Capsaicin-induced relaxation in the rat isolated external urethral sphincter: characterization of the vanilloid receptor and mediation by CGRP

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1 The potential role of capsaicin-sensitive nerves in the relaxation of the rat external urethral sphincter (REUS) was evaluated by demonstrating the existence of specific vanilloid (capsaicin) receptors and by investigating the sensory neurotransmitter(s) putatively involved in this relaxation.

2 Capsaicin (1 μ M) relaxed REUS strips precontracted with noradrenaline (NA) (0.1 mM). This effect underwent desensitization and it was absent in preparations taken from adult capsaicin-pretreated rats.

3 Capsaicin-induced relaxation of NA-precontracted REUS was mimicked by calcitonin gene-related peptide (CGRP, 0.3–10 μ M), but not by substance P (1 μ M), vasoactive intestinal polypeptide (VIP, 1 μ M), α - β methylene ATP (10 μ M), γ -aminobutyric acid (GABA, 3 mM) or galanin (1 μ M). A cross-tachyphylaxis between capsaicin (1 μ M) and CGRP (1 μ M) was observed. Both capsaicin and CGRP-induced relaxation were partially antagonized by the proposed CGRP antagonist, CGRP (8-37) (10 μ M).

4 Electrical field stimulation (EFS, 2.5 Hz, 60 V, 1 ms, trains of 5 s every 5 min) of REUS evoked a contraction characterized by a largely adrenergic slowly developing tonic contraction with superimposed fast twitches due to the striated component of the strips. Both capsaicin (1 μ M) and CGRP (0.01–1 μ M) produced an almost complete inhibition of EFS-induced tonic contraction. A cross-tachyphylaxis between capsaicin and CGRP was observed. Furthermore, these inhibitory actions were unaffected by CGRP (8-37) (10 μ M).

5 [³H]-resiniferatoxin displayed specific, saturable binding to rat urethral membranes. Data were consistent with a single site with a K_d of 105 pM and a B_{max} of 40 fmol mg⁻¹ protein. This binding was inhibited by capsaicin with a K_i of 0.6 μ M and it was reduced by approximately 80% in preparations taken from rats that had undergone surgical ablation of the major pelvic ganglion 4 days earlier.

6 In conclusion we have demonstrated the existence of vanilloid receptors on capsaicin-sensitive nerves innervating the rat urethra mainly through the major pelvic ganglion. The activation of this set of nerves could lead to a local release of CGRP that in turn elicits a remarkable urethral relaxation. Such a mechanism could be of relevance in physiological conditions to facilitate urine expulsion during micturition and in pathological conditions to help removal of noxious stimuli following mechanical/chemical irritation of the lower urinary tract.

Keywords: Neurogenic inflammation; sensory neuropeptides; micturition; resiniferatoxin

Introduction

The rat urethra has a rather complex anatomical organization with bundles of striated muscle fibres interlaced with smooth muscle (El-Badawi & Schenk, 1974) and a dense network of autonomic and somatic innervation (Watanabe & Yamamoto, 1979). Noradrenergic nerves are thought to be the major excitatory input to the urethra (Levin & Wein, 1979; Gosling, 1986; Chen & Brading, 1992) determining the continence of the bladder outlet in the periods between micturitions. However, during urination, the urethral sphincter has to relax to allow urine expulsion and a series of neural mechanism(s) should be targeted to contrast sympathetic tone. Recent reports indicate that one of these mechanisms could be the generation and release of nitric oxide from nonadrenergic-noncholinergic nerves (Persson *et al.*, 1992; Parlani *et al.*, 1993). Immunohistochemical studies combined with retrograde tracing techniques indicate the presence in the urethral wall of nerves containing substantial amounts of calcitonin gene-related peptide (CGRP) and tachykinins (Su *et al.*, 1986). These nerves have their cell bodies in dorsal root ganglia (L6-S1) and their CGRP content is substantially reduced following systemic capsaicin desensitization (Su *et al.*, 1986). Activation of these capsaicin-sensitive primary afferents can be involved in the neurogenic inflammation that follows mechanical irritation of the urethral meatus (Nord-

ling *et al.*, 1990; Abelli *et al.*, 1991). The aims of this investigation were: (a) to assess whether or not the activation of capsaicin-sensitive nerves can relax urethral strips precontracted by exogenous noradrenaline or electrical field stimulation of sympathetic nerves; (b) to investigate the putative neurotransmitter involved in such inhibitory effects and (c) to demonstrate the existence of vanilloid (capsaicin) receptors in rat urethral tissues by using a binding assay with the tritiated ultrapotent capsaicin analogue, resiniferatoxin (RTX) (Szallasi & Blumberg, 1990).

