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Expression of Rho-kinase and its functional role in the contractile activity of the mouse vas deferens

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1 The effects of two Rho-kinase inhibitors, Y-27632 and fasudil, were investigated on the contractions produced by electrical field stimulation (EFS, 40 V, 1 mS, 2, 4, 8 and 16 Hz, for 20 s), KCl (30 – 60 mM), phenylephrine (Phe) (10^{-5} – 10^{-4} M), adenosine-3′, 5′-triphosphate (ATP) (10^{-4} – 10^{-3} M) and α,β-methylene ATP (10^{-5} M).

2 EFS produced frequency-dependent reproducible contractile activity, which was almost abolished by guanethidine (10^{-5} M, for 1 h). This contraction consisted of two components (a phasic initial contraction followed by a tonic one), and it was inhibited by Y-27632 and fasudil (both at 10^{-5} M). However, these inhibitors had no effect on resting tension of the tissue.

3 Contractions elicited by KCl (30 – 60 mM) were insensitive to guanethidine (10^{-5} M, for 1 h), but suppressed by Y-27632 (10^{-5} M) and fasudil (10^{-5} M). In addition, the contractions induced by Phe (an α_1-adrenoceptor agonist) and ATP (a purinergic agent) were inhibited significantly by Y-27632 (10^{-5} M). Phasic contractions evoked by the selective P2X purinoceptor agonist α,β-methylene ATP were also suppressed by Y-27632 (10^{-5} M).

4 Western blot analysis revealed that the mouse vas deferens expresses Rho-kinase (ROKα, ROCK-2 isoform) protein with a molecular weight of approximately 160 kDa. As a positive control, the presence of this protein was also shown in homogenates of smooth muscle from the rat mesenteric artery.

5 In conclusion, Rho-kinase protein is expressed in the mouse vas deferens, and it mediates neurogenic contractile activity as well as the contractions induced by KCl, Phe, ATP and α,β-methylene ATP. Owing to the suppressive effects of Rho-kinase inhibitors on the contractile activity of the vas deferens, the possibility that these compounds might impair ejaculation must be taken into account when considering them as potential agents in the treatment of erectile dysfunction.
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Keywords: Adenosine triphosphate; electrical field stimulation; fasudil; Rho kinase; Y-27632; vas deferens

Abbreviations: ATP, adenosine-3′, 5′-triphosphate; MLCK, myosin light-chain kinase; MP, myosin phosphatase

Introduction

It has been reported that electrical field stimulation (EFS) of vas deferens induced two types of contractions: the first rapid contraction was accompanied by the release of adenosine-3′, 5′-triphosphate (ATP) and the other tonic component by noradrenaline (Brown *et al*., 1983; Bourreau *et al*., 1991). It is known that P2X purinergic receptors and α_1-adrenoceptors are mainly responsible for this contractile activity (Boland *et al*., 1992; Ravelic & Burnstock, 1998; Amobi *et al*., 2002). It has been reported that in $P2X_1$ knockout mice, electrically induced contractions are significantly reduced (Mulryan *et al*., 2000). On the other hand, P2Y receptors have been reported to mediate relaxation in the mouse vas deferens (Boland *et al*., 1992) and guinea-pig caeci (Hoyle *et al*., 1990). However, these purinoceptors may also induce contraction in the pulmonary artery and renal glomeruli (Chootip *et al*., 2002; Jankowski *et al*., 2003). It has been recently demonstrated that α_1-adrenoceptor- and purinoceptor-mediated responses involve the Rho/Rho-kinase pathway in the heart (Andersen *et al*., 2002), renal glomeruli (Jankowski *et al*., 2003), urinary bladder (Wibberley *et al*., 2003) and corpus cavernosum (Rees *et al*., 2001).

Rho-induced signalling triggers contractile responses of various tissues through Ca^{2+} sensitization, a mechanism allowing phosphorylation of myosin light-chain (MLC) and the subsequent regulation of contractile force to be independent of changes in intracellular Ca^{2+} concentration (Somlyo & Somlyo, 1994). Inhibition of Rho-kinase by (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) and fasudil can block contractile activity of the gastric fundal, ileal and other smooth muscles (Swärd *et al*., 2000; Tahara *et al*., 2002; Büyükafşar & Levent, 2003). Recently, it has been demonstrated that these inhibitors produce cavernosal smooth muscle relaxation and thus may be helpful in the treatment of erectile dysfunction (Chitaley *et al*., 2001; Rees *et al*., 2001; Wang *et al*., 2002; Büyükafşar & Ün, 2003). However, effects of the Rho-kinase inhibitors have yet to be investigated in vas deferens, which produces rhythmic contractions for ejaculation. Therefore, in the present study we examined these inhibitors on EFS-, KCl-, phenylephrine (Phe)-, ATP- and α,β-methylene ATP-induced contractions to gain an insight into the possible contribution

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of Rho/Rho-kinase signalling in the physiological control of the mouse vas deferens reactivity. Moreover, the expression of the Rho-kinase protein was detected by Western blotting.

