British Journal of Pharmacology (2005) 146, 854–861
www.nature.com/bjp

Rho kinase inhibitors reduce neurally evoked contraction of the rat tail artery *in vitro*

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1 The effects of Rho kinase inhibitors (Y27632, HA-1077) on contractions to electrical stimulation and to application of phenylephrine, clonidine or α,β-methylene adenosine 5′-triphosphate (α,β-mATP) were investigated in rat tail artery *in vitro*. In addition, continuous amperometry and intracellular recording were used to monitor the effects of Y27632 on noradrenaline (NA) release and postjunctional electrical activity, respectively.

2 Y27632 (0.5 and 1 μM) and HA-1077 (5 μM) reduced neurally evoked contractions. In contrast, the protein kinase C inhibitor, Ro31-8220 (1 μM), had little effect on neurally evoked contraction.

3 In the absence and the presence of Y27632 (0.5 μM), the reduction of neurally evoked contraction produced by the α-adrenoceptor antagonists prazosin (10 nM) and idazoxan (0.1 μM) was similar.

4 The P2-purinoceptor antagonist, suramin (0.1 mM), had no inhibitory effect on neurally evoked contraction in the absence or the presence of Y27632 (1 μM). In the presence of Y27632, desensitization of P2X-purinoceptors with α,β-mATP (10 μM) increased neurally evoked contractions.

5 Y27632 (1 μM) and H-1077 (5 μM) reduced sensitivity to phenylephrine and clonidine. In addition, Y27632 reduced contractions to α,β-mATP (10 μM).

6 Y27632 (1 μM) had no effect on the NA-induced oxidation currents or the purinergic excitatory junction potentials and NA-induced slow depolarizations evoked by electrical stimulation.

7 Rho kinase inhibitors reduce sympathetic nerve-mediated contractions of the tail artery. This effect is mediated at a postjunctional site, most likely by inhibition of Rho kinase-mediated 'Ca^{2+} sensitization' of the contractile apparatus.
British Journal of Pharmacology (2005) **146,** 854–861. doi:10.1038/sj.bjp.0706377;
published online 22 August 2005

Keywords: Amperometry; α_1-adrenoceptor; α_2-adrenoceptor; electrophysiology; noradrenaline release; rat tail artery; Rho kinase; sympathetic nerve

Abbreviations: ATP, adenosine 5′-triphosphate; α,β-mATP, α,β-methylene adenosine 5′-triphosphate; e.j.p., excitatory junction potential; NA, noradrenaline

Introduction

Sympathetic nerve-mediated contractions of the rat tail artery are largely blocked by α-adrenoceptor antagonists, with both α_1- and α_2-adrenoceptors contributing to the postjunctional response (Bao *et al*., 1993; Yeoh *et al*., 2004a, b). A synergistic role for both adenosine 5′-triphosphate (ATP) and neuropeptide Y coreleased with NA, particularly during short trains of stimuli, has also been suggested (Bradley *et al*., 2003).

The postjunctional mechanisms whereby exogenously applied α_1-adrenoceptor agonists increase intracellular Ca^{2+} concentration in vascular smooth muscle of the tail artery are complex and involve store-released Ca^{2+}, depolarization-induced Ca^{2+} entry and capacitive Ca^{2+} entry (Chen & Rembold, 1995). In addition, α_1-adrenoceptor activation increases the sensitivity of the contractile apparatus to Ca^{2+} (Chen & Rembold, 1995). In rat tail artery, α_1-adrenoceptor-mediated 'Ca^{2+} sensitization' is due primarily to small GTPase RhoA-mediated activation of Rho kinase (Mueed *et al*., 2004), which in turn phosphorylates and inhibits the activity of myosin light-chain phosphatase (see Somlyo & Somlyo, 2003).

A role for RhoA/Rho kinase-mediated 'Ca^{2+} sensitization' in α_2-adrenoceptor-mediated contractions of rat tail artery has not been identified. However, α_2-adrenoceptor-mediated vasoconstriction in both rat aorta (Carter *et al*., 2002) and porcine palmar lateral vein (Roberts, 2004) is, in part, dependent on Rho kinase activation because contractions evoked by α_2-adrenoceptor agonists are inhibited the Rho kinase antagonist Y27632.

This study investigated the effects of Rho kinase inhibitors (Y27632 and HA-1077) on neurally evoked contractions of the rat tail artery. In addition, the effects of these agents on contractions to the α-adrenoceptor agonists, phenylephrine and clonidine, and to the P2X-purinoceptor agonist, α,β-methylene adenosine 5′-triphosphate (α,β-mATP), were determined. To investigate the possibility that Rho kinase inhibitors act prejunctionally to change neurotransmitter release, the effects of Y27632 on both noradrenaline (NA) release and nerve-evoked postjunctional electrical activity were investigated using continuous amperometry and intracellular electrical recording, respectively. The findings indicate that Rho kinase inhibitors reduce the contractile response to both nerve stimulation and α-adrenoceptor agonists without changing NA

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release from the perivascular sympathetic axons or neurally evoked electrical activity of the vascular smooth muscle.

Methods

All experimental procedures conformed to the National Health and Medical Research Council of Australia guidelines and were approved by the University of New South Wales Animal Care and Ethics Committee.

Female Wistar rats (46 rats aged 8–12 weeks old) were anaesthetized (100 mg kg^{-1}, pentobarbitone intraperitoneally (i.p.)) and killed by exsanguination. The proximal part of the tail artery was dissected and maintained in physiological saline solution containing the following (in mM): Na^+, 150.6; K^+, 4.7; Ca^{2+}, 2; Mg^{2+}, 1.2; Cl^-, 144.1; $H_2PO_4^-$, 1.3; HCO_3^-, 16.3; and glucose, 7.8. This solution was gassed with 95% O_2/5% CO_2 (to pH 7.2) and heated to 36°C.