Methods

Organ bath studies

Male albino rats (Wistar-Morini strain, 360–400 g) were killed by cervical dislocation. An incision was made in the lower abdomen; the bladder was exposed and the proximal urethra was freed from the surrounding tissues. The pubic bone was cut and then the external urethral sphincter (EUS) was disconnected from the perineal muscles and removed *in toto*. Thereafter, the preparation was placed in oxygenated Krebs solution and strips (approximately 7 mm by 3 mm) were taken from the middle region of the EUS (Parlani *et al.*, 1992). Samples were mounted in a 5 ml organ bath containing Krebs solution (37°C) continuously aerated with a mix-

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ture of 96% O₂ and 4% CO₂. The Krebs solution contained (mM): NaCl 119, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2 and glucose 11. The preparations were connected with a silk thread to an isometric strain gauge under a constant load of 1 g. Contractile activity was recorded by means of a Basile 7050 unirecord polygraph. The preparations were allowed to equilibrate for at least 60 min. Based on preliminary experiments assessing the concentration-response curve to the motor effect of noradrenaline (NA), 0.1 mM was chosen as a supramaximal concentration of the adrenoceptor agonist. Exposure to NA (0.1 mM) produced a contraction that reached a steady state within 5–15 min and then lasted for at least an additional 30–45 min. The effect of drugs was studied when a stable tone was established. The inhibitory actions were measured as mg of relaxation or % of inhibition of the NA (0.1 mM)-induced contractile response before exposure to drugs. In other experiments electrical field stimulation (EFS) of the EUS was carried out by means of two platinum wire electrodes placed at the top and at the bottom of the organ bath and connected to a Grass S11 stimulator. Square wave pulses were delivered at 60 V, 2.5 Hz (trains of 5 s every 5 min). The pulse width was 1 ms. A scale of the frequency (range between 1.5 and 5 Hz) revealed that 2.5 Hz was the frequency triggering maximal responses. This frequency of EFS was selected to evaluate the effect of pharmacological interventions. After three consecutive reproducible responses (accepted variation <10%) had been obtained, drugs were added to the organ bath. The effect of drugs was evaluated on the subsequent response (5 min later) and expressed as % inhibition of the basal contraction.

To prevent desensitization, CGRP concentration-response curves were carried out in a non-cumulative manner. In each preparation a maximum of three concentrations was tested.

An incubation time of 10 min was selected to study the effect of the putative CGRP antagonist, CGRP (8-37). In each preparation the effect of the antagonist was tested on one type of contraction (NA or EFS) and one type of agonist (capsaicin or CGRP or papaverine). Statistical comparison was performed between control and treated group.

Surgical procedures

Denervation of the EUS was carried out by bilateral removal of the major pelvic ganglion, as described previously (Conte *et al.*, 1989). After the surgical operation, the rats were kept for 4 days in separate cages to allow nerve degeneration. To prevent infections, each rat received amikacin (2.5 mg kg⁻¹, i.m.) and gentamicin (2 mg kg⁻¹, i.p.) once daily.

Systemic capsaicin desensitization

Capsaicin (50 mg kg⁻¹, dissolved in a vehicle containing 10% Tween 80, 10% ethanol and 80% saline) was administered subcutaneously 4 days before the experiment. Control rats received the vehicle as described previously (Santicioli *et al.*, 1985).