Methods

Tissue preparation

Male albino mice weighing approximately 25 – 30 g were killed by a blow to the head and exsanguination. The two vasa deferentia were rapidly removed and stripped of adhering fat and connective tissue. Regardless of epididymal and prostatic portions of the vas deferens, the whole tissue of approximately 2 – 2.5 cm was used in the experiment. Each vas deferens was mounted vertically between two platinum wire electrodes connected to the Biopac stimulator (Biopac system Inc., CA, U.S.A.) in a 10 ml organ bath at an initial tension of 0.2 g. The bath, which was maintained at 37°C, contained Krebs' solution (composition in mM; NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11) gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac system Inc., CA, U.S.A.).

Organ bath experiments

Following the equilibration of 45 min, vasa deferentia were contracted with EFS (40 V, 2, 4, 8 and 16 Hz, 1 mS, for 20 s), KCl (30 and 60 mM), ATP (10^{-3} and 10^{-4} M), α,β-methylene ATP (10^{-5} M) or Phe (10^{-4} and 10^{-5} M). The contractions by the chemical stimuli were allowed to reach a plateau level except for ATP and α,β-methylene ATP, which evoked phasic contractions. After the first contraction series was obtained, Y-27632 (10^{-5} M, for 30 min), fasudil (10^{-5} M, for 30 min) or guanethidine (10^{-5} M, for 1 h) was added to the organ bath for incubation. Thereafter, the tissues were contracted in the same manner. Corresponding control series without the inhibitors were also performed. Concentrations and incubation duration of the agents used in this study were determined after preliminary experiments.

Western blot analysis for Rho-kinase (ROCK-2)

Pairs of whole vasa deferentia from different mice ($n = 5$) were isolated immediately after death, and the connective tissue was dissected out in a Petri dish containing ice-cold Krebs solution. The semen inside the lumen was removed by gently squeezing the vas deferens. In addition, the rat mesenteric artery was also isolated and used for positive control. The tissues were homogenized with the lysis buffer solution (Tris-HCl (pH = 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride 10 μM, leupeptin 10 μg ml^{-1}, pepstatin 1 μg ml^{-1}, benzamidine 1 mM). The homogenate was centrifuged at $900 \times g$ for 10 min at 4°C to remove nuclei and unlysed cells, and the supernatant was removed for protein assays (with the Lowry method) and Western blot analysis. Equal amounts of proteins (500 μg) were loaded in wells, electrophoresed on 8% SDS – polyacrylamide (PAGE) gels for 90 min at 6 – 8°C and then transferred onto a polyvinylidene diflouride membrane (PVDF) for 3 h at 6 – 8°C. The membrane was blocked with the blocking agent of ECL Advance kit (Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05% Tween-20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against ROCK-2 (ROKα, Polyclonal IgG, sc-1851, Santa Cruz Biotechnology Inc., CA, U.S.A.) at 1 : 250 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey anti-goat, 1 : 500, Santa Cruz Biotechnology Inc., CA, U.S.A.). The blots were then detected with the ECL Advance kit (Amersham Biosciences, Freiburg, Germany).

Statistical evaluations

All data represent means ± standard error of the mean (s.e.m.) of n observations. The first contractile responses were expressed as the percentage of the second contractile series. For statistical comparison, one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test was used. The student's t-test was also used when appropriate. A P-value less than 0.05 was considered significant. Graphs were drawn using the GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA, U.S.A.).

Chemicals used

(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) and fasudil (HA-1077) were obtained from Tocris Cookson Ltd (Bristol, U.K.) and KCl was obtained from Merck Co (Darmstadt, Germany). L-Phe hydrochloride, ATP, α,β-methylene ATP and guanethidine sulfate were obtained from Sigma Chemical Co. (St Louis, U.S.A.). All the chemicals were dissolved in distilled water. The primary antibody for ROCK-2 and HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). The ECL Advance kit was purchased from Amersham Biosciences (Freiburg, Germany). The kit was used according to the manufacturer's guidelines.

Results

Contractile effects of EFS, KCl, Phe, ATP and α,β-methylene ATP in the mouse vas deferens

EFS (40 V, 2, 4, 8 and 16 Hz, 1 mS, for 20 s) caused frequency-dependent contractions with two phases: phasic initial contraction followed by a tonic one (Figures 1 – 4). KCl (30 and 60 mM, Figure 5), Phe ($10^{-5} - 10^{-4}$ M, Figure 6), ATP ($10^{-4} - 10^{-3}$ M, Figures 6 and 7) and α,β-methylene ATP (10^{-5} M, Figure 8) also produced reproducible contractions. KCl and Phe induced tonic contractions, but ATP and α,β-methylene ATP produced phasic contractions. There was desensitization in response to 10^{-4} M ATP in that the contraction declined to 36% of the control series. However, at a higher concentration (10^{-3} M), there was no desensitization to ATP and the second series of contractions was 108.4 ± 14.6% of the control series. To a smaller extent, the response to α,β-methylene ATP also decreased in the second series to 88.1 ± 7.5% of control.

