# Expression of Rho-kinase (ROCK-1 and ROCK-2) and its substantial role in the contractile activity of the sheep ureter

[1]Adnan Levent & *,[1]Kansu Büyükafşar

[1]Department of Pharmacology, Medical Faculty, Mersin University Campus, Yenişehir 33169, Mersin, Turkey

**1**   Expression of two isoforms of Rho-kinase (ROCK) and its functional role in the physiological control of smooth muscle contraction in the sheep ureter were investigated. Helical strips of the ureteric smooth muscle were stimulated by electrical field stimulation (EFS, 60 V, 1 mS, 2, 4, 8, 16 and 32 Hz, for 20 S), KCl (80 mM), carbachol (CCh, $10^{-8}$–$10^{-4}$ M) or phenylephrine (Phe, $10^{-8}$–$10^{-4}$ M).

**2**   EFS produced a reproducible contractile activity, which was abolished by tetrodotoxin ($3 \times 10^{-6}$ M), a $Na^+$ channel blocker. A muscarinic receptor antagonist, atropine ($2 \times 10^{-6}$ M), and an adrenergic neuron blocker, guanethidine ($10^{-5}$ M), significantly suppressed the contraction induced by EFS. However, this contraction was augmented in the presence of $N^G$-nitro-L-arginine (L-NA, $10^{-4}$ M), a nitric oxide synthase inhibitor.

**3**   Two Rho-kinase inhibitors, Y-27632 ($5 \times 10^{-5}$ M) and fasudil ($5 \times 10^{-5}$ M), markedly attenuated the EFS-elicited contraction. CCh and Phe produced concentration-dependent contraction in the sheep ureter. $pD_2$ values for Phe and CCh were $5.04 \pm 0.11$ and $5.00 \pm 0.22$, respectively. Y-27632 ($5 \times 10^{-5}$ M) and fasudil ($5 \times 10^{-5}$ M) also significantly inhibited CCh- and Phe-induced contractions. Moreover, these ROCK inhibitors produced relaxations in the KCl-elicited contraction in a concentration-dependent manner. $pD_2$ values for Y-27632 and fasudil were, respectively, $5.17 \pm 0.07$ and $4.58 \pm 0.08$ ($P < 0.001$).

**4**   Furthermore, the influences of these agents were also tested on spontaneous phasic contractions of the tissue. Among Y-27632, fasudil, TTX, L-NA, guanethidine and atropine, only the ROCK inhibitors ($10^{-6}$–$10^{-5}$ M) were able to suppress the spontaneous contractile activity.

**5**   Western blot analysis has revealed that both isoforms of Rho-kinase (ROCK-1 and ROCK-2) are expressed in the sheep ureter. Densitometric analysis has indicated that these enzymes are less expressed in the sheep ureter than are in the sheep aorta in a significant manner.

**6**   These results show that a contractile enzyme, Rho-kinase, is expressed, and it mediates agonist- and EFS-induced contractions as well as spontaneous contractile activity of the isolated sheep ureter. Since Y-27632 and fasudil depressed the contractions, it seems plausible to postulate that Rho-kinase inhibitors may be beneficial in the treatment of renal colic.

## Introduction

One of the most important functions of the pyloureteral complex is to ensure the unidirectional transport of urine from kidney to the urinary bladder. It has been proposed that there might be some putative pacemakers responsible for initiation of peristaltic waves in the renal pelvis as well as ureteral smooth muscle (Santicioli & Maggi, 1998). On the other hand, the ureter is innervated by a large population of afferents that produce an intense pain following distention of the ureter by ureteral calculi. Electrical field stimulation (EFS) of the ureter also induces pain and muscular hyperalgesia in rats (Cervero, 1994). Furthermore, the implantation of an experimental calculus in rats have caused hyperalgesia and abdominal stretching, a hallmark of visceral pain (Giamberardino et al.,

1995a). Stretching of the ureter may cause the release of prostanoids and tachykinins which amplify ureteral motility (Laird et al., 1997; Lang et al., 2002). The drugs with spasmolytic and/or anti-inflammatory effects can be beneficial in the treatment of renal colic (Giamberardino et al., 1995b). Moreover, installation of capsaicin resulted in a pain-relieving effect in patients (Bultitude, 1995). Several mechanisms are proposed in the physiological control of ureteral peristalsis and smooth muscle tone (Santicioli & Maggi, 1998). Nevertheless, the cellular mechanisms underlying neurogenic and myogenic contractions are not exactly elucidated.

