

Different Effect of Rho Kinase Inhibition on Calcium Signaling in Rat Isolated Large and Small Arteries

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Key Words

Aorta · Calcium · Myogenic tone · Myosin light chain · Resistance mesenteric artery · Rho kinase · Vascular smooth muscle contraction

Abstract

In addition to its role in the regulation of artery contraction, Rho kinase (ROCK) was reported to be involved in the cytosolic calcium response to vasoconstrictor agonists in rat aorta and superior mesenteric artery (SMA). However, it remains to be determined whether ROCK also contributes to calcium signaling in resistance arteries, which play a major role in blood pressure regulation. The investigation of the effect of ROCK inhibition on the calcium and contractile responses of rat resistance mesenteric artery (RMA), in comparison with aorta and SMA, indicated that the calcium response to noradrenaline was inhibited by the ROCK inhibitor Y-27632 in aorta and SMA but not in RMA. The effect of Y-27632 on the calcium signal was unaffected by cytochalasin-D. ROCK activation in noradrenaline-stimulated arteries was confirmed by the inhibition of myosin light chain phosphorylation by Y-27632. Moreover, noradrenaline-induced calcium signaling was similarly inhibited by nimodipine in aorta, SMA and RMA, but nimodipine sensitivity of the contraction increased

from the aorta to the RMA, suggesting that the contraction was controlled by different sources of calcium. In pressurized RMA, Y-27632 and H-1152 depressed pressure-induced calcium responses and abolished myogenic contraction. These results stress the important differences in calcium signaling between conductance and resistance arteries.

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Introduction

In smooth muscles, changes in the cytosolic calcium concentration play a key role in regulating diverse cellular processes, including growth, differentiation and contraction. The increase in the cytosolic calcium concentration by agonist stimulation and the subsequent formation of the calcium-calmodulin complex leads to the activation of myosin light chain (MLC) kinase which phosphorylates the MLC LC₂₀ resulting in smooth muscle contraction [1]. Dephosphorylation of LC₂₀ by the MLC phosphatase evokes smooth muscle relaxation.

Vasoconstrictor agonists stimulate G protein-coupled receptors in the plasma membrane. On the one hand, G $\alpha_{q/11}$ stimulation promotes the activation of phospholipase C β (PLC β), which catalyzes the formation of inositol-

1,4,5-triphosphate (IP₃) and mobilizes external and internal calcium to increase the cytosolic calcium concentration and trigger the smooth muscle contraction [1]. On the other hand, the stimulation of G $\alpha_{12/13}$, together with G $\alpha_{q/11}$, induces the activation of the RhoA/Rho kinase (ROCK) pathway [2, 3], which favors the phosphorylated state of LC₂₀, at a constant level of intracellular calcium by inhibiting the activity of MLC phosphatase [4]. This characterizes the well-known calcium sensitization process [5].

Resistance arteries play a prominent role in the regulation of local blood flow. Their tone is controlled not only by chemical mediators but also by transmural pressure. Increased intraluminal pressure causes resistance artery contraction, which has been shown to involve the activation and the translocation of RhoA to the plasma membrane, probably within caveolae, where RhoA is able to activate the ROCK-dependent sensitization of the contraction to calcium [6]. The pressure signal is proposed to be transduced from the extracellular matrix by integrins, which can recruit focal-adhesion kinase, tyrosine kinases such as c-Src kinase and phospholipids [7] and further stimulate RhoGTPase or cytoskeletal proteins [8].

We have demonstrated for the first time that ROCK is involved in agonist-activated calcium entry in rat aorta and superior mesenteric artery (SMA) [9]. This observation has been confirmed in noradrenaline-activated rat penile small artery [10], but the contribution of ROCK to calcium signaling in resistance mesenteric artery (RMA) is unknown. In addition, the involvement of ROCK in the change in cytosolic calcium induced by increased transmural pressure in resistance artery is still conflicting.

ROCK-sensitive calcium entry has been shown to be distinct from voltage (VOC)- or store-operated calcium channels [9]. Ghisdalet al. [9] demonstrated that in aorta and SMA, ROCK activation by vasoconstrictor agonists is involved in the opening of non-selective calcium channels. These channels most probably belong to the family of the TRP (transient receptor potential) channel proteins, which are known to be expressed in vascular smooth muscle [11, 12]. Activation of non-selective cation channels could then contribute to the increase in cytosolic calcium concentration and/or lead to the depolarization of the cell membrane resulting in VOC activation.

The objective of the present study was to investigate the involvement of ROCK in the change in cytosolic calcium evoked by an agonist or by mechanical stimulation in small resistance arteries compared to observations performed in large arteries. To this aim, the role of ROCK in the calcium signal induced by noradrenaline was in-

vestigated in three types of arteries: aorta, an elastic conducting artery, SMA, a muscular distributing artery and a small RMA. In the latter, the contribution of ROCK to the change in cytosolic calcium evoked by increased transmural pressure was determined.

Our results indicated that ROCK is not involved in the change in cytosolic calcium in response to noradrenaline in RMA, which is contrary to what is observed in aorta and SMA. However, ROCK contributes to the change in cytosolic calcium associated with the myogenic response in pressurized RMA.

Materials and Methods

Tissue Preparation

Male Wistar rats (mean body weight of 250 g) were anesthetized and sacrificed by decapitation, in accordance with institutional guidelines for the use of experimental animals. Aorta, SMA or 4th-order RMA were quickly removed and immersed in buffered saline solution (composition in mM: NaCl 137; KCl 6; MgCl₂ 1.2; CaCl₂ 2; glucose 10, and HEPES 10 at pH 7.4 with Tris). Arteries were cleaned of all fat and connective tissue. The endothelium was carefully removed from aorta and SMA by gently rubbing the lumen of the artery with forceps tips, but not from most RMA to prevent damage to small resistance arteries. In some RMA, the endothelium was removed by passing a wire several times through the lumen.

Measurement of Contractile Tension and Cytosolic Calcium Concentration in Aorta, SMA and RMA

Cytosolic calcium was measured in 2-mm rings from the aorta, SMA and RMA (mean internal diameter at 100 mm Hg: 315 $\pm$ 9 μ m, n = 33) loaded with 5 (aorta/SMA) or 3.5 μ M (RMA) fura-2 acetoxymethyl ester (fura-2 AM) dissolved in physiological solution (PSS; composition in mM: NaCl 122; KCl 5.9; NaHCO₃ 15; MgCl₂ 1.2; CaCl₂ 1.25, and glucose 11) containing 0.05% Cremophore EL for 3 h (aorta/SMA) or 90 min (RMA) at room temperature. After the fura-2 loading period, rings were mounted under a tension of 20 (aorta), 8 (SMA) and about 2 mN in RMA (as determined by the normalization procedure [13]) in a 3-ml bath, which is part of a fluorimeter (CAF110; JASCO, Tokyo, Japan), perfused with PSS containing 100 μ M N ω -nitro-L-arginine (NNA), to prevent the production of NO [14], and gassed with 95% O₂ and 5% CO₂ at 37°C. Fluorescence signals at 340 nm (F₃₄₀) and 380 nm (F₃₈₀) and the F₃₄₀/F₃₈₀ ratio were simultaneously recorded with the contractile tension, which was measured by an isometric force transducer using data acquisition hardware (MacLab) and data recording software (Chart v3.3; ADInstruments Pty Ltd., Castle Hill, N.S.W., Australia). After washing, artery rings were first stimulated with 100 mM PSS KCl solution (composition in mM: NaCl 27; KCl 100; NaHCO₃ 15; MgCl₂ 1.2; CaCl₂ 1.25, and glucose 11) in order to assess vessel integrity. Washout and a resting period of 10 min were allowed before starting the experiment.