[³H]-RTX binding assay

Binding experiments were carried out with a crude, post-nuclear membrane fraction obtained from urethra of Sprague-Dawley rats as described for other tissues (Szallasi & Blumberg, 1990; 1992). Briefly, the urethra was disrupted by the aid of a Polytron tissue homogenizer in ice-cold buffer (pH 7.4), containing (in mM): KCl 5, NaCl 5.8, MgCl₂ 2, CaCl₂ 0.75, sucrose 137 and HEPES 10. The homogenate was centrifuged for 10 min at 1 000 g (4°C), the pellet discarded, and the supernatant was then centrifuged at 35,000 g for 30 min. The resulting pellet was resuspended in the above described buffer and stored at -20°C until assayed. Aliquots (100 µg) of the membrane protein in 0.5 ml of this buffer containing 0.25 mg ml⁻¹ bovine serum albumin (Sigma, Cohn

fraction V) were incubated in triplicate with [³H]-RTX and nonradioactive ligands at 37°C for 30 min. Nonspecific binding was determined in the presence of 100 nM nonradioactive RTX. After the binding reaction had been terminated by chilling the assay mixture on ice, 100 µg alpha₁-acid glycoprotein was added to each tube to reduce nonspecific binding (Szallasi *et al.*, 1992). Bound and free [³H]-RTX were then separated by centrifuging the membranes in a Beckman 12 microfuge, and the counts in the resulting pellet determined by scintillation counting.

Binding data from saturation experiments with increasing concentrations (7–500 pM) of radioactive ligand were analysed by the collection of computer programmes LIGAND (Biosoft Cambridge, UK); inhibitory constants were determined by the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Drugs

Drugs used were: noradrenaline, atropine sulphate (Fluka), acetylcholine, papaverine, GABA, L-NOARG, α,β-methylene ATP (Sigma), capsaicin (Serva), CGRP, SP, NKA, VIP, galanin, hCGRP(8-37) (Peninsula), phentolamine (Ciba Geigy), tetrodotoxin (Sankyo), amikacin (Bristol), gentamicin (Shering), pentothal sodium (Abbott). A stock solution (10 mM) of capsaicin was prepared in absolute ethanol and then appropriate dilutions in distilled water were made; all other drugs were dissolved directly in distilled water.

[³H]-RTX (37 Ci mmol⁻¹) was synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD, U.S.A. Nonradioactive RTX and resiniferonol 9,13,14-orthophenylacetate (ROPA) were purchased from LC Services (Woburn, MA, U.S.A.).

Statistics

All data in the text are means ± s.e. Statistical evaluation was performed by using Student's *t* test for paired or unpaired data when applicable or by analysis of variance (ANOVA) followed by Tukey's test.

Results

Effect of capsaicin on noradrenaline-induced tonic contraction of rat external urethral sphincter

In basal conditions specimens of rat external urethral sphincter were either quiescent (about 30%) or displayed a small-amplitude (20–100 mg) contractile activity (about 70%). After 30 min of incubation all preparations exhibited no spontaneous contractile activity. In these conditions, the administration of capsaicin (1 µM) or CGRP (0.3 µM) produced a small relaxation of 42 ± 12 mg (*n* = 7) and 35 ± 8 mg (*n* = 6), respectively. On the other hand, substance P (10 µM) was ineffective (*n* = 4), while neurokinin A (10 µM) elicited a small and transient contraction the amplitude of which was 61 ± 10 mg (*n* = 6).

The administration of noradrenaline (NA) (0.1 mM) elicited a contraction with an amplitude of 351 ± 20 mg (*n* = 25) reaching a steady-state within 5–15 min. The NA-induced tonic contraction remained stable for at least 30–45 min (*n* = 6) and it was abolished by phentolamine (3 µM, *n* = 4), but was unaffected by atropine (3 µM, *n* = 4) or tetrodotoxin (0.6 µM, *n* = 4).

Administration of capsaicin (1 µM) during NA-induced tonic contraction resulted in a rapid relaxation reaching a maximal effect (82 ± 5 mg corresponding to an inhibition of 32 ± 3%, *n* = 13) within 1–2 min (Figure 1). Capsaicin-induced relaxation lasted for at least 15–20 min and then the urethral tone slowly returned toward the previous contracted level. A second (or in one case a third) administration of capsaicin (1 µM) was without any relaxant effect, suggesting

Figure 1 Typical tracings showing the effect of capsaicin (Caps) and calcitonin gene-related peptide (CGRP) on noradrenaline (NA)-induced tonic contraction of control (a) and systemic capsaicin-desensitized (b) rats. In (a) is also shown the desensitization that occurred with a second application of capsaicin. Tension and time scales apply to all tracings. In the left upper tracings the two bars indicate a lag of about 20 min.

desensitization ($n = 4$) (Figure 1). Capsaicin-induced relaxation was unaffected by atropine ($3 \mu\text{M}$, $n = 4$) or tetrodotoxin ($0.6 \mu\text{M}$, $n = 4$).