Mechanical responses

Arterial ring segments (~1.5 mm long) taken from 10 to 20 mm distal to the base of the tail were mounted isometrically between stainless-steel wires (50 μm diameter) in a four-chamber myograph (Multi Myograph Model 610M, Danish Myo Technology, Denmark). To normalize the basal conditions and the isometric contractions, the measured force was converted to the effective pressure exerted on the luminal surface of the artery using Laplace's equation (see Mulvany & Halpern, 1977). The basal distending pressure was set at ~10.0×10^3 N m^{-2} (~75 mmHg), so that the arteries are at the peak of their length–force relationship (Yeoh *et al*., 2004a). After setting up, all tissues were exposed to four applications of phenylephrine (3 μM) for 6 min, each of which was followed by an 8 min washout interval. During this period, the peak amplitude of contractions to successive applications of phenylephrine increased to a plateau level.

Electrical stimuli (25 V, 1 ms pulse width) produced by a Grass SD9 Stimulator (Grass Instruments, U.S.A.) were applied through platinum plate electrodes mounted on either side of the artery. We have previously demonstrated that these stimulus parameters are supramaximal for evoking contraction and produce responses that are completely blocked by tetrodotoxin (Yeoh *et al*., 2004a). To demonstrate the effects of the Rho kinase inhibitors on nerve-evoked contractions, the arteries were stimulated at 6 min intervals with trains of 100 stimuli at 1 Hz. After the third or fourth train of stimuli, Y27632 (1 μM) or HA-1077 (5 μM) was added and left in contact with the artery for four trains of stimuli. The same protocol was used to assess the effects of the protein kinase C (PKC) inhibitor, Ro31-8220 (1 μM). Data from the fourth train of stimuli in the presence of the kinase inhibitors were used to assess their effects.

A second series of experiments investigated the effects of Y27632 on contractions evoked by different frequencies of stimulation. The arteries were given repeated cycles of stimulation composed of single trains of 25 stimuli at 1, 5 and 10 Hz, each separated by a 4 min interval. After the second cycle, Y27632 (1 μM) was added. The P2-purinoceptor antagonist suramin (0.1 mM) or the P2X-purinoceptor agonist α,β-mATP (10 μM) was added following the fourth cycle of stimulation and left in contact with the artery for two further cycles. Data from the second (control), fourth (Y27632) and sixth (Y27632 + suramin or α,β-mATP) cycles were analysed. The effects of suramin in the absence of Y27632 were determined in arteries from a separate group of rats.

A third series of experiments examined the effects of the α_1-adrenoceptor antagonist, prazosin, and the α_2-adrenoceptor antagonist, idazoxan, on contractions evoked 100 stimuli at 1 Hz in the presence of Y27632 (0.5 μM). In these experiments, arteries were stimulated at 15 min intervals and Y27632 was added following the third period of stimulation and either prazosin (10 nM) or idazoxan (0.1 μM) was added following the fifth period of stimulation. The effects of the antagonists in the presence of Y27632 were assessed by comparing data from the fifth and seventh stimulation periods. The effects of prazosin and idazoxan in the absence of Y27632 were determined in arteries from a separate group of rats.

Concentration–response curves for the α-adrenoceptor agonists, phenylephrine (0.03–300 μM) and clonidine (0.003–10 μM), were determined in the absence and the presence of Y27632 (1 μM). In these experiments, the arteries were exposed to each concentration for 7 min followed by 9 min washes before the next addition of the agonist. The effect of HA-1077 (5 μM) on contractions to phenylephrine (3 μM) and clonidine (0.1 μM) was also determined. In these experiments, arteries were exposed to the agonist for 6 min at intervals of 20 min, with HA-1077 applied following the second application period. The effects of HA-1077 were determined by comparing responses to the second and third applications of agonist. In addition, the effect of Y27632 on contractions to α,β-mATP (10 μM) was determined. In these experiments α,β-mATP was added for ~3 min at 30 min intervals, with Y27632 added following the second application of α,β-mATP. The effects of Y27632 were determined by comparing responses to the second and third applications of α,β-mATP.

Electrochemistry and electrophysiology experiments

For both the electrochemistry and electrophysiology experiments, ~15 mm segments of artery from 10 to 30 mm distal to the base of the tail were pinned to the Sylgard-coated base of a 1 ml recording chamber, which was continuously perfused (3–5 ml min^{-1}) with physiological saline solution (see above). The proximal end of the artery was drawn into a suction electrode and the perivascular nerves excited by electrical stimuli (20 V, 1 ms pulse width). As increasing the stimulus voltage did not increase the amplitude of the signals recorded, these stimulus parameters are assumed to be supramaximal. Recordings were made at sites 1–2 mm distal to the mouth of the stimulating electrode.

Electrochemical recording

The release of endogenous NA was monitored using continuous amperometry as described previously (Dunn *et al*., 1999; Brock & Tan, 2004). Briefly, a Nafion-coated carbon fibre electrode (7 μm diameter) was mounted so that the first 100–200 μm from the tip of the fibre was in contact with the adventitial surface of the artery. The electrode was connected to an AMU130 Nanoamperometer (Radiometer-Analytical SA, Villeurbanne Cedex, France) and a potential difference of +0.3 V was applied between the recording electrode and an

Ag/AgCl pellet placed in the recording chamber medium. The current required to maintain this voltage was monitored.

In these experiments, the physiological saline contained the α_1-adrenoceptor antagonist, prazosin (0.1 μM), to inhibit neurally evoked contractions due to released NA. During the experiments, the arteries were stimulated at 1 min intervals with 10 stimuli at 10 Hz. Y27632 (1 μM) was added to the superfusing solution following the 20th train of stimuli and left in contact with the artery for a further 20 trains. At the end of the experiments, the Ca^{2+} channel blocker, Cd^{2+} (0.1 mM), was added to verify that the signals recorded were due to Ca^{2+}-dependent release of NA.