In a first set of experiments (n = 3–10), the artery was incubated for 10 min in the presence of the ROCK inhibitor Y-27632

(3–10 μM), or the same volume of water as control experiments, before the noradrenaline-response curve was performed by increasing the concentration of noradrenaline from 10 nM up to 10 μM every 2 min, which corresponds to the plateau phase of the noradrenaline-induced calcium signal, in the continuous presence of the inhibitor or water.

In a second set of experiments ($n = 3\text{--}11$), the artery ring was incubated with nimodipine (0.1 μM), or the same volume of solvent as control experiment, before contraction was induced by a single concentration of noradrenaline (1 μM for aorta and SMA and 3 μM for RMA). In some experiments, Y-27632 (10 μM) was injected into the bath 3 min after the injection of noradrenaline, which corresponds to the plateau phase of the noradrenaline-induced calcium signal.

In a third set of experiments, in order to test the effect of cytochalasin-D, aorta ($n = 5\text{--}9$) and SMA ($n = 4\text{--}12$) were incubated for 1 h with cytochalasin-D (20 μM) during the fura-2 loading period, before mounting the vessel in the bath. Contraction was induced by a single concentration of noradrenaline (1 μM). Y-27632 (10 μM) was added 3 min after the injection of noradrenaline during the plateau phase of the calcium response induced by noradrenaline. RMA ($n = 6$) was mounted in the organ bath and first stimulated with noradrenaline (10 μM) and then incubated with cytochalasin-D (20 μM) for 20 min before a second response to noradrenaline (10 μM) was produced. Controls were performed in the absence of cytochalasin-D (with dimethyl sulfoxide, DMSO) or in the absence of Y-27632 (with water).

The calcium signal was calibrated at the end of each experiment. The maximal ratio (R_{max}) was obtained by adding ionomycin (10 μM) in PSS KCl solution, while the minimal ratio (R_{min}) was measured in the presence of EGTA (2.6 mM). After washing, autofluorescence of the artery was measured at 340 and 380 nm by quenching fura-2 fluorescence with MnCl_2 (6.6 mM). Autofluorescence values were subtracted from experimental values measured at 340 and 380 nm in order to calculate the cytosolic calcium concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) by the application of the Grynkiewicz equation, as described previously [9, 15].

Measurement of Diameter and Cytosolic Calcium in Pressurized RMA

Rat RMA (mean external diameter at 75 mm Hg of intraluminal pressure: $345 \pm 5 \mu\text{m}$, $n = 21$) were dissected, mounted onto two glass cannulae in the vessel chamber of a pressure myograph (Living Systems Instruments, www.livingsys.com) filled with physiological buffered salt solution (PBS; composition in mM: NaCl 130; KCl 3.7; NaHCO_3 14.9; KH_2PO_4 1.2; MgSO_4 1.2; CaCl_2 1.6; glucose 11, and HEPES 5) containing 100 μM NNA, oxygenated with 95% O_2 and 5% CO_2 , placed on the stage of an inverted microscope, stretched until they appeared straight at 100 mm Hg and pressurized to 75 mm Hg as basal pressure. The lumen diameter and the cytosolic calcium concentration were simultaneously monitored using the IonOptix Fluorescence hardware and the IonWizard 6.0 (IonOptix) data recording software. After a 30-min resting period at 37°C, the artery was stimulated first with a PBS solution enriched with KCl (PBS KCl; composition in mM: NaCl 33.7; KCl 50; NaHCO_3 14.9; KH_2PO_4 1.2; MgSO_4 1.2; CaCl_2 1.6; glucose 11, and HEPES 5) and then, after a 10-min washing period, with phenylephrine 1 μM on the plateau of which we added 1 μM acetylcholine in order to check the endothelial function. Vessels that did not respond to these first stimulations were dis-

carded. After 20 min of stabilization, the bath solution was replaced by a fura-2-AM solution (3.5 μM) for 45 min in the dark at room temperature without oxygenation. The fura-2-loaded vessel was then washed three times with PBS. After stabilization at 37°C, the artery was stimulated with 1 μM phenylephrine. The addition of acetylcholine (1 μM) on the plateau phase of the phenylephrine-induced calcium signal and contraction completely relaxed the vessel diameter without affecting the calcium signal, as expected as the recording system used to measure the calcium signal only detected front surface fluorescence from the external smooth muscle cell layer. The vessel was stabilized by an additional 10-min resting period before the experiments.

In a first set of experiments ($n = 3\text{--}8$), the concentration-response curve of noradrenaline was obtained by adding cumulative concentrations of noradrenaline from 10 nM up to 10 μM . After washing, the vessel was incubated with 3–10 μM of the ROCK inhibitor (Y-27632) for 10 min and the concentration-response curve was again recorded in the continuous presence of the inhibitor. Controls were performed in the absence of the inhibitor.

In a second set of experiments ($n = 3\text{--}11$), a pressure-diameter-calcium curve was made where the diameter and the calcium signal were recorded simultaneously in response to stepwise pressure increases (steps of 25 mm Hg) from 25 to 100 mm Hg. After washing, the vessel was incubated with the inhibitor (3–10 μM of Y-27632 or 1 μM of H-1152) for 10 min and the pressure-diameter-calcium response curve was again determined in the continuous presence of the inhibitor. Controls were performed in the absence of the inhibitor.

A last pressure curve was made at the end of each experiment in a calcium-free medium supplemented with 2 mM EGTA in order to determine the passive diameter of the artery. Autofluorescence of the vessel was assessed before the fura-2-AM incubation. The myogenic tone (MT) was quantified as the percentage of the difference between active (AD) and passive (PD) diameter:

$$\text{MT} = [(\text{PD} - \text{AD})/\text{PD}] \times 100.$$

Determination of LC_{20} and Ezrin/Radixin/Moesin Phosphorylation

Phospho-MLC (p LC_{20} ; in aorta and RMA, $n = 8\text{--}11$) and phospho-ezrin-radixin-moesin (pERM; in RMA, $n = 6$) were determined by Western blot. Prior to Western blot assessment, aorta or RMA rings (4 mm) were stimulated with 1 (aorta) or 10 μM (RMA) noradrenaline for 2 min in the absence or presence of the inhibitor (10 μM of Y-27632 with a 10-min preincubation) in PSS solution supplemented with 100 μM NNA. Aorta and RMA rings were snap-frozen in liquid nitrogen. In order to avoid dephosphorylation of phosphoproteins, artery rings were fixed in 10% acetone/trichloroacetic acid for 1 h at -80°C and 20 min at -20°C , and washed in cold acetone. Proteins were then extracted with an urea-containing buffer (urea, 9.5 M; IGEPAL CA-630 2%; Pharmalytes GE Healthcare 1%; 2-mercaptoethanol 5%) supplemented with 1% Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, Mass., USA). Proteins were resolved by SDS page on 4–12% Bis-Tris gels NuPAGE (Invitrogen, Merelbeke, Belgium) and transferred to polyvinylidene difluoride membranes. Membranes were probed with anti-p LC_{20} (1:1,000) antibody, anti-pERM (1/500) antibody and anti-actin (1:2,000) antibody as loading control. Bands were detected with fluorescent

secondary antibodies and quantified with an Odyssey infrared imaging system (Li-Cor, Westburg, Leusden, The Netherlands). The expression of pLC₂₀ and pERM was normalized to actin expression for each sample.

