British Journal of Pharmacology (1998) 123, 435–442 © 1998 Stockton Press All rights reserved 0007–1188/98 $12.00

Effect of *Tityus serrulatus* scorpion venom on the rabbit isolated corpus cavernosum and the involvement of NANC nitrergic nerve fibres

Cleber E. Teixeira, Antonio C. Bento, Rodrigo A.B. Lopes-Martins, Simone A. Teixeira, [1]Vera von Eickestedt, Marcelo N. Muscará, [2]Eliane C. Arantes, [2]Jose R. Giglio, [3]Edson Antunes & Gilberto de Nucci

Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, PO Box 6111, 13081-970, Campinas (SP); [1]Arthropods Laboratory, Butantan Institute, São Paulo (SP) and [2]Department of Biochemistry, Ribeirão Preto Medical School, University of São Paulo, 14049, Ribeirão Preto (SP), Brazil

1 The effect of *Tityus serrulatus* scorpion venom and its toxin components on the rabbit isolated corpus cavernosum was investigated by use of a bioassay cascade.

2 *Tityus serrulatus* venom (3–100 μg), acetylcholine (ACh; 0.3–30 nmol) and glyceryl trinitrate (GTN; 0.5–10 nmol) dose-dependently relaxed rabbit isolated corpus cavernosum preparations precontracted with noradrenaline (3 μM). The selective soluble guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ; 30 μM) increased the basal tone of the rabbit isolated corpus cavernosum and abolished the relaxations induced by the agents mentioned above. Methylene blue (30 μM) also inhibited the relaxations induced by *Tityus serrulatus* venom but, in contrast to ODQ, the inhibition was irreversible.

3 The non-selective NO synthase (NOS) inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10 μM) and N^{G}-iminoethyl-L-ornithine (L-NIO; 30 μM) also increased the tone of the rabbit isolated corpus cavernosum and markedly reduced both ACh- and *Tityus serrulatus* venom-induced relaxations without affecting those evoked by GTN. The inhibitory effect was reversed by infusion of L-arginine (300 μM), but not D-arginine (300 μM). The neuronal NOS inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM, 100 μM) did not affect either the tone of the rabbit isolated corpus cavernosum or the relaxations induced by ACh, bradykinin (Bk), *Tityus serrulatus* venom and GTN. TRIM was approximately 1,000 times less potent than L-NAME in inhibiting rabbit cerebellar NOS *in vitro*, as measured by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline.

4 The protease inhibitor aprotinin (Trasylol; 10 μg ml^{-1}) and the bradykinin B_2 receptor antagonist Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7, Oic^8]-BK; 50 nM) did not affect the rabbit isolated corpus cavernosum relaxations induced by *Tityus serrulatus* venom. The ATP-dependent K^+ channel antagonist glibenclamide (10 μM) and the Ca^{2+}-activated K^+ channel antagonists apamin (0.1 μM) and charybdotoxin (0.1 μM) also failed to affect the venom-induced relaxations. Similarly, the K^+ channel blocker tetraethylammonium (TEA; 10 μM) had no effect on the venom-induced relaxations.

5 Capsaicin (3 and 10 nmol) relaxed the rabbit isolated corpus cavernosum in a dose-dependent and non-tachyphylactic manner. Ruthenium red (30 μM), an inhibitor of capsaicin-induced responses, markedly reduced the relaxations caused by capsaicin, but failed to affect those induced by *Tityus serrulatus* venom. L-NAME (10 μM) had no effect on the capsaicin-induced relaxations of the rabbit isolated corpus cavernosum.

6 The sodium channel blocker tetrodotoxin (TTX; 1 μM) abolished the relaxations of the rabbit isolated corpus cavernosum induced by *Tityus serrulatus* venom without affecting those evoked by capsaicin, ACh and GTN. Tetrodotoxin (1 μM) also promptly reversed the response to the venom when infused during the relaxation phase.

7 The bioassay cascade of the toxin components purified from *Tityus serrulatus* venom revealed that only fractions X, XI and XII caused dose-dependent relaxations of the rabbit isolated corpus cavernosum and these were markedly reduced by either TTX (1 μM) or L-NAME (10 μM).

8 Our results indicate that *Tityus serrulatus* scorpion venom (and the active fractions X, XI and XII) relaxes rabbit corpus cavernosum via the release of NO. This release is specifically triggered by the activation of capsaicin-insensitive cavernosal non-adrenergic non-cholinergic (NANC) fibres, that may possibly be nitrergic neurones. *Tityus serrulatus* venom may therefore provide an important tool for understanding further the mechanism of NANC nitrergic nerve activation.

Keywords: Scorpion venom; nitrergic nerves; tetrodotoxin; non-adrenergic non-cholinergic nerves; nitric oxide; ODQ

Introduction

Scorpion toxins have been extensively used to study the activation of both Na^+ (Barhanin *et al*., 1982; Nagy, 1988; Yatani *et al*., 1988; Kirsch *et al*., 1989) and K^+ (Blaustein *et al*., 1991; Rogowski *et al*., 1994; Vatanpour & Harvey, 1995) channels. *Tityus serrulatus* is the most dangerous scorpion of the subfamily Tityinae because of the high toxicity of its venom and its widespread distribution in populous urban centres of southeastern Brazil (Bucherl & Diniz, 1978). The most important symptoms of the human envenomation by *Tityus*

[3] Author for correspondence.

serrulatus are intense local pain and an immediate local burning sensation which may last from a few minutes to several hours. These symptoms may be accompanied by autonomic dysfunctions (mydriasis, salivation, sphincter relaxation) and cardiovascular disorders characterized by short-lived hypotension followed by a prolonged increase in blood pressure, arrhythmia and bradycardia (Corrado *et al*., 1974). *Tityus serrulatus* venom is known to act on nerve endings to stimulate the release of either acetylcholine (Gomez *et al*., 1973; Oliveira *et al*., 1989; Vatanpour & Harvey, 1995) or catecholamines (Corrado *et al*., 1974; Moss *et al*., 1974; Langer *et al*., 1975) from different organs and tissues. Interestingly, the venom of the African scorpion *Leiurus quinquestriatus quinquestriatus* relaxes the rat isolated anococcygeus muscle via nitric oxide (NO) release, possibly due to persistent depolarization of peripheral non-adrenergic non-cholinergic (NANC) nerves (Gwee *et al*., 1995). Since NANC nerve stimulation causes corpus cavernosum relaxation (Ignarro *et al*., 1990), we have investigated the effects of *Tityus serrulatus* scorpion venom on the rabbit isolated corpus cavernosum.