Electrophysiological recording

Intracellular recordings were made as described previously (Brock & Tan, 2004). To avoid excitatory junction potentials (e.j.p.'s) with an early fast component recorded in cells close to the neuromuscular junctions at the adventitial–medial boarder (see Cassell *et al*., 1988), recordings were made from cells located deeper in the media in which e.j.p.'s decayed monoexponentially, reflecting the electrical behaviour of the smooth muscle syncytium. Data were obtained in single impalements during which recordings were made in control and test solutions. Membrane potentials were determined upon withdrawal of the microelectrode.

During the experiments, the arteries were stimulated at 1 min intervals with trains of five stimuli at 1 Hz, with Y27632 (1 μM) added to the superfusing solution following the 10th train of stimuli and left in contact with the tissues for 20 min.

Data analysis

All data were digitized (sampling frequencies of 10–200 Hz) and collected with a PowerLab recording system and the programs Chart or Scope (ADInstruments, Castle Hill, NSW, Australia). For the mechanical responses, the peak amplitude of the contractions to electrical stimulation and to the applied agonists was measured. Measurements of electrically evoked contractions were made just prior to drug addition (T_1) and in the presence of the drug at the times indicated above (T_2) and the changes expressed as T_2/T_1 ratios. To account for time-dependent changes in the amplitude of the signals, comparisons were made with T_2/T_1 ratios determined at the same time points in control experiments where no drug was added. In the experiments investigating the effects of the neurotransmitter antagonists in the presence of Y27632, comparisons were made with data obtained in control experiments in which only Y27632 was added.

The electrochemical and electrophysiological data were analysed using the computer program Igor Pro (Wavemetrics, Lake Oswego, OR, U.S.A.). For both the electrochemical and electrophysiological experiments, three responses recorded just before (T_1) and 16–20 min following addition of Y27632 (T_2) were averaged before measurements were made. In the electrochemistry experiments, stimulation produced a negative-going artefact (revealed in Cd^{2+}) that lasted up to 100 ms. Therefore, the amplitude of the NA-induced oxidation currents was determined by taking the average value recorded between 100 and 200 ms following the last stimulus in the train. In the electrophysiology experiments, the mean amplitude of the e.j.p.'s evoked during the train of stimuli and the peak amplitude of the slow depolarization that followed the train of stimuli were compared. For both electrophysiology and electrochemistry experiments, the T_2/T_1 ratios determined in drug-treated arteries were compared with those determined in control experiments where no drugs were added. The exponential decay of the NA-induced oxidation currents and e.j.p.'s was fitted using the curve-fitting functions in the computer program Igor Pro.

Most pairwise statistical comparisons were made using Student's paired or unpaired two-tailed *t*-tests. Owing to unequal variance, comparison of T_2/T_1 ratios for the electrically evoked contractions of control and α,β-mATP-treated arteries was made with Mann–Whitney *U*-tests. The concentration–response curves for phenylephrine and clonidine in the absence and the presence of Y27632 were compared by repeated measures ANOVA. EC_{50} values for the α-adrenoceptor agonists were derived from best fits to the Hill equation using Igor Pro. For all statistical tests, $P<0.05$ was taken as a significant difference. The data compared using parametric tests are presented as mean $\pm$ s.e.m., whereas those compared using nonparametric tests are presented as median and interquartile range. In all cases, n refers to the number of arteries studied.

Drugs

Y27632, HA-1077 (fasudil) and suramin were supplied by Tocris Cookson Ltd (Bristol, U.K.) and α,β-mATP, clonidine HCl, idazoxan HCl, L-phenylephrine HCl, prazosin HCl and Ro31-8220 were supplied by Sigma Chemical Company (Castle Hill, NSW, Australia). Prazosin was prepared as a 1 mM stock solution in 10% (w v^{-1}) dimethylsulphoxide in water. All other drugs apart from suramin were prepared as 1 mM stock solutions in water. Suramin was dissolved directly in the physiological saline.

Results

Effects of Y27632 and HA-1077 on electrically evoked contractions

Figure 1a and b show the effects of Y27632 (1 μM, $n=4$) on contractions evoked by 100 stimuli at 1 Hz. Following addition of Y27632, there was a marked reduction in the amplitude of responses to nerve stimulation that reached a plateau level after $\sim$20 min (Figure 1b). In comparison with the change observed in untreated control arteries ($T_2/T_1=0.92\pm0.03$, $n=5$, paired *t*-test $P=0.06$), Y27632 significantly reduced the amplitude of the contractions ($T_2/T_1=0.23\pm0.03$, unpaired *t*-test $P<0.001$). Similarly, in comparison with control arteries, HA-1077 (5 μM, $n=5$) significantly reduced the amplitude of contractions evoked by 100 stimuli at 1 Hz ($T_2/T_1=0.18\pm0.02$, paired *t*-test $P<0.001$).

Figure 1c shows T_2/T_1 ratios for contractions of control ($n=5$) and Y27632 (1 μM, $n=5$) treated arteries evoked by 25 stimuli at 1, 5 and 10 Hz. At all frequencies of stimuli, Y27632 significantly reduced the amplitude of contraction, but the inhibitory effect of this agent was much less at the higher frequencies of stimulation studied.

