboxane A₂-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J*. 2005; 389:763–774. [PubMed: 15823093]
33. Tsai MH, Jiang MJ. Rho-kinase-mediated regulation of receptor-agonist-stimulated smooth muscle contraction. *Pflüg Arch*. 2006; 453:223–232.
34. Jeon SB, Kim G, Kim JI, Seok YM, Kim SH, Suk K, Shin HM, Lee YH, Kim IK. Flavone inhibits vascular contraction by decreasing phosphorylation of the myosin phosphatase target subunit. *Clin Exp Pharmacol Physiol*. 2007; 34:1116–1120. [PubMed: 17880363]
35. Sun Q, Yue P, Ying Z, Cardounel AJ, Brook RD, Devlin R, Hwang JS, Zweier JL, Chen LC, Rajagopalan S. Air pollution exposure potentiates hypertension through reactive oxygen species-mediated activation of Rho/ROCK. *Arterioscler Thromb Vasc Biol*. 2008; 28:1760–1766. [PubMed: 18599801]
36. Freitas MR, Eto M, Kirkbride JA, Schott C, Sassard J, Stoclet JC. Y27632, a Rho-activated kinase inhibitor, normalizes dysregulation in α 1-adrenergic receptor-induced contraction of Lyon hypertensive rat artery smooth muscle. *Fundam Clin Pharmacol*. 2009; 23:169–178. [PubMed: 19298234]
37. Johnson RP, El-Yazbi AF, Takeya K, Walsh EJ, Walsh MP, Cole WC. Ca^{2+} sensitization via phosphorylation of myosin targeting subunit at threonine-855 by Rho kinase contributes to the arterial myogenic response. *J Physiol*. 2009; 587:2537–2553. [PubMed: 19359365]
38. Seok YM, Azam MA, Okamoto Y, Sato A, Yoshioka K, Maeda M, Kim I, Takuwa Y. Enhanced Ca^{2+} -dependent activation of phosphoinositide 3-kinase class II α isoform-Rho axis in blood vessels of spontaneously hypertensive rats. *Hypertension*. 2010; 56:934–941. [PubMed: 20921425]
39. Wang T, Kendig DM, Smollock EM, Moreland RS. Carbachol-induced rabbit bladder smooth muscle contraction: roles of protein kinase C and Rho kinase. *Am J Physiol*. 2010; 297:F1534–F1542.
40. Hayashi Y, Semba S, Yazawa M, Brautigan DL, Eto M. Defining the structural determinants and a mechanism for inhibition of myosin light chain phosphatase by protein kinase C-potentiated inhibitor protein of 17 kDa. *J Biol Chem*. 2001; 276:39858–39863. [PubMed: 11517233]
41. Eto M, Kitazawa T, Matsuzawa F, Aikawa S, Kirkbride JA, Isozumi N, Nishimura Y, Brautigan DL, Ohki SY. Phosphorylation-induced conformational switching of CPI-17 produces a potent myosin phosphatase inhibitor. *Structure*. 2007; 15:1591–1602. [PubMed: 18073109]
42. Eto M. Regulation of cellular protein phosphatase-1 (PP1) by phosphorylation of the CPI-17 family, C-kinase-activated PP1 inhibitors. *J Biol Chem*. 2009; 284:35273–35277. [PubMed: 19846560]
43. Yanagisawa T, Okada Y. KCl depolarization increases Ca^{2+} sensitivity of contractile elements in coronary arterial smooth muscle. *Am J Physiol*. 1994; 267:H614–H621. [PubMed: 8067417]
44. Mita M, Yanagihara H, Hishinuma S, Saito M, Walsh MP. Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem J*. 2002; 364:431–440. [PubMed: 12023886]
45. Sakamoto K, Hori M, Izumi M, Oka T, Kohama K, Ozaki H, Karaki H. Inhibition of high K^{+} -induced contraction by the ROCKs inhibitor Y-27632 in vascular smooth muscle: possible involvement of ROCKs in a signal transduction pathway. *J Pharmacol Sci*. 2003; 92:56–69. [PubMed: 12832856]