The increase in cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was regarded to be the principle mechanism in smooth muscle contraction. Upon activation of calmodulin, $Ca^{2+}$ activates myosin light chain kinase (MLCK) to phosphorylate the myosin light chain (MLC) to induce smooth

*Author for correspondence; E-mail: kbuyukafsar@mersin.edu.tr

muscle contraction (Kamm & Stull, 1985). However, the studies in which $[Ca^{2+}]_i$ and concomitant contractile force were detected have shown that $Ca^{2+}$ concentration does not always parallel the degree of MLC phosphorylation and subsequent contraction of the muscle. In other words, regulation of MLC phosphorylation and contractile force is independent of changes in $[Ca^{2+}]_i$. This is due to a phenomenon, the so-called $Ca^{2+}$ sensitization (Somlyo & Somlyo, 1994), raising the possibility that there must be some additional pathways, which can modulate the contractile process at a constant $[Ca^{2+}]_i$ (Fukata *et al.*, 2001). Indeed, it has been reported that a monomeric small GTP-binding protein, Rho, and its downstream effector, Rho-kinase (ROCK, ROK), play a substantial role in the $Ca^{2+}$ sensitization (Somlyo & Somlyo, 2003). The activation of hetero-trimeric G proteins such as $G_q$ ($G_{11}$) and $G_{12/13}$ following the stimulation of G protein-coupled receptors leads to some cellular events. On activation, $G_q$ or $G_{11}$ proteins cause phosphatidylinositol 4,5-bis-phosphate hydrolysis to diacyl-glycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$). However, following the activation of another heterotrimeric G protein, $G_{12/13}$, the signal is transmitted to the Rho/Rho-kinase pathway (Fukata *et al.*, 2001). This novel pathway provides an alternative vasoconstrictor mechanism, which is independent of $[Ca^{2+}]_i$ elevation (Somlyo & Somlyo, 1994). Indeed, it has been well established that the smooth muscle contraction can be induced also in the absence of an increase in the $[Ca^{2+}]_i$ (Bradley & Morgan, 1987).

It has been reported that Rho/Rho-kinase pathway could be involved in the contractile activity of various tissues such as aorta (Uehata *et al.*, 1997), mesenteric artery (Büyükafşar *et al.*, 2004), ileum (Sward *et al.*, 2000), gastric fundus (Büyükafşar & Levent, 2003), corpus cavernosum (Büyükafşar & Ün, 2003), urinary bladder (Wibberley *et al.*, 2003), vas deferens (Büyükafşar *et al.*, 2003) and uterus (Tahara *et al.*, 2002). However, this signalling pathway has not been studied in ureteric smooth muscle. For that reason, first we detected the expression of ROCK proteins of both isoforms by Western blotting in the tissue, and then investigated the functional importance of this enzyme by using its selective inhibitor, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxa-mide dihydrochloride monohydrate (Y-27632) (Ishizaki *et al.*, 2000), in the contractions, which are induced by EFS, KCl, phenylephrine and carbachol. We also tested another Rho kinase inhibitor, fasudil in these contractions. Furthermore, possible contribution of the enzyme to the physiological control of spontaneous contractile activity of the tissue was also examined, which would provide a further insight into mechanisms underlying pyeloureteral motility, and so display any physiological and pharmacological relevence of Rho GTPase signalling in the ureteric smooth muscle contraction. Moreover, since decreasing of calculus-induced ureteral motility and stretch may result in a remedy for the visceral pain, we aimed to point out Y-27632 as a possible antispasmodic agent in the ureteral colic.

## Methods

### Tissue preparation

Sheep ureters were obtained from a local slaughterhouse in Mersin, Turkey. The ureter was cut in the middle section of the whole tissue and then cleared of fat and other connective tissues. It was helically cut by placing onto a steel rod. Strips (about 2 cm long and 2–3 mm width) were prepared and suspended between two ring electrodes connected to the Biopac stimulator (Biopac system Inc., CA, U.S.A.) in an organ bath filled with Krebs' solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, glucose 11, $Na_2EDTA$ 0.3) gassed with 95% $O_2$ and 5% $CO_2$ under an initial tension of 0.5 g, which was chosen as the optimum resting tension after preliminary experiments. The bath temperature was maintained at 37°C. 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.).

### Experimental protocol

Following an equilibration period of 1 h, the helical strips of the sheep ureter were contracted by EFS (60 V, 1 mS, 2, 4, 8, 16 and 32 Hz, for 20 S), carbachol ($10^{-8}$–$10^{-4}$ M, cumulatively) or phenylephrine ($10^{-8}$–$10^{-4}$ M, cumulatively). These contraction series were regarded as the first series of contraction. After washing with fresh Krebs solution, the strips were incubated for 1 h. Thereafter, they were contracted with the same manner (the second series). In another series of experiments between the first and second series, the agents, which would be tested, namely, atropine ($2 \times 10^{-6}$ M for 20 min), guanethidine ($10^{-5}$ M for 60 min), $N^G$-nitro-L-arginine (L-NA, $10^{-4}$ M for 20 min), fasudil ($5 \times 10^{-5}$ M for 30 min), Y-27632 ($5 \times 10^{-5}$ M for 30 min) and tetrodotoxin (TTX, $3 \times 10^{-6}$ M for 15 min), were incubated. In the other series of experiments, effects of Y-27632 ($10^{-8}$–$10^{-4}$ M, cumulatively) and fasudil ($10^{-8}$–$10^{-4}$ M, cumulatively) were also tested on KCl (80 mM)-induced contraction. Finally, the compounds were also tested on spontaneous contractile activity of the tissue. The compounds were incubated for the above-mentioned durations. The corresponding control series without the agents were also performed. The concentrations and incubation duration of the agents used in this study were determined after preliminary experiments.

