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Capsaicin-induced nitric-oxide-dependent relaxation in isolated dog urethra

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Abstract

Capsaicin (5×10^{-8} to 5×10^{-5} M) produced a non-adrenergic and non-cholinergic phasic relaxation in a concentration-dependent manner in isolated dog urethral preparations precontracted by noradrenaline. The mode of action of capsaicin was investigated with special reference to the possible involvement of endogenous nitric oxide (NO). A marked tachyphylaxis was observed in the responses to capsaicin. Pretreatment with N^G-nitro-L-arginine-methyl-ester (L-NAME) prevented or markedly reduced the inhibitory effect of L-NAME. Methylene blue inhibited the capsaicin-induced relaxation. In preparations stored at 4°C for 72 h, the reduction in the capsaicin-induced relaxation was significantly greater than that in the relaxation induced by either electrical field stimulation or by sodium nitroprusside. We conclude that capsaicin produces an endogenous-NO-dependent relaxation in the isolated dog urethra via mechanisms that deteriorate during cold storage of the preparations. © 1997 Elsevier Science B.V.

Keywords: Capsaicin; Urethra, dog; Nitric oxide (NO); Smooth muscle relaxation

1. Introduction

A non-adrenergic, non-cholinergic (NANC)-nerve-mediated relaxation has been demonstrated in the isolated urethra of various species, including humans (Andersson et al., 1983, 1992) and dogs (Hashimoto et al., 1993; Takeda and Lepor, 1995). Although the identities of the transmitters contributing to the NANC-nerve-mediated relaxation have yet to be confirmed, endogenous nitric oxide (NO) may be responsible for the main part of this response (Andersson et al., 1992; Persson and Andersson, 1992; Andersson, 1993; Andersson and Persson, 1994). Immunohistochemical evidence of the presence of NO synthase-positive nerves and neurons in urethras isolated from rats (Alm et al., 1993), pigs (Persson et al., 1993, 1995) and dogs (Takeda and Lepor, 1995) further supports the idea of a functional role for NO in the NANC-nerve-mediated relaxation of the urethra. However, the involvement of other transmitters cannot be excluded. In fact, it has been demonstrated in the urethras isolated from dogs (Hashimoto et al., 1993) and pigs (Werkström et al., 1995) that the NANC-nerve-mediated relaxation has two components: a rapidly developing first phase followed by a longer-lasting second phase. The first component is mediated by nerve-derived NO (Hashimoto et al., 1993; Werkström et al., 1995), but the second one is NO-independent, suggesting that other transmitters may be involved.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) selectively activates sensory nerves by binding to neuronal sites and stimulating cationic channels (Docherty et al., 1991). Capsaicin-sensitive primary afferent neurons are capable of releasing several neuropeptides — for example, calcitonin gene-related peptide (CGRP) and substance P — as transmitters or modulators (Holzer, 1988, 1991; Maggi, 1991). The peptides released from the peripheral nerve endings of these capsaicin-sensitive primary afferents may be involved in the neurogenic inflammation induced by mechanical or chemical irritation of the urethra (Nording et al., 1990; Abelli et al., 1991). Indeed, Su et al. (1986) demonstrated, by combining immunohistochemistry with radioimmunoassay and retrograde tracing techniques, that not only are substantial amounts of CGRP and substance P localized in the dorsal root ganglion (sensory) neurons that innervate the bladder and urethra of the rat and guinea-pig, but that these peptides are markedly reduced by systemic pretreatment with capsaicin. The presence of substance P/CGRP-immunoreactive nerve terminals has also been demonstrated in the dog urethra (Tamaki et al., 1992).

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The afferent neurons that innervate the lower urinary tract, whose cell bodies are found in the lumbosacral dorsal root ganglia and the dorsal horn of the spinal cord, show both nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity and NO synthase immunoreactivity (McNeill et al., 1992; Dun et al., 1993; Vizzard et al., 1993, 1994). This suggests a role for NO in afferent transmission from the lower urinary tract (Andersson and Persson, 1994). Such observations have given rise to speculations about an interaction between NO and sensory neuropeptides.

