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Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries

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The present study was aimed at investigating whether, besides its pivotal role in Ca^{2+}-independent contraction of smooth muscle, Rho-kinase is involved in the mechanisms underlying the Ca^{2+} signal activated by noradrenaline in arteries. In rat aorta and mesenteric artery, the Rho-kinase inhibitor Y-27632 (10 μM) completely relaxed the contraction evoked by noradrenaline (1 μM) and simultaneously inhibited the Ca^{2+} signal by 54 ± 1 % (mesenteric artery) and 71 ± 15 % (aorta), and the cell membrane depolarisation by 56 ± 11 % (mesenteric artery). A similar effect was observed in arteries contracted by AlF_4^-, while in KCl-contracted arteries, Y-27632 decreased tension without changing cytosolic Ca^{2+}. The same effects were observed with another inhibitor of Rho-kinase (HA1077) but not with an inhibitor of protein kinase C (Ro-31-8220). Effects of Y-27632 were not prevented by incubating the artery in 25 mM KCl, with K^+ channel blockers or with the Ca^{2+} channel blocker nimodipine. Y-27632 did not affect either the increase in the production of inositol phosphates activated by noradrenaline, or the release of Ca^{2+} from non-mitochondrial stores evoked by InsP_3 in permeabilised aortic cells, or the Ca^{2+} signals evoked by thapsigargin or caffeine. The capacitative Ca^{2+} entry activated by thapsigargin was not impaired by Y-27632, but the entry of Ba^{2+} activated by noradrenaline in the presence of nimodipine was blocked by 10 μM Y-27632. These results indicate that Rho-kinase is involved in noradrenaline activation of a Ca^{2+} entry distinct from voltage- or store-operated channels in rat arteries.

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Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is the main regulator of contraction in smooth muscle cells. A rise in $[Ca^{2+}]_{cyt}$ leads to the activation of myosin light chain kinase (MLCK). Phosphorylation of myosin light chain (MLC) results in muscle contraction while dephosphorylation promotes smooth muscle relaxation. However, Ca^{2+}-independent contractions have been described, and ascribed to Ca^{2+} sensitisation of contractile proteins (Kitazawa *et al.* 1989). The small GTPase Rho and its effector, Rho-kinase, play an important role in the Ca^{2+}-independent regulation of smooth muscle contraction (Hirata *et al.* 1992). Evidence for the involvement of Rho-kinase is provided by the observations that addition of the catalytic subunit of Rho-kinase to permeabilised vessels results in contraction (Kureishi *et al.* 1997) and that the Rho-kinase inhibitor Y-27632 inhibits the contraction induced by phenylephrine or GTPγS (Uehata *et al.* 1997). Rho-kinase-dependent contraction is mediated through the increase in the level of phosphorylated MLC via the inhibition of MLC phosphatase (Fukata *et al.* 2001).

In vascular smooth muscle, cGMP-evoked relaxation is associated with an increase in smooth muscle myosin light chain phosphatase activity (Wu *et al.* 1996), and with inhibition of both Rho-dependent Ca^{2+} sensitisation of the contractile proteins and actin cytoskeleton organisation (Sauzeau *et al.* 2000). In addition, cGMP has been shown to inhibit the agonist-evoked Ca^{2+} signal (Ghisdal *et al.* 2000). However, it is not known whether inhibition of the Ca^{2+} signal could be related to the inactivation of Rho.

The objective of the present study was to investigate the role of Rho-kinase in the mechanisms underlying the increase in $[Ca^{2+}]_{cyt}$ evoked by noradrenaline in rat aorta and mesenteric artery. α-Adrenergic receptors are known to activate the G_q–phospholipase C (PLC) pathway (Exton, 1994). The increase in $[Ca^{2+}]_{cyt}$ evoked by noradrenaline originates from inositol trisphosphate (InsP_3)-evoked Ca^{2+} release from the sarcoplasmic reticulum (SR) and from extracellular Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCs) and receptor and store-operated non-selective cationic channels (McFadzean & Gibson, 2002). The results indicate that Rho-kinase is involved in the G protein-dependent activation of non-selective cation channel in rat aorta and mesenteric arteries.

METHODS

Male Wistar rats (about 250 g) were used. The rats were anaesthetised with diethyl ether and killed by decapitation. The aorta and the superior mesenteric artery were rapidly removed, immersed in physiological solution (composition (mM): NaCl 122, KCl 5.9, $NaHCO_3$ 15, glucose 10, $MgCl_2$ 1.25 and $CaCl_2$ 1.25, supplemented with indomethacin (10 μM) and gassed with a mixture of 95 % O_2–5 % CO_2), and carefully cleaned of all fat and connective tissue. All experiments were carried out in accordance with national guidelines.

Measurement of contractile tension and cytosolic Ca^{2+} concentration

Artery rings were inverted, endothelium was gently removed and the rings were incubated for 3–3.5 h at room temperature in physiological solution (composition as above) containing 5 μM fura-2 acetoxymethyl ester (fura-2-AM) and 0.05 % Cremophor EL, as described (Ghisdal *et al.* 2000). After the loading period, the rings were mounted between two hooks under a tension of 8 mN (mesenteric artery) or 20 mN (aorta) in a 3 ml cuvette continuously perfused with physiological solution supplemented with N^G-nitro-L-arginine (L-NNA, 100 μM) at 37 °C. The cuvette was part of a fluorimeter (CAF, JASCO, Tokyo, Japan) which allowed estimation of the calcium signal. The muscle tone was measured using an isometric force transducer. The artery segment was incubated for 30 min in physiological solution and was thereafter stimulated with 100 mM KCl solution (composition (mM): NaCl 27, KCl 100, $NaHCO_3$ 15, glucose 10, $MgCl_2$ 1.25, $CaCl_2$ 1.25, indomethacin 0.01). After washing and a further 15 min recuperation, the preparation was stimulated as required. When possible, control responses were recorded before incubation of the tissue with the inhibitor and application of a second stimulation. The amplitude of the second response was compared with the response measured before treatment. Control experiments without inhibitor were performed. Ca^{2+}-free solution was prepared from physiological solution without Ca^{2+} supplemented with EGTA (0.1 mM). The increase in Ca^{2+} signal was measured with reference to the signal measured immediately before the stimulation, except for increase following re-addition of Ca^{2+} to a Ca^{2+}-free solution, which was measured with reference to the baseline signal in Ca^{2+}-containing solution. At the end of the experiment, the fura-2-Ca^{2+} signal was calibrated as previously described (Ghisdal *et al.* 2000). In some experiments, in particular when Ba^{2+} was used instead of Ca^{2+}, the Ca^{2+} signal was expressed as the ratio of fluorescence at 340 and 380 nm (F_{340}/F_{380}), corrected for the autofluorescence measured at 340 and 380 nm after addition of $MnCl_2$ (10 mM). The changes in fluorescence ratio were expressed either as percentages of the basal values or were normalised to the change evoked by the KCl stimulation. The rates of Ca^{2+} or Ba^{2+} entry were measured by the average slope of the change in fluorescence ratio calculated during the first minute after the addition of the cation into the perfusion solution (MacLab, AD Instruments Pty Ltd, Castle Hill, Australia).

Simultaneous measurement of contractile tension and membrane potential

Smooth muscle cell membrane potential was recorded in a segment of the superior mesenteric artery, 2 mm length, inverted and mounted in a myograph as described (Ghisdal *et al.* 2000). A glass microelectrode filled with 1.5 M KCl (resistance 50–80 MΩ) was advanced through the luminal surface of the arterial segment. Potential differences were measured with reference to the earthed bath by means of a Dagan amplifier (Minneapolis, MN, USA).

Determination of inositol phosphates

The artery segments were incubated in modified physiological solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.25, KH_2PO_4 1.2, EDTA 0.5, $NaHCO_3$ 25, Hepes 3.3, glucose 10, Tris-HCl 20, pH 7.4) supplemented with indomethacin (10 μM) and L-NNA (100 μM) at 37° C for 1 h. Subsequently, artery segments were incubated for 4 h at 37 °C in fresh buffer containing 20–25 μCi ml^{-1} of [^{3}H]*myo*-inositol. At the end of the incubation, [^{3}H]inositol-labelled artery segments were washed in buffer for 10 min and then transferred to physiological solution containing 10 mM LiCl plus noradrenaline (0.1–1 μM) and Y-27632 (10 μM) as required. Incubation was carried out for 30 min and stopped by rapid freezing of the tissue samples. Inositol phosphates were determined as described (Ghisdal *et al.* 2000). Data were normalised to the protein content of each sample.