Drugs

Fura-2 AM, H-1152 and ionomycin were obtained from Calbiochem (Bierges, Belgium). Noradrenaline, NNA, EGTA, cytochalasin-D, DMSO and Cremophore-EL were from Sigma-Aldrich (Bornem, Belgium). Y-27632 was from Tocris Bioscience (Bristol, UK). Nimodipine was a gift from Bayer AG (Leverkusen, Germany). Primary antibodies: rabbit polyclonal anti-phospho-LC20 came from Sigma-Aldrich; rabbit polyclonal anti-phospho-ezrin (Thr⁵⁶⁷)/radixin (Thr⁵⁶⁴)/moesin (Thr⁵⁵⁸) was from Cell Signaling (Bioké, Leiden, The Netherlands); goat polyclonal anti-actin came from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies: goat anti-rabbit (CF770) was from VWR (Leuven, Belgium) and donkey anti-goat (Alexa Fluor) came from Invitrogen.

Statistical Analysis

Data are presented as means $\pm$ SEM from at least 3 artery samples. Calcium signal and contractile responses were normalized to the first KCl response induced in the same artery ring. The noradrenaline concentration producing 50% of the maximal effect (EC₅₀) was calculated by non-linear curve fitting using a sigmoidal dose-response function. The calcium/contraction relationships were analyzed using a linear regression comparing slopes in the absence/presence of Y-27632. The effect of inhibitor was analyzed using either two-way ANOVA when stimulation-response curves were compared or one-way ANOVA when responses to a single stimulation were compared with Bonferroni's post hoc test. Statistical analyses were performed with the Prism GraphPad software. Values of $p < 0.05$ indicated a significant difference.

Results

Effect of ROCK Inhibition on the Calcium Signal and the Contraction Evoked by Noradrenaline in Large and Small Arteries

The effect of ROCK inhibition on calcium and contractile isometric responses to noradrenaline was tested in aorta, SMA and RMA (fig. 1a–c). In all types of arteries, noradrenaline increased the cytosolic calcium and the contractile tension. In aorta and SMA, the calcium response was more sensitive to noradrenaline than the contraction measured after 2 min of stimulation, while in RMA both responses exhibited similar sensitivity to the α -adrenergic agonist so that the ratio of noradrenaline EC₅₀ values for the contraction and the calcium response decreased from aorta (ratio of 7.6) to RMA (ratio of 1.1; table 1).

In all arteries tested, the ROCK inhibitor Y-27632 (3–10 μ M) significantly depressed the contractile tension in

Table 1. pD₂ and ratio of EC₅₀ values of noradrenaline-induced contraction and calcium responses in aorta, SMA and RMA

	pD ₂		EC ₅₀ ratio contraction/ $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$
	contraction	$\Delta[\text{Ca}^{2+}]_{\text{cyt}}$	
Aorta	7.06 $\pm$ 0.10 (n = 10)	7.94 $\pm$ 0.11 (n = 10)	7.6
SMA	6.09 $\pm$ 0.23 (n = 9)	6.57 $\pm$ 0.33 (n = 9)	3
RMA	5.81 $\pm$ 0.08 (n = 7)	5.87 $\pm$ 0.13 (n = 7)	1.1

pD₂ values from the noradrenaline-response curves of figure 1 were given by non-linear regression in aorta, SMA and RMA in isometric configuration.

response to noradrenaline. However, the inhibitory potency of Y-27632 decreased from aorta (fig. 1a), where the contraction was completely abolished by 10 μ M Y-27632, to RMA (fig. 1c), where only the contractile response to higher concentrations of noradrenaline was depressed. Y-27632, at 3 and 10 μ M, inhibited the calcium signal induced by noradrenaline in aorta (fig. 1a) and SMA (fig. 1b), as previously reported [9]. However, ROCK inhibition did not affect the calcium response to noradrenaline in RMA (fig. 1c). The effect of Y-27632 in RMA was not affected by removal of the endothelium (online suppl. fig. 1; see www.karger.com/doi/10.1159/000341230 for all suppl. material). As already observed in aorta and SMA [9], the increase in cytosolic calcium in RMA induced by enhanced KCl concentrations in the bath was not changed in the presence of Y-27632, while the contraction was depressed (online suppl. fig. 2).

Recording of the isobaric contractile response to noradrenaline in RMA pressurized at 75 mm Hg modified the pattern of the contraction sensitivity to Y-27632, which depressed the responses to lower concentrations of noradrenaline without affecting the maximum contraction. This effect probably reflects an inhibition of the myogenic tone by Y-27632. The calcium signal measured in isobaric configuration was not affected by Y-27632, as observed in isometric condition (fig. 1d).

Plotting contraction as a function of the change in cytosolic calcium revealed that in aorta (fig. 2a), Y-27632 did not modify the slope of the calcium/contraction relationship given by linear regression of the data points, while in SMA and RMA, the steepness of the calcium/contraction relationship was decreased in the presence of Y-27632 and the slopes of the linear regression of the data points obtained with and without Y-27632 were significantly different (fig. 2b, c).