In some experiments, external urethral sphincters obtained from adult capsaicin-pretreated rats (50 mg kg^{-1} , s.c., 4 days before) were used. In these preparations the motor responses to NA were normal ($396 \pm 47 \text{ mg}$, $n = 6$, NS as compared to control), while the administration of capsaicin ($1 \mu\text{M}$) produced only negligible relaxation ($13 \pm 8 \text{ mg}$ corresponding to $3 \pm 2\%$ inhibition, $P < 0.001$ as compared to control rats, $n = 6$) (Figure 1). In urethral preparations from capsaicin-pretreated rats, capsaicin ($1 \mu\text{M}$) had no effect on basal tone ($n = 7$).

In a further set of experiments the ability of capsaicin to relax NA-precontracted urethral strips taken from rats in which the major pelvic ganglia had been surgically removed 4 days before, has been evaluated. Capsaicin ($1 \mu\text{M}$)-induced relaxation was significantly reduced as compared to urethral tissues taken from normal rat ($41 \pm 19 \text{ mg}$ and $16 \pm 3\%$ inhibition $n = 4$; $P < 0.001$ vs. control rats, $n = 4$). On the other hand the motor response to NA and the relaxation induced by CGRP ($0.3 \mu\text{M}$) or papaverine ($10 \mu\text{M}$) were similar in control and in denervated preparations ($n = 4$).

Mimicking by CGRP of capsaicin-induced relaxation of NA-precontracted EUS: antagonism by CGRP(8-37)

To obtain some insight into the neurotransmitter(s) involved in capsaicin-induced relaxation of NA-precontracted rat external urethral sphincter, the potential relaxant properties of various putative neurotransmitters were assessed. No relaxation of NA-induced tone was obtained with vasoactive intestinal polypeptide (VIP, $1 \mu\text{M}$, $n = 4$), substance P ($1 \mu\text{M}$, $n = 4$), α - β -methylene-ATP ($10 \mu\text{M}$, $n = 4$), (γ -aminobutyric acid) GABA (3 mM , $n = 4$) or galanin ($1 \mu\text{M}$, $n = 4$). On the other hand a clear and remarkable relaxation ($114 \pm 24 \text{ mg}$ corresponding to an inhibition of $35 \pm 7\%$) was elicited by CGRP ($0.3 \mu\text{M}$, $n = 5$) (Figure 1). CGRP-induced relaxation was concentration (0.3 – 1 – $10 \mu\text{M}$)-dependent with an ED_{50} of $0.94 \mu\text{M}$ (0.91 – 0.97) ($n = 6$). Relaxant response to CGRP ($0.3 \mu\text{M}$) was also observed in preparations taken from adult capsaicin pretreated rats (Figure 1). CGRP ($1 \mu\text{M}$)-induced relaxation underwent desensitization and a second application on the same NA-induced hypertone was without effect

($n = 4$) (Figure 2). Interestingly, a cross-tachyphylaxis between CGRP and capsaicin ($1 \mu\text{M}$) also occurred the latter drug being ineffective when administered following CGRP ($1 \mu\text{M}$) (Figure 2a) ($n = 4$). Control experiments indicated that a previous similar relaxation of NA-induced tone with papaverine ($10 \mu\text{M}$) did not prevent the relaxant effect of capsaicin ($1 \mu\text{M}$) (Figure 2b, $n = 3$).

The proposed selective CGRP antagonist, CGRP (8-37) ($10 \mu\text{M}$) (Chiba *et al.*, 1989) significantly reduced the relaxation induced by CGRP ($0.3 \mu\text{M}$) or by capsaicin ($1 \mu\text{M}$), but not that by papaverine (10 or $100 \mu\text{M}$), of NA-induced tonic contraction (Table 1).