$^{45}Ca^{2+}$ fluxes in β-escin permeabilised A7r5 cells

A7r5 cells were used between the 15th and the 20th passage after receipt from ECACC (European Collection of Cell Cultures, UK). The cells were cultured at 37 °C in DMEM medium (Gibco, catalogue no. 41965-039) supplemented with 2 mM glutamine and 10 % fetal bovine serum, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. The cells were seeded in 12-well dishes. Permeabilisation was carried out by replacing the culture medium with 2 ml of permeabilisation medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 30 μM β-escin, at 22 °C. The permeabilisation medium was removed after 30 min and the cells were washed twice with the same but β-escin-free medium. $^{45}Ca^{2+}$ fluxes were measured as described by Missiaen *et al.* (1992). $^{45}Ca^{2+}$ uptake was accomplished by incubation of the cells in 2 ml of loading solution containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3, 150 nM free Ca^{2+} (20 μCi ml^{-1} $^{45}Ca^{2+}$) at 22 °C for 50 min. The cells were then washed three times in an efflux medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 5 mM NaN_3, 2 μM thapsigargin. A 1 ml volume of this medium was then added to the cells and replaced every 2 min. At the end of the experiment the $^{45}Ca^{2+}$ remaining in the stores was released by incubation with 1 ml of a 1 % SDS solution for 45 min. Ca^{2+} release was expressed as the amount of Ca^{2+} leaving the stores in 2 min divided by the total store content at that time.

Drugs

Y-27632 ((+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate) was a gift from Mitsubishi Pharma Corporation (Osaka, Japan). Fura-2 acetoxymethylester (fura-2-AM), HA1077 and Ro-31-8220 were from Calbiochem (EuroBiochem, Bierges, Belgium). [^{3}H]*myo*-inositol was from NEN Life Science Products (Zaventem, Belgium), $^{45}Ca^{2+}$ was from Amersham Biosciences (Little Chalfont, UK). All other compounds were obtained from Sigma. AlF_4^- was produced by the combination of 5 mM sodium fluoride (NaF) and 30 μM aluminum chloride ($AlCl_3$).

Statistical analysis

Quantified data are presented as means ± S.E.M. The noradrenaline concentration producing 50 % of the maximal effect (EC_{50}) and the concentration of Y-27632 producing 50 % inhibition of the noradrenaline responses (IC_{50}) were calculated by non-linear curve fitting (Prism, GraphPad). Log values were used for statistical analysis. Comparisons were made by Student's *t* test. Differences with *P* values smaller than 0.05 were considered significant.

RESULTS

Effect of the Rho-kinase inhibitor Y-27632 on contraction and the Ca^{2+} signal evoked by noradrenaline, AlF_4^- and KCl

Figure 1 shows the effect of Y-27632 (10 μM) on the Ca^{2+} signal and contraction in mesenteric artery. Contraction evoked by noradrenaline was associated with a rapid increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) followed by a sustained component which was maintained for at least 10 min. Y-27632 (10 μM) produced a simultaneous decrease in contraction and in $[Ca^{2+}]_{cyt}$ (Fig. 1*A*). After 1–2 min, the contraction was completely relaxed and the Ca^{2+} signal was depressed by 54 ± 1 % (n = 5). Noradrenaline concentration–effect curves for contractile tension and Ca^{2+} signal were established in control tissues and after 15 min pre-incubation of artery rings with 10 μM Y-27632. The curves are shown in Fig. 2*A* and *B*. Y-27632 markedly depressed the contraction curve and produced a significant rightward shift of the Ca^{2+} signal curve: the noradrenaline pD_2 value ($-\log EC_{50}$) for the Ca^{2+} signal was decreased from 7.25 ± 0.16 in untreated arteries (n = 5) to 5.87 ± 0.44 in Y-27632-treated arteries (n = 5) ($P < 0.05$).

In order to exclude the possibility that Y-27632 could inhibit noradrenaline-evoked responses by interacting with the adrenergic receptor, arteries were stimulated with AlF_4^-, which activates G proteins directly (Boonen & De Mey, 1990). As illustrated in Fig. 1*B*, 10 μM Y-27632 inhibited the Ca^{2+} signal evoked by AlF_4^- by 63 ± 5.4 % (n = 4), while contraction was abolished. The effect of Y-27632 was also investigated in artery contracted by 100 mM KCl. KCl contractions were evoked in the presence of phentolamine (1 μM) to inhibit possible effects of noradrenaline, which could be released from nerve terminals. In contrast with stimulations involving G protein activation, in KCl-contracted artery Y-27632 decreased the contractile tension but did not significantly affect the Ca^{2+} signal (Fig. 1*C*). An investigation of the effects of Y-27632 in rat aorta gave similar results except that the noradrenaline-evoked Ca^{2+} signal was more sensitive to the inhibitory effect of Y-27632 in the aorta than in the mesenteric artery (in the aorta, the Ca^{2+} signal in response to 1 μM noradrenaline was inhibited by 71 ± 15 % with 10 μM Y-27632, n = 5). This was confirmed by the marked depression of the noradrenaline concentration–response curves in the aorta in the presence of the Rho-kinase inhibitor (Fig. 2*C* and *D*). In order to address the possibility that the effects of Y-27632 could be mediated via other kinases than the Rho-kinase and particularly via protein kinase C (PKC), we tested the effects of another inhibitor of Rho-kinase, HA1077 (Uehata *et al.* 1997; Sward *et al.* 2000), and of a selective inhibitor of PKC, Ro-31-8220, in aortic segments. The effects of HA1077 were similar to the effects of Y-27632 although HA1077 was slightly less potent than Y-27632: 10 μM HA1077 relaxed the contraction and inhibited the Ca^{2+} signal evoked by noradrenaline (1 μM) in aorta by 67 ± 10 % and 37 ± 10 % (n = 4), respectively (Fig. 3*A*). The increase in $[Ca^{2+}]_{cyt}$ evoked by 100 mM KCl solution was not significantly affected (the Ca^{2+} signal in the presence of 10 μM HA1077 was 91 ± 4 % (n = 3, $P > 0.05$) of the Ca^{2+} signal in the absence of the inhibitor) but the contraction was inhibited by 84 ± 4 % (n = 3). The inhibitor of PKC, Ro-31-8220

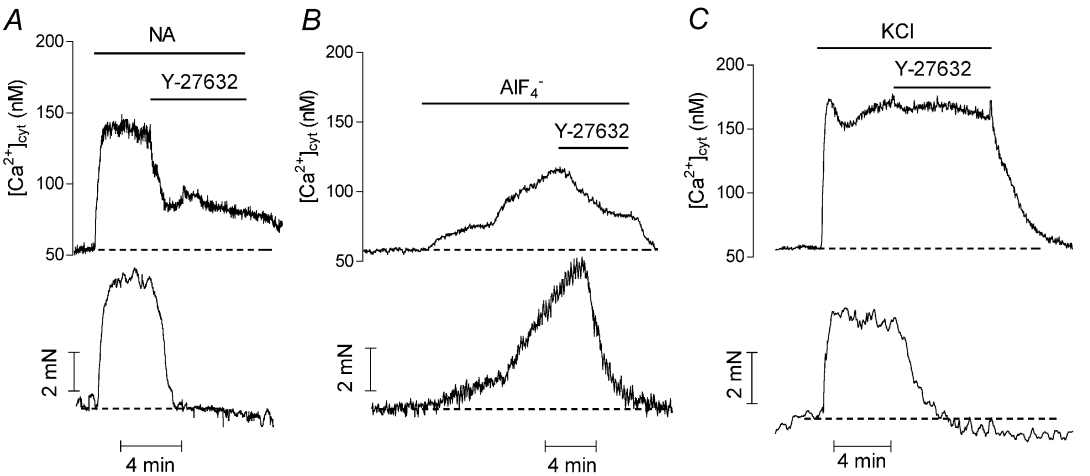

Figure 1. Representative recordings of the effect of Y-27632 on the Ca^{2+} signal and the contraction evoked by noradrenaline (*A*), AlF_4^- (*B*) or 100 mM KCl (*C*) in rat mesenteric artery rings

$[Ca^{2+}]_{cyt}$ (upper trace) and contraction (lower trace) were measured simultaneously in fura-2-loaded arteries. Traces *A*, *B* and *C* are from different arteries. Noradrenaline (NA, 1 μM), AlF_4^- (30 μM), KCl (100 mM) and Y-27632 (10 μM) were applied as indicated.