With this in mind, the present study was designed (1) to determine whether activation of capsaicin-sensitive nerves by capsaicin can induce relaxation in the isolated dog urethra and, if so, (2) to investigate whether endogenous NO is involved in this relaxant response, and (3) to examine the possible interaction between NO and the putative neuropeptides released from the capsaicin-sensitive primary afferents that innervate this tissue.

2. Materials and Methods

2.1. Tissue preparations and recording of mechanical activity

Forty-seven female mongrel dogs, weighing 7 to 12 kg, were used. The dogs were anaesthetized with sodium pentobarbital (20 mg/kg, i.v.) and exsanguinated via the femoral veins. The experimental protocol was approved by the Animal Ethics Committee, Shinshu University School of Medicine. The whole urethra was isolated and the surrounding connective tissue gently removed. The urethra was opened longitudinally under cold Krebs-bicarbonate solution (for composition, see below). Then, transverse urethral strips (approximately 2 mm in width, 5 mm in length, and of full thickness) were taken from its proximal third (between the bladder neck and 2–3 cm distal to the bladder neck). The mucosal layer was removed in some preparations using micro-scissors under a dissecting microscope and absence of the mucosa in these preparations was confirmed histologically after functional experiments. Each preparation was mounted vertically in a 10 ml organ bath and perfused at a constant rate of 4 ml/min with Krebs-bicarbonate solution of the following composition (mM): NaCl 120.0, KCl 5.9, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 5.5. The solution was maintained at 37 ± 0.5°C and aerated with a gas mixture of 95% O_2/5% CO_2 to give a pH of 7.3–7.4. Using two thin silk strings, the lower end of the preparation was connected to the bottom of the bath, and the upper end to the lever of a force-displacement transducer (Shinko Tsusin, UL-10, Miyota). The transducer could be positioned at any desired level by means of a micrometer screw, thus allowing passive changes to be made in preparation length. The isometric tension detected by the transducer was amplified and recorded on a direct-writing oscillograph (NEC San-ei, 8K, Tokyo). The resting tension was set at 0.5 ± 0.1 g, which was optimal for inducing maximal contraction. All preparations were allowed to equilibrate for at least 60 min in the oxygenated bathing medium before the experiment was started. When it was to be subjected to electrical field stimulation, the preparation was mounted in the organ bath between two platinum electrodes (3 mm long, 50 mm apart). Transmural stimulation of nerves was performed using an electrical stimulator (SEN-710, Nihon kohden, Tokyo, Japan) delivering single square pulses (pulse width 0.5 ms) at a frequency of 8 Hz at supramaximal voltage, since preliminary experiments had shown that the relaxation induced by electrical field stimulation at a frequency of 8 Hz is mostly NO-dependent, whereas at higher frequencies than 8 Hz, the electrical field stimulation-induced relaxation has NO-dependent and -independent components. The train duration was 5 s and the stimulation interval 60 or 180 s.

2.2. Experimental procedures

After the equilibration period, each experiment was started by exposing the preparations to an 80 mM high potassium Krebs-bicarbonate solution, until two similar contractions (difference < 10%) were obtained. The high potassium solution was prepared by replacing NaCl with an equimolar amount of KCl in the Krebs-bicarbonate solution. When preparations were exposed to noradrenaline (10^{-5} M), the drug elicited a contraction which corresponded to $85.7 \pm 34.4\%$ ($n = 30$) of the K^+ (80 mM)-induced contraction.

The response to capsaicin (5×10^{-8} to 5×10^{-5} M) was studied in preparations precontracted by noradrenaline (10^{-5} M), a concentration–response curve for capsaicin being thus obtained. The response to 0.05–0.5% ethanol (the vehicle for capsaicin, 5×10^{-6}–5×10^{-5} M), was determined in noradrenaline-precontracted preparations. The response to capsaicin (5×10^{-6} M) was also investigated in the presence of atropine (10^{-6} M) with propranolol (10^{-6} M), N^G-nitro-L-argininemethyl-ester (L-NAME, 10^{-4} M) alone, L-NAME (10^{-4} M) with L-arginine (L-Arg, 10^{-3} M), tetrodotoxin (3×10^{-7} M), methylene blue (10^{-5} and 10^{-4} M), indomethacin (10^{-6} M), aminoguanidine (10^{-4} M) or vasoactive intestinal polypeptide (VIP) fragment (10–28) (VIP-(10–28), 10^{-8} M, a VIP receptor antagonist). Because there was desensitization to capsaicin (see Section 3), one of several preparations obtained from the same animal served as the control while one of the above drugs was applied to one or two of the others at least 20 min before the application of capsaicin. The response to capsaicin (5×10^{-6} M) was also studied in some preparations, from which the mucosa had been removed. To investigate the effects of the degeneration of intramural nerves on the capsaicin- and electrical field stimulation-induced relaxations, some preparations deprived of their mucosa were kept in cold (4°C) Krebs-bicarbonate solution for 2 or more days. The re-