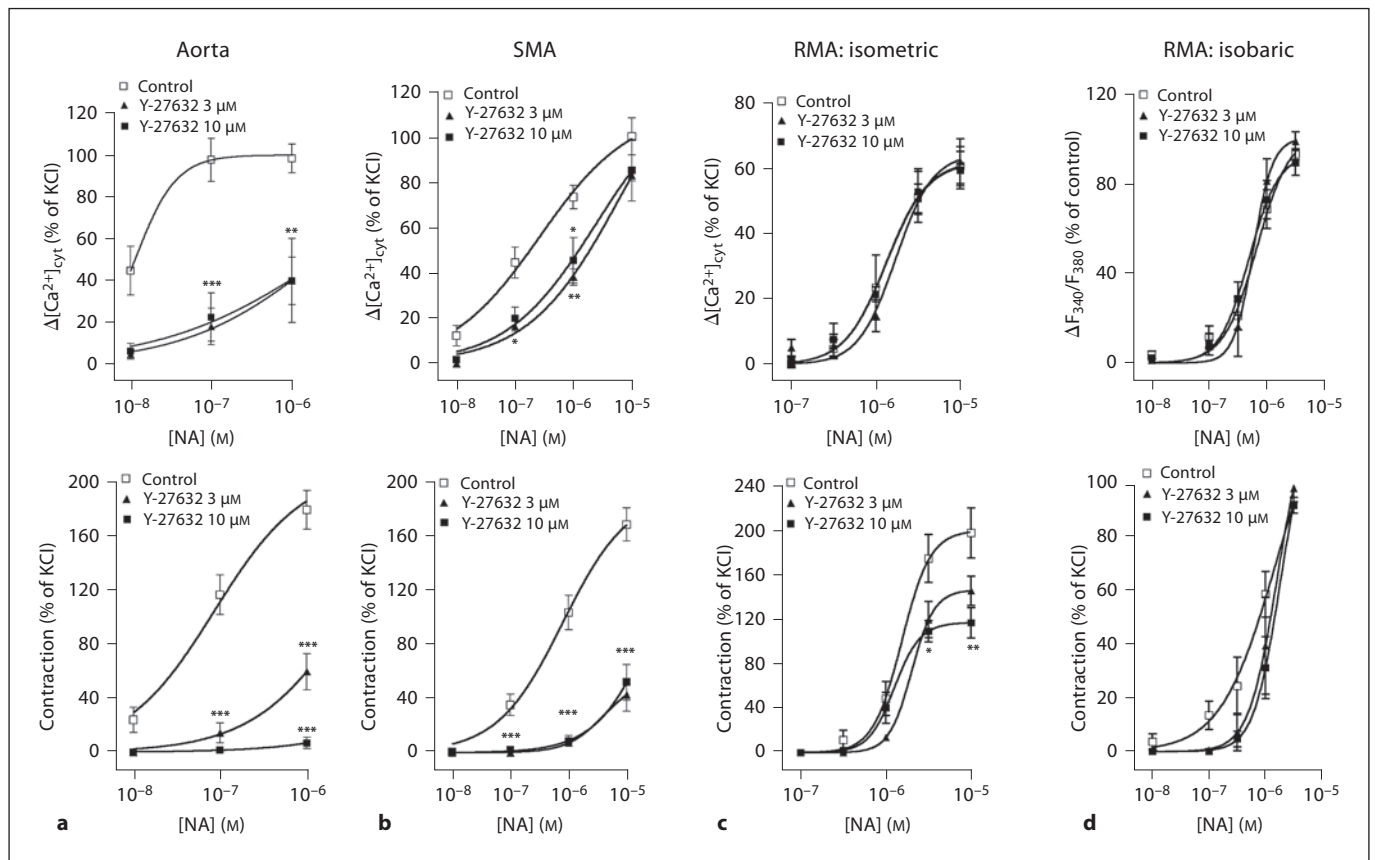


Fig. 1. Effect of the ROCK inhibitor Y-27632 on the calcium signal and the contraction induced by noradrenaline in aorta, SMA and RMA. Changes in cytosolic calcium ($\Delta[\text{Ca}^{2+}]_{\text{cyt}}$, upper graphs) and contractile tension (lower graphs) were simultaneously measured in control ($\square$) artery or in artery incubated in the presence of 3 ($\blacktriangle$) or 10 μM ($\blacksquare$) Y-27632 in aorta (**a**), SMA (**b**) and RMA in isometric (**c**) and isobaric condition (**d**). Data are expressed as

percent of the response to KCl for isometric measurements or of the control curve for RMA in isobaric condition, and are mean values from 3–10 different arteries. Vertical bars represent the SEM values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control; two-way ANOVA with a Bonferroni post hoc test. The data in aorta and SMA in the presence of Y-27632 at 10 μM have been obtained from a previous publication [9].

Effect of ROCK Inhibition on the Calcium Signal and the Contraction in Pressurized RMA

The involvement of ROCK in the myogenic response of RMA to increased pressure was investigated by performing pressure-diameter-calcium curves before and after a 10-min incubation with Y-27632 (3–10 μM). RMA were pressurized at a basal pressure of 75 mm Hg. Pressure curves in calcium and calcium-free solutions were made by increasing pressure from 25 to 100 mm Hg. The myogenic contraction started at 50 mm Hg. At 100 mm Hg, pressurized RMA developed a myogenic tone of 22% of the passive diameter measured in calcium-free medium ($n = 11$; fig. 3). Contractions were associated with the increased cytosolic calcium concentration (fig. 3). In agreement with the report by Dubroca et al. [6], the RMA

myogenic response was abolished in the presence of Y-27632. A similar level of inhibition was observed at 3 or 10 μM Y-27632 (fig. 3, right panels). In addition, ROCK inhibition also depressed the calcium response to pressure: at 100 mm Hg, the calcium response was inhibited by 53 ($n = 3$) and 57% ($n = 5$) in the presence of 3 and 10 μM Y-27632, respectively. The same effect was observed in the presence of H-1152 (1 μM), the more potent inhibitor of ROCK, which inhibited the calcium response by 52% ($n = 4$) and abolished the myogenic contraction (fig. 3b). The vessel integrity after incubation with Y-27632 was confirmed by the observation that the response to phenylephrine recorded after a 15-min washout of the inhibitor was not different from the response measured at the beginning of the experiment.

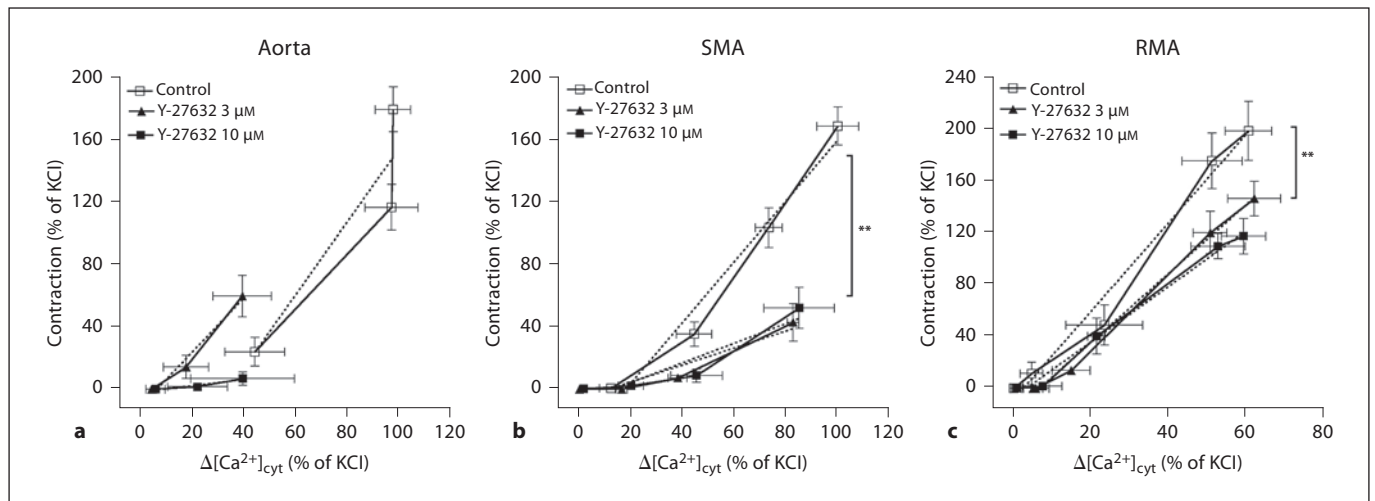


Fig. 2. Effects of Y-27632 on calcium/contraction relationship in aorta, SMA and RMA. Isometric contraction measured in control ($\square$) and 3 ($\blacktriangle$) or 10 μM ($\blacksquare$) Y-27632-treated rings in aorta (**a**), SMA (**b**) and RMA (**c**) was plotted as a function of the cytosolic

calcium concentration from data presented in figure 1. Dotted lines were obtained by linear regression of the experimental data. ** $p < 0.01$, Y-27632-treated arteries vs. control.