46. Sakurada S, Takuwa N, Sugimoto N, Wang Y, Seto M, Sasaki Y, Takuwa Y. Ca^{2+} -dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. *Circ Res.* 2003; 93:548–556. [PubMed: 12919947]
47. Urban NH, Berg KM, Ratz PH. K^{+} depolarization induces RhoA kinase translocation to caveolae and Ca^{2+} sensitization of arterial muscle. *Am J Physiol.* 2003; 285:C1377–C1385.
48. Janssen LJ, Tazzeo T, Zuo J, Pertens E, Keshavjee S. KCl evokes contraction of airway smooth muscle via activation of RhoA and Rho-kinase. *Am J Physiol.* 2004; 287:L852–L858.
49. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol.* 2005; 288:C769–C783.
50. Levent A, Büyükağar K. Expression of Rho-kinase (ROCK-1 and ROCK-2) and its substantial role in the contractile activity of the sheep ureter. *Br J Pharmacol.* 2004; 143:431–437. [PubMed: 15351780]
51. Hong SK, Kwak C, Jeong BC, Kim BS, Kim HH. Involvement of Rho-kinase in the contractile mechanism of human ureteral smooth muscle. *Neurourol Urodynam.* 2005; 24:136–141.
52. Turna B, Cinar MG, Canda AE, Orhan EC, Tiftik NR, Nazli O, Büyükağar K. Role of Rho-kinase in contractions of ureters from rabbits with unilateral ureteric obstruction. *BJU Int.* 2007; 100:1166–1171. [PubMed: 17711509]
53. Ngai PK, Walsh MP. Detection of caldesmon in muscle and non-muscle tissues of the chicken using polyclonal antibodies. *Biochem Biophys Res Commun.* 1985; 127:533–539. [PubMed: 2983725]
54. Burdyga T, Wray S. Simultaneous measurements of electrical activity intracellular [Ca^{2+}] and force in intact smooth muscle. *Pflüg Arch.* 1997; 435:182–184.
55. Burdyga T, Borisova L, Burdyga AT, Wray S. Temporal and spatial variations in spontaneous Ca events and mechanical activity in pregnant rat myometrium. *Eur J Obstet Gynecol Reprod Biol.* 2009; 144S:S25–S32.
56. Mitchell RW, Seow C, Burdyga TV, Maass-Moreno R, Pratushevich V, Ragozzino J. Relationship between myosin light chain phosphorylation and contractile capability of canine airway smooth muscle. *J Appl Physiol.* 2001; 90:2460–2465. [PubMed: 11356814]
57. Burdyga T, Wray S, Noble K. In situ calcium signaling: no calcium sparks detected in rat myometrium. *Ann N Y Acad Sci.* 2007; 1101:85–96. [PubMed: 17303831]
58. Sasaki Y, Suzuki M, Hidaka H. 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.* 2002; 93:225–232. [PubMed: 12191614]
59. Kamm KE, Stull JT. Myosin phosphorylation, force and maximal shortening velocity in neutrally stimulated tracheal smooth muscle. *Am J Physiol.* 1985; 249:C238–C247. [PubMed: 4037071]
60. Jiang MJ, Morgan KG. Agonist-specific myosin phosphorylation and intracellular calcium during isometric contractions of arterial smooth muscle. *Pflüg Arch.* 1989; 413:637–643.
61. Zimmermann B, Somlyo AV, Ellis-Davies GC, Kaplan JH, Somlyo AP. Kinetics of prephosphorylation reactions and myosin light chain phosphorylation in smooth muscle Flash photolysis studies with caged calcium and caged ATP. *J Biol Chem.* 1995; 270:23966–23974. [PubMed: 7592592]
62. Murahashi T, Fujita A, Kitazawa T. Ca^{2+} -induced Ca^{2+} desensitization of myosin light chain phosphorylation and contraction in phasic smooth muscle. *Mol Cell Biochem.* 1999; 190:91–98. [PubMed: 10098975]
63. Wilson DP, Sutherland C, Walsh MP. Ca^{2+} activation of smooth muscle contraction: evidence for the involvement of calmodulin that is bound to the Triton-insoluble fraction even in the absence of Ca^{2+} . *J Biol Chem.* 2002; 277:2186–2192. [PubMed: 11707462]
64. Tansey MG, Word RA, Hidaka H, Singer HA, Schworer CM, Kamm KE, Stull JT. Phosphorylation of myosin light chain kinase by the multifunctional calmodulin-dependent protein kinase II in smooth muscle cells. *J Biol Chem.* 1992; 267:12511–12516. [PubMed: 1319999]
65. Tansey MG, Luby-Phelps K, Kamm KE, Stull JT. Ca^{2+} -dependent phosphorylation of myosin light chain kinase decreases the Ca^{2+} sensitivity of light chain phosphorylation within smooth muscle cells. *J Biol Chem.* 1994; 269:9912–9920. [PubMed: 8144585]

66. Word RA, Tang D-C, Kamm KE. Activation properties of myosin light chain kinase during contraction/relaxation cycles of tonic and phasic smooth muscles. *J Biol Chem.* 1994; 269:21596–21602. [PubMed: 8063799]
67. Dimopoulos GJ, Semba S, Kitazawa K, Eto M, Kitazawa T. Ca^{2+} -dependent rapid Ca^{2+} sensitization of contraction in arterial smooth muscle. *Circ Res.* 2007; 100:121–129. [PubMed: 17158339]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.ceca.2011.07.003.

