

Activation by Ca^{2+} /calmodulin of an exogenous myosin light chain kinase in mouse arteries

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Activation of myosin light chain kinase (MLCK) and other kinases was studied in the arteries of transgenic mice that express an optical fluorescence resonance energy transfer (FRET) MLCK activity biosensor. Binding of Ca^{2+} /calmodulin (Ca^{2+} /CaM) induces an increase in MLCK activity and a change in FRET. After exposure to high external $[\text{K}^+]$, intracellular $[\text{Ca}^{2+}]$ (fura-2 ratio or fluo-4 fluorescence) and MLCK activity both increased rapidly to an initial peak and then declined, rapidly at first and then very slowly. After an initial peak ('phasic') force was constant or increased slowly (termed 'tonic' force). Inhibition of *rho*-kinase (Y-27632) decreased tonic force more than phasic, but had little effect on $[\text{Ca}^{2+}]$ and MLCK activation. Inhibition of PKC α and PKC β with Gö6976 had no effect. KN-93, an inhibitor of CaMK II, markedly reduced force, MLCK FRET and $[\text{Ca}^{2+}]$. Applied during tonic force, forskolin caused a rapid decrease in MLCK FRET ratio and force, but no change in Ca^{2+} , suggesting a cAMP mediated decrease in affinity of MLCK for Ca^{2+} /CaM. However, receptor (β -adrenergic) activated increases in cAMP during KCl were ineffective in causing relaxation, changes in $[\text{Ca}^{2+}]$, or MLCK FRET. At the same tonic force, MLCK FRET ratio activated by α_1 -adrenoceptors was ~60% of that activated by KCl. In conclusion, MLCK activity of arterial smooth muscle during KCl-induced contraction is determined primarily by Ca^{2+} /CaM. *Rho*-kinase is activated, by unknown mechanisms, and increases ' Ca^{2+} sensitivity' significantly. Forskolin mediated increases in cAMP, but not receptor mediated increases in cAMP cause a rapid decrease in the affinity of MLCK for Ca^{2+} /CaM.

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Abbreviations CaMK II, Ca^{2+} /CaM-dependent protein kinase II; CFP, cyan fluorescent protein; CPI-17, 17 kDa protein kinase C-potentiated phosphatase inhibitor; FRET, fluorescence resonance energy transfer; GPCR, G protein coupled receptor; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PAK, p-21 activated protein kinase; PE, phenylephrine; PKA, protein kinase A; PKC, protein kinase C; RLC, myosin regulatory light chain; YFP, yellow fluorescent protein.

Contraction of smooth muscle is believed to be primarily dependent on phosphorylation of myosin regulatory light chains (RLCs), as determined by the relative activities of Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Here, we will use the term, 'activation' (of MLCK) to refer to the increase in kinase activity of MLCK that occurs upon binding of Ca^{2+} /CaM, and the term, 'regulation' to refer to changes in the affinity of MLCK for Ca^{2+} /CaM that might result from phosphorylation of MLCK. Phosphorylation of MLCK at serine 1760 ('regulatory site A') by Ca^{2+} /CaM-dependent protein kinase II (CaMK II) or protein kinase A (PKA) is known to regulate (decrease) the affinity of MLCK for Ca^{2+} /CaM. MLCK can also be phosphorylated by protein

kinase C (PKC), p-21 activated protein kinase (PAK) and mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK) (Kamm & Stull, 2001). Activity of MLCP is regulated most importantly by *rho*-kinase and by 17 kDa protein kinase C-potentiated phosphatase inhibitor (CPI-17).

The extent and time course of activation of these kinases in smooth muscle cells of intact arteries during contraction is essentially unknown, but can be expected to be complex. For example, the rise in intracellular $[\text{Ca}^{2+}]$ that typically activates MLCK in response to G protein coupled receptor (GPCR) activation or to membrane depolarization may inevitably activate other Ca^{2+} -dependent proteins, such as CaMK II and conventional (i.e. Ca^{2+} activated) forms of PKC, potentially leading to both activation and regulation

of MLCK, and to regulation of MLCP. Furthermore, Ca^{2+} is thought also to exert an inhibitory effect on MLCP through induction of *rho*-kinase activation (Yoshioka *et al.* 2007).

Here, we use a genetically encoded (exogenous) FRET-based MLCK biosensor molecule (Isotani *et al.* 2004) in an attempt to provide information on how the endogenous MLCK might be activated and regulated, during contraction of intact arteries. At the most basic level, changes in FRET within the MLCK biosensor molecule result from conformational changes caused by the binding or release of $\text{Ca}^{2+}/\text{CaM}$, in the biosensor molecules only. The extent to which the same processes might be occurring in the endogenous MLCK molecules in the smooth muscle studied here is not actually known. Differences might occur, for example, as a result of differences in spatial localization of the two molecules. It is known, however, that the FRET ratio reported by the MLCK biosensor (in HEK 293 cells) is quantitatively linked to the concentration of Ca^{2+} and to the biosensor's ability to phosphorylate myosin regulatory light chains (Geguchadze *et al.* 2004); the half-maximal response for FRET was obtained at a pCa of 6.2, while the pCa for half-maximal phosphorylation of myosin (by the biosensor) was 6.4. If the same situation obtains in the smooth muscle cells of the arteries studied here, the biosensor FRET ratio we observe should reflect the endogenous MLCK activity, as activated by the endogenous $\text{Ca}^{2+}/\text{CaM}$. The MLCK biosensor might also be expected to provide information on possible regulation of MLCK activity during contraction. Regulation of MLCK activity (defined here as a change in the affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$ caused by phosphorylation of MLCK) should be revealed by changes in biosensor FRET ratio, when no changes in $[\text{Ca}^{2+}]$ occur (assuming that no changes in the availability of CaM occur either).

Finally, we activated contraction using both elevated external $[\text{K}^+]$ (KCl) and the α_1 -adrenoceptor agonist phenylephrine (PE). KCl has the advantage that it achieves a spatially uniform elevation of $[\text{Ca}^{2+}]$ within small arteries and thereby simplifies the analysis of spatially averaged fluorescence signals. Activation of GPCR is more physiological, but presents a more complex situation in terms of possible heterogeneity of $[\text{Ca}^{2+}]$ (Zang *et al.* 2001) and activation of many other signalling cascades.

Methods

Animals, arteries and solutions

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland, School of Medicine. Inbred Charles River, wild-type (WT) and transgenic (TG) mice were maintained on 12:12 h light–dark schedule at 22–25°C and 45–65% humidity

and fed *ad libitum* on a standard rodent diet and tap water. Adult mice (28–35 g, 12–18 weeks), were killed by inhalation of CO_2 .

As described previously, (Isotani *et al.* 2004), the TG mice express a MLCK biosensor that monitors the binding of $\text{Ca}^{2+}/\text{CaM}$ through changes in FRET between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The mesenteric arcade was dissected from the abdominal cavity, rinsed free of blood, and placed in a temperature-controlled dissection chamber (5°C) containing a solution of the following composition (in mmol l^{-1}): 3.0 Mops, 145.0 NaCl, 5.0 KCl, 2.5 CaCl_2 , 1.0 MgSO_4 , 1.0 KH_2PO_4 , 0.02 EDTA, 2.0 sodium pyruvate, and 5.0 glucose (pH 7.4). Segments, 2–3 mm in length, of third-order mesenteric arteries were dissected free. If calcium indicators were to be used, the selected artery was then further exposed to dissection solution containing either fura-2 AM (5.0 μM) or fluo-4 AM (10.0 μM) and loading was allowed to proceed for 1 h or 3 h, respectively, at room temperature. After mounting for force and fluorescence recording (below) superfusion was begun with the standard experimental solution containing (in mmol l^{-1}) 112.0 NaCl, 25.7 NaHCO_3 , 4.9 KCl, 2.0 CaCl_2 , 1.2 MgSO_4 , 1.2 KHPO_4 , 11.5 glucose, and 10.0 Hepes (pH 7.4, equilibrated with gas of 5% O_2 , 5% CO_2 , 90% N_2). Solutions containing elevated KCl were made by replacing the NaCl with KCl on an equimolar basis. PE was used in concentrations ranging from 0.1 μM to 10.0 μM . The pH and partial pressure of O_2 in the bath, within 0.5 mm of the artery, were 7.4 and 114 mmHg, respectively, measured with micro pH and O_2 sensing microelectrodes (Micro-electrodes, Inc., Londonderry, NH, USA).