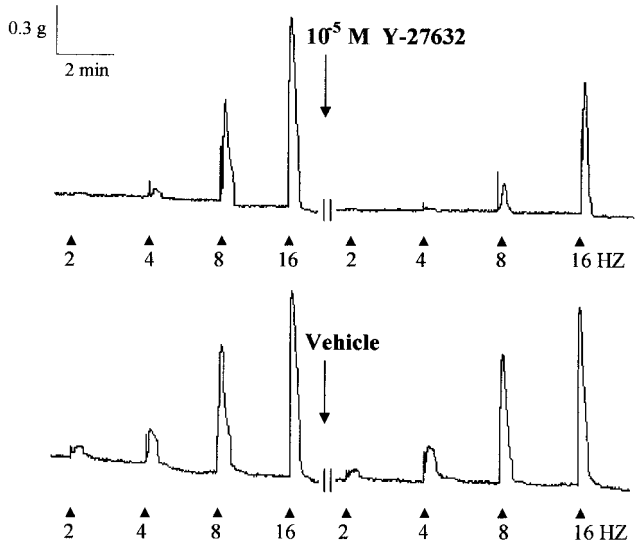

Figure 1 Tracings showing the effects of Y-27632, a Rho-kinase inhibitor and its vehicle (distilled water, 0.1–10 ml organ bath) on the electrically (40 V, 1 mS, for 20 s) induced contractile activity in the isolated whole vas deferens of mouse. After the first series of responses, Y-27632 or the vehicle was incubated for 30 min with the vas deferens before the second series was obtained. Note that both phasic and tonic contractile responses were attenuated in the presence of Y-27632.

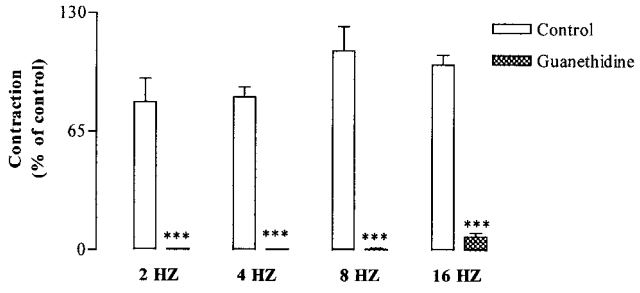

Figure 2 Abolition of EFS (40 V, 1 mS, 2, 4, 8 and 16 Hz, for 20 s)-induced contractions by guanethidine (10^{-5} M). Data represent means ± s.e.m. of five to eight observations. Contractions were expressed as percentages of control series. One-way ANOVA followed by the Bonferroni *post hoc* test was used for comparison of means. ***$P<0.001$.

Effects of guanethidine, Y-27632 and fasudil on EFS- and KCl-induced contractions

Guanethidine (10^{-5} M) abolished EFS-elicited contractile activity (Figure 2), but it had no effect on KCl-induced contraction (Figure 5). Y-27632 and fasudil (10^{-5} M) suppressed both phasic and tonic contractions produced by EFS (Figures 1, 3 and 4). A higher concentration of Y-27632 (5×10^{-5} M) also inhibited these contractions (data not shown). Moreover, KCl-induced contractions were also inhibited in the presence of these inhibitors (Figure 5). The tonic component of EFS-elicited contraction at 16 Hz was not significantly attenuated by 10^{-5} M fasudil. However, at $5x10^{-5}$ M, it markedly suppressed the contraction at 16 Hz (data not shown). Y-27632 had no effects on the resting tensions, which were 217.3 ± 20.9 and 183.7 ± 11.7 mg ($P>0.05$) in the absence and presence of Y-27632, respectively. They were 194.0 ± 22.1 mg (in the first series) and 165 ± 13.6 mg (in the presence of distilled water as vehicle, 0.1 ml of which was added to 10 ml organ bath, $P>0.05$); fasudil did not have any significant effects on the resting tone (data not shown).

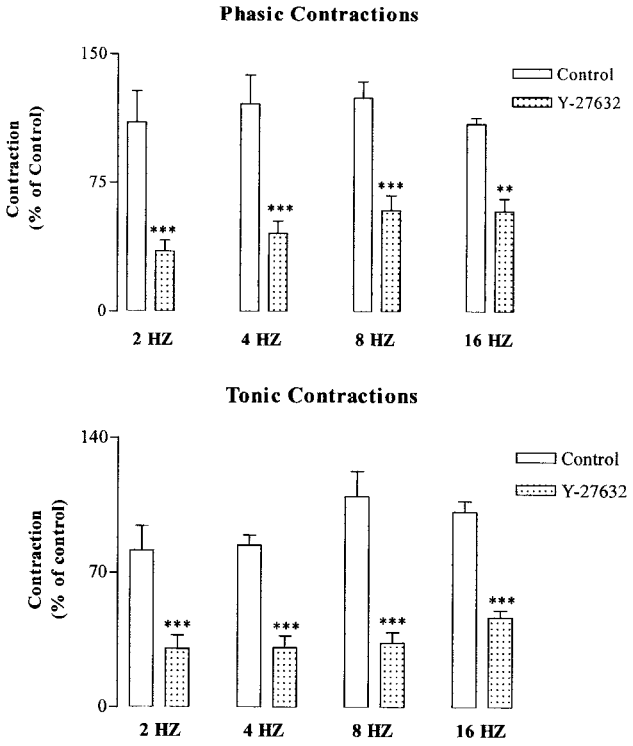

Figure 3 Suppression of the phasic and the tonic responses to EFS (40 V, 1 mS, 2, 4, 8 and 16 Hz, for 20 s) by Y-27632 (10^{-5} M, for 30 min). Data represent means ± s.e.m. of five to eight observations. Contractions were expressed as percentages of control series. One-way ANOVA followed by the Bonferroni *post hoc* test was used for comparison. **$P<0.01$, ***$P<0.001$.