(3 μM), did not affect the responses to noradrenaline and did not prevent the effects of Y-27632 (Fig. 3*B*).

The relation between the concentration of Y-27632 and its inhibitory effect on contraction and on the Ca^{2+} signal evoked by a sub-maximal concentration of noradrenaline (1 μM) in mesenteric artery is shown in Fig. 4. As observed in Figs 1 and 2, part of the Ca^{2+} signal was resistant to Y-27632 (maximum inhibition was 54 ± 0.9 %, $n = 3$), while contraction was inhibited by 91 ± 4 % ($n = 3$). However, the pA_2 ($-\log IC_{50}$) values for the effects of Y-27632 on the Ca^{2+} signal and on contraction were not different (6.29 ± 0.10 and 6.28 ± 0.03, respectively). The maximum effect was obtained at concentrations of Y-27632 higher than 3 μM. A concentration of 10 μM,

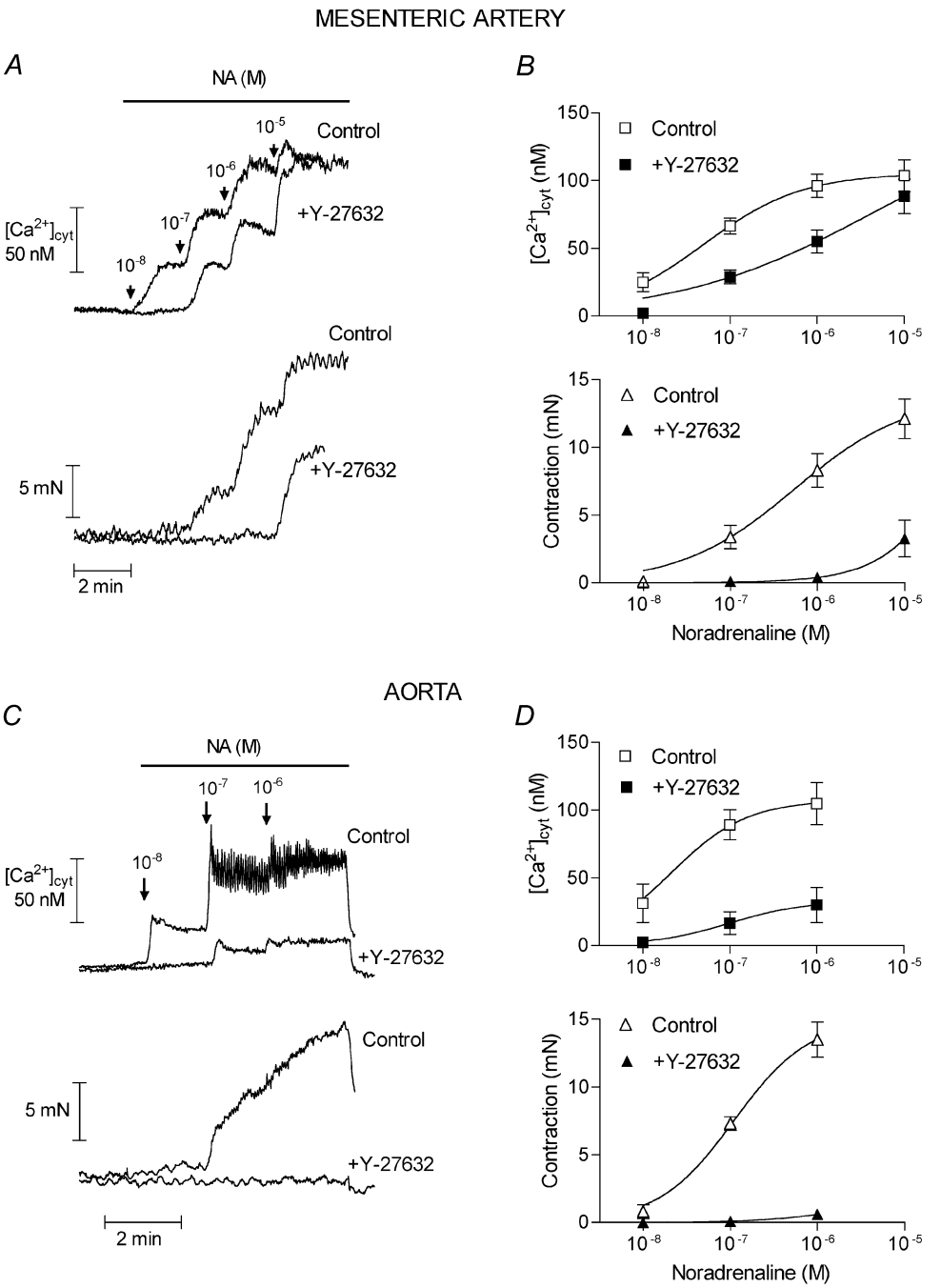

Figure 2. Effect of Y-27632 on noradrenaline concentration–response curves in fura-2-loaded mesenteric artery (*A* and *B*) and aorta (*C* and *D*)

$[Ca^{2+}]_{cyt}$ (upper traces and graphs) and contraction (lower traces and graphs) were simultaneously measured in control artery rings or in artery rings pre-incubated for 10 min in the presence of 10 μM Y-27632. *A* and *C*, typical traces obtained in untreated and Y-27632-treated segments from the same artery are superimposed. *B* and *D*, mean values from 4–5 different arteries. Vertical bars represent the S.E.M. values.

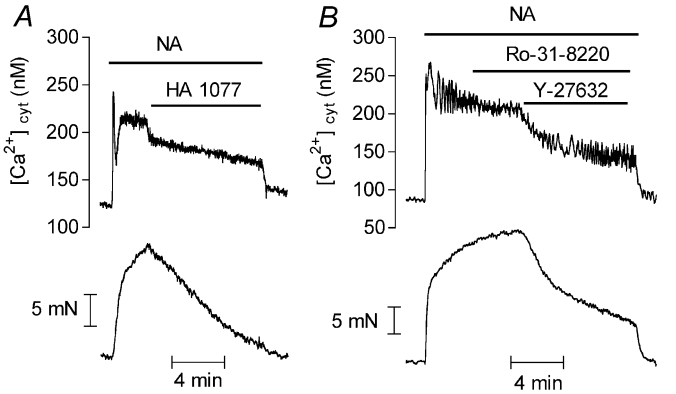

Figure 3. Representative recordings of the effect of HA1077 and Ro-31-8220 on Ca^{2+} signal (upper traces) and contraction (lower traces) evoked by noradrenaline in fura-2-loaded aorta

Noradrenaline (NA, 1 μM), HA1077 (10 μM), Ro-31-8220 (3 μM) and Y-27632 (10 μM) were applied as indicated.

which was maximally effective, was used in all following experiments.

Effect of Y-27632 on noradrenaline-evoked depolarisation

Noradrenaline-evoked contraction of mesenteric artery is accompanied by a depolarisation of vascular smooth muscle cells (Mulvany *et al.* 1982; Ghisdal *et al.* 2000). We investigated whether, in addition to its effect on the Ca^{2+} signal, Rho-kinase inactivation affected noradrenaline-evoked depolarisation in the mesenteric artery. The mean resting membrane potential of mesenteric artery smooth muscle cells was -46.2 ± 0.7 mV ($n = 14$). Noradrenaline (1 μM) depolarised smooth muscle cells to -35.8 ± 1.6 mV and simultaneously produced a contraction of 4.8 ± 0.8 mN ($n = 8$). In most cells, rhythmic oscillations of the membrane potential of 5–10 mV of amplitude were superimposed on the tonic depolarisation (Fig. 5). The pre-incubation of arteries with Y-27632 did not change the resting membrane potential (-46.0 ± 1.1 *vs.* -45.5 ± 1.1 mV, $n = 6$) but modified the response to noradrenaline. In the presence of the Rho-kinase inhibitor, the depolarisation evoked by noradrenaline presented a peak followed by a stable response (Fig. 5), which was significantly lower than in the control (cells were depolarised by 10.6 ± 1.2 mV ($n = 8$) and 6.0 ± 1.0 mV ($n = 5$) in the absence and in the presence of 10 μM Y-27632, respectively, $P < 0.05$). Simultaneously, the contractile response to noradrenaline was completely abolished (Fig. 5).