Contribution of VOC Channels to Noradrenaline-Induced Responses

To determine whether there could be a relationship between the origin of the increase in the cytosolic calcium induced by noradrenaline and its sensitivity to Y-27632, we investigated the sensitivity to the VOC channel blocker nimodipine of the calcium response in the three vascular models (fig. 4). The calcium signal (fig. 4a) induced by noradrenaline was inhibited by nimodipine (0.1 μM) by about 60% in all artery types: 63% ($n = 6$) in aorta, 60% ($n = 7$) in SMA and 61% ($n = 3$) in RMA. On the other hand, the contractile tension (fig. 4b) was only slightly inhibited in aorta (25%, $n = 6$), but it was inhibited by 63% ($n = 7$) in SMA and 88% ($n = 3$) in RMA, suggesting that noradrenaline contraction in RMA was nearly completely dependent on VOC activation.

The Actin Cytoskeleton Is Not a Prerequisite for Calcium Regulation Mechanisms in Arteries

As most of the ROCK targets are involved in actin-filament dynamics [16], we investigated whether the effect of ROCK on calcium signaling was mediated through the actin cytoskeleton. We, therefore, measured calcium entry signal after noradrenaline stimulation in aorta, SMA and RMA in the presence of an actin depolymerizing agent, cytochalasin-D (20 μM ; fig. 5). We then investigated the effect of ROCK inhibition by Y-27632 on

cytochalasin-D-treated aorta or SMA rings stimulated with noradrenaline (fig. 5).

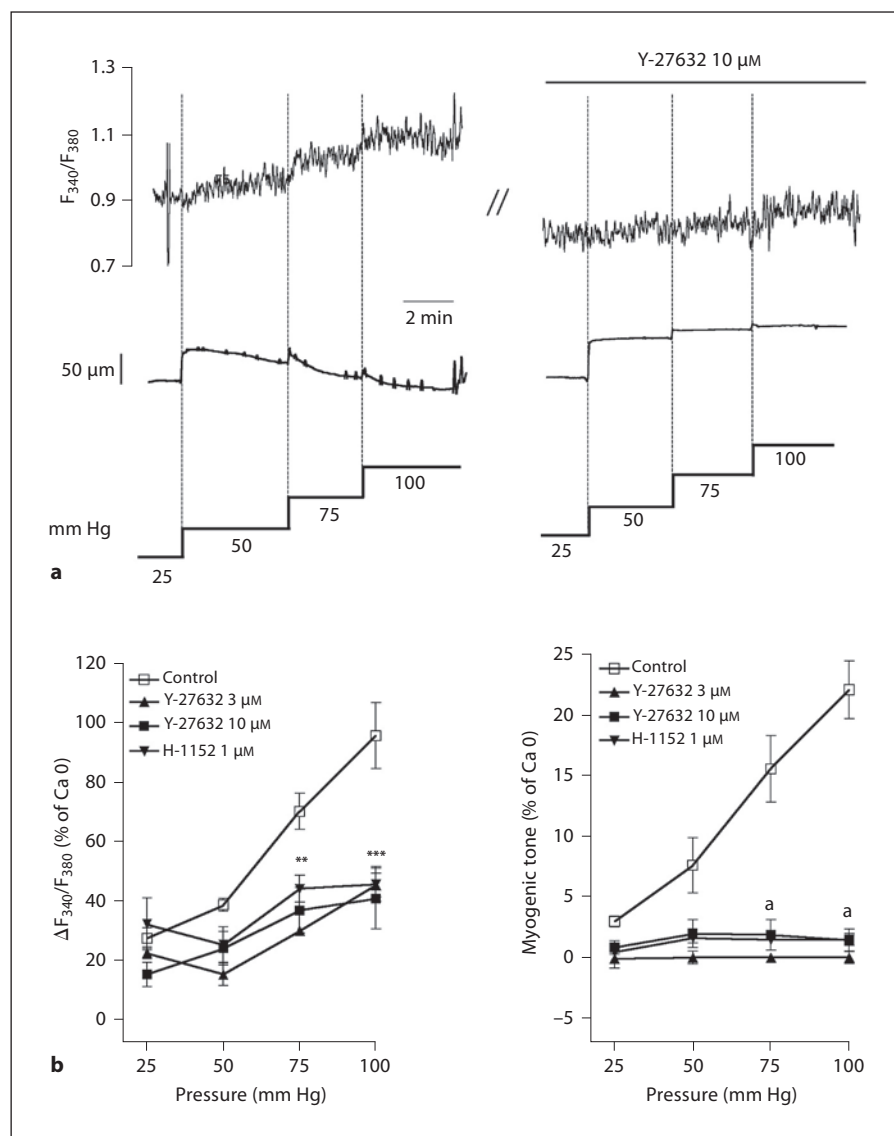
Cytochalasin-D completely abolished the artery contraction in aorta, SMA and RMA, but it did not affect the calcium signal induced by noradrenaline in any of the three artery models (fig. 5). Furthermore, in cytochalasin-D-treated aorta and SMA, the calcium response induced by noradrenaline was inhibited by Y-27632 in a similar manner as in control arteries: Y-27632 (10 μM) inhibited the noradrenaline-induced calcium signal by 62% ($n = 5$) and 69% ($n = 4$) in cytochalasin-D-treated aorta and SMA, respectively (fig. 5). These results suggested that the actin cytoskeleton was not implicated in the agonist-dependent calcium signaling pathway.

ROCK Inhibition Decreased ERM and MLC Phosphorylation in Noradrenaline-Stimulated Arteries

By inhibiting the activity of MLC phosphatase, ROCK contributes to the increased phosphorylation of LC₂₀ leading to increased developed tone [4]. To ensure that ROCK was activated in aorta as well as in RMA by noradrenaline stimulation, we measured the effect of Y-27632 (10 μM) on phospho-LC₂₀ level in noradrenaline-stimulated artery rings.

As shown in figure 6, in both aorta and RMA, LC₂₀ was significantly phosphorylated by noradrenaline. In

Fig. 3. Effect of ROCK inhibitors on the calcium signal and vessel diameter in pressurized resistance mesenteric artery. **a** Typical recording of cytosolic calcium concentration (expressed as F_{340}/F_{380}) and vessel diameter in response to pressure variation before and after incubation with 10 μM Y-27632 for 10 min. Vessel diameter at a basal pressure of 75 mm Hg was 295 μm . **b** Mean values of the change in cytosolic calcium (left panel) and myogenic tone (right panel) in response to increased pressure in the absence ($\square$) or presence of the ROCK inhibitors, 3 ($\blacktriangle$) or 10 μM ($\blacksquare$) Y-27632 or 1 μM ($\blacktriangledown$) H-1152, normalized to the responses measured in calcium-free medium. Data are means $\pm$ SEM ($n = 3$ –11 experiments). ** $p < 0.01$, *** $p < 0.001$ vs. control, except for H-1152 where the difference was significant only at 100 mm Hg (*** $p < 0.001$). ^a $p < 0.001$, vs. control; two-way ANOVA with a Bonferroni post hoc test.



the presence of the ROCK inhibitor, the level of phospho-LC₂₀ was inhibited by 59% ($n = 11$) in aorta and 84% ($n = 8$) in RMA.