Arteries were studied at 32°C, as at 37°C, fura-2 and fluo-4 are transported rapidly out of the cytoplasm of healthy smooth muscle cells. This provides a compromise between physiological conditions and the experimental necessity of retaining enough Ca^{2+} indicators to make measurements. At 32°C, arteries develop myogenic tone, which is an important physiological parameter of vessel viability (Zacharia *et al.* 2007).

When necessary, sections of aorta were also removed for biochemical measurements of MLCK content or MLCK phosphorylation by immunoblotting. Aortic sections were opened longitudinally, and immersed in the same solutions as used for the mesenteric arteries, as required.

Heart rate, indirect systolic, diastolic and mean blood pressures were recorded by tail cuff plethysmography using a blood pressure analysis system SC1000 (Hatteras Instruments Inc., Cary, NC, USA). The system utilizes a pressure transducer close to the tail-cuff on the SC1000 specimen platform. The platform temperature is precisely maintained during measurements using a low voltage silicone rubber heater. This is important to promote adequate blood flow in the tail for pulse

detection. Pulse detection is made with a photodiode detector in combination with high intensity light emitting diode. Conscious mice were conditioned to five inflation/deflation cycles by a trained operator. Subsequently, the average values for blood pressure and heart rate in each animal is obtained from 10 sequential cuff inflation/deflation cycles.

All data are expressed as means $\pm$ S.E.M. Statistical analysis was performed on raw data using SigmaStat (Systat Software Inc., San Jose, CA, USA). Statistical analysis of paired data was performed with Student's *t*-test. In all cases $P < 0.05$ was taken as the level of significance.

Rho-kinase was inhibited by Y-27632 [(R)-(-)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] (Calbiochem) and PKC was inhibited by Gö6976 (Invitrogen). CaMK II was inhibited by KN-93 (Calbiochem). Isoproterenol, forskolin and PE were obtained from Sigma, H-89 and rp-cAMP were purchased from Calbiochem.

Force measurements

Arteries were transferred to a recording chamber, where they were mounted on a confocal wire myograph (Danish Myotech Technology, Denmark). The length of the wires on the wire myograph jaws, and hence the length of the artery that generates force, was always 1.76 mm. Thus, the forces reported here from different arteries are all strictly comparable. The resting tension–internal circumference (IC) relation was determined for each vessel (Mulvany & Halpern, 1977). IC was set to $0.9 \times IC_{100}$, where IC_{100} is the internal circumference of the vessel under an effective transmural pressure of 100 mmHg (13.3 kPa).

Biochemical measurements

Sections of aorta were used in order to obtain sufficient protein for Western blots. After aortas were subjected to the particular experimental procedure, they were lysed in 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 1 mM NaF, and proteinase inhibitors. Lysates were centrifuged at 7000 *g* at 4°C for 15 min and supernatant was collected as total protein. Protein concentration was determined with BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein was separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and transferred onto Trans-Blot Nitrocellulose membrane (Bio-Rad). Polyclonal antibodies against phospho-MLCK (Ser 1760, Upstate Biotechnology, Inc., Lake Placid, NY, USA) were used following the manufacturers' protocols. Horseradish peroxidase-conjugated goat anti-rabbit and

goat anti-mouse IgG were used as secondary antibodies (Sigma). Reactions were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposure to autoradiographic film. Signalling was quantified from scanned films using ImageJ software (Scion).

Fluorescence measurements

The equipment provided (1) confocal images of Ca²⁺ activated fluorescence (fluo-4) in individual smooth muscle cells of WT arteries, (2) confocal images of MLCK biosensor fluorescence in individual smooth muscle cells of TG arteries, and (3) simultaneous measurements of spatially averaged Ca²⁺ activated fluorescence (fura-2) and MLCK biosensor fluorescence from TG arteries mounted in a widefield microscope. A Nipkow spinning disk confocal microscope equipped with excitation illumination at 488 nm or 442 nm and an intensified CCD camera (Stanford Photonics, Palo Alto, CA, USA) was used to obtain images of fluo-4 or MLCK biosensor fluorescence. For the MLCK FRET measurements the confocal microscope was also equipped with an image-splitter designed for FRET measurements of CFP/YFP type FRET indicator molecules (Dual-View, Roper Scientific, Tucson, AZ, USA). Emission filters were for CFP, 480/40 nm and for YFP, 535/35 nm (centre wavelength, bandwidth at full width at half-maximum, FWHM).

The system used for the spatially averaged (nearly) simultaneous measurements of Ca²⁺ indicator fluorescence (fura-2) and MLCK biosensor fluorescence has been described in detail recently (Wier *et al.* 2008). Briefly, because of the spectral properties of fura-2 and the MLCK biosensor, it is possible to excite fura-2 and CFP of the MLCK biosensor selectively (at slightly different times, with different excitation filters) and therefore to collect fluorescence emission selectively from each. Since the emission spectrum of fura-2 overlaps that of both CFP and YFP, emission of fura-2 can be collected with the same two emission filters that are used for the MLCK biosensor.

Spatially resolved information on [Ca²⁺] and MLCK activation in individual cells was obtained, in different arteries, with fluo-4 and confocal microscopy. Information on [Ca²⁺] and MLCK activation in the same artery was obtained with the widefield epi-fluorescence microscope. This is because our confocal microscope does not provide excitation light for both fura-2 and CFP, as would be required for simultaneous, confocal measurements of [Ca²⁺] and MLCK activation in the same artery. The confocal microscope does provide excitation light for both fluo-4 and CFP of the MLCK biosensor, but fluo-4 and CFP cannot be used together because of

extensive spectral overlap of excitation of fluo-4 and CFP (typically 488 nm and 442, respectively).

Results

In confocal images of CFP fluorescence, the MLCK biosensor appeared to be expressed in all the individual smooth muscle cells in the walls of TG arteries (Fig. 1A), although expression levels, as judged by the intensity of CFP fluorescence, varied amongst different cells. Western blots (Fig. 1B) revealed that the MLCK biosensor was expressed in aortas at a level $\sim 30\%$ of the endogenous MLCK, similar to that reported earlier for bladder smooth muscle cells in the same animals (Isotani *et al.* 2004). Because the expression levels are apparently not the same in all cells, this figure represents an average level of expression. The presence of the exogenous MLCK raises the possibility that the function of the TG arteries could be affected, as, for example, might happen if the MLCK

biosensor bound significant amounts of $\text{Ca}^{2+}/\text{CaM}$, reducing the availability of $\text{Ca}^{2+}/\text{CaM}$ for endogenous MLCK and other molecules that bind $\text{Ca}^{2+}/\text{CaM}$. No differences were detected, however, in the blood pressure (Fig. 1C) or heart rate (Fig. 1D) of TG mice compared to WT. Similarly, no differences were detected in the contractile responses of TG and WT arteries to elevated [KCl] or PE (Fig. 1E). The fact that TG animals and arteries were the same as WT animals and arteries for the characteristics examined might reflect the relatively low expression levels of the exogenous MLCK.

$[\text{Ca}^{2+}]$ and MLCK biosensor fluorescence in individual cells

Although we expected KCl to induce spatially uniform changes in $[\text{Ca}^{2+}]$ in individual smooth muscle cells (thus justifying the use of spatially averaged measurements of fura-2 and MLCK biosensor in the same artery), we confirmed this important fact by confocal imaging of