### Western blot analysis for Rho-kinase

The ureteral as well as aortic smooth muscles of sheep were homogenized with the lyses buffer solution (composition in mM; 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 $1500 \times g$ for 10 min at 4°C to remove nuclei and unlysed cells, and the supernatant was removed. It was then used for protein analysis (with Lowry method) and Western blot analysis. Equal amounts of proteins (400 μg) were loaded in wells, electrophoresed on 8% polyacrylamide–sodium dodecyl sul-fate (SDS) gels and then transferred to a nitrocellulose membrane overnight. The membrane was blocked with the blocking agent of enhanced chemiluminescence (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 primary antibodies raised against ROCK-1 (ROK$\beta$) or ROCK-2 (ROK$\alpha$, Polyclonal IgG, Santa Cruz Biotechnology Inc., CA, U.S.A.) at 1:200 dilution,

followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000, Santa Cruz Biotechnology Inc., CA, U.S.A.). The blots were then detected with the advanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany).

## Chemicals used

Ethylene glycol-bis($\beta$-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA) disodium, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), leupeptin, benzamidine, Tris–HCl, atropine sulfate, guanethidine sulfate, carbachol hydrochloride, L-NA and phenylephrine hydrochloride were obtained from Sigma Chemical Co (St Louis, U.S.A.). Y-27632 and fasudil were obtained from Tocris Cookson Ltd (Bristol, U.K.), and TTX from Alomone (Jerusalem, Israel). Potassium chloride (KCl), glycine and dodecyl sulfate sodium salt were purchased from Merck Co (Darmstadt, Germany). Primary antibodies for ROCK-1 and ROCK-2, and HRP-conjugated secondary antibody was obtained from Santa Cruz Biotechnology Inc. (CA, U.S.A.). ECL advance kit was purchased from Amersham Biosciences (Freiburg, Germany). The kit was used according to the manufacturer's guide. Potassium chloride, phenylephrine hydrochloride, endothelin-1, angiotensin-2, fasudil and Y-27632 were dissolved in distilled water.

## Statistical evaluations

All data represent means $\pm$ standard error of the mean (s.e.m.) of $n$ observations. Contractions to phenylephrine and carbachol were expressed as milliNewton (mN) of tension per g of wet tissue. The relaxations to Y-27632 and fasudil were evaluated as percent reductions of active tone induced by KCl.

For spontaneous contractions, the amplitude of the contractions at a given time was expressed as percentage of the contraction amplitude obtained at the time zero. After the application of test drugs, the spontaneous contractions were evaluated at the end of 15, 20 or 60 min in the same manner. For statistical comparison, one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or Student's *t*-test, if appropriate, was used. 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.).

## Results

### Effects of TTX, atropine, guanethidine, L-NA, fasudil and Y-27632 on EFS-elicited contractions and spontaneous contractile activity

TTX ($3 \times 10^{-6}$ M) almost abolished EFS-induced contractions in that at 2, 4, 8, 16 and 32 Hz EFS-evoked contractions were, respectively, $0 \pm 0$, $1.4 \pm 1.4$, $2.4 \pm 1.2$, $7.5 \pm 1.7$ and $7.5 \pm 1.6\%$ of the corresponding control series (Figure 1). Atropine ($2 \times 10^{-6}$ M) and guanethidine ($10^{-5}$ M) markedly inhibited these contractions (Figure 1). Electrically induced contractions were significantly augmented in the presence of L-NA ($10^{-4}$ M, Figure 1). The Rho-kinase inhibitors, Y-27632 ($5 \times 10^{-5}$ M) and fasudil ($5 \times 10^{-5}$ M), produced a significant inhibition on the EFS-elicited contractions (Figure 2 and 3). The sheep ureteral helical strips exhibited spontaneous contractile activity with the frequency of $0.2 \pm 0.007$ Hz ($n = 51$). The mean amplitude of the spontaneous contractions was $11.0 \pm 0.8$ mN ($n = 51$). None of these agents but Y-27632 ($10^{-6}$ and $10^{-5}$ M)
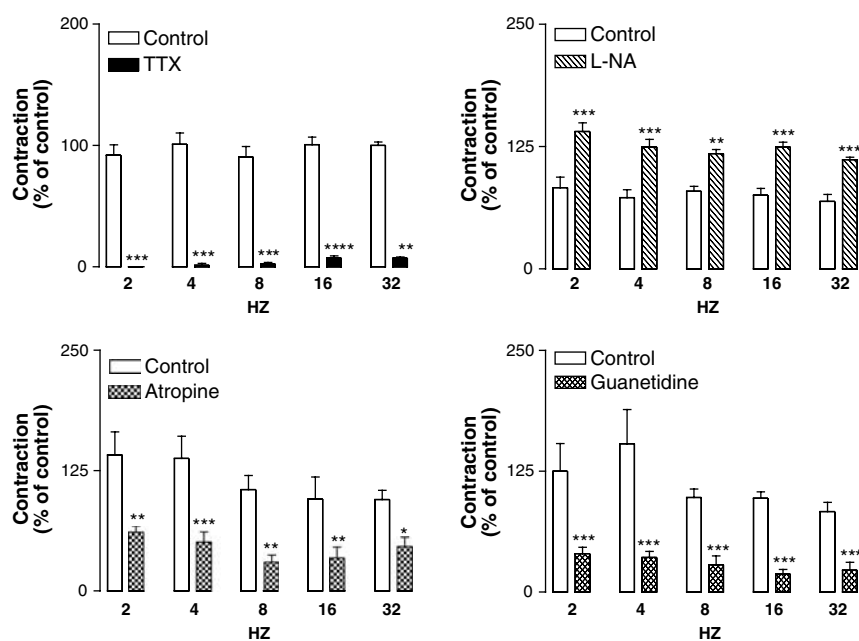