sponses to capsaicin (5×10^{-6} M), electrical field stimulation and sodium nitroprusside (10^{-5} M) were measured after a 48, 72 or 96 h period of cold storage and compared with responses recorded from control preparations (i.e., ones that had not undergone cold storage).

The relaxation response to electrical field stimulation was studied in preparations precontracted by noradrenaline (10^{-5} M). The effects were investigated of L-NAME (10^{-4} M) with and without L-Arg (10^{-3} M) and of tetrodotoxin (3×10^{-7} M), methylene blue (10^{-5} and 10^{-4} M) and indomethacin (10^{-6} M) on the electrical field stimulation-induced relaxation. In these experiments, when the variation between five consecutive responses to electrical field stimulation was less than 10%, the drugs to be investigated were infused into the organ baths. Five intermittent electrical field stimulations were applied again at least twenty min after drug administration.

To investigate the effects of capsaicin-desensitization on the electrical field stimulation-induced relaxation, either capsaicin (5×10^{-6} M) was applied 5 times at 5 min intervals to organ baths perfused with Krebs-bicarbonate solution at a constant rate of 4 ml/min, or the preparations were irrigated continuously by a capsaicin (5×10^{-6} M)-containing Krebs-bicarbonate solution during intermittent electrical field stimulation (at 60 or 180 s intervals). Then, responses to electrical field stimulation were recorded for at least 60 min after the beginning of the application of capsaicin. Control preparations were run in parallel to study the reproducibility of the electrical field stimulation-induced relaxation.

To obtain some insight into the neurotransmitters that might be involved in the capsaicin-induced relaxation of dog urethral preparations precontracted by noradrenaline, the effects of several putative neurotransmitters were evaluated. The effects of VIP (10^{-10} to 10^{-6} M), CGRP (10^{-7} M), substance P (10^{-7} M) and neurokinin A (10^{-7} M) were investigated in noradrenaline-precontracted preparations. The response to VIP (10^{-7} M) was also studied in the presence of VIP-(10–28) (10^{-8} M) or L-NAME (10^{-4} M).

2.3. Drugs

The following drugs were used: capsaicin, (−)-noradrenaline bitartrate (noradrenaline), tetrodotoxin, N^G-nitro-L-arginine-methyl-ester (L-NAME), L-arginine (L-Arg), methylene blue, indomethacin, sodium nitroprusside, calcitonin gene-related peptide (CGRP), substance P, vasoactive intestinal polypeptide (VIP), VIP fragment (10–28) (VIP-(10–28)), aminoguanidine hemisulphate (all from Sigma, St. Louis, MO, USA), atropine sulphate (Tanabe Seiyaku, Osaka, Japan), propranolol hydrochloride (Zeneca, Osaka, Japan) and neurokinin A (Research Biochemicals International, Natick, MA, USA). Stock solutions were prepared as follows and then stored at −40°C: capsaicin was dissolved in distilled absolute ethanol at a concentration of 10^{-2} M and the other drugs were dissolved in distilled water. Subsequent dilutions of the drugs were made with distilled water on the day of the experiment. With the concentrations of capsaicin used, no precipitation was observed when subsequent dilutions were made or the solutions were added to the Krebs-bicarbonate solution in the organ bath. The reported concentrations are the calculated final concentrations in the bath solution. The concentrations used were chosen on the basis of pilot experiments.