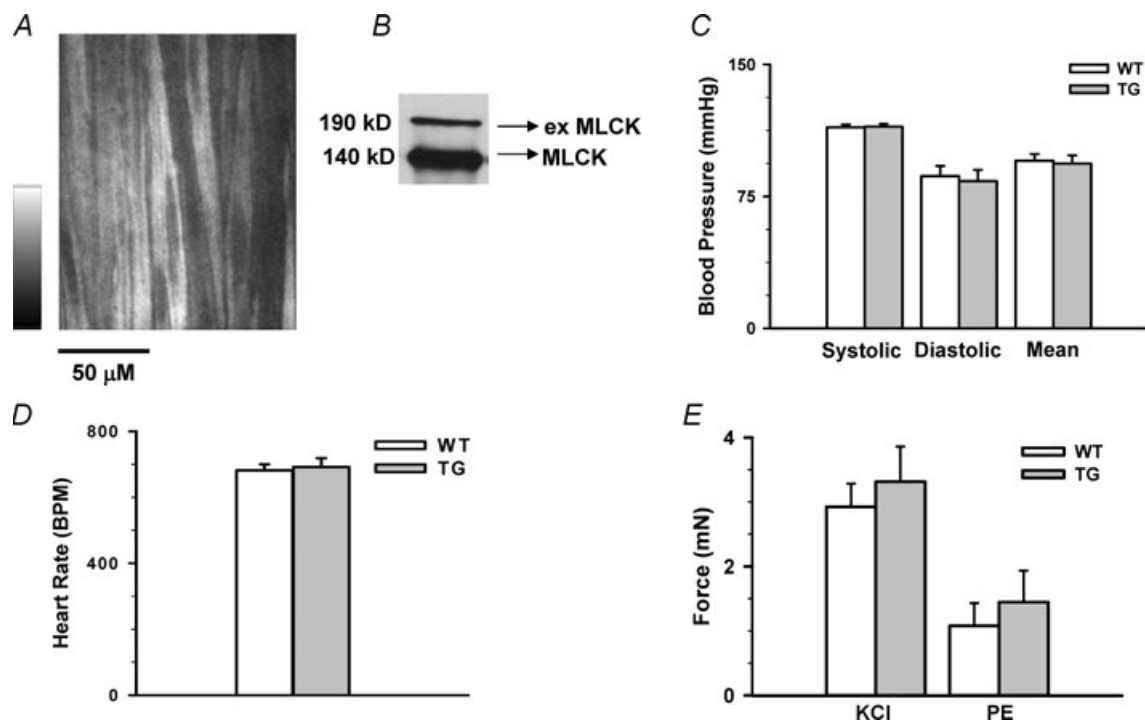


Figure 1. Visualization of MLCK biosensor in intact arteries and physiological properties of arteries containing the MLCK biosensor

A, confocal image of CFP fluorescence (as part of exogenous MLCK) in transgenic (TG) artery mounted on confocal wire myograph. Grey scale indicates increasing fluorescence intensity from 0 to 255 arbitrary units. B, Western blots of endogenous and exogenous (biosensor) MLCK. In 4 aortas, exogenous MLCK was present at 0.32 ± 0.03 of the amount of the endogenous MLCK. C, systolic arterial blood pressure, diastolic arterial blood pressure, mean arterial blood pressure and heart rate (D) do not show a statistically significant change between wild type (WT) ($n = 4$) and TG animals ($n = 5$). E, contraction in response to elevated [KCl] (60 mM) and PE (1.0 μM) does not show a statistically significant change between WT ($n = 14$) and TG ($n = 14$) arteries. Results are presented as means $\pm$ S.E.M. Results show that MLCK biosensor animals (TG) have normal blood pressure and their arteries respond to depolarization (KCl) and G protein coupled receptor (GPCR) activation similarly to WT arteries.

fluo-4 fluorescence in individual cells of WT arteries (Fig. 2A) during KCl exposure and confocal imaging of MLCK biosensor fluorescence in individual cells of TG arteries (Fig. 2D). Fluo-4, instead of fura-2, was used to check the uniformity of [Ca²⁺] changes in the artery because fura-2, cannot be used in the confocal microscope. Because fluo-4 is not a ratiometric Ca²⁺ indicator, the measurements are subject to possible errors due to artery motion. The maximum translation of the cells in the field of view during the (nominally) isometric contraction was less than 5%. Indeed, the changes in fluo-4 fluorescence (Fig. 2B) and MLCK FRET ratio (Fig. 2E) in representative individual smooth muscle cells during KCl exposure are similar to the spatially averaged fluorescence signals (Figs. 2C, F). The magnitude

and basal levels of the fluorescence signals do vary substantially amongst different cells, for unknown reasons, but the time courses of both fluo-4 and MLCK FRET ratio changes are all remarkably similar in the different cells. Thus the spatially averaged fluorescent signal can be used as an approximation to events within an (average) cell.

Time courses of MLCK FRET ratio and [Ca²⁺]

Average MLCK FRET ratio and Ca²⁺ indicator signals during KCl exposure were typically very similar in time course, declining rapidly at first after the initial peak, and then declining very slowly throughout the remainder of the exposure to KCl (Fig. 3A). In contrast to this, however,

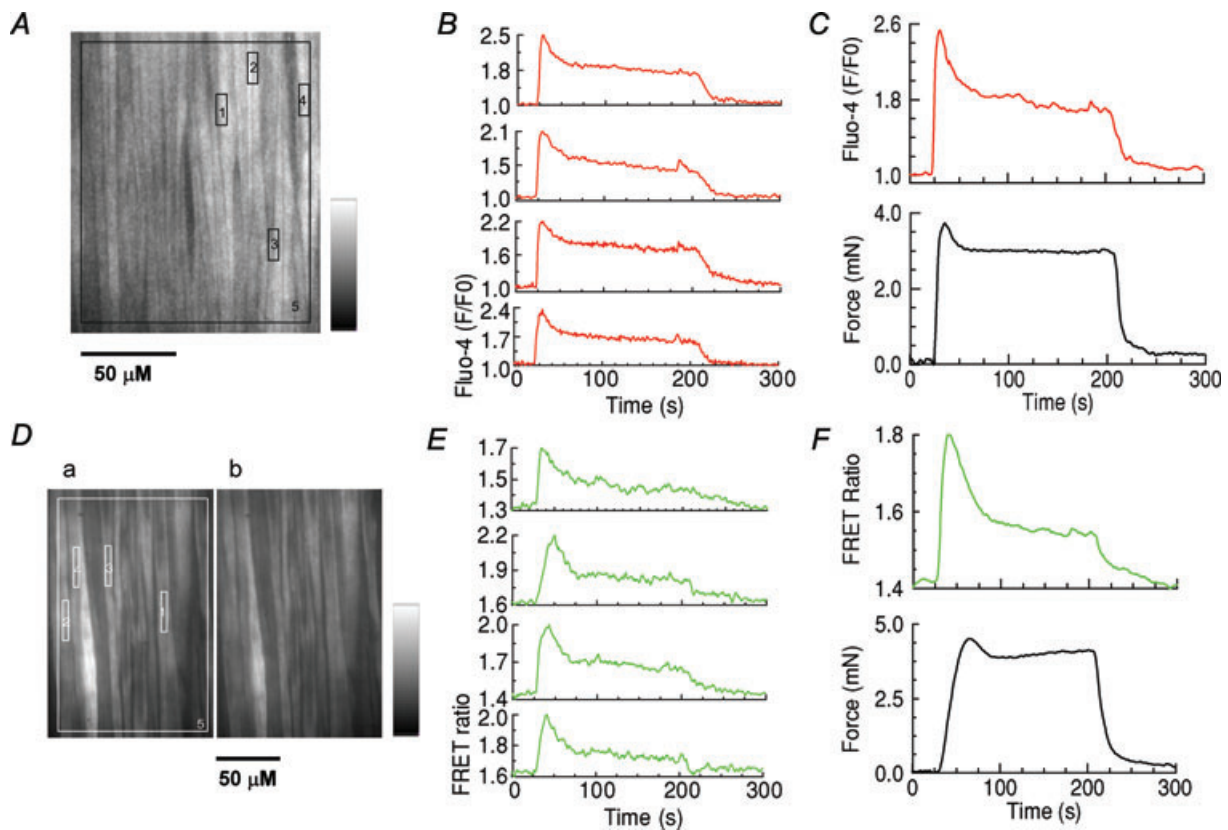


Figure 2. Confocal images of MLCK biosensor fluorescence and fluo-4 fluorescence in areas of interest (AOI) within individual smooth muscle cells of TG and WT arteries, respectively, during exposure to elevated KCl

A, image of fluo-4 fluorescence in wall of WT artery. B, normalized fluo-4 fluorescence ratios (F/F_0) from the 4 'areas-of-interest' (AOIs) marked in A. C, spatial average F/F_0 was obtained from the large white-lined AOI numbered '5' in (A) and simultaneously recorded force. D, image of MLCK biosensor fluorescence in wall of TG artery on confocal myograph; Da, CFP emission channel; Db, YFP emission channel. The numbered, white-lined rectangles are the AOIs from which the MLCK FRET ratios were obtained during KCl exposure, as shown in E. E, MLCK FRET ratios from the AOIs in Da. F, spatial average MLCK FRET was obtained from the large white-lined AOI numbered '5', and simultaneously recorded force. The figure is intended to show that the spatially averaged MLCK FRET ratio and normalized fluo-4 fluorescence ratios, provide a reasonable approximation to the corresponding signals measured in small regions of individual smooth muscle cells during cell activation by elevated KCl. Grey scale indicates increasing fluorescence intensity from 0 to 255 arbitrary units.

force typically rose slowly again after the initial peak and then was maintained constant, during a substantial period of time (Fig. 3B). We have shown previously (Wier *et al.* 2008) that the time courses of MLCK FRET ratio and Ca^{2+} (fura-2 fluorescence) are not affected by artery motion or fluorescence spectral overlap.