Effect of capsaicin or CGRP on electrical field stimulation induced tonic contraction of EUS

Electrical field stimulation (2.5 Hz , 60 V , 1 ms , trains of 5 s every 5 min) induced a contractile response characterized by two components (Figure 3): a slowly developing tonic contraction on which were superimposed a series of twitches due to the activation of the striated component of the sphincter. The tonic component was abolished by tetrodotoxin ($0.3 \mu\text{M}$, $n = 4$) and largely reduced ($84 \pm 7\%$ inhibition) by phen-tolamine ($3 \mu\text{M}$, $n = 5$).

Capsaicin ($1 \mu\text{M}$) or CGRP ($1 \mu\text{M}$) produced an almost complete inhibition of EFS-induced tonic contraction ($95 \pm 6\%$, $n = 8$, and $81 \pm 8\%$, $n = 4$, for capsaicin and CGRP, respectively, Figure 3a,b). Capsaicin-induced inhibition could be evoked only once in each preparation ($n = 4$) and it was significantly reduced in rats desensitized to capsaicin ($16 \pm 7\%$, $n = 6$, $P < 0.001$, Figure 3c). In these latter preparations the inhibitory action of CGRP ($1 \mu\text{M}$) was normal ($91 \pm 5\%$ of inhibition; $n = 4$, Figure 3d). The inhibitory effect of CGRP ($1 \mu\text{M}$) on EFS-induced tonic contraction underwent desensitization and a cross-desensitization with capsaicin ($1 \mu\text{M}$) was observed (Figure 4, $n = 4$). CGRP-induced relaxa-

Figure 2 Typical tracings showing the desensitization of the calcitonin gene-related peptide (CGRP) relaxant response and the cross-desensitization with capsaicin (Caps) (a). Panel (b) shows the specificity of this cross-desensitization: papaverine (at an equieffective concentration to CGRP) did not modify capsaicin-induced relaxation. Tension and time scales apply to all tracings.

Table 1 Effect of calcitonin gene-related peptide (CGRP (8-37)) (10 μM) on relaxation of noradrenaline (NA)-precontracted EUS induced by CGRP, capsaicin or papaverine

Drug	(μM)	n	Control		+ hCGRP (8-37)	
			Relaxation (mg)	% inhibition	Relaxation (mg)	% inhibition
CGRP	0.3	4	110 $\pm$ 18	38 $\pm$ 8	44 $\pm$ 13*	13 $\pm$ 3*
Capsaicin	1	6	88 $\pm$ 4	30 $\pm$ 3	36 $\pm$ 4**	14 $\pm$ 2**
Papaverine	10	6	153 $\pm$ 23	45 $\pm$ 6	152 $\pm$ 25	37 $\pm$ 11

Inhibition relate to the % reduction of the amplitude of the contraction. Each value is the mean $\pm$ s.e.mean.

* $P < 0.05$ vs control; ** $P < 0.01$ vs control.

Figure 3 Inhibitory effect of capsaicin (Caps) (a) and calcitonin gene-related peptide (CGRP) (b) on EFS-induced contraction of rat external urethral sphincter. In (c) and (d) the effects obtained in systemic capsaicin-pretreated rats are shown. EFS (2.5 Hz, 60 V, 1 ms, trains of 5 s every 5 min) was applied at the black horizontal bars. Tension and time scales apply to all tracings.

tion was concentration (0.01–0.1–1 μM)-dependent with an ED_{50} of 44 nM (16–120) ($n = 6$). The inhibitory effect of capsaicin (1 μM) or CGRP (0.3–1 μM) were unaffected by CGRP (8-37) at a concentration of 10 μM ($n = 4$ for each substance). CGRP (8-37) was also ineffective against a concentration of CGRP (0.01 μM) that produced an inhibition of EFS-induced contraction (30 $\pm$ 2%) similar to that exerted by CGRP (0.3 μM) on NA-induced contraction ($n = 4$).