Effects of Y-27632 on Phe-, ATP- and α,β-methylene ATP-evoked contractions

Y-27632 (10^{-5} M) depressed Phe (10^{-5} and 10^{-4} M) evoked contractions (Figure 6). The contractions induced by 10^{-4} M ATP were not changed in the presence of Y-27632; however, contractile activity induced by 10^{-3} M ATP was significantly suppressed (Figures 6 and 7). The response to 10^{-3} M ATP was 108.4 ± 14.6% of the control series ($n=5$); however, it was 36.0 ± 8.0% ($n=5$) at 10^{-4} M. In the presence of 10^{-5} M Y-27632, they were 50.8 ± 15.1% ($P<0.01$, $n=5$) and 25.2 ± 8.1% ($P>0.05$, $n=5$), respectively. Y-27632 (10^{-5} M) also attenuated α,β-methylene ATP-induced phasic contractions (Figure 8). The contraction was 88.1 ± 7.5% in control conditions and 52.4 ± 11.1% in the presence of Y-27632 ($P<0.05$).

Expression of ROCK-2 in the mouse vas deferens

Western blot analysis showed that the mouse vas deferens expressed Rho-kinase protein with a molecular weight of approximately 160 kDa. For a positive control, we also analyzed homogenates of the rat mesenteric artery and demonstrated that ROCK-2 was also expressed in this tissue (Figure 9).

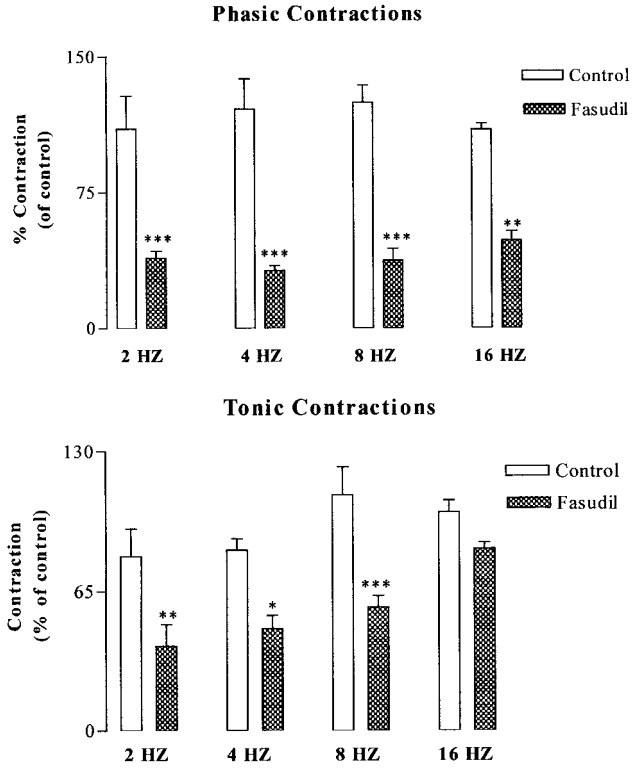

Figure 4 Suppression of the phasic and the tonic responses to EFS (40 V, 1 mS, 2, 4, 8 and 16 Hz, for 20 s) by fasudil (10^{-5} M, for 30 min). Data represent means ± s.e.m. of five to eight observations. Contractions were expressed as percentages of control series. One-way ANOVA followed by the Bonferroni *post hoc* test was used for comparison. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

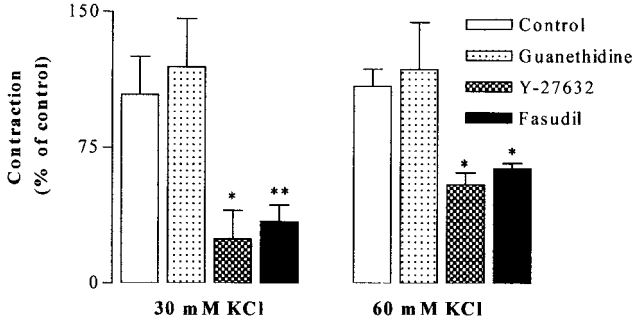

Figure 5 Effects of guanethidine (10^{-5} M, for 1 h, $n=7-9$), Y-27632 (10^{-5} M, for 30 min, $n=5-8$) and fasudil (10^{-5} M, for 30 min, $n=5-8$) on the contractions induced by KCl (30 – 60 mM). Data represent means ± s.e.m. Contractions were expressed as percentages of control series. One-way ANOVA followed by the Bonferroni *post hoc* test was used for comparison. *$P<0.05$; **$P<0.01$. Note that guanethidine did not suppress KCl-induced contraction.

Discussion

In the present study, we investigated the effects of two Rho-kinase inhibitors, Y-27632 and fasudil, on the contractile activity produced by EFS, KCl, an α-adrenoceptor agonist, Phe, a purinergic compound, ATP and a selective P2X purinergic receptor agonist, α,β-methylene ATP in the mouse vas deferens. Furthermore, we investigated whether vas deferens can express Rho-kinase protein (ROKα, ROCK-2 isosyme) by Western blotting.