Ca^{2+} sensitivity of contraction

The differences in time courses between MLCK FRET ratio and force could be regarded as *prima facie* evidence of changes in ' Ca^{2+} sensitivity of contraction'. Indeed, pharmacological inhibition of *rho*-kinase with Y-27632 markedly inhibited the tonic component of KCl induced force, but had relatively much less effect on phasic and tonic fura-2 fluorescence or phasic and tonic MLCK FRET ratio (Fig. 4A and B).

In some smooth muscles, Ca^{2+} -dependent forms of PKC modulate Ca^{2+} sensitivity through a CPI-17 mediated inhibition of MLCP (Azam *et al.* 2007). Application of Gö6976 (1.0 μM , 10 min), which selectively inhibits PKC α and PKC β (Gschwendt *et al.* 1996), had no effect, however, on the force, fluo-4 fluorescence or MLCK FRET ratio (Fig. 4C).

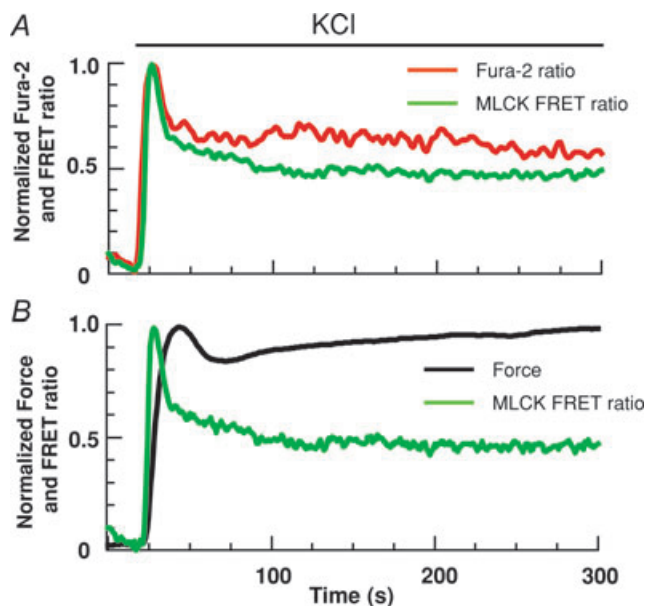


Figure 3. $[\text{Ca}^{2+}]$ and MLCK FRET ratio follow similar time courses during exposure to KCl, but force follows a different time course.

A, superimposed, simultaneous recordings of fura-2 fluorescence (red), and MLCK FRET ratio (green) during KCl exposure. Data have been normalized by subtracting the baseline (i.e. the values before exposure to KCl) and then dividing by the maximum value attained. B, superimposed recordings of MLCK FRET ratio (green) and force (black), normalized as above. The MLCK FRET ratio is the same in both (A) and (B).

It has been noted (Pfitzer, 2001) that when intracellular $[\text{Ca}^{2+}]$ rises in smooth muscle, CaMK II may be activated, phosphorylate MLCK at Site A, and provide negative feedback on the activation of MLCK. This concept is based on earlier work showing that the MLCK activity (a measure of the affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$) decreased during sustained elevation of intracellular $[\text{Ca}^{2+}]$ in uterine smooth muscle and that this phenomenon was blocked by inhibitors of CaMK II (Word *et al.* 1994). Earlier work had shown that Site A of MLCK was phosphorylated in a Ca^{2+} -dependent manner (Tansey *et al.* 1994). The similarity in time course of Ca^{2+} indicator signals and MLCK FRET ratio during KCl exposure (Fig. 3) argues against major changes in affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$ during KCl exposure. If, however, CaMK II were significantly phosphorylating MLCK and decreasing the affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$, then one would expect that application of a CaMK II inhibitor would increase MLCK FRET ratio and force, provided that Ca^{2+} remained the same. The CaMK II inhibitor KN-93 (10.0 μM), applied during KCl exposure, decreased Ca^{2+} indicator signals, MLCK FRET ratio and force all to a similar extent (Fig. 4D). The mechanisms by which KN-93 lowered intracellular $[\text{Ca}^{2+}]$ are not known, but may involve an effect on voltage-gated Ca^{2+} channels (Gao *et al.* 2006).

Activation of certain GPCRs is known to increase Ca^{2+} sensitivity of contraction, by activating *rho*-kinase and PKC signal transduction pathways. To investigate this with the system used here, we compared the MLCK FRET ratio signals activated by the α_1 -adrenoceptor (AR) agonist PE with those activated by exposure to KCl (Fig. 5). In each artery ($n = 6$), PE was used at a concentration that gave the same tonic force as that produced by our standard KCl protocol in that artery. During the tonic phase of force, the accompanying MLCK FRET ratio elicited by PE was 62% of that elicited by KCl, providing clear evidence of increased Ca^{2+} sensitivity during α_1 -AR activation, compared to KCl. Possible spatial inhomogeneities of the MLCK FRET ratio signal, due to possible agonist-induced inhomogeneities of $[\text{Ca}^{2+}]$, would not have been detected in these experiments, as discussed later.

PKA

It has long been known that 'pharmacological' elevation of cAMP results in PKA-dependent phosphorylation of MLCK (de Lanerolle *et al.* 1984) in biochemical measurements. The prevailing concept is that phosphorylation of MLCK by PKA reduces the affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$, when $\text{Ca}^{2+}/\text{CaM}$ is not bound to MLCK. Here we have used KCl activation as a means to control $[\text{Ca}^{2+}]$ during elevation of cAMP and activation of PKA after application of forskolin to intact arteries.

We have shown previously that forskolin does not change intracellular [Ca²⁺] under these circumstances (Wier *et al.* 2008). First, we examined phosphorylation of Ser 1760 (i.e. the regulatory site on MLCK) using a specific phospho-antibody (Fig. 6A). Important for the present study was the result that both the exogenous MLCK and the endogenous MLCK were both phosphorylated in the presence of forskolin, and this phosphorylation was inhibited by H-89 (Fig. 6A). Thus, changes in affinity of MLCK as a result of phosphorylation of Ser 1760 can be expected to result in changes in FRET. Furthermore, Ser

1760 was phosphorylated by exposure to forskolin also in the presence of KCl and these increases were blocked by the competitive antagonist of cAMP binding to PKA, rp-cAMP (100.0 μ M, $n = 3$). Forskolin at 1.0 μ M was applied for 2 min, after KCl had been elevated for 2 min. Arteries were pretreated, or not, with a selective PKA inhibitor (100.0 μ M rp-cAMP for 10 min).

Forskolin was applied in two fundamentally different ways to arteries during physiological experiments. In the first series of experiments (Fig. 7A), forskolin was applied when intracellular [Ca²⁺] was not simultaneously