Methods

Rabbit corpus cavernosum preparation

Male New Zealand white rabbits (1.5–2.5 kg, provided by CEMIB-UNICAMP) were anaesthetized with pentobarbitone sodium (Sagatal, 30–40 mg/kg^{-1}, i.v.) and exsanguinated via the carotid artery. Following penectomy, the rabbit corpus cavernosum was dissected in the Krebs solution and cleared of the tunica albuginea and surrounding tissues. Strips of rabbit isolated corpus cavernosum were superfused in a cascade system (Vane, 1964) with warmed (37°C) and oxygenated (95%O_2 + 5%CO_2) Krebs solution at a flow rate of 5 ml min^{-1}. The tissue responses (tension of 2.5 g) were detected with auxotonic levers attached to Harvard heart/smooth muscle isotonic transducers and displayed on a Watanabe multichannel pen recorder (model WTR 381). After a 60–90 min period of equilibration, rabbit isolated corpus cavernosum strips were precontracted with noradrenaline (3 μM) in order to increase the basal tone. The tissues were continuously infused with indomethacin (5.6 μM) to inhibit the generation of cyclo-oxygenase products.

Tityus serrulatus venom and other substances (glyceryl trinitrate, acetylcholine, bradykinin, substance P, vasoactive intestinal peptide, calcitonin gene-related peptide, cromakalim and capsaicin) were administered as single bolus injections (10–50 μl). N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), D-NAME, L-arginine, D-arginine, N^{G}-iminoethyl-L-ornithine (L-NIO), 1-(2-trifluoromethylphenyl) imidazole (TRIM), 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ), aprotinin (Trasylol), Hoe 140 (D-Arg-[Hyp^3,Thi^5,DTic^7,Oic^8]-BK), apamin, charybdotoxin, glibenclamide, ruthenium red, methylene blue, atropine, tetraethylammonium and tetrodotoxin were infused over rabbit isolated corpus cavernosum tissues 20 min before and during a bolus injection of the appropriate agonists.

Effect of L-NAME and TRIM on rabbit cerebellum nitric oxide synthase activity in vitro

The *in vitro* actions of both L-NAME and TRIM were studied in a rabbit cerebellum homogenate by measuring their ability to inhibit the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described by Forstermann *et al*., (1990). Briefly, the rabbits were anaesthetized with sodium pentobarbitone (Sagatal; 40 mg kg^{-1}, i.v.), the cerebella were rapidly removed and homogenized in five volumes of cold incubation buffer (50 mM Tris-HCl buffer, pH 7.4) containing 1 mM PMSF and 1 mM L-citrulline. The homogenates were incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$ and 10 mM L-arginine containing 100,000 d.p.m. of [2,3,4,5-^{3}H]-L-arginine monohydrochloride at room temperature (25–27°C). The NOS inhibitor, L-NAME or TRIM was then added to the homogenates to give a final concentration of 10 nM–3 mM. The protein content of the samples was determined according to the method of Peterson (1977) and the activities of cerebellar NOS are expressed as pmol L-citrulline produced min^{-1} mg^{-1} protein. The reduction in L-citrulline production caused by the inhibitors was expressed as a percentage of the maximum activity. From the semi-log concentration-activity curves, the values of pI_2($-\log_{10}$ of the molar concentration of inhibitor that causes 50% inhibition) were calculated.

Purification of Tityus serrulatus *venom*

Dried whole venom (400 mg) dissolved in ammonium bicarbonate buffer (0.01 M, pH 7.8) was purified with a 2.5 × 67.0 cm column of CM-cellulose-52 as previously described (Arantes *et al*., 1989). The resulting pools, designated fractions I-XIII, were then directly lyophilized, wetted and again lyophilized until complete removal of the salt. All of these fractions were assayed in the rabbit isolated corpus cavernosum bioassay cascade. The doses of all fractions used to carry out the bioassay cascade were previously determined by calculating the extinction coefficient (ε) of each fraction. This coefficient gives the protein content (mg ml^{-1}) in a given absorbance. Usually, it is presented as the fraction absorbance which contains 1 mg ml^{-1} protein.

Drugs and Tityus serrulatus *venom*

The venom of *Tityus serrulatus* was provided by the Butantan Institute (São Paulo). The crude venom (lot no. 041088) was obtained by electrostimulation of the telsons of scorpions in captivity and was lyophilized and stored at −20°C.

Acetylcholine, apamin, aprotinin, atropine, D-arginine, L-arginine, bradykinin, calcitonin gene-related peptide, capsaicin, charybdotoxin, cromakalim, glibenclamide, indomethacin, methylene blue, N$^{\omega}$-nitro-D-arginine methyl ester (D-NAME), N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), noradrenaline, ruthenium red, substance P, tetraethylammonium, tetrodotoxin and vasoactive intestinal peptide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^{G}-iminoethyl-L-ornithine (L-NIO) was bought from Research Biochemicals International (Nattick, MA; U.S.A.). Glyceryl trinitrate (ampoules containing 1 mg ml^{-1} in isotonic saline) and pentobarbitone sodium (Sagatal) were acquired from Lipha Pharmaceuticals (London, U.K.) and May & Baker (Dagenham, Essex, U.K.), respectively. Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8-]-BK) was a gift from Hoechst AG (Frankfurt, Germany). 1-(2-Trifluoromethylphenyl) imidazole (TRIM) and 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ) were obtained from Tocris Cookson Inc. (St. Louis, MO, U.S.A.). [^{3}H]-L-arginine was purchased from Amersham International (U.K.).

Tityus serrulatus venom and test agents were stored in stock solution at −20°C and then diluted with isotonic saline (0.9% w/v) when assayed in the rabbit isolated corpus cavernosum strips.

The composition of the Krebs solution was (in mM): NaCl 118, $NaHCO_3$ 25, glucose 5.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.17 and $CaCl_2 6H_2O$ 2.5.

Statistical analysis

The relaxations induced by *Tityus serrulatus* venom and other agents were expressed relative to the submaximal relaxation induced by GTN, which was taken to be 100%. The results are shown as the mean ± s.e.mean of n experiments. Analysis of variance and Student's paired t test were employed to evaluate the data. A P value less than 0.05 was considered to indicate significance.