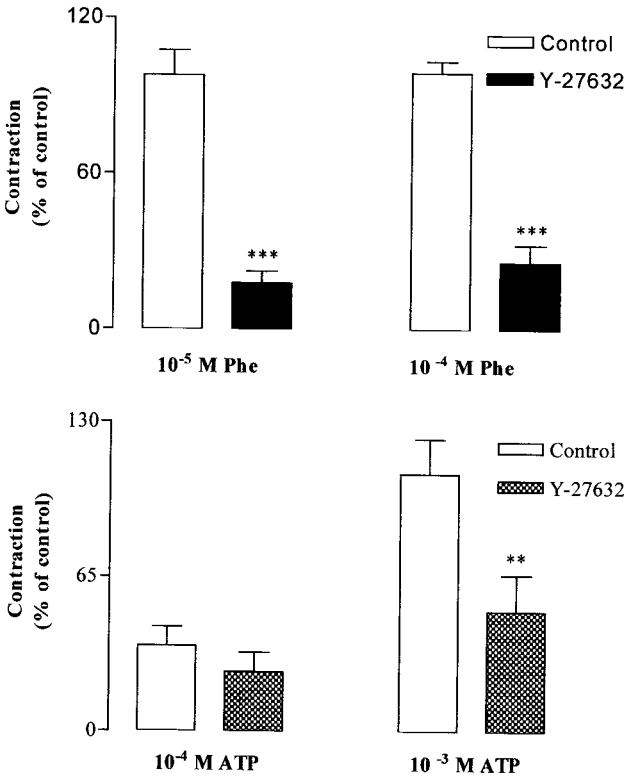

Figure 6 Attenuation of the contractile responses to Phe (10^{-5} – 10^{-4} M) and ATP (10^{-4} – 10^{-3} M) by Y-27632 and fasudil (both at 10^{-5} M, for 30 min). Data represent means ± s.e.m. of five to seven observations. Contractions were expressed as percentages of control series. One-way ANOVA followed by the Bonferroni *post hoc* test was used for comparison. **$P<0.01$; ***$P<0.001$.

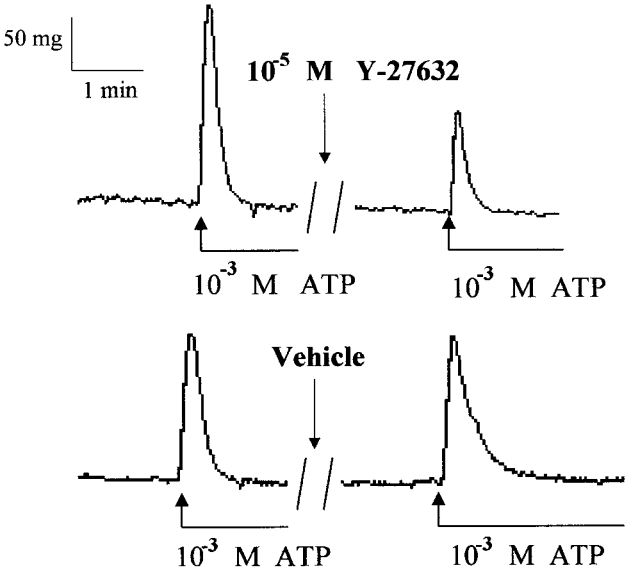

Figure 7 Original tracings showing the contractile effect of ATP and the inhibition of this response by Y-27632 (10^{-5} M, for 30 min). ATP induced phasic contractions of the vas deferens. Brackets in the tracings represent washing and incubation period with Y-27632 and its vehicle, distilled water (0.1 – 10 ml organ bath).

Increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggered by the activation of various receptors coupled to heterotrimeric G proteins activates myosin light-chain kinase

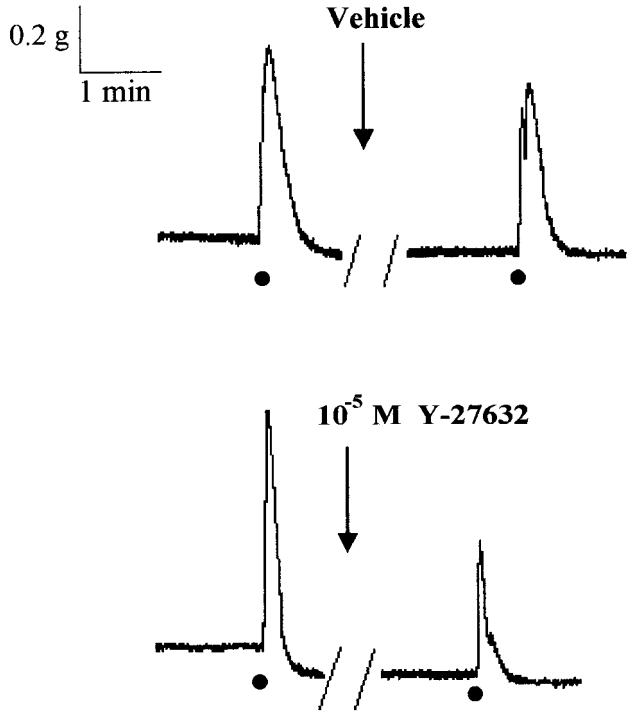

Figure 8 Original tracings showing the contractile effect of α,β-methylene ATP and the inhibition of this response by Y-27632 (10^{-5} M, for 30 min). α,β-Methylene ATP-induced phasic contractions. Dots (●) show the application of α,β-methylene ATP. Brackets in the tracings represent the washing and incubation period with Y-27632 and its vehicle, distilled water (0.1 – 10 ml organ bath).