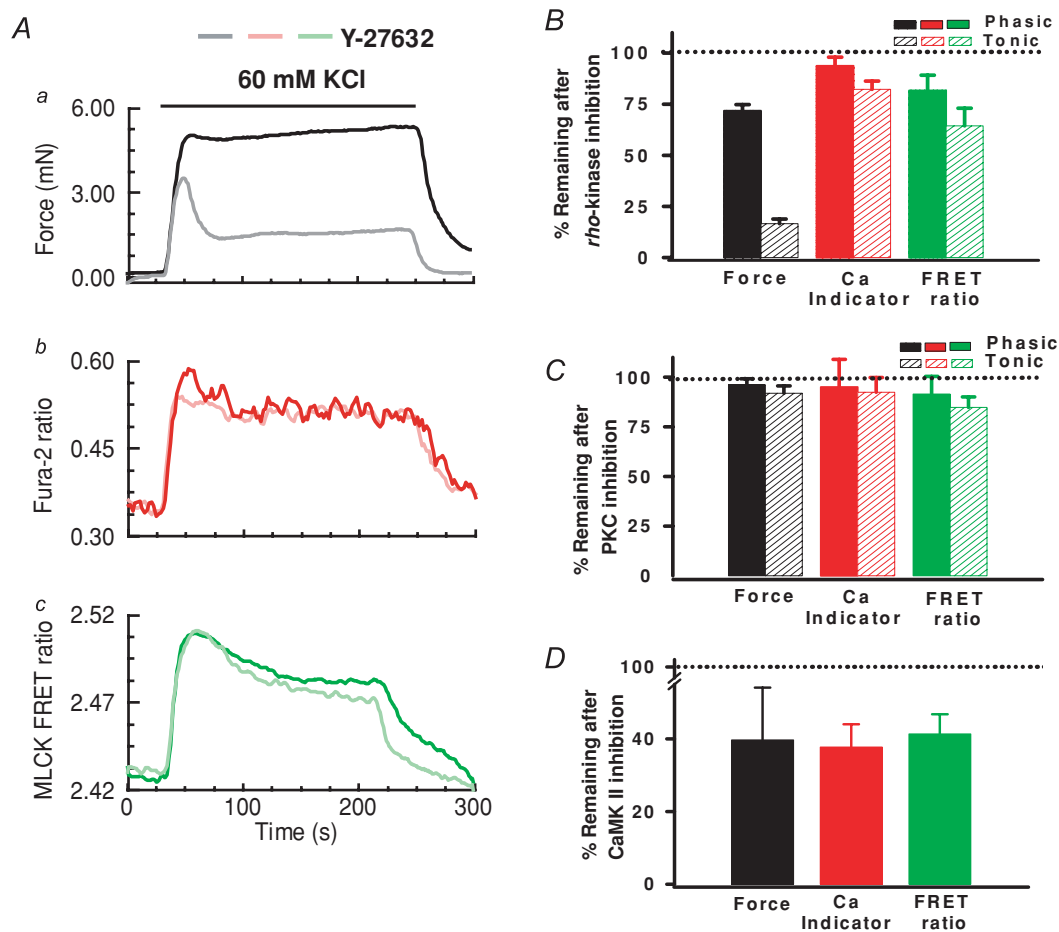


Figure 4. Kinases involved in Ca²⁺ sensitivity of smooth muscle contraction in small mesenteric arteries

A, ρ -kinase inhibition: effects of Y-27632 (3 μ M) on force (*a*), [Ca²⁺] (fura-2 ratio) (*b*), and MLCK FRET ratio (*c*). **B**, summarized data from experiments shown in **A**. Analysis shows that, pharmacological inhibition of ρ -kinase with Y-27632 significantly inhibited the tonic component of KCl induced force ($P < 0.001$, paired t -test, $n = 13$) but had relatively much less effect on phasic and tonic fura-2 fluorescence or phasic and tonic MLCK FRET ratio ($P < 0.05$, paired t -test, $n = 7$). **C**, inhibition of PKC: summarized data from experiments showing that Gö6976 (1 μ M) did not have a significant effect ($P > 0.05$, paired t -test, $n = 3-6$) on force, [Ca²⁺] (fluoro-4 fluorescence), and MLCK FRET ratio. **D**, inhibition of CaMK II: summarized data showing that KN-93 (10.0 μ M) applied during exposure to elevated KCl caused a decrease in force, [Ca²⁺] (fluoro-4 fluorescence), and MLCK FRET ratio. Analysis (paired t -test, $n = 3-5$) shows that CaMK II inhibition caused a significant decrease in force ($P < 0.01$, paired t -test), [Ca²⁺] (fluoro-4 fluorescence; $P < 0.05$, paired t -test), and MLCK FRET ratio ($P < 0.01$, paired t -test). The 'phasic' values of force, [Ca²⁺] (fura-2/fluoro-4 fluorescence), or MLCK FRET ratio were defined as the peak values reached initially after exposure to KCl, and the 'tonic' values were defined as those at the end of the exposure to KCl. Results are represented as means $\pm$ S.E.M.

elevated by exposure to elevated KCl. Previous biochemical experiments have indicated that phosphorylation of MLCK by PKA results in a change of affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$ only when $\text{Ca}^{2+}/\text{CaM}$ is not bound to MLCK (Adelstein *et al.* 1982; Nishikawa *et al.* 1984). In this set of experiments, we applied forskolin ($10.0 \mu\text{M}$) prior to exposure to KCl (Fig. 7A). The test was preceded by a control exposure to elevated K^+ , in order to provide a control recording of force, fura-2 ratio and MLCK FRET ratio. When the same concentration of external K^+ was applied in the presence of forskolin, the increase in MLCK FRET ratio was reduced, and force development was extremely small. In the presence of forskolin and elevated KCl, MLCK FRET ratio was approximately 50% of what it had been in elevated KCl in the absence of forskolin. A similar type of experiment (Fig. 7B) was also performed as follows: (1) an initial response to elevated KCl was obtained, (2) forskolin was applied coincidentally with removal of KCl, (3) KCl was re-applied to elevate $[\text{Ca}^{2+}]$ in the presence of forskolin, and, finally, (4) forskolin was then removed, in the continued presence of elevated KCl. The most important results are obtained at steps (3) and (4): presumed elevation of Ca^{2+} by KCl caused an increase in MLCK FRET ratio to about half what it was in the presence of elevated KCl alone, and removal of forskolin in the presence of elevated KCl restored the MLCK FRET ratio completely. These results are consistent with the concept that c-AMP-dependent protein kinase (PKA) can phosphorylate MLCK when the binding site on MLCK for $\text{Ca}^{2+}/\text{CaM}$ is not occupied, and decrease the affinity of that site for $\text{Ca}^{2+}/\text{CaM}$, when $\text{Ca}^{2+}/\text{CaM}$ rises subsequently.

Although it has been reported from biochemistry experiments that phosphorylation of MLCK by PKA when $\text{Ca}^{2+}/\text{CaM}$ is bound does not change affinity of MLCK

for $\text{Ca}^{2+}/\text{CaM}$, the issue had not been investigated in intact arteries. To elevate cAMP at a time when $\text{Ca}^{2+}/\text{CaM}$ should be bound to MLCK, and thus reveal in real time possible changes in affinity of MLCK for $\text{Ca}^{2+}/\text{CaM}$ immediately, we applied forskolin (0.1 to $100.0 \mu\text{M}$) during KCl induced contractions at a time when the MLCK FRET ratio and force had both reached approximately steady, elevated levels (Fig. 8B). At a concentration of $10.0 \mu\text{M}$ ($n=6$), forskolin applied at this time induced a rapid initial relaxation, followed by a much slower phase of relaxation that proceeded until the artery had relaxed completely (Fig. 8Aa). When normalized appropriately, the rates of change of MLCK FRET ratio (dFRET/dt) and force (dForce/dt) (Fig. 8Ab) were remarkably similar in time course, with the changes in MLCK FRET ratio just preceding those in force. The subsequent much slower relaxation is seen in the dForce/dt as a subsequent slowly declining phase of dForce/dt . A similar slow change in dFRET/dt did not occur. These effects of forskolin occurred over the range of $0.3 \mu\text{M}$ to $100.0 \mu\text{M}$ (Fig. 8B). Both the maximum initial rapid relaxation (open circles) and the MLCK FRET ratio change were limited to a decrease of approximately 50% over that range of concentration of forskolin, while the slow relaxation (filled triangles) proceeded to 100% (i.e. complete relaxation).

Forskolin produces an increase in $[\text{cAMP}]$ by a direct activation of adenylyl cyclase. Physiologically, increases in $[\text{cAMP}]$ in smooth muscle are produced after activation of β -adrenergic receptors (e.g. β_2 -AR). Therefore, it was important to determine whether activation of β -AR has the same effects as forskolin. During the tonic phase of force produced by KCl or by PE, we applied the β -AR agonist isoproterenol ($10.0 \mu\text{M}$) (Fig. 9A).