Results

Involvement of nitric oxide (NO) in the rabbit corpus cavernosum relaxations induced by Tityus serrulatus *venom*

Tityus serrulatus venom (3–100 μg), acetylcholine (ACh; 0.3–30 nmol), bradykinin (BK; 0.3–10 nmol) and glyceryl trinitrate (GTN; 0.5–10 nmol) caused dose-dependent relaxations of the rabbit isolated corpus cavernosum strips (not shown; $n=12$ each). The infusion of 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ; 30 μM, $n=4$), a selective inhibitor of NO-stimulated soluble guanylyl cyclase activity, abolished the relaxations induced by ACh (0.6 nmol; 66 ± 18% before and 1 ± 0.5% during ODQ infusion; $P<0.01$), BK (10 nmol; 49 ± 10% before and 0.0% during ODQ infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 85 ± 4% before and 5 ± 1% during ODQ infusion; $P<0.01$). The GTN (1.3 nmol)-induced relaxation was also significantly reduced by ODQ (98 ± 1% inhibition; $P<0.01$). The relaxations evoked by these agents were greatly restored 15 min after the ODQ infusion had stopped ($P<0.01$; Figure 1). An infusion of methylene blue (MB; 30 μM, $n=4$) significantly reduced the relaxations induced by ACh (0.6 nmol; 87 ± 3% before and 37 ± 11% during MB infusion; $P<0.05$) and *Tityus serrulatus* venom (10 μg; 96 ± 5% before and 33 ± 10% during MB infusion; $P<0.01$). The GTN (1.3 nmol)-induced relaxation was also significantly reduced by MB (32 ± 11% inhibition; $P<0.05$). However, in contrast to ODQ, the inhibitory effect of MB was irreversible (not shown).

The infusion of D-NAME (10 μM, $n=5$) did not affect either the basal tone of the rabbit isolated corpus cavernosum tissues or the relaxations induced by either ACh (0.6 nmol; 130 ± 18% before and 107 ± 14% during D-NAME infusion) or *Tityus serrulatus* venom (30 μg; 113 ± 16% before and 87 ± 18% during D-NAME infusion). However, the subsequent infusion of L-NAME (10 μM, $n=6$) further increased the tone of the rabbit isolated corpus cavernosum tissues and markedly reduced both ACh- (0.6 nmol; 107 ± 14% before and 17 ± 8% during L-NAME infusion; $P<0.01$) and *Tityus serrulatus* venom-induced relaxations (30 μg; 87 ± 18% before and 13 ± 7% during L-NAME infusion; $P<0.01$) without affecting those evoked by GTN. Infusion of L-arginine (300 μM, $n=6$), but not D-arginine (300 μM, $n=5$), partially reversed the increased tone and significantly restored the relaxations induced by ACh (0.6 nmol; 17 ± 8% before and 80 ± 13% during L-arginine infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 13 ± 7% before and 75 ± 12% during L-arginine infusion; $P<0.01$).

Similarly, the NO synthesis inhibitor N^G-iminoethyl-L-ornithine (L-NIO; 30 μM, $n=6$) increased the tone of the rabbit isolated corpus cavernosum and significantly reduced the relaxations induced by both ACh (0.6 nmol; 145 ± 27% before and 24 ± 6% during L-NIO infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 171 ± 26% before and 35 ± 10% during L-NIO infusion; $P<0.01$). At this dose, L-NIO had no significant effect on the GTN-induced relaxations. The subsequent infusion of L-arginine (300 μM, $n=6$) significantly restored both the increased tone and the rabbit isolated corpus cavernosum relaxations induced by ACh (110 ± 9%; $P<0.01$) and *Tityus serrulatus* venom (115 ± 17%; $P<0.01$) (Figure 2).

TRIM (1-(2-trifluoromethylphenyl) imidazole; 100 μM, $n=4$) had no effect on the rabbit isolated corpus cavernosum relaxations induced by *Tityus serrulatus* venom (30 μg; 90 ± 6% before and 85 ± 4% during TRIM infusion), ACh (0.6 nmol; 66 ± 18% before and 66 ± 18% during TRIM infusion), BK (10 nmol; 48 ± 11% before and 49 ± 11% during TRIM infusion) or GTN (1.3 nmol).

In vitro *NO synthase (NOS) activity*

The maximum NOS activity in the homogenates (in the absence of any inhibitor) was 5.96 ± 0.31 pmol L-citrulline min^{-1} mg^{-1} protein ($n=3$). When Ca^{2+} was omitted from the

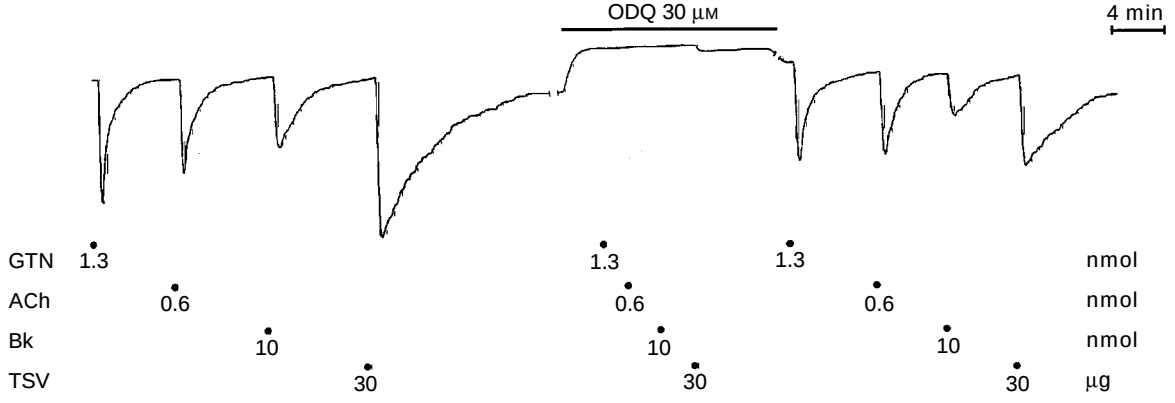

Figure 1 Inhibition by ODQ (30 μM) of the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol) and *Tityus serrulatus* venom (TSV; 30 μg). The infusion of ODQ increased the tone of the tissues and virtually abolished the relaxations induced by the above agonists. After the ODQ infusion had ended, the relaxations were greatly restored. This is a representative tracing of four experiments. Bk, bradykinin.

incubation media, the conversion of L-arginine to L-citrulline was inhibited by more than 90%, thus confirming that the enzymatic activity measured in the homogenates was due to calcium-dependent NOS. From the concentration vs NOS activity curves, the pI_2 values derived were 5.17 ± 0.76 and 2.62 ± 0.18 for L-NAME and TRIM, respectively ($n=3$).