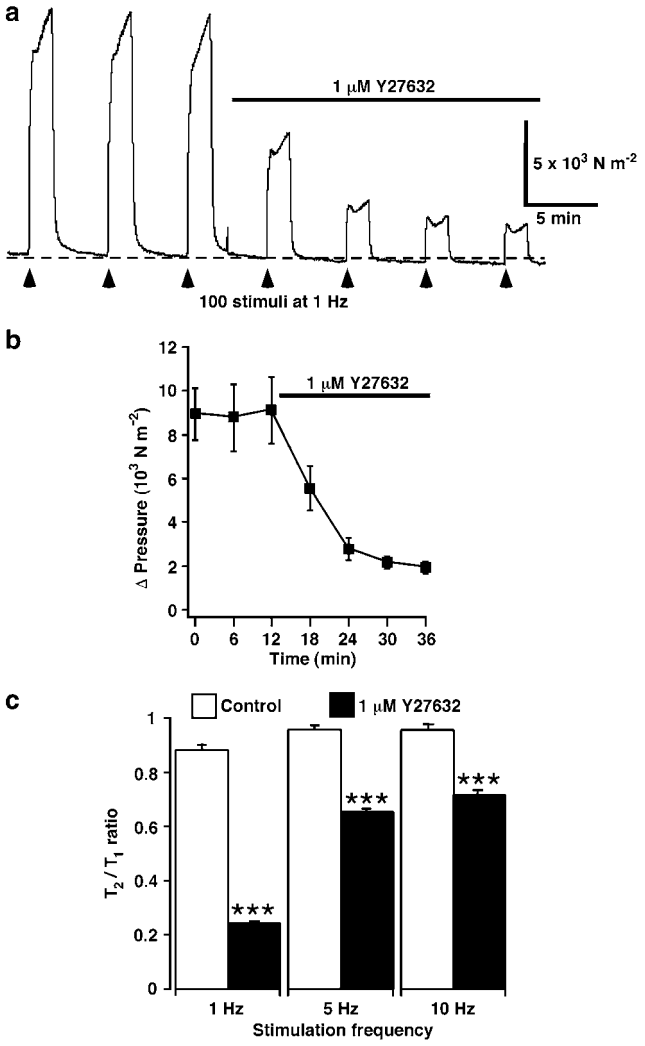

Figure 1 Effects of Y27632 (1 μM) on electrically evoked contractions of the rat tail artery. (a) Trace showing contractions to 100 stimuli at 1 Hz recorded before and during application of Y27632. (b) Graph showing the time course of the effects Y27632 on the amplitude of contractions evoked by 100 stimuli at 1 Hz ($n=4$). (c) Histogram showing T_2/T_1 ratios for contractions to 25 stimuli at 0.5, 1, 5 and 10 Hz in control ($n=5$) and Y27632- ($n=5$) treated arteries. In (c), statistical comparisons were made with paired t-tests. ***$P<0.001$.

Effects of Ro31-8220 on contractions evoked by electrical stimulation

Y27632 at relatively low concentrations has been suggested to inhibit PKC and in particular PKCdelta (IC_{50} 6 mM; Eto *et al.*, 2001). For this reason, the effect of Ro31-8220 (1 μM), which blocks multiple PKC isoforms including PKCdelta (Tan *et al.*, 2003), on contractions evoked by 100 stimuli at 1 Hz was assessed. In these experiments ($n=3$), application of Ro31-8220 for 20 min had little effect on the peak amplitude of neurally evoked contractions (control, $16.2\pm1.8\times10^3$ N m^{-2}; Ro31-8220, $15.1\pm1.6\times10^3$ N m^{-2}).

Effects of Y27632 on the blockade produced by prazosin and idazoxan

Both prazosin (10 nM) and idazoxan (0.1 μM) significantly reduced the peak amplitude of contractions to 100 stimuli in the absence and the presence of 0.5 μM Y27632 (Figure 2). In comparison with the responses of control arteries ($n=6$, T_2/T_1 0.95 ± 0.05), this concentration of Y27632 reduced the peak amplitude of contractions to 100 stimuli at 1 Hz ($n=6$, T_2/T_1 0.32 ± 0.06; unpaired t-test $P<0.001$). Proportionately, the reduction in the amplitude of contractions produced by prazosin and idazoxan did not differ significantly in the absence or the presence of Y27632 (prazosin, unpaired t-test $P=0.17$; idazoxan, unpaired t-test $P=0.78$).

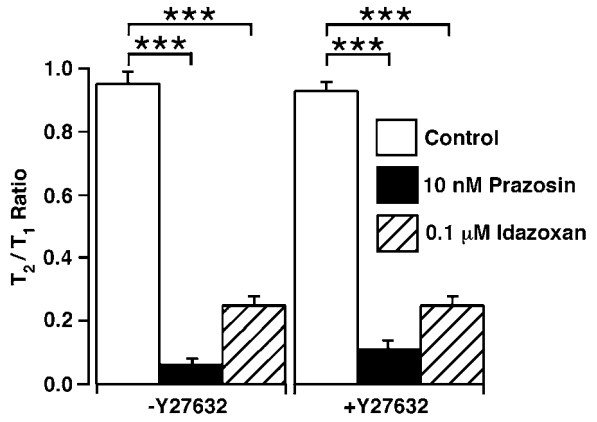

Figure 2 Effects of prazosin (10 nM) and idazoxan (0.1 μM) on contractions evoked by 100 stimuli at 1 Hz in the absence and the presence of Y27632 (1 μM). The histogram shows T_2/T_1 ratios for control arteries ($n=6$) and arteries treated with either prazosin ($n=6$) or idazoxan ($n=6$). Statistical comparisons were made with unpaired t-tests. ***$P<0.001$.

Effects of Y27632 and HA-1077 on the sensitivity to phenylephrine and clonidine

Y27632 (1 μM) significantly changed the concentration–response curves for both phenylephrine and clonidine and decreased the maximum contraction to both agents (Figure 3a and b; repeated measures ANOVA, control vs Y27632, $P<0.01$ for both comparisons); the reduction in the maximum contraction was much larger for clonidine. In addition, Y27632 increased the EC_{50} for phenylephrine (control, 1.7 ± 0.4 μM; Y27632, 6.3 ± 1.8 μM; paired t-test $P<0.05$) and clonidine (control, 0.07 ± 0.01 μM; Y27632, 0.17 ± 0.05 μM; paired t-test $P<0.05$).