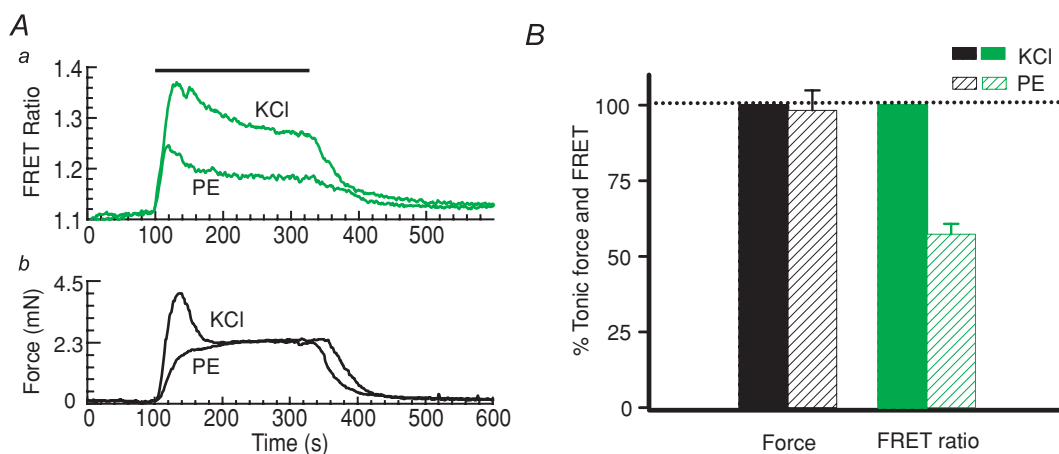


Figure 5. Increased Ca^{2+} sensitivity of force after α_1 -AR activation as compared to KCl exposure in mesenteric arteries

A, MLCK FRET ratio (a) and force (b) activated by KCl (60 mM) and PE ($1 \mu\text{M}$). B, summarized results show that during tonic phase of force elicited by KCl, MLCK FRET ratio is significantly less as compared to elicited by PE ($P < 0.01$, Mann-Whitney rank sum test, $n = 6$). Results are represented as means $\pm$ S.E.M.

Isoproterenol caused a strong reduction in PE-induced force, [Ca²⁺] (fura-2), and MLCK FRET ratio, but not in the KCl-induced force, Ca²⁺, or MLCK FRET ratio (Fig. 9B). In contrast, isoproterenol did relax arteries activated by α_1 -AR, but this was accompanied by reductions in [Ca²⁺] and MLCK FRET ratio. The fact that isoproterenol was unable to produce significant relaxation in arteries activated by KCl suggests that the mechanism of relaxation induced by β -AR activation involves primarily Ca²⁺. Thus, these data do not present evidence for β -AR-mediated changes in the affinity of MLCK for Ca²⁺/CaM.

Discussion

On the time scale used here, the time courses of [Ca²⁺] (fura-2 ratio) and activation of MLCK (MLCK FRET ratio)

appear approximately similar after elevation of [Ca²⁺] by KCl (e.g. Fig. 3A and Fig. 2 of Wier *et al.* 2008). The kinetics of the formation of Ca²⁺/CaM and of the binding of Ca²⁺/CaM to MLCK within the intracellular environment are not yet known in detail. However, a recent model predicts that half-times of activation of MLCK after elevation of [Ca²⁺] should be less than 1 s when [Ca²⁺] is above about 0.2 μ M (Fajmut *et al.* 2005). Thus, on the time scale used here, significant differences in the time courses of fura-2 ratio and MLCK FRET ratio probably represent phenomena other than kinetics of Ca²⁺/CaM binding to MLCK.

The time course of force was typically quite different from that of fura-2 ratio and MLCK FRET ratio, with force increasing (slightly) over very long periods of time when MLCK FRET ratio was decreasing. In the example of Fig. 3B, a slow, continuous decline in MLCK FRET

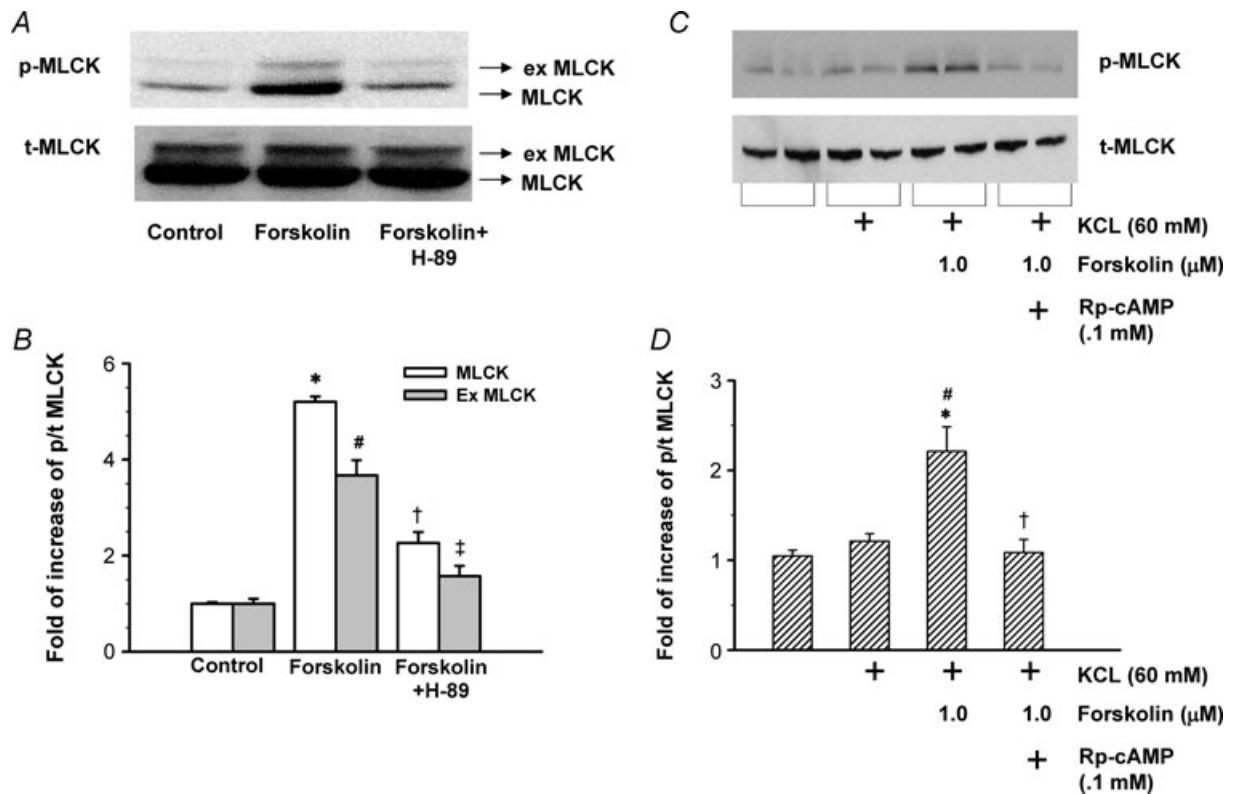


Figure 6. Forskolin causes increased phosphorylation of MLCK on Ser 1760, both exogenous biosensor and endogenous, and this effect is blocked by inhibition of PKA

A, phosphorylated MLCK (p-MLCK) and total MLCK (t-MLCK) in aorta. In each lane of the gels, upper band is the MLCK biosensor (labelled as ex MLCK), lower band is endogenous MLCK. B, summarized results are represented as means $\pm$ S.E.M. of the densitometric analysis of the bands illustrated in A. Both are significantly phosphorylated by exposure to 1 μ M forskolin (ANOVA, *, #, $P < 0.01$, forskolin treatment compared to controls, $n = 3$) and the effect is blocked by the inhibitor of PKA, H-89 (10.0 μ M) (ANOVA, †, ‡, $P < 0.01$, forskolin+H-89 compared to forskolin alone, $n = 3$). C, the competitive antagonist of cAMP binding to PKA, rp-cAMP (100.0 μ M), blocks the action of forskolin (1 μ M) to increase Ser 1760 phosphorylation during KCl exposure. D, summarized results presented as means $\pm$ S.E.M. of the densitometric analysis of the bands illustrated in C. (ANOVA, *, #, $P < 0.01$, compares control and KCl alone to KCl+forskolin respectively; †, $P < 0.05$ compares forskolin+rp-cAMP to forskolin alone $n = 3$ in each group.) KCl was elevated in all groups except control.

ratio begins about 10 s after the peak in FRET ratio and continues for another 120 s. Force, on the other hand, begins a slow increase at about 40 s after KCl and continues until the end of the record, at 300 s. Such slow changes are unlikely to represent the kinetics of force generation after activation of MLCK (much faster).

