



## SPECIAL REPORT

**Y-27632, an inhibitor of Rho-kinase, antagonizes noradrenergic contractions in the rabbit and human penile corpus cavernosum****<sup>1,2</sup>Rowland W. Rees, <sup>2</sup>David J. Ralph, <sup>3</sup>Michael Royle, <sup>1</sup>Salvador Moncada & \*<sup>1</sup>Selim Cellek**<sup>1</sup>The Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6AE; <sup>2</sup>Institute of Urology, Middlesex Hospital, London W1W 7EL, UK and <sup>3</sup>Sussex Nuffield Hospital, Brighton BN2 6DX

We have examined the effect of an inhibitor of Rho-kinase, (+)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632), on the contractions elicited by noradrenergic nerve stimulation and by phenylephrine in the human and rabbit penile corpus cavernosum. In both tissues, after treatment with scopolamine (10  $\mu$ M) and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 300  $\mu$ M), electrical field stimulation (EFS) elicited noradrenergic contractions. These contractions were inhibited by Y-27632 in a concentration-dependent manner. The compound caused concentration-dependent relaxation of phenylephrine-contracted tissues, which were treated with scopolamine (10  $\mu$ M), guanethidine (10  $\mu$ M) and L-NAME (300  $\mu$ M). These results suggest that Rho-kinase is involved in noradrenergic contractile pathway in the cavernosal smooth muscle of the penis.

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**Abbreviations:** EFS, electrical field stimulation; IP<sub>3</sub>, inositol 1,4,5 trisphosphate; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular concentration of calcium; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; SMPP-1M, smooth muscle myosin phosphatase

**Introduction** In the human penile cavernous tissue, sympathetic stimulation causes contraction of the smooth muscle. This action is elicited by noradrenaline released from sympathetic nerves acting on the postjunctional  $\alpha_1$ -adrenoceptors (for reviews see Andersson & Wagner, 1995; Cellek, 2000). The mechanism of noradrenergic contraction in the smooth muscle is based on an increase in intracellular concentrations of calcium ([Ca<sup>2+</sup>]<sub>i</sub>). This is achieved by release of Ca<sup>2+</sup> from internal stores *via* the action of inositol 1,4,5 trisphosphate (IP<sub>3</sub>), which is produced by G-protein-coupled phospholipase C (Berridge, 1993). It has also been suggested that noradrenaline can induce a change in the membrane potential which elicits the opening of voltage-gated channels in the plasma membrane and thereby allows the entry of Ca<sup>2+</sup> into the cell (Himpens *et al.*, 1995).

Recently, an additional mechanism has been proposed for smooth muscle contractility. Activation of excitatory receptors coupled to G-proteins can also cause contraction of smooth muscle by increasing Ca<sup>2+</sup>-sensitivity and without changing [Ca<sup>2+</sup>]<sub>i</sub>. This pathway involves RhoA, a small, monomeric G-protein that activates Rho-kinase. Activated Rho-kinase phosphorylates the regulatory subunit of smooth muscle myosin phosphatase (SMPP-1M). Inhibitory phosphorylation of SMPP-1M leads to sensitization of myofilaments to Ca<sup>2+</sup> (for review see Somlyo & Somlyo, 2000). A specific inhibitor of Rho-kinase, (+)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632; Uehata *et al.*, 1997; Ishizaki *et al.*, 2000), has been shown to relax vascular and non-vascular smooth muscle in different preparations (Fu *et al.*, 1998; Iizuka *et al.*, 2000; Robertson *et al.*, 2000; Sward *et al.*, 2000).

Y-27632 has been demonstrated to lower blood pressure of hypertensive but not of normotensive rats (Uehata *et al.*, 1997) suggesting that it has relaxant activity preferentially on the vascular beds with high basal tone. Since cavernosal smooth muscle is kept contracted by basal noradrenergic activity in the detumescent state, we were interested in investigating the effect of Y-27632 on this tissue.