HA-1077 (5 μM, $n=5$) also reduced the peak amplitude of the contractions evoked by phenylephrine (3 μM: control, $23.7\pm1.1\times10^3$ N m^{-2}; HA-1077, 14.8 ± 0.6 N m^{-2}; paired t-test $P<0.01$) and clonidine (0.1 μM: control, $19.8\pm2.8\times10^3$ N m^{-2}; HA-1077, $3.9\pm1.3\times10^3$ N m^{-2}; paired t-test $P<0.001$).

Effects of Y27632 on contractions to P2X-purinoceptor activation

Y27632 reduced the amplitude of contractions to α,β-mATP by about 60% (10 μM; Figure 4a and b). However, the P2-purinoceptor antagonist, suramin (0.1 mM), in the absence and the presence of Y27632 (1 μM) had no inhibitory effect on contractions to 25 stimuli at 1, 5 and 10 Hz (Figure 5a, c and d). Indeed, for four of five experiments in the absence and the presence of Y27632, suramin produced a small increase in the responses to nerve stimulation, but only the increase in

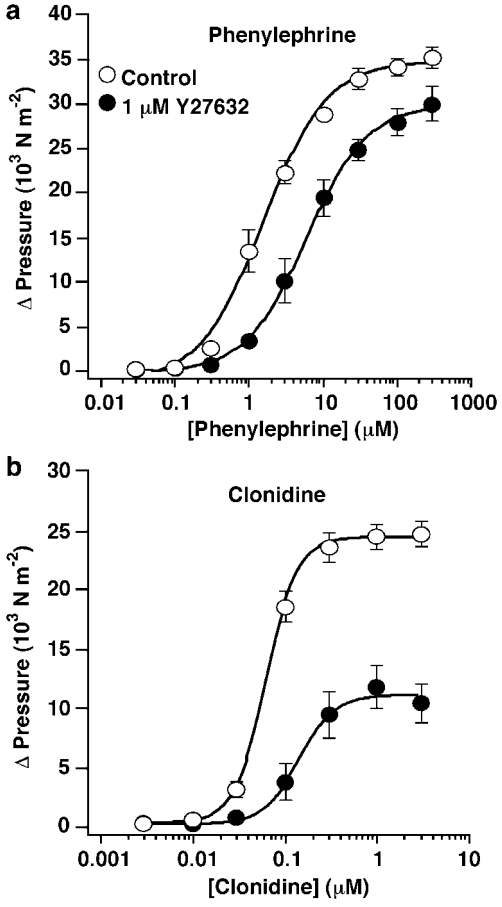

Figure 3 Effects of Y27632 (1 μM) on the sensitivity of the tail artery to the α-adrenoceptor agonists, phenylephrine and clonidine. (a and b) Concentration–response curves for phenylephrine (a) and clonidine (b) in the absence and the presence of Y27632 ($n=6$). The curves are the best fits to the Hill equation.

the response to 1 Hz stimulation in the absence of Y27632 and to 10 Hz stimulation in the presence of Y27632 was significant (Figure 5c and d). When the P2X-purinoceptors were desensitized by the continuous application of α,β-mATP (10 μM, $n=5$; Sneddon & Burnstock, 1984) in the presence of Y27632 (1 μM), the amplitude of contractions to 25 stimuli at 1, 5 and 10 Hz was increased (Figure 5b and e).

Effects of Y27632 on endogenous NA release

Trains of 10 stimuli at 10 Hz were used to evoke NA release. The stimulation artefacts during the trains of stimuli prevented the oxidation currents evoked by individual stimuli being discerned, but summation of these signals produced a large amplitude oxidation current (Figure 6a). In comparison with the control experiments, Y27632 (1 μM, $n=6$) had no effect on the amplitude of oxidation currents measured at the end of the trains of stimuli (T_2/T_1: control, 0.92 ± 0.03; Y27632, 0.93 ± 0.03; unpaired t-test $P=0.89$).

Changes in the time course of decay of the oxidation currents reflect effects on the clearance of NA (Stjärne *et al.*, 1994; Dunn *et al.*, 1999). Both in the absence and the presence of Y27632, the decay of oxidation currents was best fitted by the sum of two exponentials. Y27632 had no significant effect

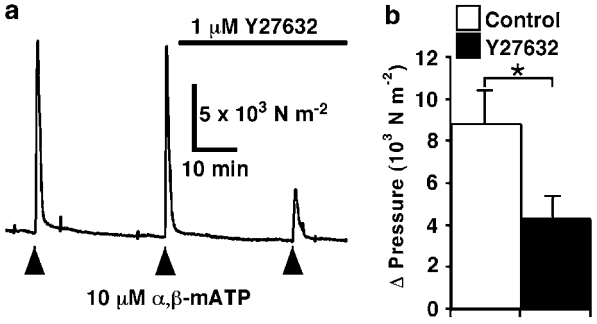

Figure 4 Effect of Y27632 (1 μM) on contractions to α,β-mATP (10 μM). (a) Trace showing contractions to α,β-mATP recorded before and during application of Y27632. During the periods when α,β-mATP was applied, the contraction to this agent peaked and then declined. (b) Histogram showing the peak increase in pressure produced by α,β-mATP in the absence and the presence of Y27632 ($n=4$). Statistical comparison was made with a paired t-test. $^*P<0.05$.

on either the fast (control, 0.45 ± 0.03 s; Y27632, 0.46 ± 0.03 s; paired t-test $P=0.25$) or the slow (control, 1.72 ± 0.23 s; Y27632, 1.43 ± 0.11 s; paired t-test $P=0.24$) time constants of decay.