Lack of effect of aprotinin, Hoe 140 and atropine

The protease inhibitor aprotinin (Trasylol; 10 μg ml^{-1}) did not significantly affect the rabbit isolated corpus cavernosum relaxations induced by either ACh ($n=4$) or *Tityus serrulatus* venom ($n=4$; Table 1).

The stable bradykinin B_2 receptor antagonist Hoe 140 (50 nM), $n=5$) virtually abolished the BK (10 nmol)-induced relaxations ($P<0.01$) without affecting those induced by *Tityus serrulatus* venom (10 μg; Table 1).

The muscarinic receptor antagonist atropine (1 μM, $n=5$) significantly reduced the ACh (0.6 nmol)-induced relaxation ($P<0.01$) but had no effect on those induced by either BK (10 nmol) or *Tityus serrulatus* venom (10 μg; Table 1).

Effect of K^+ channel blockers

The ATP-dependent K^+ channel agonist cromakalim (10 and 30 nmol) caused dose-dependent (51 ± 9 and 95 ± 19% relaxation, respectively; $n=6$) and long-lasting corpus cavernosum relaxations. Figure 3 shows that an infusion of the ATP-dependent K^+ channel antagonist glibenclamide (10 μM, $n=6$) significantly reduced the cromakalim (30 nmol)-induced relaxations without affecting those induced by either ACh (0.6 nmol) or *Tityus serrulatus* venom (10 μg; Table 2 and Figure 3). In contrast, the cromakalim (30 nmol)-induced relaxations were not significantly affected by L-NAME (10 μM; 30 ± 6% before and 27 ± 4% during L-NAME infusion, $n=4$).

The Ca^{2+}-activated K^+ channel blockers apamin (0.1 μM, $n=6$) and charybdotoxin (0.1 μM, $n=6$) also had no significant effect on the rabbit isolated corpus cavernosum relaxations induced by ACh, BK, cromakalim and *Tityus serrulatus* venom (Table 2). In addition, the K^+ channel blocker TEA (10 μM, $n=4$) failed to affect ACh-, cromakalim- and *Tityus serrulatus* venom-induced relaxations (Table 2). At these concentrations, glibenclamide, apamin, charybdotoxin and TEA had no effect on the GTN-induced relaxations (not shown, $n=6$).

Involvement of NANC mechanisms

Bolus injections of capsaicin (3 and 10 nmol) over the rabbit isolated corpus cavernosum tissues induced dose-dependent and non-tachyphylactic relaxations ($n=6$) with a similar pattern to that induced by *Tityus serrulatus* venom and other agonists. The infusion of ruthenium red (RR; 30 μM, $n=6$) did not significantly affect the relaxation induced by ACh (0.6 nmol; 88 ± 20% before and 83 ± 13% during RR infusion), BK (10 nmol; 49 ± 9% before and 55 ± 8% during RR infusion) and *Tityus serrulatus* venom (10 μg; 81 ± 17% before and 68 ± 16% during RR infusion). At this dose, RR markedly reduced the capsaicin-induced relaxations (Figure 4).

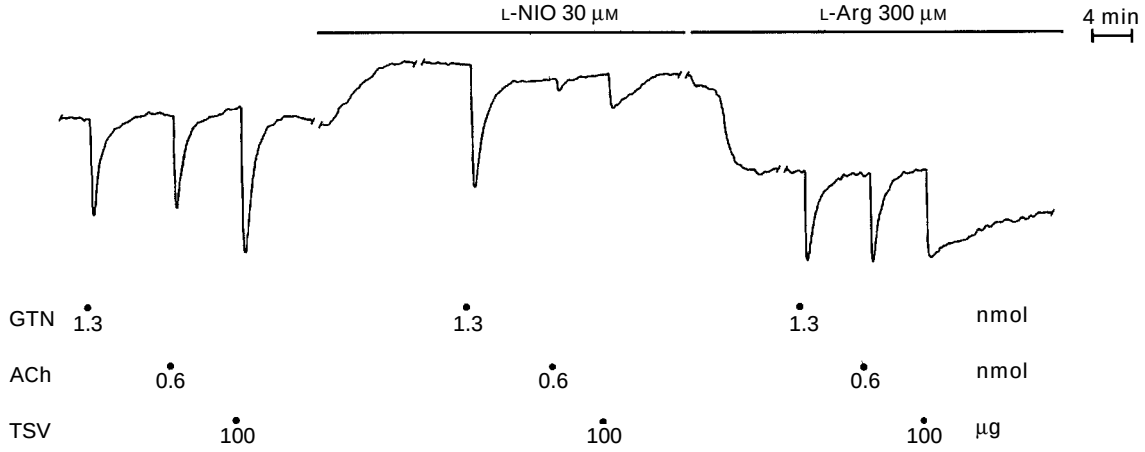

Figure 2 Effects of N^{ω}-nitro-imino-L-ornithine (L-NIO, 10 μM) and L-arginine (L-Arg; 300 μM) on rabbit corpus cavernosum (RbCC) strips. The infusion of L-NIO increased the RbCC tone and reduced the relaxation induced by both acetylcholine (ACh; 0.6 nmol) and *Tityus serrulatus* venom (TSV; 100 μg). The relaxations induced by glyceryl trinitrate (GTN; 1.3 nmol) were not significantly affected by the L-NIO infusion. Subsequent infusion of L-Arg reversed the increased RbCC tone and also partially restored the relaxations induced by ACh and TSV. This is a representative tracing of six experiments.

Table 1 Effect of aprotinin (10 μg ml^{-1}), Hoe 140 (50 nM) and atropine (1 μM) on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol), bradykinin (BK; 10 nmol) and *Tityus serrulatus* venom (TSV; 10 μg)

	RbCC relaxations (%)					
	ACh		*BK*		*TSV*	
Treatment	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
Aprotinin	141 ± 16	148 ± 46	ND	ND	84 ± 23	62 ± 34
Hoe 140	ND	ND	88 ± 5.5	16 ± 9*	98 ± 11	95 ± 10
Atropine	117 ± 7	10 ± 4*	56 ± 9	66 ± 18	95 ± 10	80 ± 6

Aprotinin, Hoe 140 and atropine were infused over the RbCC tissues at a flow rate of 0.1 ml min^{-1} for at least 20 min before injection of the agonists. The RbCC relaxations induced by ACh, BK and TSV were expressed (mean $\pm$ s.e.mean, $n=4-5$) relative to the submaximal relaxation induced by glyceryl trinitrate which was taken to be 100%. ND, not determined. * $P<0.01$, compared to the respective control.