**Methods** Tissue specimens were prepared as described previously (Cellek & Moncada, 1997). Male New Zealand rabbits (3.5–5.0 kg; *n* = 3) were sacrificed by an overdose of pentobarbitone and the penis was excised. The isolated strips of corpus cavernosum (four from each animal) were placed horizontally between two ring electrodes in tissue chambers at 37°C. The tissues were perfused at a constant flow of 1 ml min<sup>-1</sup> by means of peristaltic pumps (Miniplus 2, Gilson) with a medium of the following composition (mM): NaCl 136.9, KCl 2.7, CaCl<sub>2</sub> 1.8, MgSO<sub>4</sub> 0.6, NaHCO<sub>3</sub> 11.9, KH<sub>2</sub>PO<sub>4</sub> 0.5, glucose 11.5, indomethacin 0.01, dexamethasone 0.01 and gassed with 5% CO<sub>2</sub> in O<sub>2</sub> (pH 7.4–7.6). One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Linearcorder WR 3101 (Graphtec) for registration of isometric changes in tension. The preparations were stretched (2 to 5 mN) until they reached approximately the *in situ* length and allowed to equilibrate for 90 min. The preparations were stimulated electrically (electrical field stimulation; EFS) with 5 s trains of rectangular pulses of 50 V, 0.3 ms pulse duration and frequencies ranging from 1 to 50 Hz, delivered by Grass S88 stimulators every 120 s throughout the experiment. Drugs were applied into the medium reservoir.

Whole corpus cavernosum was obtained from patients (ages 28, 32, 43; *n* = 3) undergoing penectomy for gender reassignment in Sussex Nuffield Hospital (Brighton, U.K.).

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None of the patients had erectile dysfunction or any systemic disease. All patients gave written informed consent to the study, which was approved by the East Unit Research Ethics Committee of Mid Sussex National Health Service Hospital, U.K. The tissues were transported from the hospital to the laboratories in ice-cold medium (composition as above) in less than 3 h. On arrival, they were transferred into the medium at room temperature and cleaned of adherent tissue and blood. Four to eight strips were cut from the middle part of each corpus cavernosum. Each strip was mounted into the tissue chambers as described above.

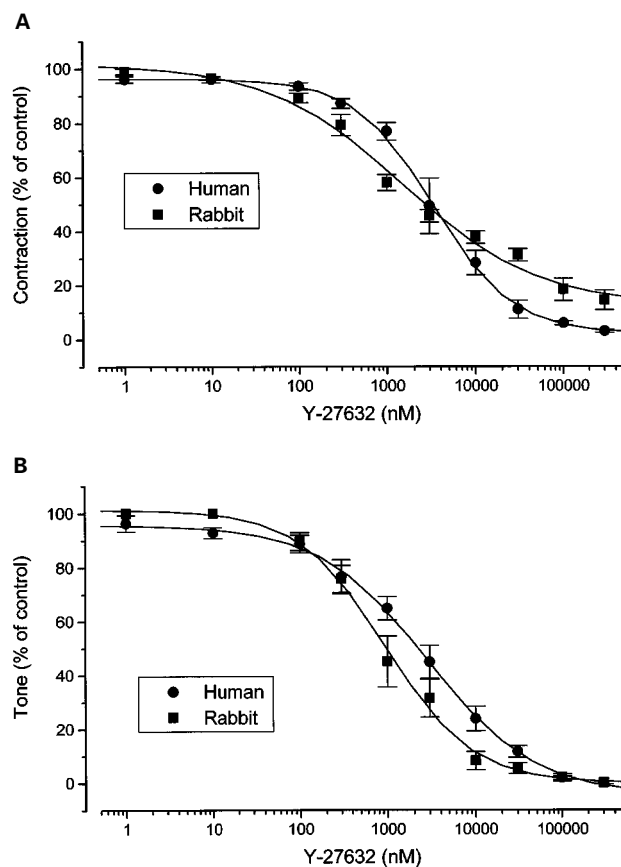
Y-27632 was provided by Welfide Inc (Osaka, Japan). All other compounds were from Sigma, U.K. Y-27632 was dissolved in phosphate-buffered saline at 4°C and added to the medium reservoir cumulatively in incremental concentrations once the effect of the compound on EFS- or phenylephrine-induced tone was stabilised. At the end of the experiment the tissues were exposed to medium without Y-27632 in order to assess the reversibility of its effect.

**Results** *Effect of Y-27632 on EFS-induced contractions* In the presence of scopolamine (10 µM) and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 300 µM) to inhibit cholinergic and nitrenergic neurotransmission respectively, the tissue was stimulated with EFS. Under these conditions EFS of the human and rabbit corpus cavernosum has been shown to elicit noradrenergic contractions (Cellek & Moncada, 1997). Since a frequency of 5 Hz was found to give optimum contractile responses for both tissues under these conditions (not shown), this frequency was used in all of the experiments. Contractions elicited with EFS were inhibited with Y-27632 (1 nM–300 µM). The inhibition was partial (97.0 ± 1.5% and 85.6 ± 3.6% inhibition at 300 µM) with an IC<sub>50</sub> of 3.3 ± 0.25 µM and 2.8 ± 0.2 µM in human and rabbit tissues respectively (*n* = 3, Figures 1A and 2). The inhibition was reversible; the contractions returned to control values following removal of Y-27632 from the medium (Figure 2).