Characterization of vanilloid (capsaicin) receptor in rat urethral tissues

[^3H]-RTX displayed specific, saturable binding to rat urethral membranes. Nonspecific binding at the K_d value represented approximately 30% of the total. Scatchard analysis of the data was consistent with a single component possessing a K_d of 105 $\pm$ 26 pM and a B_{max} of 40 $\pm$ 6 fmol mg^{-1} protein (3 determinations; mean $\pm$ s.e.mean, Figure 5). The Hill coefficient was approaching 1, suggesting that the binding sites are not cooperative. The curvilinear analysis of the data by the LIGAND programme confirmed the one-site model suggested by the Scatchard plot. Capsaicin inhibited binding with a K_i of 0.6 $\pm$ 0.3 μM (range) indicating a 6000 fold lower potency than that of RTX (not shown). Resiniferonol 9,13,14-orthophenylacetate (ROPA), the C-20 de-esterified parent compound of RTX which lacks vanilloid-like activity (Szallasi *et al.*, 1989), by contrast, did not inhibit binding up to 1 μM . In the urethra of rats that had undergone surgical ablation of major pelvic ganglia 4 days earlier, specific [^3H]-RTX binding was reduced by approximately 80% (2 determinations).

Discussion

In the rat urethra the existence of capsaicin-sensitive neuropeptide-containing sensory nerves have been firmly established by immunohistochemical techniques (Su *et al.*, 1986). Capsaicin-sensitive primary afferents subserve a dual sensory and efferent function with potential physiological and pathological relevance (Maggi & Meli, 1988). Stimulation of these urethral sensory nerves can trigger a series of reflexes important for the urethra-bladder coordination during mic-turition (Conte *et al.*, 1989; Maggi, 1993). At the same time the local release of sensory neuropeptides can produce neurogenic inflammation (Abelli *et al.*, 1991) and relax the bladder outlet thus facilitating urine expulsion (Andersson *et al.*, 1990). Following excessive mechanical or chemical irritation of the urethral meatus a hyperreactivity of this neural system could, at least in principle, lead to dyssynergia between bladder and urethral function and to incontinence due to reduced tone of the urethral sphincter.

In the present study, we have characterized further this sensory innervation of the EUS with a binding and functional pharmacological approach. The functional analysis was carried out on EUS precontracted by increasing the sympathetic tone by stimulation at pre- (EFS) or post- (exogenous NA) junctional level.

First, we have demonstrated the existence in urethral membranes of specific binding sites for [^3H]-RTX. RTX is an ultrapotent analogue of capsaicin that allowed the characterization of vanilloid (capsaicin) receptors in the soma (dorsal root ganglia) and central arms (spinal cord) of this subgroup of sensory nerves (Szallasi & Blumberg, 1990). Recently, using a modified assay in which the nonspecific binding is reduced by adding alpha-lacid glycoprotein, a plasma protein that binds RTX to the assay mixture (Szallasi & Blumberg, 1992), we have been able to demonstrate vanil-

Figure 4 Typical experiment showing the cross-desensitization between calcitonin gene-related peptide (CGRP) and capsaicin (Caps)-induced inhibition of EFS-induced tonic contraction of EUS. In (a) is shown the control response that was completely abolished by a first application of CGRP (b). In these conditions a normal response to EFS could be readily restored by addition of L-N^o-nitro-arginine 100 μ M (c). At this point a second application of CGRP (d) or of capsaicin (e) had no inhibitory effects.

Figure 5 Scatchard plot of specific [³H]-resiniferatoxin ([³H]-RTX) binding by rat urethral membranes. Points represent mean values from a single experiment. An additional two experiments gave similar results. The line was fitted using the LIGAND programme.

loid receptors (i.e. specific [³H]-RTX binding sites) in the urinary bladder (Szallasi *et al.*, 1993) and in the rat urethra (this study). The specificity of the [³H]-RTX binding sites is confirmed by their competitive displacement by capsaicin but not by ROPA, a diterpene derivative of RTX that is devoid of stimulating properties for the vanilloid receptor (Szallasi *et al.*, 1989). Moreover, specific [³H]-RTX binding was largely reduced (by approximately 80%) in urethral samples taken from rats that underwent resection of the major pelvic ganglion. This finding suggests that the majority of vanilloid receptors in the urethra are expressed on nerves that travel through the major pelvic ganglion. The remaining approximately 20% of specific [³H]-RTX binding suggests that vanilloid receptors could also be present on some fibres of other nerves projecting to the urethra such as the pudendal and/or the hypogastric.