Activation of ROCK in noradrenaline-stimulated arteries was confirmed by measuring the phosphorylation of ERM proteins, which are known targets of ROCK [17]. A previous report indicated that in rat aorta and SMA, noradrenaline-induced ERM phosphorylation is significantly depressed by Y-27632 [18]. As shown in figure 7, the same observation was obtained in RMA: in the presence of the ROCK inhibitor, the level of pERM was significantly inhibited (42%; $n = 6$).

Discussion

The present study investigated the role of ROCK in the regulation of the cytosolic calcium concentration after noradrenaline or mechanical stimulation in rat vascular smooth muscle. While ROCK is involved in noradrenaline-induced calcium signaling in aorta and SMA, our results indicated that ROCK does not participate to the change in cytosolic calcium in response to noradrenaline in RMA. However, in RMA, ROCK contributes to the pressure-induced cytosolic calcium increase and myogenic response. Furthermore, the contribution of ROCK to noradrenaline-induced contraction exhibits a trend

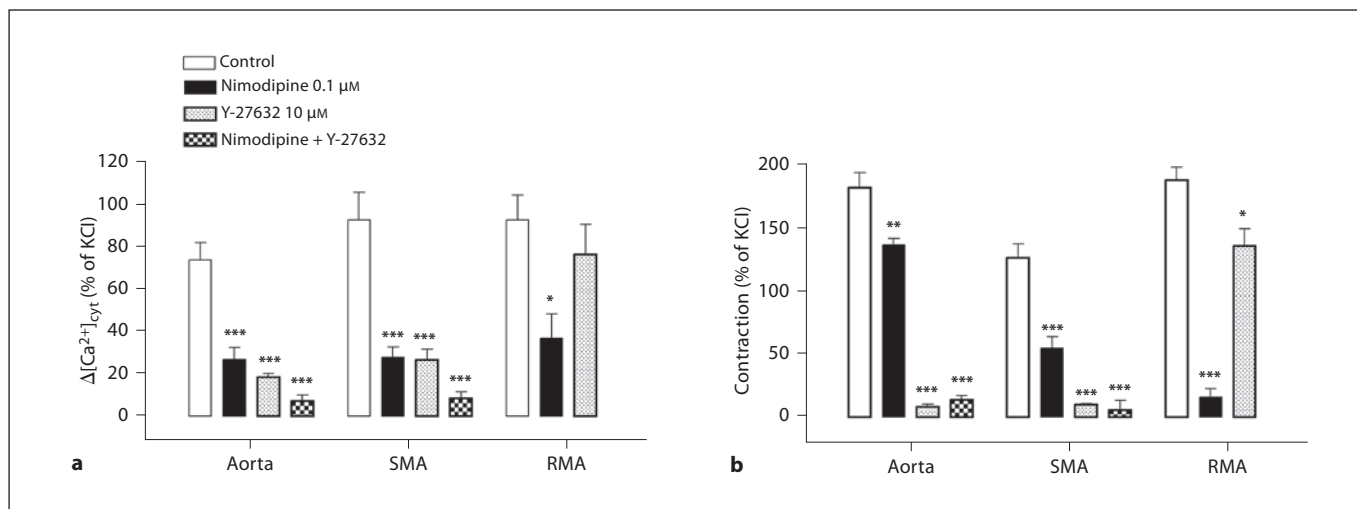


Fig. 4. Effect of nimodipine and Y-27632 on the change in cytosolic calcium and contraction induced by noradrenaline in aorta, SMA and RMA. Cytosolic calcium (**a**) and contraction (**b**) were simultaneously measured in control arteries and in the presence of nimodipine (1 μM) and/or Y-27632 (10 μM) after noradrena-

line stimulation (1 μM for aorta and SMA, and 3 μM for RMA). Data are expressed as percent of the response to KCl and are means $\pm$ SEM from 3–11 arteries. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control; one-way ANOVA with a Bonferroni post hoc test.

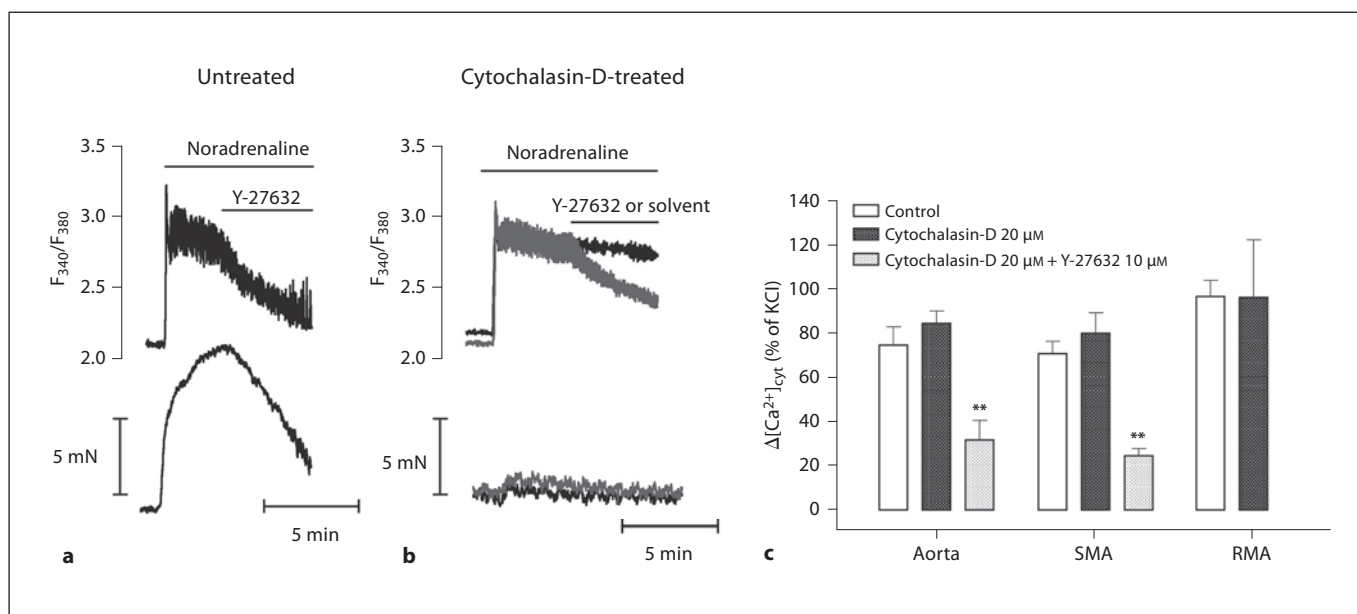


Fig. 5. Effect of cytochalasin-D on noradrenaline-induced calcium signaling in aorta, SMA and RMA. **a, b** Typical traces showing the effect of noradrenaline (1 μM) on cytosolic calcium expressed as F_{340}/F_{380} (upper panel) and contractile tension (lower panel) in control aorta (**a**) and cytochalasin-D-treated aorta (**b**). Y-27632 (10 μM) was applied as indicated. **c** Bar chart of the mean

values of noradrenaline (1 μM for aorta and SMA, and 10 μM for RMA)-induced cytosolic calcium concentration increase ($\Delta[\text{Ca}^{2+}]_{\text{cyt}}$: % of the response to KCl) in 4–12 arteries. Vertical bars represent SEM. ** $p < 0.01$, absence vs. presence of Y-27632; one-way ANOVA with a Bonferroni post hoc test.