Influence of K^+ channel blockers or increased external KCl concentration on the effects of Y-27632

In order to determine whether the Y-27632-evoked inhibition of the Ca^{2+} signal and the depolarisation in response to noradrenaline that was observed in mesenteric artery could be caused by an interaction of Rho-kinase with K^+ channels, we investigated the effect of Rho-kinase inhibition in the presence of a cocktail of the following K^+ channel blockers: 1 mM 4-aminopyridine to inhibit voltage-operated K^+ channels, 0.1 μM apamin to inhibit small-conductance Ca^{2+}-activated K^+ channels, 0.1 μM charybdotoxin to inhibit large-conductance Ca^{2+}-activated K^+ channels, and 10 μM glybenclamide to inhibit ATP-

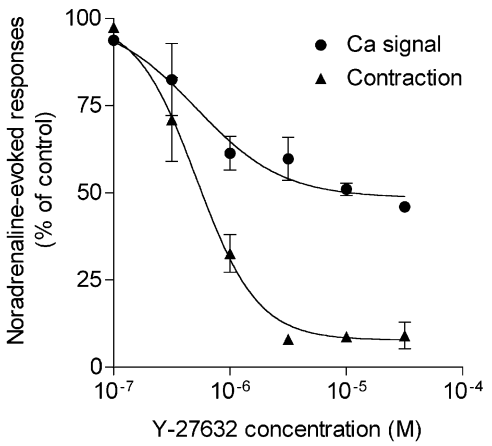

Figure 4. Concentration–inhibition curves for effects of Y-27632 on the Ca^{2+} signal and the contraction evoked by noradrenaline in mesenteric artery

Fura-2-loaded arteries were incubated with Y-27632 (10 μM) for 15 min before stimulation with 1 μM noradrenaline. Responses were expressed as a percentage of the control responses recorded before the incubation with Y-27632. Each point is the mean value of 4–5 determinations. Vertical bars represent the S.E.M. values.

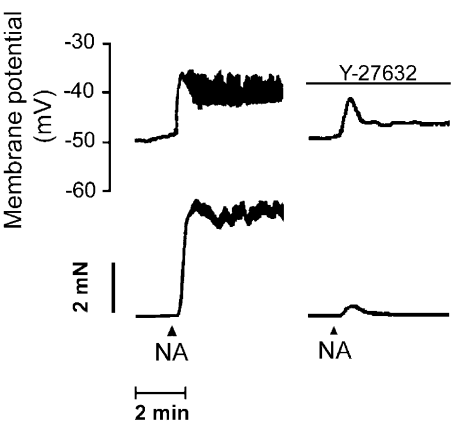

Figure 5. Effect of Y-27632 on the depolarisation evoked by noradrenaline in mesenteric artery smooth muscle cells

Representative recording of the change in membrane potential (upper trace) and in tension (lower trace) evoked by noradrenaline (NA, 1 μM) in mesenteric artery before and after incubation with Y-27632 (10 μM). Traces are from the same artery segment.

Table 1. Effect of Y-27632 on the accumulation of inositol phosphates evoked by noradrenaline in aorta and mesenteric artery

	Accumulation of inositol phosphates (% of basal control value)			
	Mesenteric artery ($n = 8$)		Aorta ($n = 5$)	
	Untreated	+Y-27632	Untreated	+Y-27632
Control	100	99 ± 5	100	105.3 ± 4.5
Noradrenaline (0.1 μM)	230 ± 21*	216 ± 16*	343 ± 18*	315 ± 22*
Noradrenaline (1 μM)	395 ± 33*	388 ± 20*	437 ± 19*	411 ± 25*

Artery rings loaded with [^{3}H]*myo*-inositol were pretreated with or without Y-27632 (10 μM) for 20 min and further incubated with noradrenaline (0.1–1 μM) for 30 min. *$P < 0.01$ *vs.* control.

dependent K^+ channels (Nelson & Quayle, 1995). The results indicated that neither K^+ channel blockers nor 25 mM KCl, which change the equilibrium potential of K^+ to less negative values, prevented the inhibitory effect of Y-27632 on the Ca^{2+} signal evoked by noradrenaline: Y-27632 inhibited the noradrenaline-evoked Ca^{2+} signal by 40 ± 9.7 % ($n = 5$), 42 ± 7.7 % ($n = 5$) and 49 ± 1.4 % ($n = 5$) in the presence of the cocktail of K^+ channel blockers, in a solution containing 25 mM KCl and in untreated mesenteric arteries, respectively.

Effect of Y-27632 on the production of inositol phosphates evoked by noradrenaline

α-Adrenergic receptors are known to be coupled with G_q proteins which activate phospholipase Cβ to increase the production of InsP_3 (Exton, 1994). Ca^{2+} signalling activated by noradrenaline in arteries is dependent on the increase in InsP_3 production (Ghisdal *et al.* 2000). In order to investigate whether the inhibitory effect of Y-27632 on the noradrenaline-dependent Ca^{2+} signal could be related to an inhibition of the PLC activity, we measured its effect on the accumulation of inositol phosphates produced by noradrenaline in the aorta and the mesenteric artery. Table 1 summarises the results and shows that no significant difference could be observed between untreated arteries and arteries incubated in the presence of Y-27632 (10 μM).

Effect of Y-27632 on the release of Ca^{2+} from non-mitochondrial stores evoked by InsP_3

To address the question of whether Rho-kinase regulates InsP_3 receptors, we measured the effect of Y-27632 on InsP_3-induced Ca^{2+} release in permeabilised monolayers of cultured aortic A7r5 cells. This cell line is a well-characterised model system for analysing InsP_3-induced Ca^{2+} release under conditions of unidirectional flux (Missiaen *et al.* 1992). After permeabilisation of the cells with β-escin and loading of non-mitochondrial stores with $^{45}Ca^{2+}$, incubation of the cells in an efflux medium led to a passive efflux of $^{45}Ca^{2+}$ out of the stores. The addition of InsP_3 to the intracellular medium produced an immediate release of $^{45}Ca^{2+}$. As shown in Fig. 6, the increase in fractional release of Ca^{2+} evoked by 0.1–10 μM InsP_3 did not differ between control cells and cells treated with 10 μM Y-27632.

Effect of Y-27632 on responses to noradrenaline in the presence of the Ca^{2+} channel blocker nimodipine

In rat aorta and mesenteric artery, the contractions evoked by noradrenaline are dependent on both intracellular and extracellular Ca^{2+}. Part of the entry of Ca^{2+} produced by noradrenaline is associated with the opening of VOCs (Godfraind & Dieu, 1981; Morel & Godfraind, 1991). In the mesenteric artery, as in the aorta, in the presence of the L-type Ca^{2+} channel blocker nimodipine (1 μM),

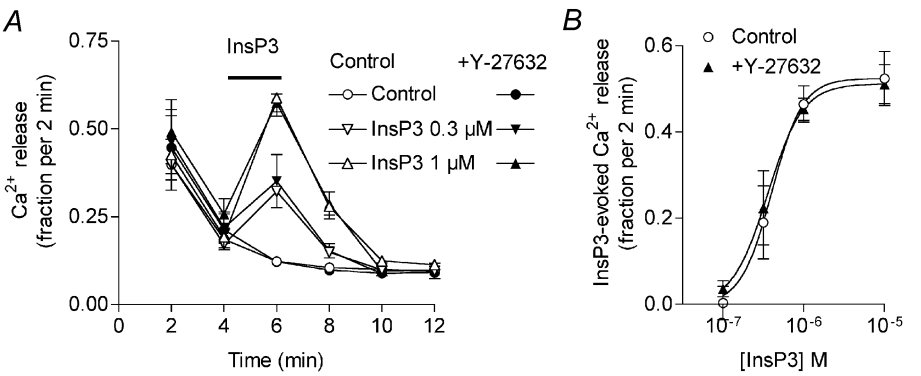

Figure 6. Effect of Y-27632 on InsP_3-evoked $^{45}Ca^{2+}$ release in permeabilised A7r5 aortic cells

A, $^{45}Ca^{2+}$-loaded Ca^{2+} stores were allowed to release Ca^{2+} passively in the efflux medium with or without Y-27632 (10 μM). InsP_3 (0.3 and 1 μM) was applied for 2 min as indicated by the horizontal bar. Ca^{2+} release was plotted as fractional release (the amount of Ca^{2+} leaving the stores in 2 min divided by the total store content at that time). Points are means from four experiments. *B*, Ca^{2+} release evoked by a 2 min application of various concentrations of InsP_3 in the absence or presence of 10 μM Y-27632.