Effects of Y27632 on postjunctional electrical activity

Trains of five stimuli at 1 Hz were used to elicit changes in membrane potential. Each stimulus during the trains evoked an e.j.p. that lasted about 1 s (Figure 6b). In addition, an NA-induced slow depolarization (Cheung, 1982) was recorded that peaked 15–20 s following the start of the train and lasted about 1 min (Figure 6b). In comparison with control experiments, Y27632 (1 μM, $n=5$) did not change the amplitude of either the e.j.p.'s evoked during the trains of stimuli (T_2/T_1: control, 0.98 ± 0.02; Y27632, 0.94 ± 0.02; unpaired t-test $P=0.20$) or the NA-induced slow depolarization (T_2/T_1: control, 0.98 ± 0.06; Y27632, 1.01 ± 0.08; unpaired t-test $P=0.86$). Y27632 also had no effect on the time constant of decay of e.j.p.'s (control, 222 ± 17 ms; Y27632, 217 ± 17 ms; paired t-test $P=0.41$) or on the resting membrane potential (control, -68 ± 1 ms; Y27632, -67 ± 1 mV; paired t-test $P=0.21$), indicating that this agent did not change the passive electrical properties of the vascular smooth muscle (see Cassell *et al.*, 1988).

Discussion

The Rho kinase inhibitor Y27632 reduced the amplitude of contractions evoked by nerve stimulation. As Y27632 had no effect on the release or clearance of endogenous NA, the effects of this agent on neurally evoked contraction appear to be accounted for by its postjunctional actions. Furthermore, as Y27632 did not change the NA-induced slow depolarizations, which are due to the activation of α_2-adrenoceptors by released NA (Itoh *et al.*, 1983; Cassell *et al.*, 1988), this agent did not appear to interfere with the activation of these membrane receptors. However, the sensitivity of the vascular smooth muscle to the α-adrenoceptor agonists, phenylephrine and clonidine, was reduced. Therefore, it is likely that the inhibitory effects of Y27632 on nerve-evoked contraction can

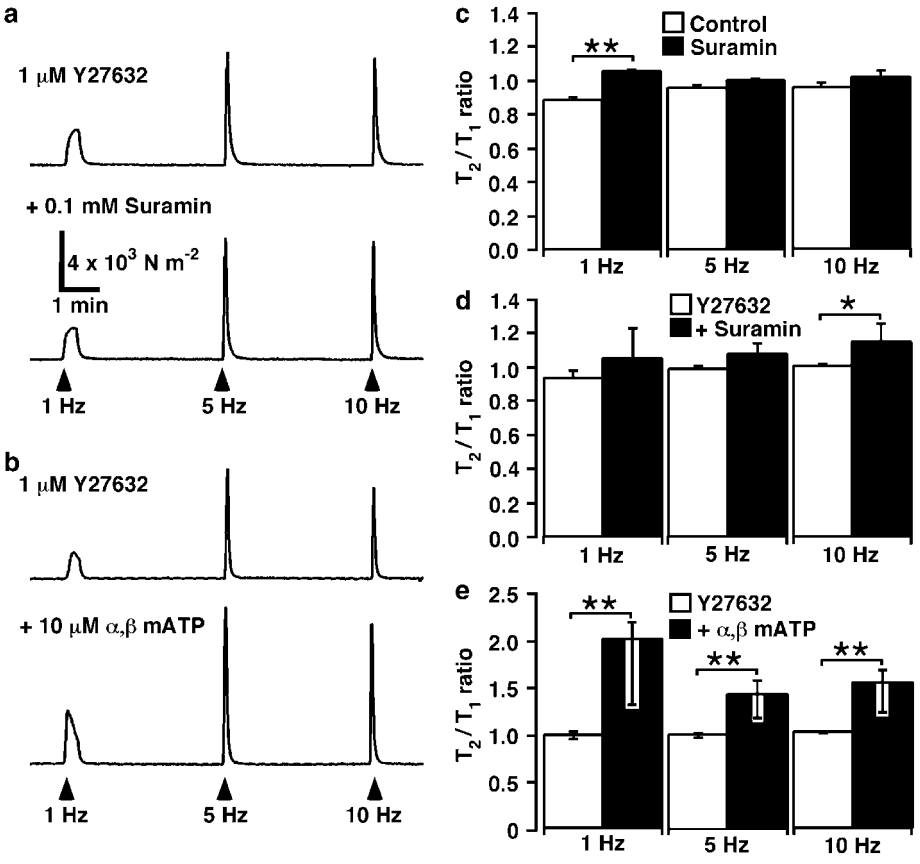

Figure 5 Effects of suramin (0.1 mM) and α,β-mATP (10 μM) on contractions to electrical stimulation in the presence of Y27632 (1 μM). (a and b) Traces showing contractions to 25 stimuli at 1, 5 and 10 Hz in the presence of Y27632 before and during application of suramin (a) or α,β-mATP (b). (c–e) Histograms showing T_2/T_1 ratios for contractions to 25 stimuli at 0.5, 1, 5 and 10 Hz in control (i.e. without Y27632 $n=5$) and suramin ($n=6$) treated arteries (c), in Y27632 ($n=5$) and Y27632 plus suramin ($n=6$) treated arteries (d) and in Y27632 ($n=5$) and Y27632 plus α,β-mATP ($n=6$) treated arteries. In (c) and (d), data are present as mean and s.e.m. and statistical comparisons were made with paired t-tests. In (e), data are presented as median and interquartile range and statistical comparisons were made with Mann–Whitney U-tests. $^*P<0.05$, $^{**}P<0.01$.