The bolus injection of substance P (SP; 0.75 and 2.2 nmol) had no effect on the rabbit isolated corpus cavernosum tissues ($n=4$, not shown), although higher doses (7.5 nmol) evoked a short-lived contraction ($10.5\pm2\%$, $n=4$) of the tissues. In doses up to 2 nmol, calcitonin gene-related peptide (CGRP) had no significant effect on the rabbit isolated corpus

Figure 3 The ATP-dependent K^+ channel antagonist glibenclamide (10 μM) virtually abolished the rabbit corpus cavernosum (RbCC) relaxation induced by the K^+ channel opener cromakalim (30 nmol) without affecting that induced by *Tityus serrulatus* venom (10 μg). The RbCC relaxations induced by acetylcholine (ACh; 0.6 nmol) and glyceryl trinitrate (GTN; 1.3 nmol) were also not affected by glibenclamide. This is a representative tracing of six experiments.

Table 2 Lack of effect of K^+ channel blockers on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol), bradykinin (BK; 1 nmol), cromakalim (CMK; 30 mnol) and *Tityus serrulatus* venom (TSV; 10 μg)

	RbCC relaxations (%)							
	Glibenclamide		*Apamin*		*Charybdotoxin*		*TEA*	
Agents	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ACh	112 ± 11	98 ± 8	99 ± 7	90 ± 8	107 ± 18	100 ± 20	64 ± 13	80 ± 11
BK	ND	ND	47 ± 3	48 ± 13	44 ± 9	43 ± 10	ND	ND
CMK	95 ± 19	11 ± 6*	57 ± 15	67 ± 21	ND	ND	48 ± 8	46 ± 13
TSV	125 ± 11	102 ± 11	83 ± 13	88 ± 11	84 ± 20	87 ± 19	60 ± 9	69 ± 14

Glibenclamide (10 μM), apamin (0.1 μM), charybdotoxin (0.1 μM) and tetraethylammonium (TEA, 10 μM) were infused over the RbCC tissues at a flow rate of 0.1 ml min^{-1} for at least 20 min before injection of the agonists. The RbCC relaxations induced by ACh, BK and TSV were expressed (mean $\pm$ s.e.mean, $n=6$) relative to the submaximal relaxation induced by glyceryl trinitrate which was taken to be 100%. ND, not determined. * $P<0.05$ compared to the respective control.

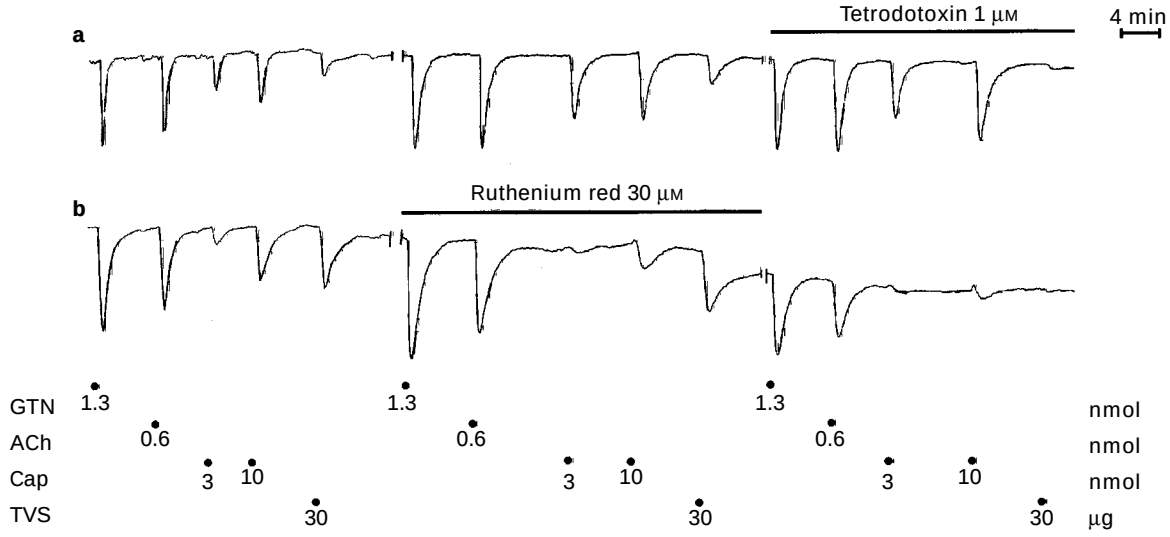

Figure 4 Effects of ruthenium red and tetrodotoxin on rabbit corpus cavernosum (RbCC). The infusion of ruthenium red (30 μM) over the second tissue (b) markedly inhibited the capsaicin-induced RbCC relaxations, without affecting those evoked by *Tityus serrulatus* venom. The sodium channel blocker tetrodotoxin (1 μM) infused over both tissues abolished the RbCC relaxations induced by *Tityus serrulatus* venom (TSV; 30 μg), but had no effect on those induced by capsaicin (Cap; 3 and 10 nmol), acetylcholine (ACh; 0.6 nmol) and glyceryl trinitrate (GTN; 1.3 nmol). This is a representative tracing of six experiments.

cavernosum tissues ($n=4$, not shown). Bolus injection of vasoactive intestinal peptide (VIP; 0.6 and 2 nmol) caused small relaxations ($8\pm2.5\%$ and $19\pm4\%$, respectively). The VIP (2 nmol)-induced relaxations were not significantly affected by L-NAME (10 μM; $16\pm6\%$ before and $14\pm5\%$ during L-NAME infusion; $n=4$, not shown).

Effect of the sodium channel blocker tetrodotoxin

An infusion of tetrodotoxin (TTX; 1 μM, $n=6$) virtually abolished the *Tityus serrulatus* venom (30 μg)-induced relaxations ($66\pm9\%$ before and $4\pm2\%$ during TTX infusion; $P<0.01$). At this concentration, TTX did not affect either the tone of the rabbit isolated corpus cavernosum tissues or the relaxations induced by capsaicin (10 nmol; $71\pm6\%$ before and $68\pm6\%$ during TTX infusion, $n=6$), BK (10 nmol; $75\pm9\%$ before and $73\pm7\%$ during TTX infusion), ACh (0.6 nmol; $79\pm1\%$ before and $90\pm10\%$ during TTX infusion) and GTN (1.3 nmol) (Figure 4). In addition, the established relaxations by *Tityus serrulatus* venom were promptly reversed by the same concentration of TTX (Figure 5).