**Figure 1** Effects of TTX (left upper panel, $3 \times 10^{-6}$ M, $n = 8$), L-NA (right upper panel, $10^{-4}$ M, $n = 11$), atropine (left lower panel, $2 \times 10^{-6}$ M, $n = 8$) and guanethidine (right lower panel, $10^{-5}$ M, $n = 8$) on the EFS (60 V, 1 mS, 2, 4, 8, 16, 32 Hz, 20 S)-elicited contractions. The contractions were expressed as percent of control (the first series). Data represent means $\pm$ s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Comparison was made by ANOVA, followed by Bonferroni *post hoc* test.
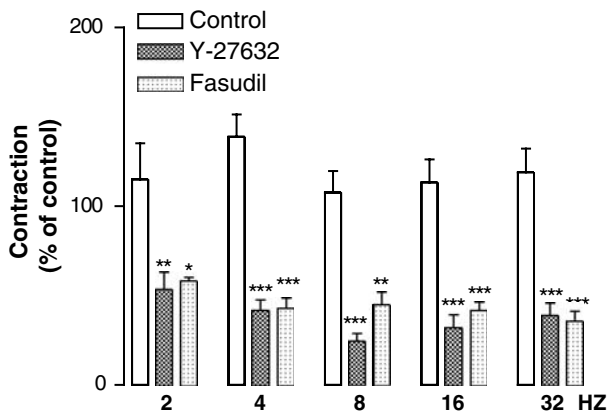
**Figure 2** Effects of Y-27632 ($5 \times 10^{-5}$ M, $n = 7$) and fasudil ($5 \times 10^{-5}$ M, $n = 5$) on the EFS (60 V, 1 mS, 2, 4, 8, 16, 32 Hz, 20 S)-elicited contractions. The contractions were expressed as percent of control (the first series). Data represent means ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Comparison was made by one-way ANOVA, followed by Bonferroni *post hoc* test.



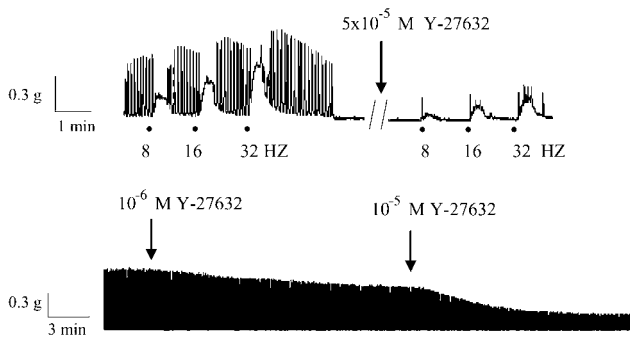**Figure 3** Original tracings showing the effects of the Rho-kinase inhibitor Y-27632 ($10^{-6}$–$5 \times 10^{-5}$ M) on EFS (60 V, 1 mS, 8, 16, 32 Hz, 20 S, upper panel)-elicited and spontaneous (lower panel) contractions in the helical strips of the sheep ureter. Note that Y-27632 suppresses not only EFS-induced contractions but also spontaneous contractile activity.

and fasudil ($10^{-6}$ and $10^{-5}$ M) had suppressing effects on the spontaneous contractions (Figure 3 and Table 1).

*Effect of Rho-kinase inhibitors, Y-27632 and fasudil, on KCl-, phenylephrine- and carbachol-induced contractions*

Y-27632 ($10^{-8}$–$10^{-4}$ M) and fasudil ($10^{-8}$–$10^{-4}$ M) produced relaxation in a concentration-dependent manner on KCl (80 mM)-elicited contraction (Figure 4). pD$_2$ values for Y-27632 and fasudil were $5.17 \pm 0.07$ and $4.58 \pm 0.08$, respectively ($P < 0.001$). Y-27632 ($5 \times 10^{-5}$ M) and fasudil ($5 \times 10^{-5}$ M) conspicuously inhibited the contractions induced by phenylephrine and carbachol (Figures 5 and 6).