2.4. Analysis of data

The effects of electrical field stimulation and of drugs are expressed as the percentage relaxation of the noradrenaline-induced contraction in each preparation. When the effects were to be evaluated of drugs other than capsaicin on the electrical field stimulation-induced relaxation, the average relaxation seen in three consecutive responses to intermittent electrical field stimulation before drug administration was compared to that after drug administration. To study the effect of capsaicin on the relaxation responses to intermittent electrical field stimulation, the average relaxation in 5 consecutive responses to intermittent electrical field stimulation was measured before and 60 min after capsaicin-treatment. Then, the mean

Table 1
The effects of drugs on the relaxation induced by capsaicin (5×10^{-6} M) in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M)

	Control (%)	*n*	With drug (%)	*n*
Atropine (10^{-6} M) + propranolol (10^{-6} M)	13.6 ± 1.5	5	13.5 ± 2.4	8
L-NAME (10^{-4} M)	11.9 ± 3.5	10	1.8 ± 0.8 [a]	10
L-NAME (10^{-4} M) + L-Arg (10^{-3} M)	11.9 ± 3.5	10	11.8 ± 2.8	10
Tetrodotoxin (3×10^{-7} M)	13.3 ± 2.8	5	14.4 ± 4.4	6
Indomethacin (10^{-6} M)	11.6 ± 2.6	4	11.9 ± 1.7	6
Methylene blue (10^{-5} M)	12.7 ± 0.7	5	12.3 ± 2.9	8
Methylene blue (10^{-4} M)	10.9 ± 0.9	5	1.1 ± 0.7 [a]	8

Results are expressed as the percentage relaxation of the noradrenaline-precontracted preparations (mean ± S.E.M.), *n* denotes the number of preparations. Abbreviations: L-NAME: N^G-nitro-L-arginine-methyl-ester; L-Arg: L-arginine.
[a] $P < 0.01$ (ANOVA followed by Fisher's PLSD).

difference between these values was calculated and compared to that for the control preparations. To analyze the effects of cold storage, the data were compared to the responses in control preparations that had not undergone cold storage.

Results are given as mean values ± standard error of the mean (S.E.M.). The n value denotes the number of preparations. Four to eight strips were taken from each animal, but only one or two strips were used for each experiment. Statistical analysis was performed using the Student's t-test for paired data, and by one-way analysis of variance (ANOVA) followed by the Fisher's Protected Least Significant Difference (PLSD) test for unpaired data. A probability level less than 0.05 was accepted as significant.

3. Results

3.1. The relaxing effects of capsaicin and its vehicle

Administration of noradrenaline (10^{-5} M) produced a plateau-like stable contraction of the urethral preparations (tension increase of 0.87 ± 0.05 g, $n = 22$). Capsaicin (5×10^{-8} to 5×10^{-5} M, $n = 5$–7 for each concentration) produced a phasic relaxation in a concentration-dependent manner of the noradrenaline-precontracted preparations (Figs. 1 and 2). The relaxation responses to capsaicin at 5×10^{-6} M averaged $12.0 \pm 1.4\%$ of the noradrenaline-induced contraction ($n = 7$). A second application of capsaicin (5×10^{-6} M, $n = 4$), given 60 min after the first challenge, did not produce any effect (Fig. 1). We also tested the effect of the ethanol. The vehicle for 5×10^{-6} M capsaicin, 0.05% ethanol, did not produce any relaxation and 10^{-5} M capsaicin produced a significantly larger relaxation than its vehicle, 0.1% ethanol ($n = 6$, $P < 0.01$). However, the vehicle for 5×10^{-5} M capsaicin, 0.5% ethanol, significantly relaxed the preparations, to an extent that was not different from the effect of 5×10^{-5} M capsaicin solution (Fig. 2).

3.2. Effects of drugs on the capsaicin-induced relaxation

Pretreatment with atropine (10^{-6} M) plus propranolol (10^{-6} M, $n = 8$) did not affect the relaxation induced by capsaicin (5×10^{-6} M, Table 1). By contrast, L-NAME (10^{-4} M, $n = 10$) significantly reduced the relaxing effect of capsaicin (Fig. 3, Table 1). The addition of L-Arg (10^{-3} M, $n = 10$) prevented or markedly reduced the inhibitory effect of L-NAME (Fig. 3, Table 1). Methylene blue at 10^{-4} M ($n = 8$), but not at 10^{-5} M ($n = 8$), inhibited the capsaicin-induced relaxation (Table 1). Tetrodotoxin (3×10^{-7} M, $n = 6$) did not inhibit the capsaicin-induced relaxation (Fig. 3, Table 1). Neither indomethacin (10^{-6} M, $n = 6$, Table 1) nor aminoguanidine hemisulphate (10^{-4} M, $n = 4$, data not shown) had any significant effect on the capsaicin-induced relaxation.