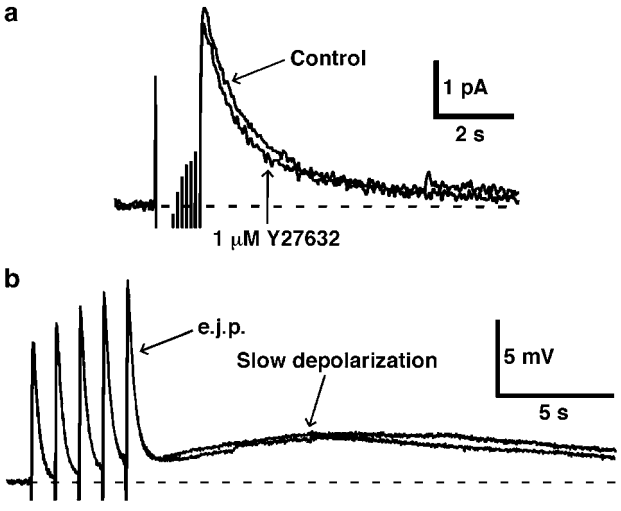

Figure 6 Examples of electrochemical and electrophysiological records. (a) Overlaid traces showing NA-induced oxidation currents evoked by 10 stimuli at 10 Hz before and during the addition of Y27632 (1 μM). (b) Overlaid traces showing excitatory junction potentials (e.j.p.'s) and the NA-induced slow depolarization produced by five stimuli at 1 Hz before and during application of Y27632 (1 μM).

be entirely accounted for by a reduction in neurotransmitter-induced 'Ca^{2+} sensitization' of the contractile apparatus.

While this study primarily reports findings for Y27632, we also confirmed the inhibitory effects of another Rho kinase antagonist, HA-1077, on contractions to both nerve stimulation and the α-adrenoceptor agonists, phenylephrine and clonidine. In contrast, the PKC inhibitor Ro31-8220 had little effect on nerve-evoked contractions and it has previously been reported that this agent does not change the sensitivity of the tail artery to phenylephrine (Mueed *et al*., 2004), so the possibility that the effects of Y27632 are mediated through inhibition of PKC can be excluded. Together, these findings suggest that the effects of Y27632 observed in this study were mediated through inhibition of Rho kinase.

The inhibitory actions of Y27632 on phenylephrine-induced contractions are similar to those reported by Mueed *et al*. (2004). The marked inhibitory effect of Y27632 on contractions produced by clonidine suggests that Rho kinase also plays a major role in the response to α_2-adrenoceptor activation. A similar conclusion has been made for both rat aorta (Carter *et al*., 2002) and porcine palmar lateral vein (Roberts, 2004), where Y27632 greatly attenuated the contractions to α_2-adrenoceptor activation.

The concentrations of the α-adrenoceptor antagonists used in this study are approximately 10 times higher than the pA_2 values for prazosin at α_1-adrenoceptors and idazoxan at α_2-adrenoceptors (see Brock *et al*., 1997). While the contractions to clonidine were more markedly inhibited by Y27632 than those to phenylephrine, proportionately the reductions of neurally evoked contractions produced by prazosin and idazoxan did not differ in the absence or the presence of Y72632. This finding suggests that Y27632 does not change the relative contributions of α_1- and α_2-adrenocepor activation to neurally evoked contraction. The degrees of blockade produced by idazoxan and by prazosin summed to more than 100% both in the absence and the presence of Y27632. Previously, we have shown that the blockade produced by combined application of prazosin and idazoxan in the absence of Y27632 is similar in magnitude to that produced by prazosin alone (see Yeoh *et al*., 2004a, b). The nonadditive nature of the blockades produced by prazosin and idazoxan further supports the idea that α_2-adrenoceptor activation has synergistic actions on α_1-adrenoceptor-mediated contraction in the tail artery (Xiao & Rand, 1989; Brock *et al*., 1997).

As reported previously (Bao & Stjärne, 1993), blocking the postjunctional actions of ATP at P2X-purinoceptors can produce an increase, rather than a decrease, in neurally evoked contractions of the tail artery (see also Yeoh *et al*., 2004b). Furthermore, we have previously shown that the combined blockade of both P2-purinoceptors (with suramin) and α-adrenoceptors (with phentolamine) produced a similar inhibition of nerve-evoked contraction as that produced by α-adrenoceptor blockade alone (Yeoh *et al*., 2004b). These findings suggest that P2X-purinoceptors do not contribute significantly to contractions evoked by the patterns of electrical stimulation employed in this study. However, Y27632 did reduce the contraction produced by the P2X-purinoceptor agonist, α,β-mATP. A similar inhibitory effect of Y27632 on P2X-purinoceptor-mediated contraction has been reported for mouse vas deferens (Buyukafsar *et al*., 2003). As Y27632 did not change the amplitude of e.j.p.'s, which result from the actions of neurally released ATP at $P2X_1$-purinoceptors (McLaren *et al*., 1998), this agent does not appear to interfere with the activation of $P2X_1$-purinoceptors or the release of ATP. Thus, it is likely that downstream activation of Rho kinase contributes to P2X-purinoceptor-mediated contraction.

In conclusion, Rho kinase inhibitors inhibit nerve-mediated activation of the vascular smooth muscle of the tail artery. This effect appears to be mediated at a postjunctional site, reducing both α_1- and α_2-adenoceptor-mediated activation of the vascular smooth muscle. While not directly investigated, we assume that the activation of these adrenoceptors by released NA results in Rho kinase-mediated 'Ca^{2+} sensitization' of contractile apparatus (see Mueed *et al*., 2004).

This work was supported by the National Health and Medical Research Council of Australia (Project Grant No. 350903, Fellowship Grant No. 350904). We thank Elspeth McLachlan for her comments on the manuscript.