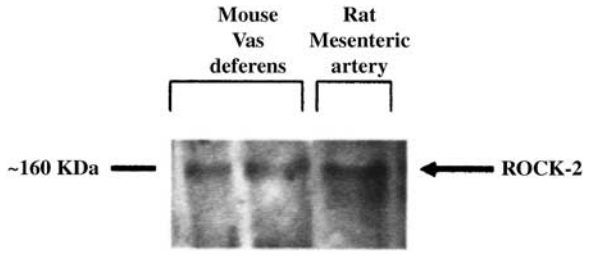

Figure 9 Western blotting for Rho-kinase (ROCK-2, ROKα) in the mouse vas deferens and rat mesenteric artery. Homogenates of the tissues were submitted to SDS – PAGE with 8% polyacrylamide and then transferred onto a PVDF. The membrane was blocked with an ECL advance blocking agent in Tris-buffered solution containing 0.05% TBS-T for 1 h. It was then probed with a primary antibody raised against ROCK-2 (polyclonal IgG) at 1 : 250 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey anti-goat, 1 : 500). Proteins bound with the antibodies were then visualized by the ECL Advance kit.

(MLCK) to phosphorylate the MLC through the binding of Ca^{2+} to calmodulin (Kamm & Stull, 1985; Somlyo & Somlyo, 2000). Although $[Ca^{2+}]_i$ plays a crucial role for contractile machinery in the smooth muscle, it has been reported that the regulation of MLC phosphorylation and contractile force can be independent of changes in $[Ca^{2+}]_i$, that is, at a constant $[Ca^{2+}]_i$, contractile force can be sustained. This is referred to as Ca^{2+} sensitization (Somlyo & Somlyo, 1994). A small GTPase, Rho and its downstream effector, Rho-kinase (p160Rho, ROCK-2, ROKα) could mediate the Ca^{2+} sensitization (Somlyo & Somlyo, 2000). It has been suggested that the major mechanism by which Rho-kinase causes the Ca^{2+} sensitization is the inhibition of a myosin phosphatase (Swärd *et al*., 2000; Fukata *et al*., 2001). Antagonism of Rho-kinase by Y-27632 or fasudil may cause vasodilatation (Uehata *et al*., 1997), inhibition of uterine contraction (Tahara *et al*., 2002), negative inotropic responses in the heart (Andersen *et al*., 2002), cytoprotection in ischemic brain damage (Satoh *et al*., 2001) and penile erection (Chitaley *et al*., 2001; Rees *et al*., 2001; Büyükafşar & Ün, 2003).

In this study, EFS-induced contraction was abolished in the presence of guanethidine, indicating that noradrenergic nerves mediate this contraction. There are also many reports confirming this finding in the literature. In addition, Phe, ATP and α,β-methylene ATP all produced contractile activity. At a lower concentration (10^{-4} M), there was desensitization to ATP. This might imply that a different kind of subreceptor for ATP could mediate the desensitization, as this phenomenon can develop against particular purinergic receptors (Ravelic & Burnstock, 1998). It has been reported that noradrenaline and ATP are colocalized in the adrenergic nerve endings and coreleased (Fedan *et al*., 1981; Burnstock & Sneddon 1985). α_1-Adrenoceptors and P2X purinoceptors, which are reported to be coupled with Rho/Rho-kinase signalling (Rees *et al*., 2001; Andersen *et al*., 2002; Wibberley *et al*., 2003), mediate electrically induced contraction in vas deferens (Burt *et al*., 1998; Ravelic & Burnstock, 1998). In support, α,β-methylene ATP, which is a selective P2X purinoceptor agonist induced phasic contractile response in this study. We evaluated phasic as well as tonic contractile responses to EFS; the former is known to be mediated by the release of ATP and the latter by noradrenaline. Y-27632 and fasudil significantly attenuated these contractions, showing that purinergic and adrenergic components could involve Rho/Rho-kinase signalling. Accordingly, the contractions induced by exogenously administered Phe, ATP and α,β-methylene ATP were dramatically inhibited by Y-27632, indicating that both α_1-adrenoceptor- and P2X purinoceptor-induced signalling could be associated with the Rho/Rho-kinase pathway. Furthermore, the contraction evoked by a P2X receptor agonist, α,β-methylene ATP, was attenuated by Y-27632 in the rat urinary bladder (Wibberley *et al*., 2003). On the other hand, P2Y receptors have also been reported to mediate the contractile effect of ATP, which could take place through the pathway of Rho-kinase signalling in the rat renal glomeruli (Jankowski *et al*., 2003). In the present study, any purinergic receptors other than P2X purinoceptors could not be excluded in the mediation of ATP-induced contraction. In addition, whether the inhibition of EFS-induced contraction by the Rho-kinase inhibitors was presynaptic or postsynaptic has not been examined. Further studies are needed to elucidate this possibility since it has been recently suggested that Rho/Rho-kinase signalling may be involved in neurotransmitter release (Büyükafşar & Levent, 2003).

KCl-induced contraction was also inhibited by Y-27632 and fasudil. In our previous study, we obtained such an action in the mouse gastric fundus (Büyükafşar & Levent, 2003). Inhibition of KCl-evoked contraction by the Rho-kinase inhibitors is interesting, and reveals that Ca^{2+} ions might also induce a Ca^{2+} sensitization since the effect of KCl depends on calcium influx through voltage-operated Ca^{2+} channels. This seems to be an important finding because Ca^{2+} influx may trigger its sensitization, rather like Ca^{2+}-induced Ca^{2+} release.