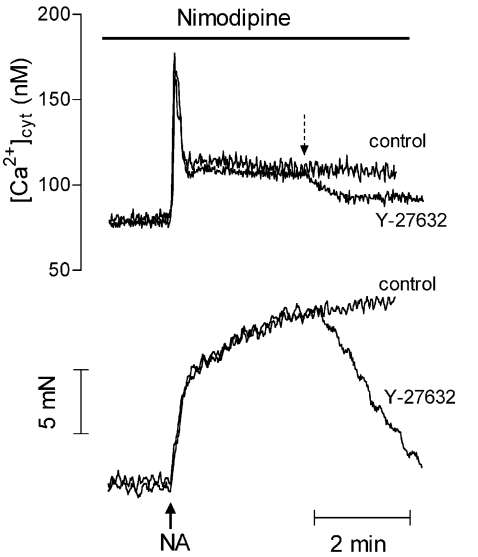

Figure 7. Inhibition of the responses to noradrenaline by Y-27632 in nimodipine-treated aorta

Representative recording of the effect of noradrenaline (1 μM NA) on $[Ca^{2+}]_{cyt}$ (upper trace) and contractile tension (lower trace) in fura-2-loaded aortic segments pretreated with the Ca^{2+} channel blocker nimodipine (1 μM). Y-27632 (10 μM) or vehicle were added at the time indicated by the dashed arrow. Control and Y-27632 traces were from different segments of the same aorta.

noradrenaline produced a transient peak of $[Ca^{2+}]_{cyt}$ followed by a sustained phase which plateaued at about 30–45 % of the response measured in the absence of nimodipine (in the absence and in the presence of nimodipine, noradrenaline increased the Ca^{2+} signal in mesenteric artery by 114 ± 20 nM, $n = 6$, and 35 ± 14 nM, $n = 5$, ($P < 0.05$), and in aorta by 83 ± 7 nM, $n = 8$ and 37 ± 4 nM, $n = 8$ ($P < 0.05$), respectively) (Fig. 7). Simultaneously, in agreement with a previous report (Godfraind & Dieu, 1981), contraction was less affected by nimodipine in the aorta where it was reduced by 25 %, from 19 ± 0.9 mN ($n = 8$) to 15 ± 1.2 mN ($n = 8$, $P > 0.05$), than in the mesenteric artery, where contraction was inhibited by 57 %, from 7.8 ± 0.8 mN ($n = 9$) to 3.4 ± 0.8 mN ($n = 5$, $P < 0.05$). In the presence of 10 μM Y-27632, the plateau phase of the nimodipine-resistant increase in $[Ca^{2+}]_{cyt}$ was depressed by 67 ± 8 % in the mesenteric artery ($n = 3$, $P < 0.05$ compared with the inhibition produced by Y-27632 in the absence of nimodipine) and by 63 ± 5 % in the aorta ($n = 7$, not significantly different from the effect of Y-27632 in the absence of nimodipine) (Fig. 7).

Effect of the Rho-kinase inhibitor Y-27632 on responses to noradrenaline in Ca^{2+}-free solution and after re-addition of Ca^{2+}

The components of the Ca^{2+} signal evoked by noradrenaline were further investigated in aorta perfused with Ca^{2+}-free solution. Perfusion with Ca^{2+}-free solution produced a small decrease in the basal F_{340}/F_{380} ratio, which was reversed when Ca^{2+} was re-added to the perfusion solution (not shown). Noradrenaline applied after 90 s perfusion of the aorta with Ca^{2+}-free solution produced a large but transient increase in Ca^{2+} signal (peak was 141 ± 23 nM Ca^{2+}, $n = 12$) associated with a sustained contraction that was depressed compared with the tension developed in Ca^{2+}-containing solution (9.6 ± 0.8 mN, $n = 12$, and 19 ± 0.9 mN, $n = 7$, respectively, $P < 0.01$). The re-addition of Ca^{2+} to the bathing solution produced a slow increase in $[Ca^{2+}]_{cyt}$ which reached a steady level of 67 ± 6.7 nM above the baseline ($n = 12$). Figure 8 shows that in aortas pre-

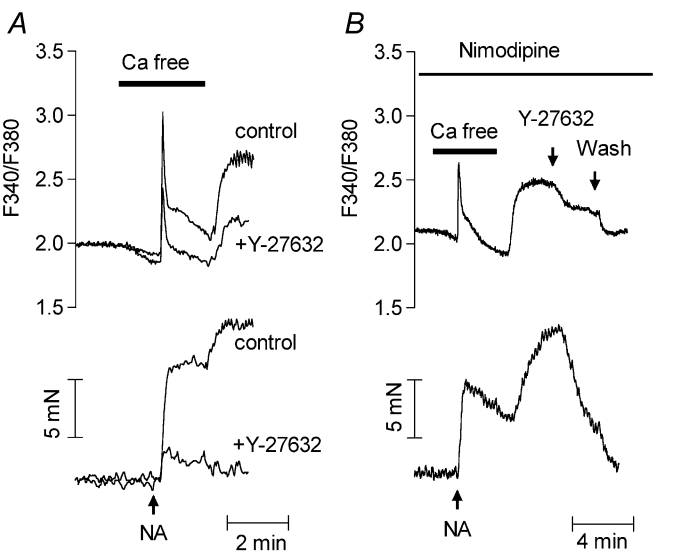

Figure 8. Effect of Y-27632 on the Ca^{2+} signal and the contraction evoked by noradrenaline in aorta in Ca^{2+}-free solution

A, representative recording of the effect of pre-incubation of fura-2-loaded aorta with Y-27632 on the Ca^{2+} signal (upper traces) and contraction (lower traces). Physiological solution was changed to Ca^{2+}-free solution as indicated by the horizontal line. Noradrenaline (NA, 1 μM) was applied as indicated by the arrow. Traces obtained before and after incubation with Y-27632 (10 μM) are superimposed. They were obtained from the same artery ring. *B*, representative recording of Ca^{2+} signal (upper trace) and contraction (lower trace) showing the effect of Y-27632 (10 μM) applied during the re-addition of Ca^{2+} to the perfusion solution in a fura-2-loaded aorta pretreated with nimodipine (1 μM). Noradrenaline (NA, 1 μM) and Y-27632 (10 μM) were applied as indicated.

incubated with Y-27632, the peak Ca^{2+} response evoked by noradrenaline in Ca^{2+}-free solution was significantly depressed (40 ± 5 % inhibition at peak, $n = 7$) (Fig. 9*A*). The rate of Ca^{2+} entry evoked by the re-addition of Ca^{2+} to the solution was decreased by 59 %, from 1.37 ± 0.27 F_{340}/F_{380} ratio units s^{-1} to 0.64 ± 0.10 ratio units s^{-1} ($n = 6$, $P < 0.05$), and the steady-state Ca^{2+} level was inhibited by 75 ± 5 % ($n = 6$) (Fig. 9*B*).