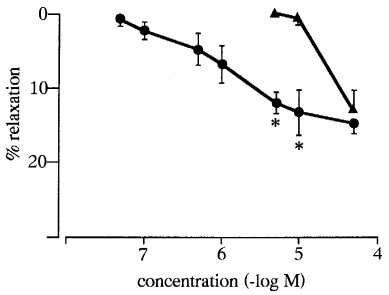

Fig. 2. Concentration–response curve for the capsaicin-induced relaxation (●) and the relaxation induced by its vehicle, ethanol (▲), in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M). Data points for vehicle, shown at concentrations of 5×10^{-6}, 10^{-5} and 5×10^{-5} M, indicate effects of 0.05%, 0.1% and 0.5% ethanol solutions, respectively. Each point shows percentage relaxation of the noradrenaline-induced tension and represents the mean ($n = 5$–7) with S.E.M. shown by the vertical bar. * $P < 0.01$ (capsaicin vs. vehicle, ANOVA followed by Fisher's PLSD).

3.3. Effects of capsaicin and of electrical field stimulation in mucosa-free preparations

To evaluate the possible modulatory role of the urothelium, capsaicin (5×10^{-6} M) was applied to some preparations ($n = 5$) which had been deprived of their mucosa. The relaxation response to capsaicin in such preparations was by $10.8 \pm 2.2\%$, which did not differ significantly from the corresponding value ($12.0 \pm 1.7\%$) in control preparations with the mucosa intact ($n = 5$). There was no significant difference between mucosa-free and mucosa-in-

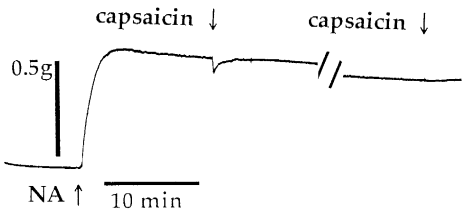

Fig. 1. Original tracing showing the effect of capsaicin (5×10^{-6} M) and the effect of a second administration of capsaicin (at the same concentration) 60 min after the first in an isolated dog urethral preparation precontracted by noradrenaline (NA, 10^{-5} M).

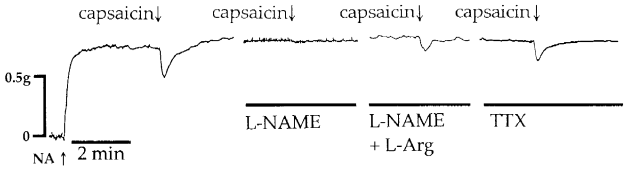

Fig. 3. Original tracing showing the relaxation induced by capsaicin (5×10^{-6} M) in the absence or presence of N^G-nitro-L-arginine-methyl-ester (L-NAME, 10^{-4} M) alone, L-NAME (10^{-4} M) with L-arginine (L-Arg, 10^{-3} M), or tetrodotoxin (TTX, 3×10^{-3} M) in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M). Each treatment was made on different preparation.

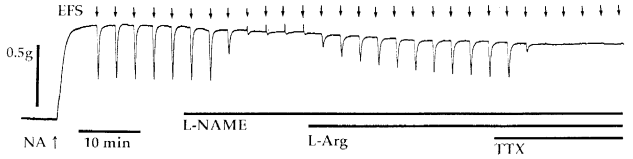

Fig. 4. Original tracing showing relaxation induced by electrical field stimulation (EFS, at ↓, single square pulses, pulse width 0.5 ms, 8 Hz at supramaximal voltage, 5 s train duration, stimulation interval 3 min), in an isolated dog urethral preparation precontracted by noradrenaline (NA, 10^{-5} M). Responses are shown in the absence of drugs and then in the presence of N^G-nitro-L-arginine-methylester (L-NAME, 10^{-4} M) alone, L-NAME (10^{-4} M) with L-arginine (L-Arg, 10^{-3} M), and finally L-NAME (10^{-4} M) with L-Arg (10^{-3} M) and tetrodotoxin (TTX, 3×10^{-7} M).

tact preparations in terms of their electrical field stimulation-induced relaxation.