We do not know which step(s) in the pathway from the activation of RhoA to Rho-kinase is involved. Perhaps, Ca^{2+} activates RhoA protein or its downstream effector Rho-kinase to induce Ca^{2+} sensitization. In the literature, there is a controversy over the inhibition of KCl-induced contractile responses by Rho-kinase inhibitors. In this and our previous study (Büyükafşar & Levent, 2003), we demonstrated that KCl-induced responses were inhibited in the presence of Y-27632 or fasudil. Moreover, Y-27632 induced concentration-dependent relaxations in strips of small and large mesenteric arteries, precontracted with K^+ depolarization (Asano & Nomura, 2003). Further, it has been reported that membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase (Mita *et al*., 2002). On the other hand, there are numerous reports suggesting that K^+ depolarization-induced response could be insensitive or more resistant to Rho-kinase inhibitors than the contractions induced by agonists (Uehata *et al*., 1997; Anabuki *et al*., 2000; Weber & Webb, 2001; Wibberley *et al*., 2003). Further studies are necessary to elucidate this controversy. The inability of guanethidine to inhibit KCl-induced contraction may exclude the possibility that K^+ ion can cause the release of noradrenaline from adrenergic nerve endings in the vas deferens.

With respect to the expression of ROCK-2 protein, this is the first demonstration that mouse vas deferens expresses Rho-kinase with a molecular weight of approximately 160 kDa. However, the expression of another known isoform of Rho-kinase, ROCK-1 (ROKβ), was not investigated in this study. So, this remains to be detected. It was previously reported that guinea-pig vas deferens expressed RhoA protein (Fujita *et al*., 1995), which is the upstream activator of Rho-kinase (Fukata *et al*., 2001), strengthening our results.

In conclusion, the mouse vas deferens expresses ROCK-2 protein, and this enzyme can mediate EFS-, Phe-, ATP- and α,β-methylene ATP- as well as KCl-induced contractile responses. Another deduction from this study is that the use of Rho-kinase inhibitors in the treatment of erectile dysfunction may cause an impaired ejaculation, as EFS-evoked sympathetic contractions are known to be involved in the physiological ejaculation (Sato *et al*., 1991; Kihara *et al*., 1997; de Groat & Booth, 1980).

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References

AMOBI, N.I., GUILLEBAUD, J., KAISARY, A.V., TURNER, E. & SMITH, I.C. (2002). Discrimination by SZL49 between contractions evoked by noradrenaline in longitudinal and circular muscle of human vas deferens. *Br. J. Pharmacol.,* **136,** 127–135.

ANABUKI, J., HORI, M., HAYAKAWA, K., AKAHANE, S., OZAKI, H. & KARAKI, H. (2000). Muscarinic stimulation does not induce RhoA/ROCK-mediated Ca^{2+} sensitization of the contractile element in chicken gizzard smooth muscle. *Pflugers Arch.,* **441,** 189–199.

ANDERSEN, G.G.O., QVIGSTAD, E., SCHIANDER, I., AASS, H., OSNES, J.B. & SKOMEDAL, T. (2002). Alpha(1)-AR-induced positive inotropic response in heart is dependent on myosin light chain phosphorylation. *Am. J. Physiol. Heart Circ. Physiol.,* **283,** H1471–H1480.

ASANO, M. & NOMURA, Y. (2003). Comparison of inhibitory effects of Y-27632, a Rho kinase inhibitor, in strips of small and large mesenteric arteries from spontaneously hypertensive and normotensive Wistar – Kyoto rats. *Hypertens. Res.,* **26,** 97–106.

BOLAND, B., HIMPENS, B., VINCENT, M.F., GILLIS, J.M. & CASTEELS, R. (1992). ATP activates P2X-contracting and P2y-relaxing purinoceptors in the smooth muscle of mouse vas deferens. *Br. J. Pharmacol.,* **107,** 1152–1158.

BOURREAU, J.P., ZHANG, Z.D., LOW, A.M. & DANIEL, E.E. (1991). Ryanodine and the adrenergic, purinergic stimulation in the rat vas deferens smooth muscle smooth muscle: functional and radioligand binding studies. *J. Pharmacol. Exp. Ther.,* **256,** 1063–1071.

BROWN, D.A., DOCHERTY, J.R., FRENCH, A.M., MCDONALD, A., MCGRATH, J.M. & SCOTT, N.C. (1983). Separation of adrenergic and nonadrenergic contractions to field stimulation in the rat vas deferens. *Br. J. Pharmacol.,* **79,** 379–393.

BURNSTOCK, G. & SNEDDON, P. (1985). Evidence for ATP and noradrenaline as cotransmitters in sympathetic nerves. *Clin. Sci.,* **68,** 89–92.

BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1998). $Alpha_{1A}$-adrenoceptor mediated contraction of rat prostatic vas deferens and the involvement of ryanodine stores and Ca^{2+} influx stimulated by diacylglycerol and PKC. *Br. J. Pharmacol.,* **123,** 317–325.

BÜYÜKAFŞAR, K. & LEVENT, A. (2003). Involvement of Rho/Rho-kinase signalling in the contractile activity and acetylcholine release in the mouse gastric fundus. *Biochem. Biophys. Res. Commun.,* **303,** 777–781.