References

BAO, J.X., GONON, F. & STJÄRNE, L. (1993). Frequency- and train length-dependent variation in the roles of postjunctional α_1- and α_2-adrenoceptors for the field stimulation-induced neurogenic contraction of rat tail artery. *Naunyn Schmiedebergs Arch. Pharmacol.,* **347,** 601–616.

BAO, J.X. & STJÄRNE, L. (1993). Dual contractile effects of ATP released by field stimulation revealed by effects of α,β-methylene ATP and suramin in rat tail artery. *Br. J. Pharmacol.,* **110,** 1421–1428.

BRADLEY, E., LAW, A., BELL, D. & JOHNSON, C.D. (2003). Effects of varying impulse number on cotransmitter contributions to sympathetic vasoconstriction in rat tail artery. *Am. J. Physiol. Heart Circ. Physiol.,* **284,** H2007–H2014.

BROCK, J.A., MCLACHLAN, E.M. & RAYNER, S.E. (1997). Contribution of α-adrenoceptors to depolarization and contraction evoked by continuous asynchronous sympathetic nerve activity in rat tail artery. *Br. J. Pharmacol.,* **120,** 1513–1521.

BROCK, J.A. & TAN, J.H. (2004). Selective modulation of noradrenaline release by α_2-adrenoceptor blockade in the rat-tail artery *in vitro*. *Br. J. Pharmacol.,* **142,** 267–274.

BUYUKAFSAR, K., LEVENT, A. & ARK, M. (2003). Expression of Rho-kinase and its functional role in the contractile activity of the mouse vas deferens. *Br. J. Pharmacol.,* **140,** 743–749.

CARTER, R.W., BEGAYE, M. & KANAGY, N.L. (2002). Acute and chronic NOS inhibition enhances α_2-adrenoreceptor-stimulated RhoA and Rho kinase in rat aorta. *Am. J. Physiol. Heart Circ. Physiol.,* **283,** H1361–H1369.

CASSELL, J.F., MCLACHLAN, E.M. & SITTIRACHA, T. (1988). The effect of temperature on neuromuscular transmission in the main caudal artery of the rat. *J. Physiol.,* **397,** 31–49.

CHEN, X.L. & REMBOLD, C.M. (1995). Phenylephrine contracts rat tail artery by one electromechanical and three pharmacomechanical mechanisms. *Am. J. Physiol.,* **268,** H74–H81.

CHEUNG, D.W. (1982). Two components in the cellular response of rat tail arteries to nerve stimulation. *J. Physiol.,* **328,** 461–468.

DUNN, W.R., BROCK, J.A. & HARDY, T.A. (1999). Electrochemical and electrophysiological characterization of neurotransmitter release from sympathetic nerves supplying rat mesenteric arteries. *Br. J. Pharmacol.,* **128,** 174–180.

ETO, M., KITAZAWA, T., YAZAWA, M., MUKAI, H., ONO, Y. & BRAUTIGAN, D.L. (2001). Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. *J. Biol. Chem.,* **276,** 29072–29078.

ITOH, T., KITAMURA, K. & KURIYAMA, H. (1983). Roles of extrajunctional receptors in the response of guinea-pig mesenteric and rat tail arteries to adrenergic nerves. *J. Physiol.,* **345,** 409–422.

MCLAREN, G.J., BURKE, K.S., BUCHANAN, K.J., SNEDDON, P. & KENNEDY, C. (1998). Evidence that ATP acts at two sites to evoke contraction in the rat isolated tail artery. *Br. J. Pharmacol.,* **124,** 5–12.

MUEED, I., BAINS, P., ZHANG, L. & MACLEOD, K.M. (2004). Differential participation of protein kinase C and Rho kinase in α_1-adrenoceptor mediated contraction in rat arteries. *Can. J. Physiol. Pharmacol.,* **82,** 895–902.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19–26.

ROBERTS, R.E. (2004). The role of Rho kinase and extracellular regulated kinase-mitogen-activated protein kinase in α_2-adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein. *J. Pharmacol. Exp. Ther.,* **311,** 742–747.

SNEDDON, P. & BURNSTOCK, G. (1984). ATP as a co-transmitter in rat tail artery. *Eur. J. Pharmacol.,* **106,** 149–152.

SOMLYO, A.P. & SOMLYO, A.V. (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.,* **83,** 1325–1358.

STJÄRNE, L., BAO, J.X., GONON, F. & MSGHINA, M. (1994). Nerve activity-dependent variations in clearance of released noradrenaline: regulatory roles for sympathetic neuromuscular transmission in rat tail artery. *Neuroscience,* **60,** 1021–1038.

TAN, M., XU, X., OHBA, M., OGAWA, W. & CUI, M.Z. (2003). Thrombin rapidly induces protein kinase D phosphorylation, and protein kinase C delta mediates the activation. *J. Biol. Chem.,* **278,** 2824–2828.

XIAO, X.H. & RAND, M.J. (1989). α_2-Adrenoceptor agonists enhance vasoconstrictor responses to α_1-adrenoceptor agonists in the rat tail artery by increasing the influx of Ca^{2+}. *Br. J. Pharmacol.,* **98,** 1032–1038.

YEOH, M., MCLACHLAN, E.M. & BROCK, J.A. (2004a). Tail arteries from chronically spinalized rats have potentiated responses to nerve stimulation *in vitro*. *J. Physiol.,* **556,** 545–555.

YEOH, M., MCLACHLAN, E.M. & BROCK, J.A. (2004b). Chronic decentralization potentiates neurovascular transmission in the isolated rat tail artery, mimicking the effects of spinal transection. *J. Physiol.,* **561,** 583–596.

(Received June 13, 2005
Revised July 8, 2005
Accepted July 26, 2005
Published online 22 August 2005)