*Expression of Rho-kinase in the sheep ureter and aorta*

Western blot analysis revealed that both isoforms of ROCK (ROCK-1 and ROCK-2) were expressed in the sheep ureteric smooth muscle at the molecular weight about 160 kDa. When compared with expression levels in the sheep aorta, the ureteral

**Table 1** Influence of $N^G$-nitro-L-arginine (L-NA), tetrodotoxin, atropine, guanethidine, fasudil and Y-27632 on the spontaneous contractile activity of the isolated sheep ureteral helical strips

| | |
|---|---|
| Control | $90.9 \pm 2.8\%$, $n = 12$ |
| L-NAME ($10^{-4}$ M) | $96.2 \pm 2.9\%$, $n = 7$, NS |
| Control | $94.3 \pm 2.1\%$, $n = 15$ |
| TTX ($3 \times 10^{-6}$ M) | $93.1 \pm 5.2\%$, $n = 4$, NS |
| Control | $90.9 \pm 2.8\%$, $n = 12$ |
| Atropin ($10^{-6}$ M) | $97.1 \pm 2.1\%$, $n = 7$, NS |
| Control | $76.1 \pm 7.5\%$, $n = 7$ |
| Guanethidine ($10^{-5}$ M) | $89.8 \pm 3.8\%$, $n = 7$, NS |
| Control | $94.3 \pm 2.1\%$, $n = 15$ |
| Y-27632 ($10^{-6}$ M) | $55.7 \pm 3.0\%$***, $n = 7$ |
| Y-27632 ($10^{-5}$ M) | $15.2 \pm 3.1\%$***, $n = 6$ |
| Fasudil ($10^{-6}$ M) | $67.1 \pm 3.4\%$***, $n = 4$ |
| Fasudil ($10^{-5}$ M) | $43.0 \pm 2.4\%$***, $n = 4$ |

The amplitude of contractions at a given time was expressed as the percentage of the contraction amplitude obtained at time zero. After the application of L-NA and atropine, the spontaneous contractions were evaluated at the end of 20 min; for Y-27632, fasudil and TTX at the end of 15 min and for guanethidine at the end of 60 min. For comparison, one-way ANOVA followed by Bonferroni *post hoc* test was used. ***$P < 0.001$, NS: not significant.
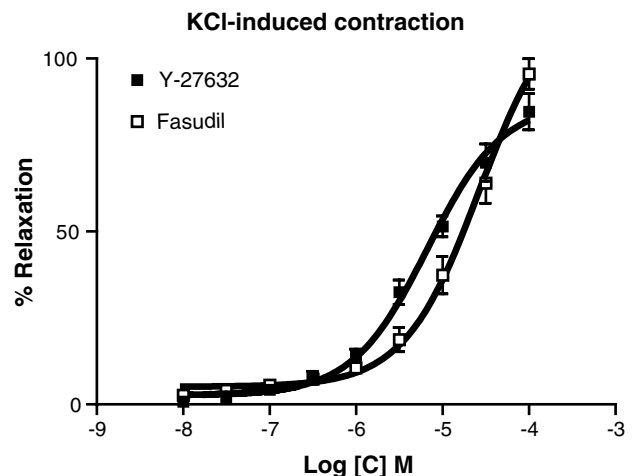


**Figure 4** Effect of Y-27632 ($10^{-8}$–$10^{-4}$ M, cumulatively, $n = 7$) and fasudil ($10^{-8}$–$10^{-4}$ M, cumulatively, $n = 6$) on KCl (80 mM)-induced tonic contraction. Relaxations were expressed as percent reductions in the KCl-induced contraction. Data represent means ± s.e.m.

smooth muscle expressed these proteins significantly less (Figure 7).

## Discussion

In this study, we examined the possible involvement of Rho/Rho-kinase pathway in the spontaneous as well as EFS, KCl, phenylephrine and carbachol-induced contractions of the sheep ureter. Furthermore, we detected the expression of two isoforms of Rho-kinase in this tissue, and compared its expression level to that in the sheep aortic smooth muscle.