In the presence of nimodipine, the peak Ca^{2+} signal evoked by noradrenaline in Ca^{2+}-free solution was reduced by 27 ± 8 % and the effect of re-addition of Ca^{2+} was depressed by 29 ± 10 % ($n = 7$) compared with untreated arteries. However, the peak response and Ca^{2+} signal evoked by re-addition of Ca^{2+} to the Ca^{2+}-free solution were still inhibited by Y-27632 (Fig. 9). In the presence of nimodipine (Fig. 8*B*), as in its absence (not shown), the application of 10 μM Y-27632 during the plateau phase of the Ca^{2+} signal evoked by re-addition of Ca^{2+} to the Ca^{2+}-free solution inhibited the Ca^{2+} signal with the same potency as that obtained after pre-incubation of the artery with the Rho-kinase inhibitor,

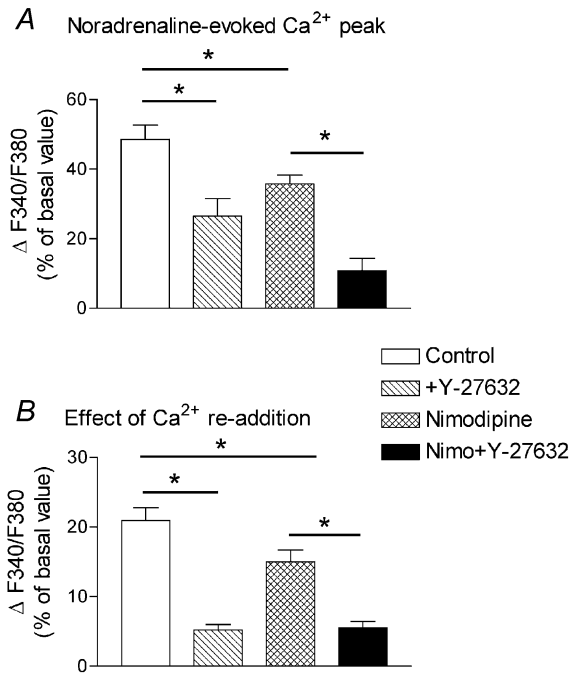

Figure 9. Effect of Y-27632 on the different components of the Ca^{2+} signal evoked by noradrenaline in fura-2-loaded aorta in the absence or presence of nimodipine

Columns represent the mean values of the increase in F_{340}/F_{380} ratio measured in Ca^{2+}-free solution at the time of the peak Ca^{2+} response to noradrenaline (1 μM) (*A*), or after re-addition of Ca^{2+} (1.25 mM) to the Ca^{2+}-free solution (*B*), in untreated aorta, or in aorta pretreated with Y-27632 (10 μM), nimodipine (1 μM), or nimodipine plus Y-27632. The protocol of the experiment was as represented in Fig. 8*A*. The effect of re-addition of Ca^{2+} was measured with reference to the baseline fluorescence ratio measured in Ca^{2+}-containing solution. Increases in the F_{340}/F_{380} ratio were expressed as a percentage of the basal F_{340}/F_{380} value and are means from 7–13 determinations ± S.E.M. * $P < 0.05$.

suggesting that this effect was not related to the inhibition of the peak of Ca^{2+}.

Effect of the Rho-kinase inhibitor Y-27632 on Ba^{2+} entry

In order to exclude any intervention of Ca^{2+} release or storage in the Ca^{2+} signal and to be free of the activation of Ca^{2+}-dependent feedback mechanisms, we used Ba^{2+} instead of Ca^{2+} to monitor the entry of divalent cations activated by noradrenaline (Inoue *et al.* 2001). Fura-2-loaded aortas were treated with nimodipine to block VOCs and incubated for 5 min in Ca^{2+}-free solution. Addition of Ba^{2+} (1 mM) to the Ca^{2+}-free solution increased the F_{340}/F_{380} ratio above the basal value by 18 ± 3 % of the basal signal ($n = 6$). When arteries were stimulated by 1 μM noradrenaline during incubation in the Ca^{2+}-free solution, the increase in F_{340}/F_{380} ratio evoked by Ba^{2+} was significantly higher than in unstimulated aortas (29 ± 1.8 % of the basal ratio, $n = 7$, $P < 0.05$ compared with unstimulated aortas) (Fig. 10). Y-27632 did not affect the increase in F_{340}/F_{380} ratio due to Ba^{2+} in unstimulated arteries but it significantly decreased the rate and the amplitude of the entry of Ba^{2+} in noradrenaline-stimulated arteries (Fig. 10). The same inhibition of Ba^{2+} entry was observed when Y-27632 was applied before noradrenaline (not shown) or after the Ca^{2+} peak evoked by noradrenaline in Ca^{2+}-free solution (as shown in Fig. 10), indicating that the inhibition was unrelated to the amplitude of the Ca^{2+} peak evoked by noradrenaline in Ca^{2+}-free solution.

Effect of Y-27632 on agonist-independent responses and on capacitative Ca^{2+} entry

In order to investigate whether the Ca^{2+} signal evoked by agonist-independent stimuli was also sensitive to Y-27632, we tested the responses of aorta to caffeine and to thapsigargin. In Ca^{2+}-containing solution, caffeine (10 mM) produced a rapid increase in $[Ca^{2+}]_{cyt}$ which peaked at 148 ± 19 nM Ca^{2+} (86 ± 17 % of the KCl-evoked response, $n = 7$) followed by a lower plateau phase (97 ± 12 nM Ca^{2+}, $n = 7$) associated with a small and transient contraction (2.4 ± 1.1 mN, 23 ± 9 % of the contraction evoked by KCl, $n = 7$, not shown). The Ca^{2+} signal evoked by caffeine was not affected by Y-27632 (data not shown). It has been shown that translocation of Rho to the plasma membrane is a necessary step for the activation of Rho-kinase by carbachol, phenylephrine or GTPγS (Fujihara *et al.* 1997; Gong *et al.* 1997; Miyazaki *et al.* 2002). In order to exclude the possibility that the lack of effect of Y-27632 could be due to the non-activation of the Rho A–Rho-kinase pathway in the absence of agonist, the artery was first stimulated with noradrenaline and then caffeine was added to the bathing solution (Fig. 11*A* and *B*). In the presence of noradrenaline, caffeine did not produce a further large change in Ca^{2+} signal: $[Ca^{2+}]_{cyt}$ increased transiently to 158 ± 15 nM and stabilised at 95 ± 18 nM ($n = 4$). In Y-27632-treated aorta, the Ca^{2+} signal evoked by noradrenaline alone was depressed by 52 ± 8.3 %

($n = 4$), as expected from the previous results, while the response to noradrenaline plus caffeine was not impaired (the Ca^{2+} signal in the presence of Y-27632 was 116 ± 13 % of the response in the absence of Y-27632, $n = 4$).

Thapsigargin evokes the release of intracellular Ca^{2+} stores by blocking the activity of the Ca^{2+} pump of the SR. All experiments with thapsigargin were performed in the presence of nimodipine in order to observe capacitative Ca^{2+} entry. In Ca^{2+}-containing solution, the addition of thapsigargin (1 μM) increased $[Ca^{2+}]_{cyt}$ by 56 ± 18 nM ($n = 4$) (Fig. 11*C* and *D*) without producing a marked contraction (not shown). The response to thapsigargin was not modified after incubation with Y-27632. Capacitative Ca^{2+} entry was estimated from the increase in Ca^{2+} signal produced by the addition of Ca^{2+} into the bathing solution after 6 min incubation in Ca^{2+}-free solution in the presence of thapsigargin (Berridge, 1995). Perfusion with Ca^{2+}-free solution caused a slow decrease in the Ca^{2+} signal. Noradrenaline was applied for 3 min during the incubation of the artery in the Ca^{2+}-free solution, in order to ensure that Rho-kinase was activated (Fig. 11*C* and *D*). Under this condition, noradrenaline produced only a small, transient peak in Ca^{2+} signal, varying between 2 and 30 % of the response to KCl ($P < 0.05$ compared with the Ca^{2+} signal measured in the absence of thapsigargin). The re-addition of Ca^{2+} to the solution produced a large increase in Ca^{2+} signal above the basal level, which was not affected by Y-27632.