BÜYÜKAFŞAR, K. & ÜN, İ. (2003). Effects of the Rho-kinase inhibitors, Y-27632 and fasudil on the corpus cavernosum from diabetic mice. *Eur. J. Pharmacol.,* **472,** 235–238.

CHITALEY, K., WINGARD, C.J., WEBB, R.C., BRANAM, H., STOPPER, V.S., LEWIS, R.W. & MILLS, T.M. (2001). Antagonism of Rho-kinase stimulates rat penile erection *via* a nitric oxide independent pathway. *Nat. Med.,* **7,** 119–122.

CHOOTIP, K., NESS, K.F., WANG, Y., GURNEY, A.M. & KENNEDY, C. (2002). Regional variation in P2 receptor expression in the rat pulmonary arterial circulation. *Br. J. Pharmacol.,* **137,** 637–646.

DE GROAT, W.C. & BOOTH, A.M. (1980). Physiology of male sexual function. *Ann. Intern. Med.,* **92,** 329–331.

FEDAN, J.S., HOGABOOM, G.K., O'DONNELL, J.P., COLBY, J. & WESTFALL, D.P. (1981). Contribution by purines to the neurogenic response of the vas deferens of the guinea pig. *Eur. J. Pharmacol.,* **69,** 41–53.

FUJITA, A., TAKEUCHI, T., NAKAJIMA, H., NISHIO, H. & HATA, F. (1995). Involvement of heterotrimeric GTP-binding protein and rho protein, but not protein kinase C, in agonist-induced Ca^{2+} sensitization of skinned muscle of guinea pig vas deferens. *J. Pharmacol. Exp. Ther.,* **274,** 555–561.

FUKATA, Y., AMANO, M. & KAIBUCHI, K. (2001). Rho – Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol. Sci.,* **22,** 32–39.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramine antagonizes responses to P2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and *Taenia caeci*. *Br. J. Pharmacol.,* **99,** 617–621.

JANKOWSKI, M., SZCZEPANSKA-KONKEL, K., KALINOWSKI, L & ANGIELSKI, S. (2003). Involvement of Rho-kinase in P2Y-receptor-mediated contraction of renal glomeruli. *Biochem. Biophys. Res. Commun.,* **302,** 855–859.

KAMM, K.E. & STULL, J.T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.,* **25,** 593–620.

KIHARA, K., SATO, K. & OSHIMA, H. (1997). Involvement of the adrenal medulla in ejaculatory reactions in the dog. *Int. J. Androl.,* **20,** 104–111.

MITA, M., YANAGIHARA, H., HISINUMA, S., SAITO, M. & WALSH, M.P. (2002). Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem. J.,* **364,** 431–440.

MULRYAN, K., GITTERMAN, D.P., LEWIS, C.J., VIAL, C., LECKIE, B.J., COBB, A.L., BROWN, J.E., CONLEY, E.C., BUELL, G., PRITCHARD, C.A. & EVANS, R.J. (2000). Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. *Nature,* **403,** 86–89.

RAVELIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

REES, R.W., RNALPH, D.J., ROYLE, M., MONCADA, S. & CELLEK, S. (2001). Y-27632, an inhibitor of Rho-kinase, antagonizes noradrenergic contraction in the rabbit and human penile corpus cavernosum. *Br. J. Pharmacol.,* **133,** 455–458.

SATO, K., KIHARA, K., ANDO, M., SATO, T. & OSHIMA, H. (1991). Seminal emission by electrical stimulation of the spermatic nerve and epididymis. *Int. J. Androl.,* **14,** 461–467.

SATOH, S., UTSUNOMIYA, T., TSURUI, K., KOBAYASHI, T., IKEGAKI, I., SASAKI, Y. & ASANO, T. (2001). Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. *Life Sci.,* **69,** 1441–1453.

SOMLYO, A.P. & SOMLYO, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature,* **372,** 231–236.

SOMLYO, A.P. & SOMLYO, A.V. (2000). Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.,* **522,** 177–185.

SWÄRD, K., DREJA, K., SUSNJAR, M., HELLSTRAND, P., HARTSHORNE, D.J. & WALSH, M.P. (2000). Inhibition of Rho-associated kinase blocks agonist-induced Ca^{2+} sensitization of myosin phosphorylation and force in guinea-pig ileum. *J. Physiol.,* **522,** 33–49.

TAHARA, M., MORISHIGE, K., SAWADA, K., YOSHIHIDE, I., KAWAGISHI, R., TASAKA, K. & MURATA, Y. (2002). RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology,* **143,** 920–929.

UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, T., INUI, J., MAEKAWA, M. & NARUMIYA, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature,* **389,** 990–994.

WANG, H., ETO, M., STEERS, D.S., SOMLYO, A.P. & SOMLYO, A.V. (2002). RhoA-mediated Ca^{2+} sensitization in erectile function. *J. Biol. Chem.,* **277,** 30614–30621.

WEBER, D.S. & WEBB, R.C. (2001). Enhanced relaxation to the rho-kinase inhibitor Y 27632 in mesenteric arteries from mineralocorticoid hypertensive rats. *Pharmacology,* **63,** 129–133.

WIBBERLEY, A., CHEN, Z., HU, E., HIEBLE, J.P. & WESTFALL, T.D. (2003). Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. *Br. J. Pharmacol.,* **138,** 757–766.

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