Bioassay cascade of fractions purified from Tityus serrulatus *venom*

The bioassay cascade of the fractions I-XIII revealed that fractions I-IX and XIII (up to 80 μg) had no effect on the rabbit isolated corpus cavernosum tissues (not shown; $n=6$). In contrast, fractions X (1.2–12 μg), XI (1.4–14 μg) and XII (1–10 μg) caused dose-dependent relaxations. These fractions corresponded to 4.01, 3.58 and 2.97% of whole venom, respectively. *Tityus serrulatus* venom (30 μg; $n=8$) caused $65\pm8\%$ whereas fraction X (3.8 μg, $n=8$), fraction XI (4.2 μg, $n=8$) and fraction XII (3.0 μg, $n=8$) caused $44\pm8\%$, $56\pm12\%$ and $54\pm10\%$ of relaxation, respectively. The infusion of either L-NAME (10 μM; $n=4$) or TTX (1 μM; $n=4$) reduced by $>90\%$ the relaxations of rabbit isolated corpus cavernosum induced by these fractions.

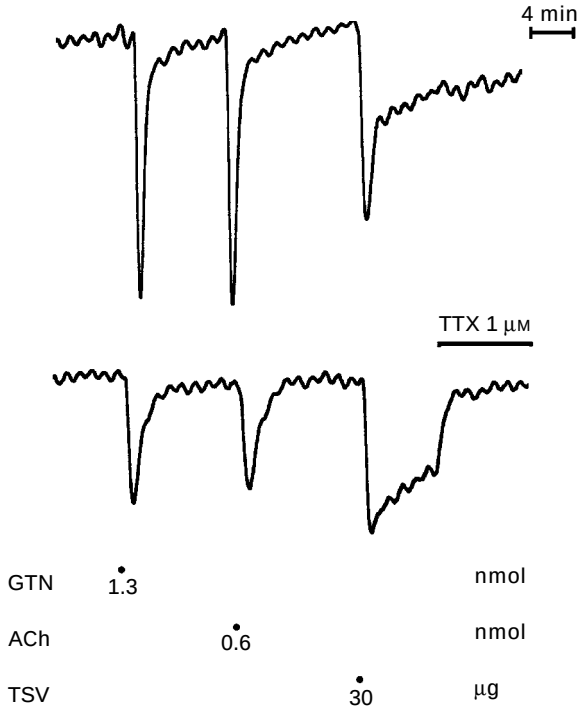

Figure 5 Infusion of the sodium channel blocker tetrodotoxin (TTX, 1 μM) over the second tissue promptly reversed the established RbCC relaxation induced by *Tityus serrulatus* venom (TSV; 30 μg). This is a representative tracing of four experiments.

Discussion

Our results indicate that *Tityus serrulatus* scorpion venom relaxes the rabbit corpus cavernosum via the release of NO. The involvement of NO was confirmed by the findings that the non-specific NOS inhibitors L-NAME (Moore *et al*., 1989) and L-NIO (Rees *et al*., 1990) markedly reduced the *Tityus serrulatus* venom-induced relaxations and that this inhibition was reversed by L-arginine (but not D-arginine). Methylene blue (Gruetter *et al*., 1981; Rapoport & Murad, 1983) and ODQ (Garthwaite *et al*., 1995), inhibitors of soluble guanylate cyclase, also markedly reduced the relaxation induced by *Tityus serrulatus* venom, further supporting the involvement of NO. In contrast to MB, the inhibition by ODQ was reversible. Whether this finding reflects a weaker binding of the inhibitor to soluble guanylate cyclase remains to be established.

NO activates Ca^{2+}-dependent K^+ channels in rabbit aorta (Bolotina *et al*., 1994). Cromakalim relaxed the rabbit isolated corpus cavernosum and this effect was selectively blocked by glibenclamide, indicating the presence of functional ATP-dependent K^+ channels in the erectile tissue. However, the finding that *Tityus serrulatus* venom-induced relaxations were not affected by either the Ca^{2+}-dependent K^+ channel antagonists, apamin (Burgess *et al*., 1980) and charybdotoxin (Gimenez-Gallego *et al*., 1986), or by glibenclamide, excludes the involvement of these K^+ channels in the venom transduction mechanism. The failure of TEA to affect venom-induced relaxations further supports the contention that K^+ channels do not play a role.

Similar to the scorpion venom, *Phoneutria nigriventer* spider venom also relaxes rabbit isolated corpus cavernosum via the release of NO. However, this release is secondary to tissue kallikrein activation (Lopes-Martins *et al*., 1994). Since neither the protease inhibitor aprotinin (Vogel & Werle, 1970) nor the BK antagonist Hoe 140 (Wirth *et al*., 1991) affected the *Tityus serrulatus* venom-induced relaxation, we may rule out this intermediate step in NO release.

The erectile tissues from different animal species are innervated by both adrenergic excitatory and cholinergic inhibitory nerve fibres (see Andersson & Wagner, 1995). The failure of the muscarinic receptor antagonist atropine to affect the *Tityus serrulatus*-induced relaxations indicates that the venom does not act either by activating cholinergic fibres nor through the presence of ACh-like substances in the venom itself.

The erectile tissues are also richly innervated by NANC inhibitory nerve fibres (Gillespie, 1972; Klinge & Sjostrand, 1974) and this is believed to play a pivotal role in the neural mechanisms involved in penile erection through the release of NO (Ignarro *et al*., 1990; Pickard *et al*., 1991; Kim *et al*., 1991; Rajfer *et al*., 1992). Thus, the potential sources of NO production in the rabbit isolated corpus cavernosum preparation employed in this study are both NANC neurones and the endothelium, which covers the network of sinusoidal capillaries supplying the cavernosal tissue.

The classical sodium channel blocker tetrodotoxin specifically inhibited the *Tityus serrulatus* venom-induced rabbit isolated corpus cavernosum relaxations, strongly indicating that NO release by the venom is preceeded by nerve activation, possibly involving the NANC system. Indeed, the addition of *Tityus serrulatus* venom (or tityustoxin) to the peripheral cut

end of either sciatic or saphenous nerve of the rat releases a neurogenic permeability factor from sensory fibres, which is responsible for the increase in vascular permeability and oedema formation in the areas supplied by the nerves (Garcia-Leme *et al*., 1977).