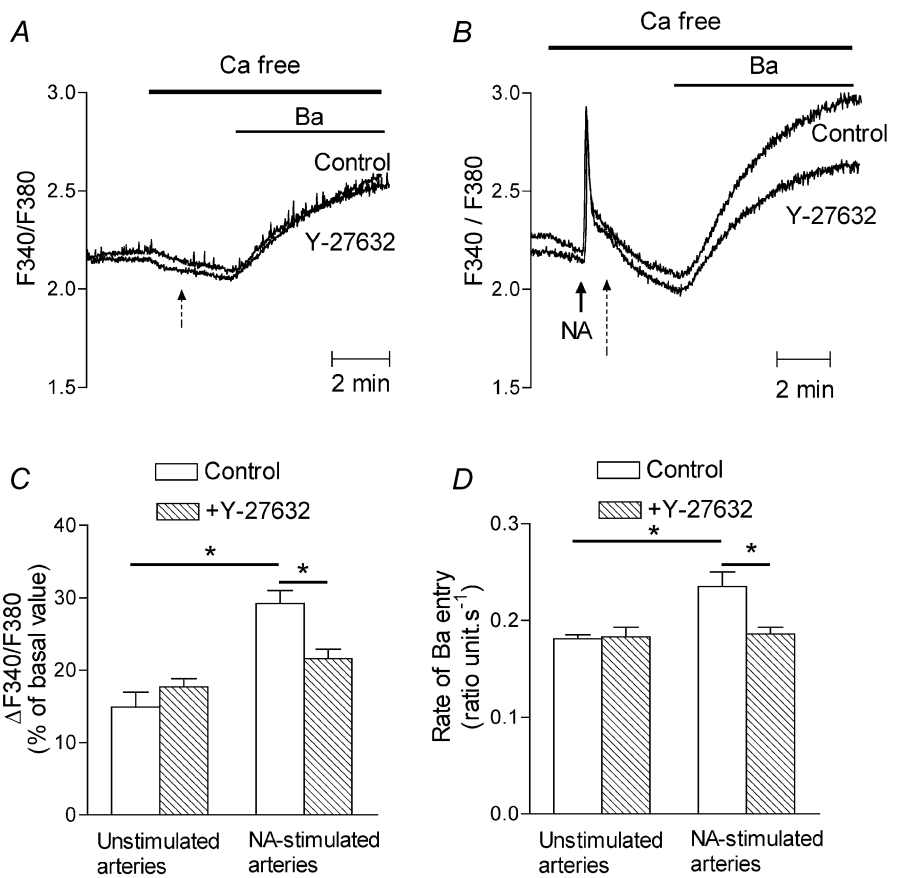

Figure 10. Effect of Y-27632 on Ba^{2+} entry in fura-2-loaded aorta

A, superimposed representative recordings of the changes in the F_{340}/F_{380} ratio evoked by the addition of Ba^{2+} (1 mM) into nimodipine-containing Ca^{2+}-free perfusion solution. Vehicle (control) or Y-27632 (10 μM) was added at the time indicated by the dashed arrow. Traces were from different aortas. *B*, superimposed representative recordings of the changes in F_{340}/F_{380} ratio evoked by the addition of Ba^{2+} (1 mM) to nimodipine-containing Ca^{2+}-free perfusion solution after stimulation with 1 μM noradrenaline (NA). Vehicle (control) or Y-27632 (10 μM) was added at the time indicated by the dashed arrow. Traces were from different aortas. *C*, bar graph showing the mean values of the change in the F_{340}/F_{380} ratio evoked by Ba^{2+} expressed as a percentage of the basal F_{340}/F_{380} value in unstimulated aorta ($n = 6$) and in aorta stimulated by 1 μM noradrenaline (NA, $n = 7$) in the absence and presence of Y-27632 (10 μM). *D*, bar graph showing the mean values of the rate of Ba^{2+} entry expressed as change in F_{340}/F_{380} ratio s^{-1} in unstimulated aorta ($n = 6$) or in aorta stimulated by 1 μM noradrenaline (NA, $n = 7$) in the absence (□) and presence of Y-27632 (10 μM) (▨). * $P < 0.05$.

DISCUSSION

The present results indicate that in rat aorta and mesenteric artery, Rho-dependent kinase is involved in the Ca^{2+} entry activated by noradrenaline or by direct stimulation of G proteins, in addition to its role in the Ca^{2+} sensitisation of the contractile proteins.

In agreement with previous reports, the Rho-kinase inhibitor Y-27632 inhibited contraction of the aorta and of the mesenteric artery (Uehata *et al.* 1997). Depression of KCl-evoked contraction confirms that Rho-kinase is activated by depolarisation in rat caudal artery (Mita *et al.* 2002). Interestingly, the Ca^{2+} signal evoked by noradrenaline or direct activation of G protein, but not that evoked by high KCl, was significantly inhibited by the Rho-kinase inhibitors Y-27632 and HA1077.

Y-27632 displayed the same potency in inhibiting contraction and the Ca^{2+} signal activated by noradrenaline. The IC_{50} value of Y-27632 (500 nM) is in agreement with its reported IC_{50} value for the inhibition of the Rho-dependent kinases p160ROCK and its isozyme ROCK-II in *in vitro* assay and for the relaxation of rabbit aorta (Uehata *et al.* 1997; Davies *et al.* 2000). Another inhibitor of Rho-kinase, HA1077 (Uehata *et al.* 1997; Sward *et al.* 2000), produced the same pattern of effects as Y-27632 and the effects of the two inhibitors were not additive (data not shown). In addition, the PKC inhibitor Ro-31-8220 did

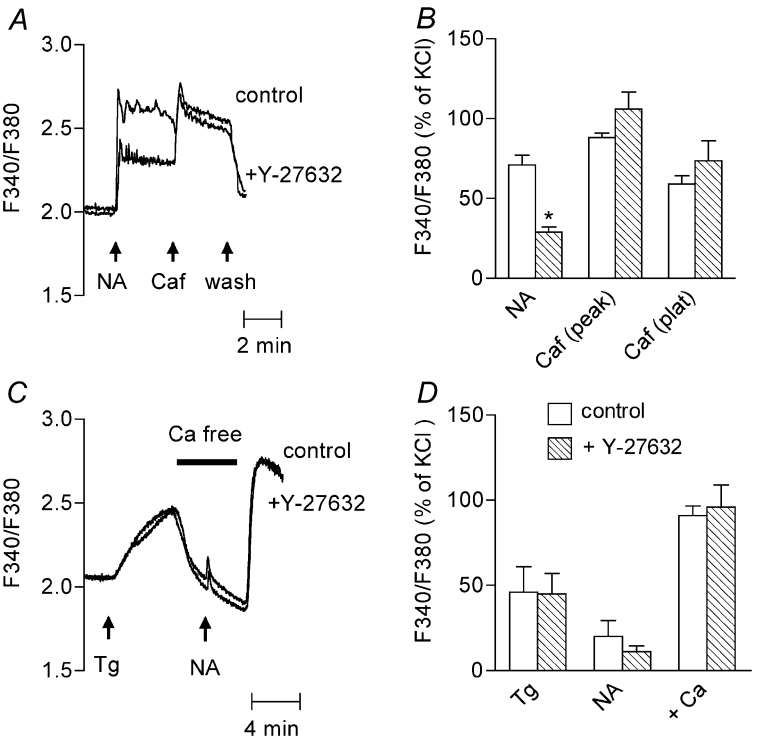

Figure 11. Effect of Y-27632 on the Ca^{2+} signal evoked by caffeine or thapsigargin in fura-2-loaded aorta

A, representative recording of the effect of pre-incubation of the aorta with Y-27632 (10 μM) on the Ca^{2+} signal evoked by caffeine. Noradrenaline (NA, 1 μM) was applied 3 min before stimulation with caffeine (Caf, 10 mM). Traces obtained before and after incubation with Y-27632 are superimposed. They were obtained from the same aortic ring. *B*, bar graph showing mean values of the Ca^{2+} signal evoked by noradrenaline (NA); caffeine measured at the peak (Caf peak) and the plateau of the response (Caf plat) in control (□) and in Y-27632-treated aorta (▨). Data were normalised to the response to 100 mM KCl and are means from 4 determinations ± S.E.M. * $P < 0.05$, Y-27632-treated *vs.* untreated aortas. *C*, representative recording of the effect of Y-27632 on Ca^{2+} signal evoked by thapsigargin. Thapsigargin (Tg, 1 μM) and noradrenaline (NA, 1 μM) were applied as indicated by the arrows. The physiological solution was changed to a Ca^{2+}-free solution as indicated by the horizontal bar. Traces obtained without and with Y-27632 (10 μM) are superimposed. They were obtained from different aortic rings. *D*, bar graph showing mean values of Ca^{2+} signal evoked by thapsigargin (Tg), noradrenaline (NA) measured at the peak of the response in Ca^{2+}-free solution, and after re-admission of Ca^{2+} (1.25 mM) to the solution (+Ca) in control (□) and in Y-27632-treated aorta (▨). Effect of re-addition of Ca^{2+} was measured with reference to the baseline fluorescence ratio measured in Ca^{2+}-containing solution. Data were normalised to the response to 100 mM KCl and are means from 4 determinations ± S.E.M.

not mimic the effect of Y-27632 and did not prevent it. We cannot exclude the possibility that Y-27632 inhibits other kinases or interacts with other targets, but, taken together, these observations suggest that the effects of Y-27632 on contraction and the Ca^{2+} signal are mediated through its interaction with Rho-dependent kinase.