EFS of the sheep ureteral smooth muscle induced contraction, which was abolished by the sodium channel blocker TTX, indicating that the mediators responsible for the contractile activity could be released from nerves. The mammalian ureter
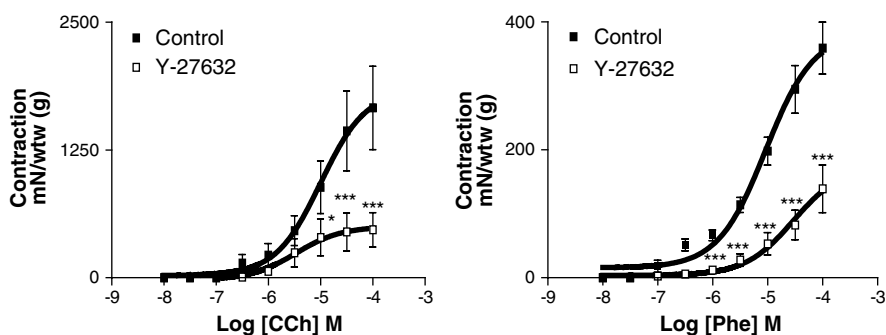
**Figure 5** Effects of carbachol, a cholinergic agonist (CCh, $10^{-8}$–$10^{-4}$ M, left panel, $n = 7$), and phenylephrine, an α-adrenoceptor agonist (Phe, $10^{-8}$–$10^{-4}$ M, right panel, $n = 7$) in the absence and presence of the Rho-kinase inhibitor, Y-27632 ($5 \times 10^{-5}$ M). Contractions were expressed as mN of the wet tissue weight (g). Data represent means $\pm$ s.e.m. $*P < 0.05$, $***P < 0.001$. Comparison was made by one-way ANOVA, followed by Bonferroni *post hoc* test.
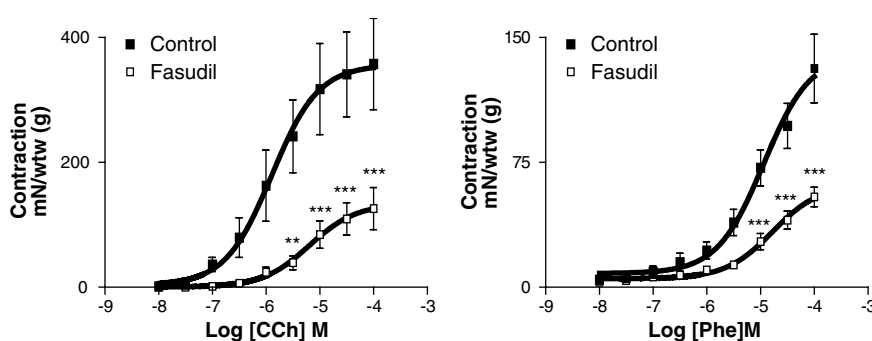


**Figure 6** Effects of carbachol, a cholinergic agonist (CCh, $10^{-8}$–$10^{-4}$ M, left panel, $n = 7$), and phenylephrine, an α-adrenoceptor agonist (Phe, $10^{-8}$–$10^{-4}$ M, right panel, $n = 4$), in the absence and presence of the Rho-kinase inhibitor, fasudil ($5 \times 10^{-5}$ M). Contractions were expressed as mN of the wet tissue weight (g). Data represent means $\pm$ s.e.m. $**P < 0.01$, $***P < 0.001$. Comparison was made by one-way ANOVA, followed by Bonferroni *post hoc* test.
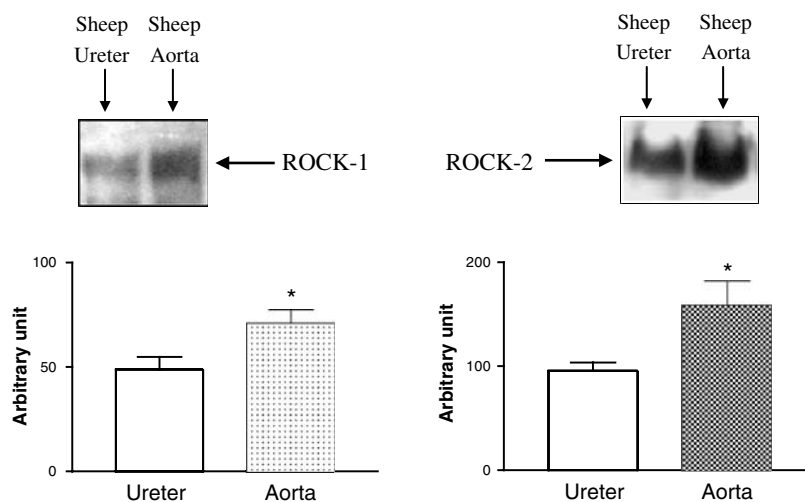


**Figure 7** Demonstration of the expressions of ROCK-1 and ROCK-2 in the sheep ureteric ($n = 4$) and aortic ($n = 4$) smooth muscle homogenates by Western blotting. The homogenates were submitted to SDS–PAGE with 8% polyacrylamide and then transferred to a nitrocellulose membrane (PVDF). The membrane was blocked with an ECL Advance blocking agent 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-1 and ROCK-2 (Polyclonal IgG) at 1 : 200 dilution, followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000). Proteins bound with the antibodies were then visualized by the ECL Advance kit. Densitometric analysis of the protein bands was evaluated by a computer program (Scion image, U.S.A.).