NANC activation leads to the release of neuropeptides such as substance P, CGRP and VIP. Capsaicin, a substance known to activate primary sensory neurones (Jancsó *et al*., 1977) causes the release of these neuropeptides from NANC terminals in different tissues (Holzer, 1991). However, it is unlikely that the venom activates NANC primary sensory C fibres to cause the release of neuropeptides, since bolus injections of substance P and CGRP were unable to relax rabbit isolated corpus cavernosum tissues whereas VIP caused small relaxations. The observation that capsaicin caused NO-independent and tetrodotoxin-insensitive relaxations reinforces the concept, that rabbit isolated corpus cavernosum relaxation by *Tityus serrulatus* venom does not result from the activation of capsaicin-sensitive sensory neurones. Furthermore, ruthenium red, an inhibitor of capsaicin-induced stimulation of sensory neurones (Buckely *et al*., 1990; Amann & Maggi, 1991), markedly reduced capsaicin-induced relaxations but failed to affect those induced by the venom. The mechanism by which capsaicin relaxes the rabbit isolated corpus cavernosum is not yet clear but it may involve a direct vasorelaxing effect on the cavernosal tissues, independent of both endothelium and nerve stimulation.

We propose therefore that *Tityus serrulatus* venom acts selectively on NANC fibres, possibly nitrergic neurones, and that the NO generated in the nerve diffuses through the nerve endings to relax the adjacent vascular smooth muscle. Indeed, neuronal NOS (bNOS) has been detected in both the rat (Burnett *et al*., 1992) and human (Burnett *et al*., 1993) penis by use of a specific NOS antibody and immunohistochemistry. The findings that TRIM, a specific neuronal NOS inhibitor in the mouse (Handy *et al*., 1995), did not affect the NO release induced by *Tityus serrulatus* venom, may reflect its reduced potency on the rabbit enzyme.

Several protein toxins purified from South American scorpions venoms have been described and classified either as α or β toxins, according to their effects on voltage-dependent Na^+ channels of excitable cells (Barhanin *et al*., 1982). The former prolong the action potential, whereas the latter do not depend on the membrane potential for to their action. Interestingly, a peptide isolated from fraction XI (MW 7230) delays the inactivation of Na^+ channels of the B fibres of the rabbit vagus nerve (Arantes *et al*., 1993), whereas fraction XIII, which does not cause rabbit isolated corpus cavernosum relaxation, contains a β toxin (Jonas *et al*., 1986). Thus, our results indicate that activation of Na^+ channels in NANC fibres is essential for NO release.

C.E.T. is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

References

AMANN, R. & MAGGI, C.A. (1991).Ruthenium red as a capsaicin antagonist. *Life Sci.,* **49,** 849 – 856.

ANDERSSON, K.-E. & WAGNER, G. (1995). Physiology of penile erection. *Physiol.Rev.,* **75,** 191 – 236.

ARANTES, E.C., PRADO, W.A., SAMPAIO, S.V. & GIGLIO, J.R. (1989). A simplified procedure for the fractionation of Tityus serrulatus venom: isolation and partial characterization of TsTX-IV, a new neurotoxin. *Toxicon,* **27,** 907 – 916.

ARANTES, E.C., RICCIOPPO-NETO, F., SAMPAIO, S.V., VIEIRA, C.A. & GIGLIO, J.R. (1993). The delay of the inactivation of Na^+ channels by TsTX-V, a new neurotoxin from Tityus serrulatus scorpion venom. *Toxicon,* **31,** 108 – 109.

BARHANIN, J., GIGLIO, J.R., LEOPOLD, P., SCHMID, A., SAMPAIO, S.V. & LAZDUNSKI, M. (1982). *Tityus serrulatus* venom contains two classes of toxins: *Tityus* γ toxin is a new tool with a very affinity for studying the Na^+ channel. *J. Biol. Chem.,* **257,** 12553 – 12558.

BLAUSTEIN, M.P., ROGOWSKI, R.S., SCHNEIDER, M.J. & KRUEGER, B.K. (1991). Polypeptide toxins from the venoms of Old World and New World scorpions preferentially block different potassium channels. *Mol. Pharmacol.,* **40,** 932 – 942.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature,* **368,** 850 – 853.

BUCHERL, W. & DINIZ, C.R. (1978). Arthropod venoms. In *Handb. Exp. Pharmacol.,* ed. Bettini, S. 371 – 394. Berlin, Heidelberg, New York: Springer-Verlag.

BUCKLEY, T.L., BRAIN, S.D. & WILLIAMS, T.J. (1990). Ruthenium red selectively inhibits oedema formation and increased blood flow induced by capsaicin in rabbit skin. *Br. J. Pharmacol.,* **99,** 7 – 8.

BURGESS, G.M., CLARET, M. & JENKINSON, D.H. (1980). Effects of quinine and apamin on the calcium-dependent potassium permeability of mammalian hepatocytes and red cells. *J. Physol.,* **317,** 67 – 90.

BURNETT, A.L., LOWENSTEIN, C.J., BREDT, D.S., CHANG, T.S.K. & SNYDER, S.H. (1992). Nitric oxide: a physiologic mediator of penile erection. *Science,* **257,** 401 – 403.

BURNETT, A.L., TILLMAN, S.L., CHANG, T.S.K., EPSTEIN, J.I., LOWENSTEIN, C.J., BREDT, D.S., SNYDER, S.H. & WALSH, P.C. (1993). Immunohistochemical localization of nitric oxide synthase in the autonomic innervation of the human penis. *J. Urol.,* **150,** 73 – 76.

CORRADO, A.P., RICCIOPPO-NETO, F. & ANTONIO, A. (1974). The mechanism of the hypertensive effect of Brazilian scorpion venom (*Tityus serrulatus* Lutz e Mello). *Toxicon,* **12,** 145 – 150.

FORSTERMANN, U., GORSKY, L.D., POLLOCK, J.S., SCHMIDT, H.H.H.W., HELLER, M. & MURAD, F. (1990). Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. *Biochem. Biophys. Res. Commun.,* **168,** 727 – 732.

GARCIA-LEME, J., PIMENTA, A.F., RAULINO-FILHO, M. & DINIZ, C.R. (1977). Sensory nerves and inflammation: evidence for the release of a neurogenic permeability factor by tityustoxin. *J. Pathol.,* **124,** 165 – 176.

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.,* **48,** 184 – 188.

GILLESPIE, J.S. (1972). The rat anococcygeous muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.,* **45,** 404 – 416.