Fig. 6. Effect of Y-27632 on MLC phosphorylation in noradrenaline-stimulated aorta and RMA. Arteries were stimulated for 2 min with noradrenaline ($1 \mu\text{M}$ in aorta (a–c) and $10 \mu\text{M}$ in RMA (b–d)) in the absence or presence of Y-27632 ($10 \mu\text{M}$). Artery extracts were subjected to Western blot analysis for evaluating the amount of MLC phosphorylation (pLC_{20}). **a, b** Mean values of pLC_{20} normalized to the expression of actin in non-stimulated (control) and noradrenaline-stimulated arteries in the absence (NA) or in the presence of Y-27632 (NA + Y-27632) from 8–11 arteries. Vertical bars represent the SEM. *** $p < 0.001$, control vs. stimulated condition. ^a $p < 0.05$, ^c $p < 0.001$, vs. without inhibitor; one-way ANOVA with a Bonferroni post hoc test. **c, d** Typical Western blot of aorta and RMA extracts. Membranes were blotted with anti- pLC_{20} (1:1,000) and anti-actin (1:2,000) antibody as loading control.

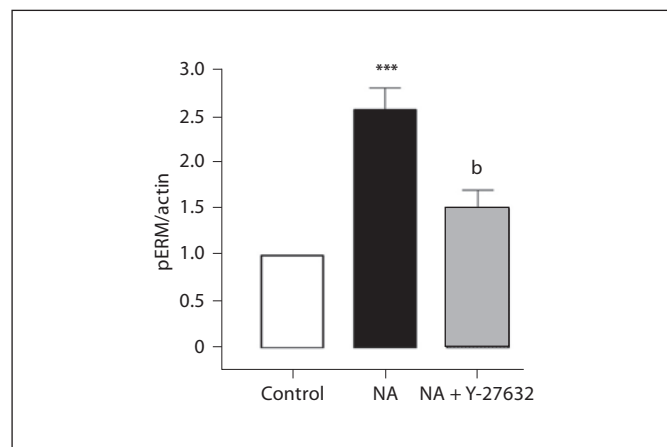
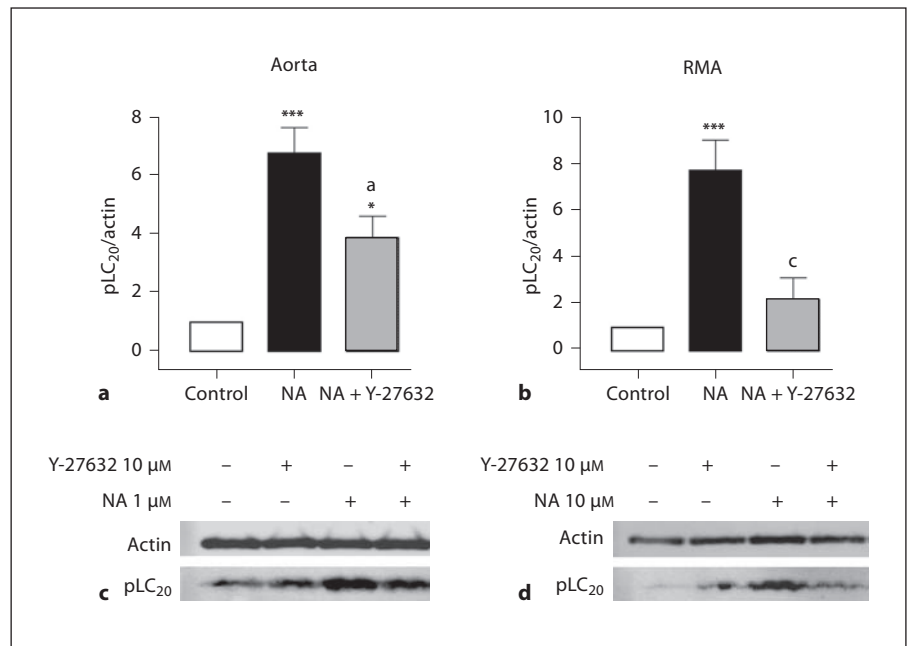


Fig. 7. Effect of Y-27632 on ERM protein phosphorylation in noradrenaline-stimulated RMA. Arteries were stimulated for 2 min with noradrenaline ($10 \mu\text{M}$) in the absence or presence of Y-27632 ($10 \mu\text{M}$). Artery extracts were subjected to Western blot analysis to evaluate the amount of pERM. Membranes were blotted with anti-pERM (1:500) and anti-actin (1:2,000) antibody as loading control. Mean values of pERM normalized to the expression of actin in non-stimulated (control) and in noradrenaline-stimulated arteries in the absence (NA) or presence of Y-27632 (NA + Y-27632), from 6 artery extracts. Vertical bars represent the SEM. *** $p < 0.001$, control vs. stimulated; ^b $p < 0.01$, vs. without inhibitor; one-way ANOVA with a Bonferroni post hoc test.

from the aorta to the RMA opposite to the contribution of VOC channels to the contraction.

To study the contribution of ROCK to calcium responses, we used the ROCK inhibitors Y-27632 and H-1152. Y-27632 has a 100 times greater potency for inhibiting ROCK than for conventional protein kinase C isoforms, which results in a relatively good selectivity [19]. H-1152 has ten times greater affinity for ROCK than Y-27632, with an even weaker activity against other kinases [20]. The inhibitor concentration was chosen on the basis of concentration-effect relationships in order to obtain maximum inhibition of the contraction to noradrenaline [9, 21] (see also online suppl. fig. 3).

Contribution of ROCK to the Contractile Response to Noradrenaline

The inhibition of ROCK did not affect the contractile response to noradrenaline similarly in the three types of arteries tested: Y-27632 ($10 \mu\text{M}$) nearly completely abolished the contraction to noradrenaline in aorta and SMA, but only slightly depressed the contractile response (by 26%) in RMA. Asano and Nomura [22] previously reported similar differences in the sensitivity of noradrenaline contraction to Y-27632 between SMA and RMA. The weak inhibitory potency of Y-27632 on noradrenaline-induced contraction in RMA was not in line with the marked inhibition of LC_{20} phosphorylation. Therefore, in

RMA, in the presence of Y-27632, the force appeared to be uncoupled from LC₂₀ phosphorylation. The kinetics of these responses should be analyzed to further characterize the relationship between contraction and LC₂₀ phosphorylation.