is innervated by afferent sensory, noradrenergic, cholinergic, nitrergic and some other nerves. Acetylcholine, noradrenaline and tachykinins such as substance P and neurokinin A exert as excitatory neurotransmitters, whereas calcitonine gene-related peptide and nitric oxide (NO) act as inhibitory neurotransmitters (Santicioli & Maggi, 1998). Accordingly, in the present study, atropine and guanethidine inhibited EFS-induced contraction. Moreover, L-NA augmented these contractions, indicating that concomitant release of NO upon EFS may modulate the contractile effects of excitatory neurotransmitters. However, myogenic spontaneous contractile activity of the ureter is somewhat different. This contraction was not inhibited in the presence of TTX, excluding any neurogenic components. Neither was it affected by atropine or guanethidine, indicating that neither cholinergic nor adrenergic components are involved in this contractile activity. In support, it has been demonstrated that spontaneous waves of the renal pelvic smooth muscle are resistant to cholinergic, noradrenergic and neuronal blockers, suggesting that this activity is purely myogenic in origin (Seki & Suzuki, 1990). Finally, the NOS inhibitor, L-NA had no influence on spontaneous contraction, excluding any modulation of NO in the contractions. In addition, NO synthase blockers did not affect the motility of the guinea-pig (Maggi et al., 1995) and sheep ureter (Garcia-Pascual et al., 1996). It is know that functional capsaicin-sensitive sensory afferents and endogenous release of both tachykinins and prostanoids are essential in the maintenance of normal peristalsis (Lang et al., 2002). In confirmation, Patacchini et al. (1998) have reported that a tachykinin, neurokinin A, produced concentration-dependent contraction of human and guinea-pig ureter, which was inhibited by a selective $NK_2$ receptor antagonist. In this study, however, we did not have any decisive findings over the main mechanism(s) of the myogenic spontaneous contractions. Nevertheless, since Y-27632 and fasudil had suppressor effects on the contractile activity, the Rho/Rho-kinase pathway seems to be involved in the spontaneous contractions, regardless of the nature of excitatory mediator(s) or upstream mechanisms. It has been proposed that there might be some putative pacemakers responsible for the initiation of peristaltic waves in the renal pelvis as well as ureteral smooth muscle (Maggi et al., 1988; Lammers et al., 1996). It has been reported that normal ureteral peristalsis is physiologically due to the myogenic properties of pyeloureteral smooth muscle; however, in pathological conditions such as those occurring during a passage of a stone or a bacterial infection, local efferent and function of capsaicin-sensitive sensory nerves may possibly affect ureteral peristalsis (Santicioli & Maggi, 1998). In the present study, however, we did not try any exogenously applied tachykininergic compounds like neurokinin A or substance P. Therefore, we did not examine the effect of Y-27632 and fasudil on these agents-induced contractions. However, it has been demonstrated that Rho/Rho-kinase signalling is also involved in the tachykinin receptor-induced

signal transduction in the rat urinary bladder smooth muscle (Wibberley et al., 2003). Therefore, it is possible that Y-27632 and fasudil would be able to inhibit the effects of excitatory tachykinin agonists in the sheep ureteric smooth muscle.

With respect to the agonists, phenylephrine-induced contraction was suppressed by Y-27632 and fasudil in the sheep ureter, as shown in the mouse vas deferens (Büyükafşar et al., 2003), indicating that α-adrenoceptors could be involved in the Rho/Rho-kinase signalling. Indeed, it has been reported by many researchers that phenyephrine- and noradrenaline-induced contractions were dramatically inhibited by Rho-kinase inhibitors (see for reviews, Fukata et al., 2001; Wettschureck & Offermanns, 2002). Adrenaline, noradrenaline and phenylephrine produced concentration-dependent increases in both the phasic activity and basal tone of ureteral preparations, which were mediated by α-adrenoceptors (Hernandez et al., 1992). On the other hand, carbachol-induced contraction was also attenuated in the presence of Y-27632 and fasudil. This shows that cholinergic receptors may be coupled with this novel signalling pathway. In the isolated canine ureteric spiral preparation, it has been demonstrated that carbachol induced contraction, and this contraction could be mediated by $M_3$ cholinergic receptor (Tomiyama et al., 2003). These are consistent with the data obtained in the EFS series, where atropine and guanethidine inhibited EFS-evoked contraction.

It has been known that Rho-signalling can be activated by the stimulation of heterotrimeric G protein-coupled receptors (Somlyo & Somlyo, 2000). However, in the present study, Y-27632 and fasudil relaxed the ureteral smooth muscle, which was contracted by KCl. It has been known that $K^+$ stimulation induces an increase in intracellular free $Ca^{2+}$ concentration of smooth muscles. Although we did not check whether KCl might cause the release of any excitatory neurotransmitters from the nerves supplying the tissue, it may act on the smooth muscle as in gastric fundus (Büyükafşar & Levent, 2003) and vas deferens (Büyükafşar et al., 2003). Consequently, $Ca^{2+}$ entry may activate Rho-signalling pathway, like $Ca^{2+}$-induced $Ca^{2+}$ sensitization in smooth muscle cells. In order to ascertain this hypothesis, further studies are needed.

In conclusion, the results of this descriptive study indicate that Rho-kinase of both isoforms are expressed in the sheep ureteric smooth muscle, and it plays a substantial role in the spontaneous activity as well as the contractions induced by EFS, KCl, phenylephrine and carbachol. Since Y-27632 and fasudil have the marked inhibitions on these contractions, the ROCK inhibitors seem to be potential therapeutic agents in the renal colic treatment.

## References

BRADLEY, A.B. & MORGAN, K.G. (1987). Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. *J. Physiol. (Lond.)*, **385**, 437–448.