GIMENEZ-GALLEGO, G., NAVIA, N.A., REUBEN, J.P., KATZ, G.M., KACZOROWSKI, G.J. & GARCIA, M.L. (1986). Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 3329 – 3333.

GOMEZ, M.V., DAI, M.E.M. & DINIZ, C.R. (1973). Effect of scorpion venom, tityustoxin, on the release of acetylcholine from incubated slices of rat brain. *J. Neurochem.,* **20,** 1051 – 1061.

GRUETTER, C.A., BARRY, B.K., MCNAMARA, D.B., GRUETTER, D.Y., KADOWITZ, P.J. & IGNARRO, L.J. (1981). Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and carcinogenic nitrosoamine. *J. Cyclic Nucleotide Res.,* **5,** 211 – 224.

GWEE, M.C.E., CHEAH, L.S. & GOPALAKRISHNAKONE, P. (1995). Involvement of the L-arginine-nitric oxide synthase pathway in the relaxant responses of the rat isolated anococcygeus muscle to a scorpion (*Leiurus quinquestriatus quinquestriatus*) venom. *Toxicon,* **33,** 1141 – 1150.

HANDY, R.L.C., WALLACE, P., GAFFEN, Z.A., WHITEHEAD, K.J. & MOORE, P.K. (1995). The antinociceptive effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase *in vitro*, in the mouse. *Br. J. Pharmacol.,* **116,** 2349 – 2350.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.,* **43,** 143 – 201.

IGNARRO, L.J., BUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.,* **170,** 843 – 850.

JANCSÓ, G., KIRÁLY, E. & JANCSÓ-G'BOR, A. (1977). Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature,* **270,** 741 – 743.

JONAS, P., VOGEL, W., ARANTES, E.C. & GIGLIO, J.R. (1986). Toxin γ of the scorpion *Tityus serrulatus* modifies both activation and inactivation of sodium permeability of nerve membrane. *Pflügers Arch.,* **407,** 92 – 99.

KIM, N., AZADZOI, K.M., GOLDSTEIN, I. & SAENZ DE TEJADA, I. (1991). A nitric oxide-like factor mediates nonadrenergic-noncholinergic neurogenic relaxation of penile corpus cavernosum smooth muscle. *J. Clin. Invest.,* **88,** 112 – 118.

KIRSCH, G.E., SKATTEBOL, A., POSSANI, L.D. & BROWN, A.M. (1989). Modification of sodium channel gating by an α scorpion toxin from *Tityus serrulatus*. *J. Gen. Physiol.,* **93,** 67 – 83.

KLINGE, E. & SJÖSTRAND, N.O. (1974). Contraction and relaxation of the retractor penis muscle and the penile artery of the bull. *Acta Physiol. Scand.,* suppl. **420,** 1 – 88.

LANGER, S.Z., ADLER-GRASCHINSKY, E., ALMEIDA, A.L. & DINIZ, C.R. (1975). Prejunctional effects of a purified toxin from the scorpion *Tityus serrulatus*. *Naunyn-Schmiedeberg's Arch Pharmacol.,* **287,** 243 – 259.

LOPES-MARTINS, R.A.B., ANTUNES, E., OLIVA, M.L., SAMPAIO, C., BURTON, J. & DE NUCCI, G. (1994). Pharmacological characterization of rabbit corpus cavernosum relaxation mediated by tissue kallikrein-kinin system. *Br. J. Pharmacol.,* **113,** 81 – 86.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1989). L-N^G-nitro arginine (L-NOARG) a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.,* **99,** 408 – 412.

MOSS, J., THOA, N.B. & KOPIN, I.K. (1974). On the mechanism of scorpion toxin-induced release of norepinephrine from peripheral adrenergic neurons. *J. Pharmacol. Exp. Ther.,* **190,** 39 – 48.

NAGY, K. (1988). Mechanism of inactivation of single sodium channels after modification by chloramine-T, sea anemone toxin and scorpion toxin. *J. Membr. Biol.,* **106,** 29 – 40.

OLIVEIRA, M.J., FONTANA, M.D., GIGLIO, J.R., SAMPAIO, S.V., CORRADO, A.P. & PRADO, W.A. (1989). Effects of the venom of the Brazilian scorpion *Tityus serrulatus* and two of its fractions on the isolated diaphragm of the rat. *Gen. Pharmacol.,* **20,** 205 – 210.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.,* **83,** 346 – 356.

PICKARD, R.S., POWELL, P.H. & ZAR, M.A. (1991). The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle. *Br. J. Pharmacol.,* **104,** 755 – 759.

RAJFER, J., ARONSON, W.J., BUSH, P.A., DOREY, F.J. & IGNARRO, L.J. (1992). Nitric oxide as mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N. Engl. J. Med.,* **326,** 90 – 94.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ. Res.,* **52,** 352 – 357.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **101,** 746 – 752.

ROGOWSKI, R.S., KRUEGER, B.K., COLLINS, J.H. & BLAUSTEIN, M.P. (1994). Tityustoxin Kα blocks voltage-gated noninactivating K^+ channels and unblocks inactivating K^+ channels blocked by α-dendrotoxin in synaptosomes. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 1475 – 1479.

VANE, J.R. (1964). The use of isolated organs for detecting active substances in the circulating blood. *Br. J. Pharmacol. Chemother.,* **23,** 360 – 373.

VATANPOUR, H. & HARVEY, A.L. (1995). Modulation of acetylcholine release at mouse neuromuscular junctions by interaction of three homologous scorpion toxins with K^+ channels. *Br. J. Pharmacol.,* **114,** 1502 – 1506.

VOGEL, R. & WERDE, E. (1970). Kallikrein inhibitors. In *Handb. Exp. Pharmacol.,* ed. Erdos, E.G. **Vol. XXV,** 213 – 249. New York: Springer.

WIRTH, K., HOCK, F.J., ALBUS, U., LINZ, W., ALPERMANN, H.G., ANAGNOSTOPOULOS, H., HENKE, ST., BREIPOHL, G., KONIG, W., KNOLLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin antagonist: *in vivo* studies. *Br. J. Pharmacol.,* **102,** 774 – 777.

YATANI, A., KIRSCH, G.E., POSSANI, L.D. & BROWN, A.M. (1988). Effects of New World scorpion toxins on single-channel and whole cell cardiac sodium currents. *Am. J. Physiol.,* **254,** H443 – H451.

(Received May 30, 1997
Revised October 17, 1997
Accepted October 20, 1997)