Ca²⁺ sensitivity of contraction

The small difference in the normalized fura-2 ratio and the MLCK FRET ratio that develops about 10 s after KCl (Fig. 3A) could indicate a slightly decreased affinity of MLCK for Ca²⁺/CaM, possibly mediated by Ca²⁺/CaM-dependent kinase II (CaMK II). Because the differences in time courses of fura-2 ratio and MLCK FRET ratios during KCl that we observed are small, however, our data are not consistent with a scheme in

which CaMK II has a large effect in 'desensitization of contraction' to Ca²⁺, as seems to occur in tracheal smooth muscle (Stull *et al.* 1990). Also, if significant CaMK II-mediated desensitization of contraction were occurring, one might have expected inhibition of CaMK II to cause an increase in MLCK activation and force, not a decrease (Fig. 4D). However, because [Ca²⁺] also decreased, net MLCK activation might have decreased, despite an increased affinity for Ca²⁺/CaM. Evaluation of this possibility would require a quantitative analysis beyond the scope of the present work. Nevertheless, force, Ca²⁺, and MLCK FRET ratio all decreased to approximately the same extent (Fig. 4D). Thus, the present data do not support the idea that CaMK II causes significant desensitization of contraction when [Ca²⁺] increases. Previous biochemical studies on a similar (i.e. vascular) smooth muscle (swine carotid artery) have reached similar conclusions to ours (Van Riper *et al.* 1995; Rokolya & Singer, 2000). In fact, it has been suggested that, rather than desensitize contraction to Ca²⁺, CaMK II activates contraction, through a mitogen activated protein kinase (MAPK)-dependent activation of MLCK (Kim *et al.* 2000). Our data could be consistent with such a mechanism, although the slow increase in force that would be attributable to this mechanism would seem to be small. Furthermore, our data show that this slow increase in force, without concomitant changes in MLCK activation may be due to *rho*-kinase mediated modulation of MLCP, as we discuss next.

Pharmacological inhibition of *rho*-kinase decreased strongly the tonic or slowly increasing component of KCl induced force generation in these arteries, without significant effects on [Ca²⁺] or MLCK activation (Fig. 4A, B). Because the initial peak of KCl-induced force was less affected, we suggest that *rho*-kinase is being activated *during* the KCl exposure. The effects on the initial peak force might be due to inhibition of a constitutive activity of *rho*-kinase. Activation of *rho*-kinase after GPCR activation, and the resulting 'Ca²⁺ sensitization' through inhibition of the targeting subunit, MYPT1, of MLCP are well characterized (Somlyo & Somlyo, 2003). It is now being recognized, however, that depolarization (KCl) is also a stimulus for 'Ca²⁺ sensitization' (Ratz *et al.* 2005; Dimopoulos *et al.* 2007). Ca²⁺-dependent activation of *rho* and *rho*-kinase has been reported in rabbit aortic smooth muscle cells (Sakurada *et al.* 2003) and depolarization induced contraction of rat caudal arteries also involves *rho*-kinase (Mita *et al.* 2002). *Rho*-kinase also activates CPI-17, the PKC (conventional and novel, c/n) activated 17 kDa inhibitor of MLCP. The importance of CPI-17 in mediating Ca²⁺ sensitization varies markedly with the type of smooth muscle, and its importance in rat mesenteric arteries is not yet clear. However, the inhibitor of conventional (i.e. Ca²⁺ activated) PKCs, Gö6976, had no effect (Fig. 4C), suggesting that in this tissue, CPI-17

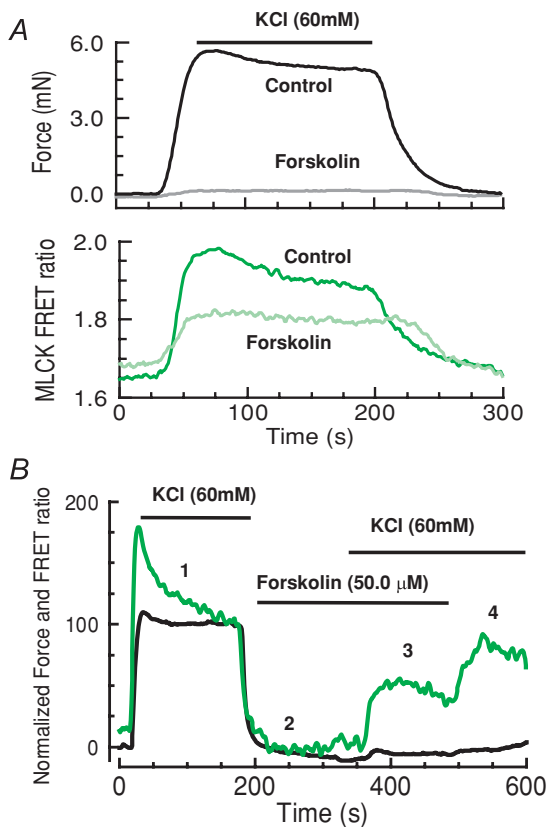


Figure 7. Forskolin causes relaxation and decreases MLCK FRET ratio, but does not change [Ca²⁺]

A, forskolin was applied before and during exposure to KCl. In the presence of forskolin, the force induced by KCl was very small (grey trace) while MLCK FRET ratio was reduced to approximately one-half the control value. B, simultaneous recording of force and MLCK FRET ratio (green). Experiment was performed as follows: (1) an initial response to elevated KCl was obtained, (2) forskolin was applied coincidentally with removal of KCl, (3) KCl was re-applied to elevate [Ca²⁺] in the presence of forskolin, and (4) forskolin was removed, in the continued presence of elevated KCl.

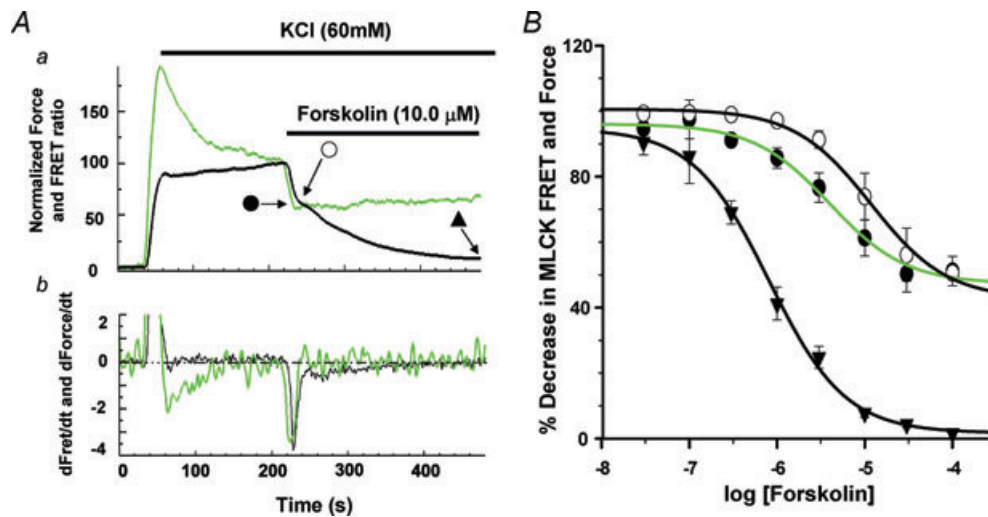


Figure 8. Concentration dependence of forskolin's effect to cause a rapid decrease in force and MLCK FRET ratio during KCl exposure

Aa, force and MLCK FRET ratio during experiment, normalized by subtracting baselines and dividing by the respective values just before the exposure to forskolin. Forskolin caused a very rapid, but incomplete decrease in MLCK FRET ratio, and a corresponding change in force. Force continued to decline, in a much slower phase that went to complete relaxation. Ab, Derivatives of normalized FRET and force with respect to time. Note the similarity in the rates of change of MLCK FRET ratio and force upon application of forskolin, with the change in FRET ratio preceding the force. B, concentration dependence of the effects of forskolin on FRET ratio and force. Open circles plot the magnitude of the initial relaxation, filled circles plot the magnitude of the change in FRET ratio, and filled triangles plot the value of force finally achieved, all as functions of the concentration of forskolin. Results are presented as means $\pm$ s.e.m. Symbols and arrows in Aa show how the measurements were made; $n = 6$ in each group.

is not prominently activated, either by Ca²⁺-dependent PKCs nor by *rho*-kinase, during membrane depolarization induced contractions.