*Effect of Y-27632 on elevated tone with phenylephrine* The tone of the tissue was elevated with 10 and 1 µM phenylephrine (EC<sub>80</sub> for human and rabbit tissues respectively) in the presence of scopolamine (10 µM), guanethidine (10 µM) and L-NAME (300 µM). Y-27632 (1 nM–100 µM) caused relaxation of the tissue in a concentration-dependent manner (EC<sub>50</sub>: 2.2 ± 0.25 µM and 0.99 ± 0.3 µM for human and rabbit tissues respectively) (*n* = 3, Figure 1B). The tone returned to control level after Y-27632 was removed from the medium (not shown).

**Discussion** Noradrenergic neurotransmission plays an important role in the regulation of cavernosal tone. In the detumescent state, penile corpus cavernosum is kept contracted by release of noradrenaline from sympathetic nerves acting on postjunctional α-adrenoceptors (Andersson & Wagner, 1995). During erection the noradrenergic pathway seems to be under control of the nitrenergic pathway (Cellek & Moncada, 1997). In diabetic erectile dysfunction, noradrenergic neurotransmission takes over, resulting in enhanced noradrenergic and diminished nitrenergic responses (Cellek *et al.*, 1999).

Noradrenaline elicits its contractile effect in the cavernosal smooth muscle *via* α<sub>1</sub>-adrenoceptors. Indeed, contractile

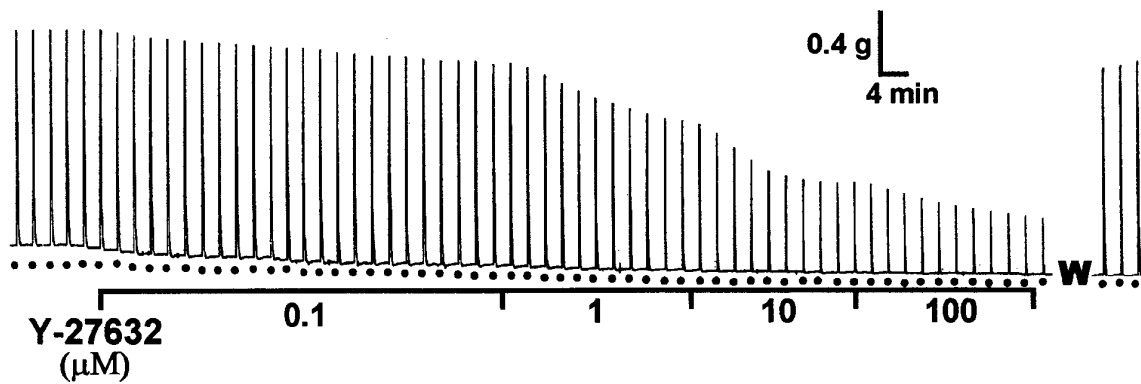


**Figure 1** Effect of Y-27632 on EFS-induced noradrenergic contractions (A) and phenylephrine-induced tone (B) in the human (circles) and rabbit (squares) corpus cavernosum (*n* = 3 for both).

responses elicited either by EFS or by exogenous noradrenaline in the human corpus cavernosum are completely inhibited by prazosin (Andersson & Wagner, 1995). Binding of noradrenaline to the α<sub>1</sub>-receptor activates G-protein-coupled phospholipase-C, which initiates IP<sub>3</sub>-dependent Ca<sup>2+</sup> release from internal stores to start the contractile process (Berridge, 1993; Himpens *et al.*, 1995).

Other mechanisms have been proposed for the contractile action of noradrenaline in smooth muscle. In vascular smooth muscle, noradrenaline can induce a change in the membrane potential, which elicits the opening of voltage-gated channels in the plasma membrane and thereby allows the entry of extracellular Ca<sup>2+</sup> into the cell (Himpens *et al.*, 1995). The effect of Ca<sup>2+</sup>-channel blockers to reduce [Ca<sup>2+</sup>]<sub>i</sub> and to relax the vascular smooth muscle is consistent with this mechanism. In the urogenital smooth muscle, however, Ca<sup>2+</sup>-channel blockers do not affect noradrenaline-induced contractions (Vila *et al.*, 1985). Moreover, these contractions have been shown not to involve a change in the membrane potential (Large, 1984) and are not affected by reducing extracellular Ca<sup>2+</sup> concentrations (Elemide & Oriowo, 1986). It appears that IP<sub>3</sub>-dependent Ca<sup>2+</sup> release from internal stores is responsible for smooth muscle contraction in the urogenital smooth muscle (Vila *et al.*, 1985).