Ca^{2+} signalling activated by α-adrenoceptors in arteries is dependent on the activation of PLCβ by G_q proteins (Exton, 1994). PI4 phosphate 5 kinase, which is involved in the synthesis of the PLC substrate, PIP2, has been reported to be a target of Rho-kinase (Oude Weernink *et al.* 2000). However, prolonged inactivation of Rho protein is required to observe the inhibition of inositol phosphate production in N1E-115 neuroblastoma cells (Zhang *et al.* 1996) or in mouse fibroblasts (Chong *et al.* 1994). The present results showed that inhibition of Rho-kinase produced a drop in the noradrenaline-activated Ca^{2+} signal within a few minutes but did not affect the production of inositol phosphates. Inhibition of the initial rapid increase in InsP_3 production evoked by agonists after 5 s of stimulation (Berridge, 1983) cannot be excluded from the present data but would not be consistent with the observation that Y-27632 evenly inhibited the Ca^{2+} response to noradrenaline 10 min after beginning of stimulation.

In vascular smooth muscle cell, the increase in cytosolic Ca^{2+} after noradrenaline stimulation originates from both intracellular stores, mainly located in the SR, and the extracellular compartment, in proportions varying according to the artery. Ca^{2+} entry occurs through several pathways: VOC, receptor-operated and store-operated Ca^{2+} channels (McFadzean & Gibson, 2002). Y-27632 inhibited the nimodipine-resistant entry of Ba^{2+} in noradrenaline-stimulated arteries. Since Ba^{2+} is not taken up by Ca^{2+} exchangers and pumps, this result shows unequivocally that Y-27632 inhibits the activation of a cationic channel distinct from the VOC. The observations that Y-27632 affected noradrenaline-evoked Ca^{2+} and Ba^{2+} entry similarly when added before or after store emptying, and that InsP_3 production and Ca^{2+} release were not affected, indicate that Rho-kinase might regulate a cationic channel directly. This pathway is distinct from the capacitative Ca^{2+} entry, or store-operated Ca^{2+} entry, which was demonstrated by the effect of re-addition of Ca^{2+} to the medium after store depletion by thapsigargin in Ca^{2+}-free medium (Berridge, 1995). The latter Ca^{2+} signal was sensitive to low concentrations of Gd^{3+} (not shown), which is known to be an inhibitor of capacitative Ca^{2+} entry (Broad *et al.* 1999), but was not affected by Y-27632. The saturation of the effect of Y-27632 on the fura-2–Ca^{2+} signal compared with the contractile response probably reflects the selective inhibition of non-selective cationic channels while other Ca^{2+} sources activated by noradrenaline were unaffected.

In aorta bathed in Ca^{2+}-free solution, noradrenaline produced a large but transient Ca^{2+} signal, sensitive to thapsigargin but also depressed by nimodipine and by Y-27632. Inhibition of the peak Ca^{2+} response in Ca^{2+}-free solution by Y-27632 might suggest that Rho-kinase is involved in the mechanisms underlying the release of intracellular Ca^{2+}. This conclusion was not substantiated by the observation that the release of $^{45}Ca^{2+}$ from non-mitochondrial stores evoked by InsP_3 in β-escin-permeabilised A7r5 aortic cells was not affected by Y-27632. In addition, in freshly isolated cells from rat aorta, Y-27632 (10 μM) did not affect the intracellular Ca^{2+} release evoked by 50 μM InsP_3 monitored by the activation of Ca^{2+}-dependent K^+ current (authors' unpublished data). Nevertheless, the limitations of these two observations, the former performed with cultured cells which may not be representative of aorta or mesenteric artery, and the latter using maximal concentrations of InsP_3, do not allow us to disregard the possibility that Y-27632 produced a change in InsP_3 receptors. The capacity for Ca^{2+} release of the SR did not appear to be regulated by Rho-kinase since Ca^{2+} signals evoked by thapsigargin or by caffeine were not impaired by Y-27632. More probably, both Rho-kinase-activated non-selective cationic channels and VOCs contribute to Ca^{2+} store refilling, as reported for VOCs in several excitable cells (McCarron *et al.* 2000; Lee *et al.* 2001).

The non-selective cationic channel regulated by Rho-kinase could be similar to the cationic conductance activated by noradrenaline in rabbit portal vein smooth muscle cells (Byrne & Large, 1988). This conductance is highly permeable to divalent cations (Byrne & Large, 1988; Wang & Large, 1991). Its proposed physiological role is to produce membrane depolarisation with subsequent opening of VOCs and also to allow direct influx of Ca^{2+} ions. The observation that Y-27632 inhibited noradrenaline-evoked depolarisation in rat mesenteric artery smooth muscle cells is in agreement with a role for this conductance in depolarisation and its regulation by Rho-kinase. It is indeed improbable that the inhibition by Y-27632 of the depolarisation evoked by noradrenaline resulted from an interaction of Rho-kinase with K^+ channels, because blockade of most K^+ channels with a cocktail of blockers, or a marked increase in the reversal potential of K^+ did not prevent the effects of the Rho-kinase inhibitor. The Rho-kinase activated cationic channel does not appear to control resting membrane potential, which was not affected by Y-27632.

Although inhibition of the Rho A–Rho-kinase pathway does not appear to affect the Ca^{2+} signal in porcine coronary artery (Nobe & Paul, 2001) or in guinea-pig smooth muscle (Lucius *et al.* 1998), there are several reports suggesting that Rho-kinase could be involved in

Ca^{2+} signalling in rat aorta (Takizawa *et al.* 1993), and, more recently, in tracheal smooth muscle (Ito *et al.* 2002) and in cultured human aortic endothelial cells (Yokoyama *et al.* 2002). There is now compelling evidence for the involvement of the Trp family of channel proteins in the Ca^{2+} permeable cation channels activated by G-coupled receptors. The mammalian homologue Trp6 has been shown to function as a Ca^{2+} entry channel independently of store-operated channels (Boulay *et al.* 1997) and to be an important element of the native current activated by α_1-adrenoceptor stimulation in rabbit portal vein (Inoue *et al.* 2001). In rabbit vena cava, a putative non-selective cationic channel activated by $InsP_3$ channel-mediated Ca^{2+} release and involved in store refilling may be encoded at least in part by the *Trp1* gene (Lee *et al.* 2002). Further experiments should investigate whether these channel proteins could be a component of the Rho-kinase-regulated channel identified in aorta and mesenteric artery smooth muscle. Differences in the sensitivity of Ca^{2+} entry to Rho-kinase inhibition between tissues could arise from the expression of different channel proteins.

In conclusion, the present results showed that, in rat aorta and mesenteric artery smooth muscle cells, in addition to its role in Ca^{2+} sensitisation, Rho-kinase is involved in noradrenaline-evoked activation of Ca^{2+} entry, distinct from voltage-operated Ca^{2+} channels or thapsigargin-activated store-operated channels. This Ca^{2+} entry contributes to depolarisation and to the increase in intracellular $[Ca^{2+}]$.

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