Fig. 1. The temporal relationship between electrical activity, the Ca^{2+} transient and force induced by brief stimulation with high $[\text{K}^+]$ or carbachol. (A and B) A series of grey-scale images showing changes in fluo-4 fluorescence in ureteric segments induced by 1 s local application of high $[\text{K}^+]$ (120 mM KCl) or CCh (100 μM) in the absence and presence of extracellular Ca^{2+} ; (C) membrane potential (top trace), F/F_0 ratio (middle traces) recorded in region 1 (red) and region 2 (blue), and isometric force (bottom trace) were recorded simultaneously in intact fluo 4-loaded rat ureteric segments. Data are representative of 5 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2.

The temporal relationship between electrical activity, the Ca^{2+} transient and force induced by prolonged application of high $[\text{K}^+]$ or carbachol. Membrane potential (top trace), F/F_0 ratio (middle trace) and isometric force (bottom trace) were recorded simultaneously in intact fluo 4-loaded rat ureteric strips. The responses to high $[\text{K}^+]$ (120 mM KCl) or CCh (10 μM) are shown. Data are representative of 4 independent experiments.

Fig. 3.

The effect on the Ca^{2+} transient and force or shortening of high $[K^+]$ -induced depolarization, carbachol in Ca^{2+} -free solution, or carbachol added during steady-state K^+ -induced contraction. (A) Intact fluo 4-loaded rat ureteric strips were treated initially with high $[K^+]$ (120 mM KCl) in the presence of extracellular Ca^{2+} . Removal of K^+ and extracellular Ca^{2+} (0 Ca^{2+}) induced relaxation to baseline, whereupon addition of CCh (100 μ M) induced a transient increase in $[Ca^{2+}]_i$ (upper trace) and force (lower trace). Washout of CCh and replacement of extracellular Ca^{2+} was followed by another K^+ -induced contraction. Addition of CCh, once steady-state levels of $[Ca^{2+}]_i$ and force had been achieved, elicited a brief Ca^{2+} transient, which was greater in amplitude than that induced by high $[K^+]$ depolarization, and a phasic contraction, which was equal to that induced by high $[K^+]$ depolarization. The phasic contraction associated with Ca^{2+} release was followed by a slight but statistically significant increase in tonic force, which was not accompanied by a steady-state increase in $[Ca^{2+}]_i$. (B and C) Superimposed normalized traces of the initial components of the Ca^{2+} transients and force induced by high $[K^+]$ (B) and CCh in Ca^{2+} -free solution (C); (D) a series of pseudo-color images showing time-dependent changes in fluo-4 fluorescence and shortening of the ureteric segment clamped at the bottom induced by brief (1 s) high $[K^+]$ stimulation, applied at the red arrow; (E) superimposed traces of the normalized Ca^{2+} transient and muscle shortening expressed as a change of length in relation to the length at rest (L_0). Panel A is representative of 7 and panel D of 4 independent experiments.

Fig. 4.

The temporal relationship between the Ca^{2+} transient, LC_{20} phosphorylation and force evoked by high [K⁺]-induced depolarization or carbachol applied in Ca^{2+} -free solution. Intact fluo 4-loaded rat ureteric strips were treated with high [K⁺] (120 mM KCl) in the presence of extracellular Ca^{2+} (A) or CCh (100 μM) in the absence of extracellular Ca^{2+} (C) and F/F_0 ratio (top trace) and isometric force (bottom trace) were simultaneously measured. In separate experiments, tissues were quick frozen at the indicated times for quantification of LC_{20} phosphorylation by urea-glycerol gel electrophoresis (middle trace); (B and D) superimposed traces of the normalized Ca^{2+} transient, LC_{20} phosphorylation and force induced by high [K⁺] (B, taken from A) or CCh (D, taken from C).

Fig. 5.

Immunostaining of ROK α and ROK β in intact rat ureteric preparations. Representative grey-scale images of rat ureteric smooth muscle bundles labelled with anti-ROK α and anti-ROK β (Texas red). Specificity of immunostaining was verified by omitting the primary antibody (negative control).