Contribution of ROCK to the Cytosolic Calcium Increase Induced by Noradrenaline

ROCK contribution to the calcium signal decreased along the vascular tree, from a marked contribution in the aorta to no detectable participation in the resistance artery. The origin of the tissue-selective contribution of ROCK to the noradrenaline-induced calcium signal cannot be found in the difference in the expression or activation of ROCK: indeed, the activation of ROCK in RMA was demonstrated by the effect of ROCK inhibition on noradrenaline-stimulated LC₂₀ phosphorylation and confirmed by the ROCK-dependent phosphorylation of ERM proteins in noradrenaline-stimulated RMA.

The cause for the difference in ROCK contribution to calcium signaling could be related to the mobilization of calcium from different sources according to the type of artery. The increased contribution of VOC-dependent calcium entry to the contraction from proximal to more distal arteries [22, 23] was confirmed by the increased sensitivity of the contraction to nimodipine from the aorta to the RMA. However, this progressive increase in the inhibition of the contractile response by nimodipine was associated with the same level of inhibition of the calcium signal in all three arteries, in such a way that in aorta the calcium response was more sensitive to nimodipine than the contraction, while the difference disappeared in RMA. The discrepancy between the contraction and the calcium signal observed in aorta suggests that a component of cytosolic calcium does not participate to the contraction. Such intracellular compartmentalization of calcium cannot be identified in isolated arteries with the fura-2 technique, which detects the bulk cytosolic calcium rather than that localized in the compartment [24, 25]. The difference between arteries then suggests that compartmentalization of calcium in a cell domain where it is not available for the contractile machinery is more important in aorta than in SMA and in RMA. Furthermore, while calcium entry through VOC, which was not dependent on ROCK, is the main determinant of noradrenaline-induced contraction in RMA, other additional calcium signaling pathways are activated in aorta and SMA. In those arteries, the additive effect of nimodipine and Y-27632 indicated that ROCK-dependent calcium entry is distinct from VOC. A previous report showed

that this ROCK-dependent calcium entry is not activated by store depletion [9]. The best candidates for driving non-voltage- and non-store-dependent calcium influx are the non-selective cation channels of the TRP canonical family, which have been reported to be expressed in different types of arteries [11, 12, 26]. By producing sodium and calcium influx, the activation of these channels also causes membrane depolarization and thereby indirectly contributes to the development of the contraction. However, neither the mechanism of their activation nor their function has been identified definitively.

Another hypothesis was that modifications in cytoskeleton proteins could be responsible for the difference in ROCK contribution to agonist-evoked calcium responses between the different arteries. It is known that ROCK is involved in cytoskeleton dynamics and that the actomyosin cytoskeleton regulates tension development independently of cross-bridge cycling [27]. Actin might also play a prominent role in controlling TRP channel activity by modulating their interaction with regulatory proteins or controlling their internalization [28]. The application of the actin depolymerizing agent cytochalasin-D completely abolished the contractile tension in response to noradrenaline in all arteries, without affecting the calcium signal, in agreement with the report by Saito et al. [29] in rat aorta and by Shaw et al. [30] in pressurized RMA. In addition, the calcium signal evoked by noradrenaline in cytochalasin-D-treated aorta and SMA was still depressed by Y-27632. Thus, it appears that actin mobilization is not the key factor involved in the ROCK-calcium signaling pathway in arteries after agonist stimulation. Other cytoskeleton components could be involved in the effect of ROCK [31]. It has been suggested in HEK cells that the upper activator of MLC, MLC kinase, modulates the activation process of TRPC5 [32], which reveals a potential interaction between ROCK, MLC kinase, myosin and calcium channels that is worth being investigated in the future.

The Calcium/Contraction Relationship in Response to Noradrenaline

In aorta, the calcium/contraction relationship was not affected by ROCK inhibition (fig. 2) suggesting that ROCK did not influence calcium sensitivity in aorta, which is contrary to what was observed in SMA and RMA. This result did not agree with the inhibition of noradrenaline-stimulated LC₂₀ phosphorylation in the presence of Y-27632 in aorta as in RMA. This discrepancy could be related to the marked inhibition by Y-27632 of the cytosolic calcium responsible for the contraction in aorta. Furthermore, responses obtained in the presence

of Y-27632 in aorta are very low thus preventing the correct interpretation of the calcium/contraction relation. In addition, as mentioned before, compartmentalization of cytosolic calcium in aorta might prevent the detection of a localized increase in calcium.

Involvement of ROCK in the Myogenic Responses

Changing pressure from 25 to 100 mm Hg in rat RMA led to a decrease in artery diameter in response to increased intraluminal pressure that was associated with the increased cytosolic calcium concentration. The myogenic response was abolished in the presence of the ROCK inhibitors Y-27632 (3–10 μ M) or H-1152 (1 μ M), in agreement with the contribution of calcium sensitization through MYPT1 phosphorylation by ROCK leading to increased LC₂₀ phosphorylation [33]. Activation of the RhoA-ROCK pathway by increased transmural pressure has been shown by Dubroca et al. [6] and could result from Src activation induced by integrin clustering [8]. The abolition of the myogenic response to increased transmural pressure by Y-27632 is in contrast to the lower contribution of ROCK in noradrenaline-induced contraction in RMA.

The present study showed for the first time that the calcium signal induced by high intravascular pressure was significantly depressed by the ROCK inhibitor Y-27632 at 3 and 10 μ M, while VanBavel et al. [34] in rat RMA and Bolz et al. [7] in hamster gracilis artery did not find any effect of ROCK inhibition on the pressure-induced calcium increase. Schubert et al. [36] in rat tail artery and Gokina et al. [35] in rat cerebral small artery observed an increase in the pressure-induced calcium signal after ROCK inhibition [7, 34–36]. In addition, the same effect was observed with the other ROCK inhibitor H-1152 (1 μ M), which is devoid of the inhibitory effect of protein kinase C.

Mechanical stimulation caused by the increased intraluminal pressure is known to depolarize resistance

artery smooth muscle cells and to activate VOC, which are fundamental to the arteriolar myogenic response [37], as demonstrated by the inhibition of the responses in the presence of calcium channel blockers [38, 39]. The exact mechanism of the depolarization leading to VOC activation and myogenic response is currently not well understood but has been proposed to involve the activation of stretch-activated channels [39–41]. As Y-27632 did not affect the calcium entry through VOC (online suppl. data 2), the inhibition of pressure-induced calcium signaling could be related to the involvement of ROCK in pressure-induced depolarization. Since the calcium response induced by high pressure was depressed by Y-27632, the absence of an effect of ROCK inhibition on noradrenaline-induced calcium signaling indicates that the mechanism leading to depolarization should be different in pressurized and agonist-stimulated RMA.

In summary, the present results show that ROCK is involved in noradrenaline-induced calcium signaling in aorta and SMA but not in small RMA. However, ROCK contributes to pressure-induced calcium signaling and contraction in mechanically activated small resistance mesenteric arteries, which play a major role in blood pressure regulation, in agreement with the implication of ROCK in the pathophysiology of vascular disease. The tissue-selective contribution of ROCK to the calcium responses to vasoconstrictor agonist stimulation reflects important differences in the signaling pathway activated in conductance and resistance arteries.

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