The affinity of the vanilloid receptor for RTX and capsaicin in the urethra (105 μ M and 0.5 μ M respectively) is similar to that determined in the urinary bladder (30 μ M and 0.5 μ M) (Szallasi *et al.*, 1993). Likewise, both urethral and urinary bladder membranes bound [³H]-RTX in a non-cooperative fashion. This behaviour contrasts to the apparent positive cooperativity of [³H]-RTX binding in dorsal root ganglia, as well as dorsal horn membranes (Szallasi & Blumberg, 1993). These findings suggest that vanilloid receptors in the bladder and the urethra are probably identical.

In good agreement with binding data, capsaicin at a concentration of 1 μ M (that is, about two times the K_i for the vanilloid receptor) elicited a remarkable relaxation of EUS precontracted by adding exogenous noradrenaline or by EFS. Similar results were obtained in the female rat urethra

(Andersson *et al.*, 1990) and in the male rat proximal urethra (Maggi *et al.*, 1987). Specificity of this relaxant effect of capsaicin was confirmed by its desensitization (the response could be evoked only once in each preparation), by its absence in preparations taken from systemic capsaicin-pretreated animals and by its reduction in urethral strips from ganglionectomized animals. Experiments designed to identify the putative neurotransmitter(s) that mediates these inhibitory responses to capsaicin ruled out the involvement of VIP, GABA, galanin, purines and substance P, since none of these endogenous substances exerted relaxant effect on NA-precontracted EUS. On the other hand, exogenous CGRP mimicked the capsaicin inhibitory response both on NA- and on EFS-induced urethral hypertone as well as in resting conditions. As expected, the inhibitory effects of exogenous CGRP (that should be unrelated to the presence of sensory nerves) were unaffected in EUS obtained from capsaicin-pretreated or ganglionectomized rats.

Since CGRP is usually co-stored with tachykinins in sensory nerves (Maggi & Meli, 1988), it could be hypothesized that the functional response to capsaicin could be the resultant of the corelease of several sensory neuropeptides. However, substance P has been shown to contract slightly longitudinal and circular strips of female rat urethra (Andersson *et al.*, 1990) and in our hands neurokinin A, but not substance P, had a small contractile effect on basal tone (this study). These motor responses to tachykinins could, in principle, slightly counteract the CGRP-induced relaxation and explain the tendentially minor action of capsaicin, as compared to CGRP, on NA-induced contraction. However, when the urethral tone was increased by means of EFS, a complete inhibition of the tonic response was obtained either with capsaicin or with CGRP. In the female rat urethra, CGRP was equipotent with capsaicin in circular but not in longitudinal urethral strips (Andersson *et al.*, 1990). As a whole, we believe that tachykinins have a very marginal (if any) role and that CGRP release can fully explain capsaicin-induced relaxation of NA-precontracted EUS. This hypothesis is further indicated by two other evidence: (a) a cross-desensitization was noticed between CGRP and capsaicin-induced relaxation and (b) CGRP (8-37) inhibited in a significant manner both capsaicin- and CGRP-induced relaxation of NA-contracted REUS. The peptide fragment CGRP (8-37) has been characterized as a competitive antagonist of at least a subclass of CGRP receptor (Chiba *et al.*, 1989; Maggi *et al.*, 1991; Barthó *et al.*, 1991). It is interesting to note that CGRP (8-37) had no antagonist action on the inhibition by capsaicin or CGRP (either 0.01, 0.3 or 1 μ M) of EFS-induced tonic contraction. CGRP is significantly more effective in relaxing EFS- than NA-induced contraction of EUS. It is tempting to speculate that CGRP could antagonize EFS-induced contraction also through prejunctional (CGRP (8-37) resistant) receptors. Further experiments are obviously necessary to assess the potential involvement in EFS- (but not NA-) induced motor responses of a CGRP

(8-37)-resistant receptor, such as the proposed CGRP₂ subtype (Quirion *et al.*, 1993).

In conclusion we have demonstrated the existence of vanilloid receptors on capsaicin-sensitive nerve endings (mainly passing through major pelvic ganglia) in the rat EUS. We postulate that their activation leads to CGRP release, that in turn elicits a remarkable urethral relaxation. Such a mechanism could be of relevance in functional antagonism of sympathetic tone during micturition and could help in removal of noxious stimuli following chemical or mechanical

irritation of urethra and the bladder. Further studies are necessary to evaluate whether a dysfunction of this system could also be involved in urinary pathologies such as urinary incontinence or detrusor instability.

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