Fig. 6.

The effect of H-1152 on the temporal relationship between electrical activity, the Ca^{2+} transient and force induced by high $[K^+]$ depolarization. (A) Electrical activity (top trace), the Ca^{2+} transient (middle trace) and force (bottom trace) before and after 10 min pre-treatment with H-1152 (100 nM). (B) Action potentials recorded at the onset of high $[K^+]$ depolarization in the absence and presence of H-1152 shown on a faster time scale. Note that H-1152 significantly reduced the amplitude of the plateau component of the action potential (B), resulting in a decrease in the amplitude of the phasic component of the Ca^{2+} transient and force (A). Data are representative of 4 independent experiments.

Fig. 7.

The effect of H-1152 on LC₂₀ and MYPT1 phosphorylation induced by high $[\text{K}^+]$. (A) Force (bottom trace) and the Ca^{2+} transient (top trace) induced by 120 mM KCl in the absence and presence of H-1152 (100 nM) applied 10 min before the addition of K^+ ; middle trace shows LC₂₀ phosphorylation measured in a separate set of experiments in which contractile responses were terminated at the indicated times and LC₂₀ phosphorylation levels were determined in the absence and presence of H-1152. (B) Representative Western blots of phosphorylated LC₂₀, MYPT1-T697 (pT697), and MYPT1-T855 (pT855) recorded in resting muscle (1) and at 40 s of high $[\text{K}^+]$ -induced contraction in the absence (2) and presence (3) of H-1152 (100 nM). (C) Cumulative data quantifying the phosphorylation of LC₂₀, MYPT1-T697 and MYPT1-T855 recorded before (1) and at 40 s of high $[\text{K}^+]$ -induced contraction in the absence (2) and presence (3) of H-1152. Values represent means $\pm$ SEM ($n = 4$). *Statistically significant differences (Student's t test) from the resting level ($P < 0.01$).

Fig. 8.

The effect of H-1152 on the temporal relationship between electrical activity, the Ca^{2+} transient and force induced by carbachol. (A) Electrical activity (top trace), the Ca^{2+} transient (middle trace) and force (bottom trace) induced by CCh (100 μM) before and after 10 min pre-treatment with H-1152 (100 nM). (B) Action potentials recorded at the onset of CCh-induced depolarization in the absence and presence of H-1152 shown on a faster time scale. Note that H-1152 significantly reduced the duration of the plateau component of the action potential, resulting in a decrease in the amplitude of the phasic component of the Ca^{2+} transient and force (A). Data are representative of 4 independent experiments.

Fig. 9.

The effect of H-1152 on the carbachol-induced Ca^{2+} transient, LC_{20} phosphorylation and force. (A) Superimposed records of the Ca^{2+} transients (top trace), LC_{20} phosphorylation (middle trace) and force (bottom trace) induced by CCh (100 μM) in Ca^{2+} -free solution in the absence and presence of H-1152 (100 nM). (B) Representative Western blots of LC_{20} phosphorylation measurements at two different times during the CCh-induced contraction in the absence and presence of H-1152 (points 1 and 2 shown in A). (C) Rate constants of LC_{20} dephosphorylation and relaxation were calculated from mono-exponential fits of the relaxation and dephosphorylation data in A.

Fig. 10.

The effect of H-1152 on LC₂₀ and MYPT1 phosphorylation induced by carbachol in the presence of nifedipine. (A) The Ca²⁺ transient (top trace) and force (bottom trace) evoked by CCh (100 μ M) in the absence or presence of H-1152 (100 nM) applied 10 min before the addition of CCh; middle trace shows LC₂₀ phosphorylation measured in a separate set of experiments in which contractile responses were terminated at the indicated times and LC₂₀ phosphorylation levels were measured in the absence and presence of H-1152. (B) Representative Western blots of phosphorylated LC₂₀, MYPT1-T697 (pT697) and MYPT1-T855 (pT855) recorded in resting muscle (1) and at 40 s of CCh-induced contraction in the absence (2) and presence (3) of H-1152 (100 nM). (C) Cumulative data quantifying the phosphorylation of LC₂₀, MYPT1-T697 and MYPT1-T855 recorded before (1) and at 40 s of CCh-induced contraction in the absence (2) and presence (3) of H-1152. Values represent means $\pm$ SEM ($n = 4$). *Statistically significant differences (Student's *t* test) from the resting level ($P < 0.01$).