Ancillary regulatory mechanisms, which could modify the sensitivity of the contractile and regulatory proteins to [Ca<sup>2+</sup>]<sub>i</sub>, are suggested to be operating in the smooth muscle



**Figure 2** Effect of Y-27632 on EFS-induced contractions of rabbit corpus cavernosum. 'W' denotes the washing period for 140 min in the absence of Y-27632. Each train of EFS (50 V, 5 Hz, 0.3 ms pulse duration, for 5 s, every 120 s) is shown with a closed circle. The mechanogram is an original recording of the responses of one preparation and is representative of all experiments in this series ( $n=3$ ).

(Somlyo & Somlyo, 2000). This 'sensitizing mechanism' can cause contraction of smooth muscle by increasing  $\text{Ca}^{2+}$ -sensitivity without necessarily changing  $[\text{Ca}^{2+}]_i$ . One such mechanism is the Rho-kinase pathway, which has been suggested to be the major  $\text{Ca}^{2+}$ -sensitizing mechanism in the smooth muscle (Somlyo & Somlyo, 2000). Rho-kinase is activated by RhoA, a small, monomeric G-protein believed to be coupled to excitatory receptors (e.g.  $\alpha_1$ -adrenoceptor). Activated Rho-kinase phosphorylates the regulatory subunit of smooth muscle myosin phosphatase (SMPP-1M). Inhibitory phosphorylation of SMPP-1M leads to sensitization of myofilaments to  $\text{Ca}^{2+}$  (Somlyo & Somlyo, 2000).

The role of Rho-kinase pathway in the regulation of vascular and non-vascular smooth muscle tone has been elucidated *in vitro* and *in vivo* using a specific inhibitor of Rho-kinase, Y-27632. Oral administration of the compound to spontaneously hypertensive, renal hypertensive and DOCA-salt hypertensive rats caused a persistent fall in blood pressure while the same oral dose did not affect the blood pressure of normotensive rats (Uehata *et al.*, 1997). Inhalation of Y-27632 inhibited acetylcholine- or ovalbumin-induced increase in lung resistance of guinea-pig (Iizuka *et al.*, 2000). Application of Y-27632 into perfused rat lungs caused no change in basal perfusion pressure but was found to inhibit hypoxic pulmonary vasoconstriction (Robertson *et al.*, 2000). Thus, Rho-kinase inhibitor, Y-27632, has been shown to antagonise contractile mechanisms in vascular and non-vascular smooth muscle preparations, particularly in conditions where an elevated tone exists.

In order to elucidate the role of Rho-kinase pathway in the cavernosal smooth muscle contraction, we applied Y-27632 to isolated penile corpus cavernosum strips from human and rabbit contracted either with EFS or phenylephrine. Both

type of contractions were inhibited by Y-27632 in a concentration-dependent manner, suggesting that the Rho-kinase pathway is involved in the noradrenergic contractile response of human and rabbit penile cavernosal smooth muscle. These results support the findings of Chitale *et al.* (2001) who showed the relaxant effect of Y-27632 on rat cavernous tissue *in vitro*. To our knowledge, our results are the first to describe the effect of a Rho-kinase inhibitor in human cavernous tissue.

Phasic (transient) contractile responses elicited by EFS were more resistant to inhibition by Y-27632 than the tonic (sustained) contraction by phenylephrine ( $\text{IC}_{50}$  for Y-27632 was  $3.3 \pm 0.25 \mu\text{M}$  for EFS-induced contractions and  $2.2 \pm 0.25 \mu\text{M}$  for phenylephrine-induced contraction in human cavernous tissue;  $P < 0.05$ ). This is in accordance with the previous reports in the guinea-pig ileum and human trachea in which Y-27632 has been found to be effective against the tonic but not the transient phase of contraction (Yoshii *et al.*, 1999; Sward *et al.*, 2000). Our results further support the role of Rho-kinase pathway in regulating the smooth muscle tone in conditions where a tonic contraction or a high basal tone are involved. Indeed, recently intracavernous application of Y-27632 has been reported to elicit penile erection in rats (Chitale *et al.*, 2001), suggesting that Rho-kinase is critically involved in maintaining basal noradrenergic tone in a detumescent state.

Rho-kinase antagonism represents a potential therapeutic use for the treatment of erectile dysfunction where elevated noradrenergic tone has been reported (Taub *et al.*, 1993; for review see Cellek, 2000). Moreover, Rho-kinase inhibitors would not require a functional nitrgic system and could possibly be more effective in patients with full loss of nitrgic function who are unable to benefit from phosphodiesterase-5 inhibitors (Rendell *et al.*, 1999).

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