The Ca²⁺ sensitivity of contractions activated by α_1 -AR appeared markedly higher than that of contractions

activated by KCl (Fig. 5), as MLCK FRET ratios were markedly lower with α_1 -AR activation than with KCl. Indeed the concept that GPCR agonists activate increases in 'Ca²⁺ sensitivity is well established (Somlyo & Somlyo, 2003). However, the interpretation of our observations

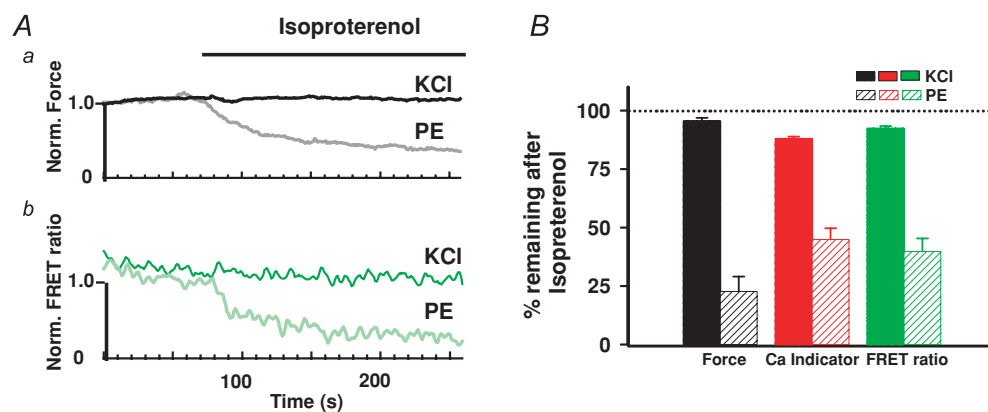


Figure 9. β -AR activation by isoproterenol caused a large reduction in the force, [Ca²⁺] and MLCK FRET ratio caused by exposure to PE but not to KCl

Aa, effect of isoproterenol on tonic force after exposure to KCl and PE. Force was normalized to the value existing at the time of exposure to isoproterenol (10.0 μ M). Ab, corresponding FRET ratios, normalized as above. Isoproterenol caused a reduction in PE-induced force and FRET ratio, but not in the KCl-induced force or FRET ratio. B, summarized results of effects of isoproterenol on KCL and PE induced force, Ca²⁺ indicator signal and MLCK FRET ratio in 6 arteries. All the mean values shown were significantly different from the controls (i.e. 100%) ($P < 0.05$, Mann-Whitney rank sum test, $n = 6$). Results are represented as means $\pm$ s.e.m.

is complicated by the fact that Ca^{2+} signalling activated by α_1 -AR can be heterogeneous, consisting under some circumstances of asynchronous propagating Ca^{2+} waves in different cells (Zang *et al.* 2001; Zacharia *et al.* 2007). Corresponding heterogeneity of the MLCK FRET ratio signals, if it had occurred, would not have been detectable in the spatially averaged fluorescence measurements made here. Averaging spatially inhomogeneous MLCK FRET fluorescence changes would be expected to result in a lower FRET signal than the peak values attained in individual cells.

PKA

Understanding the possible role of PKA in physiological regulation of MLCK has proven extremely difficult; PKA is involved in numerous cellular processes that affect intracellular $[\text{Ca}^{2+}]$ (and thus affect activation), such as entry of Ca^{2+} through voltage-dependent Ca^{2+} channels, availability of K^+ channels, Ca^{2+} uptake and release in the SR, and Ca^{2+} extrusion from the cell. Furthermore, activation of PKA by cAMP will be accompanied by other actions of cAMP, such as the direct activation of nucleotide-gated ion channels, and activation of Epacs (guanine nucleotide exchange factor directly activated by cAMP) (Schmidt *et al.* 2007). Application of forskolin, thus increasing cAMP, during GPCR induced contraction certainly decreases $[\text{Ca}^{2+}]$ by these mechanisms and this decrease in $[\text{Ca}^{2+}]$ is a major contributor to relaxation. In contrast, we have shown previously (Wier *et al.* 2008), as have others (Porter *et al.* 2006), that application of forskolin during KCl exposure does not change the $[\text{Ca}^{2+}]$. Since $[\text{Ca}^{2+}]$ does not change after application of forskolin, but MLCK FRET ratio decreases by up to 50%, the most straightforward interpretation of that result is that 'pharmacological' elevation of cAMP, through activation of adenylate cyclase (AC), decreases the affinity of the MLCK biosensor for Ca^{2+} /CaM. Exposure of arteries to forskolin also causes increased phosphorylation of Ser 1760 on MLCK, and the effect is blocked by a competitive antagonist (rp-cAMP) of the cAMP-dependent activation of PKA.

The fact that MLCK FRET ratio could not be decreased by more than about 50% by forskolin might indicate that Ser 1760 phosphorylated MLCK retains an appreciable affinity for Ca^{2+} /CaM. The rapid reduction in MLCK FRET ratio was accompanied closely in time by a rapid reduction in force, also to about 50% of the 'control' level, prior to application of forskolin. Other processes, apparently, activated a very much slower relaxation, which could proceed to complete relaxation, at the highest concentrations of forskolin used. Since intracellular $[\text{Ca}^{2+}]$ does not change under these circumstances, we suggest that these processes are most likely to be the

well-known stimulatory effect of cAMP on MLCP (Somlyo & Somlyo, 2003).

We attempted to determine whether PKA mediated phosphorylation of MLCK is an important mechanism of force relaxation in response to physiological (e.g. GPCR mediated), as opposed to pharmacological, increases in cAMP. This appears not to be the case in tracheal smooth muscle (Miller *et al.* 1983). In the arteries studied here, presumed full activation of β -AR by high concentration of isoproterenol caused only very minor relaxation and change in $[\text{Ca}^{2+}]$ and MLCK FRET ratio, when $[\text{Ca}^{2+}]$ was elevated by exposure to KCl. In contrast, isoproterenol caused large decreases in $[\text{Ca}^{2+}]$, MLCK FRET ratio and force in arteries contracted by activation of adrenoceptors (α_1 -AR). Taken together, these results indicate that the major (physiological) mechanism of β -receptor/cAMP mediated relaxation in these arteries is a decrease in $[\text{Ca}^{2+}]$, not regulation of MLCK or even of MLCP. If cAMP/PKA-dependent stimulation of MLCP occurred, it was clearly insufficient to cause any relaxation, when $[\text{Ca}^{2+}]$ was constantly elevated. Finally, the results indicate that there is something very different about the production of cAMP by direct activation of adenyl cyclase and by receptor mediated production of cAMP. We speculate that the concentrations of cAMP achieved by forskolin may be higher, and/or the spatial localization of the cAMP is different. Nevertheless, this remains a difficult issue to resolve because, as opposed to the case of KCl exposure, $[\text{Ca}^{2+}]$ decreases during physiological stimuli that increase $[\text{Ca}^{2+}]$ and cause relaxation. When both $[\text{Ca}^{2+}]$ and MLCK FRET ratio decrease, any change in affinity of MLCK for Ca^{2+} /CaM will be difficult to quantify, given that MLCK FRET ratio should decrease also as a result of less available Ca^{2+} /CaM.

Summary

After depolarization, the activation of MLCK, as judged by the FRET ratio, appears to follow the $[\text{Ca}^{2+}]$ closely. There is little evidence of regulation of MLCK by other kinases. On the other hand, the time course of force differs from that of $[\text{Ca}^{2+}]$ and MLCK activation. While the kinetics of the force generating reactions may contribute to this difference, *rho*-kinase also contributes, probably through the well-known modulation of MLCP activity. The mechanism(s) of activation of *rho*-kinase are not known, but could be Ca^{2+} dependent. Ca^{2+} activated forms of PKC seem not to be involved. The affinity of MLCK for Ca^{2+} /CaM can be regulated by 'pharmacological' increases in cAMP/PKA, even when Ca^{2+} /CaM is already bound, but not by physiological increases after β -adrenoceptor activation. Analysis of the MLCK FRET ratio supports the concept that Ca^{2+} sensitivity of contraction activated by α_1 -AR is markedly higher than that during contraction

activated by depolarization, even though Ca²⁺ sensitivity also increases after depolarization.

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Author contributions

HR and JZ performed the functional experiments on the isolated arteries, analyzed these data and contributed to writing the manuscript. ML performed and analyzed the biochemical experiments. HR measured physiological parameters of intact mice. WGW participated in experiments and was primarily responsible for experimental design, analysis of data, and

writing of the manuscript. All authors read and approved the manuscript.

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