BULTITUDE, M.I. (1995). Capsaicin in treatment of loin pain/haematuria syndrome. *Lancet*, **345**, 921–922.

BÜYÜKAFŞAR, K., ARIKAN, O., ARK, M., SEÇILMIŞ, A., ÜN, İ. & ŞINGIRIK, E. (2004). Rho-kinase expression and its contribution to the control of perfusion pressure in the isolated rat mesenteric vascular bed. *Eur. J. Pharmacol.*, **485**, 263–268.

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

BÜYÜKAFŞAR, 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.

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

CERVERO, F. (1994). Sensory innervation of the viscera: peripheral bases of visceral pain. *Physiol. Rev.,* **74,** 95–138.

FUKATA, Y., MUTSUKI, A. & KAIBUCHI, K. (2001). Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganisation of non-muscle cells. *Trends Pharmaco.l Sci.,* **22,** 32–39.

GARCIA-PASCUAL, A., COSTA, G., LABADIA, A., PERSSON, K. & TRIGUERO, D. (1996). Characterization of nitric oxide synthase activity in sheep urinary tract: functional implications. *Br. J. Pharmacol.,* **118,** 905–914.

GIAMBERARDINO, M.A., VALENTE, R., DE BIGONTINA, P., IEZZI, S. & VECCHIET, L. (1995a). Effects of spasmolytic and/or non-steroidal antiinflammatory drugs on muscle hyperalgesia of ureteral origin in rats. *Eur. J. Pharmacol.,* **278,** 97–101.

GIAMBERARDINO, M.A., VALENTE, R., DE BIGONTINA, P. & VECCHIET, L. (1995b). Artificial ureteral calculosis in rats: behavioural characterization of visceral pain episodes and their relationship with referred lumbar muscle hyperalgesia. *Pain,* **61,** 459–469.

HERNANDEZ, M., PRIETO, D., SIMONSEN, U., RIVERA, L., BARAHONA, M.V. & GARCIA-SACRISTAN, A. (1992). Noradrenaline modulates smooth muscle activity of the isolated intravesical ureter of the pig through different types of adrenoceptors. *Br. J. Pharmacol.,* **107,** 924–931.

ISHIZAKI, T., UEHATA, M., TAMECHIKA, I., KEEL, J., NONOMURA, K., MAEKAWA, M. & NARUMIYA, S. (2000). Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol. Pharmacol.,* **57,** 976–983.

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,** 593–603.

LAIRD, J.M.A., ROSA, C. & CERVERO, F. (1997). Effects of artificial calculosis on rat ureter motility: peripheral contribution to the pain of ureteral colic. *Am. J. Physiol.,* **41,** R1409–R1416.

LAMMERS, W.J.E.P., AHMAD, H.R. & ARAFAT, K. (1996). Spatial and temporal variations in pacemaking and conduction in the isolated renal pelvis. *Am. J. Physiol.,* **270,** F567–F574.

LANG, R.J., DAVIDSON, M.E. & EXINTARIS, B. (2002). Pyeloureteral motility and ureteral peristalsis: essential role of sensory nerves and endogenous prostaglandins. *Exp. Physiol.,* **87,** 129–146.

MAGGI, C.A., PARLANI, M., ASTOLFI, M., SANTICIOLI, P., ROVERO, P., ABELLI, V., SOMMA, V., GIULIANI, S., REGOLI, D., PATACCHINI, R. & MELI, A. (1988). Neurokinin receptors in the rat lower urinary tract. *J. Pharmacol. Exp. Ther.,* **246,** 308–315.

MAGGI, C.A., SANTICIOLI, P. & GIULIANI, S. (1995). Role of cyclic AMP and protein kinase A in K channel activation by CGRP in the guinea-pig ureter. *J. Autonom. Pharmacol.,* **15,** 403–419.

PATACCHINI, R., SANTICIOLI, P., ZAGORODNYUK, V., LAZZERI, M., TURINI, D. & MAGGI, C.A. (1998). Excitatory motor and electrical effects produced by tachykinins in the human and guinea-pig isolated ureter and guinea-pig renal pelvis. *Br. J. Pharmacol.,* **125,** 987–996.

SANTICIOLI, P. & MAGGI, C.A. (1998). Myogenic and neurogenic factors in the control of pyeloureteral motility and ureteral peristalsis. *Pharmacol. Rev.,* **50,** 683–721.

SEKI, N. & SUZUKI, H. (1990). Electrical properties of smooth muscle cell membrane in renal pelvis of rabbits. *Am. J. Physiol.,* **259,** F888–F894.

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. (Lond.),* **522,** 177–185.

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.

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

TOMIYAMA, Y., WANAJO, I., YAMAZAKI, Y., MURAKAMI, M., KOJIMA, M. & SHIBATA, N. (2003). Functional muscarinic cholinoceptors in the isolated canine ureter. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **367,** 348–352.

UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, K., 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.

WETTSCHURECK, N. & OFFERMANNS, S. (2002). Rho/Rho-kinase signalling in physiology and pathophysiology. *J. Mol. Med.,* **80,** 629–638.

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.