3.4. Effects of drugs on the electrical field stimulation-induced relaxation

Electrical field stimulation (a 5 s train of stimuli at 8 Hz, applied repeatedly at 3 min intervals) elicited reproducible transient relaxations in noradrenaline (10^{-5} M)-precontracted preparations (Fig. 4). Pretreatment with atropine (10^{-6} M) plus propranolol (10^{-6} M, $n=8$) did not have any significant effect on the electrical field stimulation-induced relaxation (Table 2). By contrast, L-NAME (10^{-4} M, $n=13$) significantly inhibited the electrical field stimulation-induced relaxation. The addition of L-Arg (10^{-3} M, $n=6$) counteracted the effect of L-NAME (Fig. 4). Tetrodotoxin (3×10^{-7} M, $n=5$) almost completely (97.8 ± 3.2% of control relaxation) suppressed the electrical field stimulation-induced relaxation (Table 2). Indomethacin (10^{-6} M, $n=6$) had no effect on the electrical field stimulation-induced relaxation (Table 2). Methylene blue at 10^{-4} M ($n=5$), but not at 10^{-5} M ($n=9$), significantly inhibited the electrical field stimulation-induced relaxation (Table 2).

3.5. Effect of capsaicin on the electrical field stimulation-induced relaxation

Neither repeatedly applied capsaicin (5×10^{-6} M, 5 times at 5 min intervals, $n=7$) nor continuous irrigation with capsaicin (5×10^{-6} M, $n=4$) affected the relaxations induced by intermittent electrical field stimulation, applied at 60 or 180 s intervals during a 60-min-series (data not shown).

3.6. Effect of cold storage on sodium nitroprusside-, electrical field stimulation- and capsaicin-induced relaxations

The relaxation induced by sodium nitroprusside (10^{-5} M) was not significantly decreased by a 72 h period of cold storage. On the other hand, the responses to capsaicin and the responses to electrical field stimulation were both significantly smaller when the preparations were kept at 4°C for 72 h (Fig. 5). In preparations kept under cold storage for 72 h, the capsaicin-induced relaxations (5×10^{-6} M) were significantly smaller than the relaxations induced by either electrical field stimulation or sodium nitroprusside (Fig. 5). In preparations stored at 4°C for 96 h, both the capsaicin-induced and the electrical field stimulation-induced relaxations were significantly smaller than the relaxation induced by sodium nitroprusside (Fig. 5).

3.7. Effects of some neuropeptides that might mediate the capsaicin-induced relaxation

VIP (10^{-10} to 10^{-6} M, $n=5$ for each concentration) produced a relaxation that was concentration-dependent (Fig. 6). VIP-(10–28) (10^{-8} M, $n=4$), a VIP receptor antagonist, did not inhibit the relaxation induced by VIP (10^{-7} M, Fig. 6). Likewise, pretreatment with L-NAME (10^{-4} M, $n=5$) did not inhibit the VIP (10^{-7} M)-induced relaxation (Fig. 6). Little or no relaxation (0.9 ± 0.8% of the precontracted tension) was obtained with CGRP (10^{-7}

Table 2
The effects of drugs on the relaxation induced by electrical field stimulation in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M)

	Control (%)	With drug (%)	n
Atropine (10^{-6} M) + propranolol (10^{-6} M)	62.6 ± 28.1	72.5 ± 25.8	8
L-NAME (10^{-4} M)	64.7 ± 7.8	4.5 ± 1.2 [a]	13
L-NAME (10^{-4} M) + L-Arg (10^{-3} M)	69.0 ± 9.3	64.0 ± 11.9	6
Tetrodotoxin (3×10^{-7} M)	77.4 ± 20.3	2.2 ± 3.2 [a]	5
Indomethacin (10^{-6} M)	55.5 ± 14.8	57.8 ± 13.2	6
Methylene blue (10^{-5} M)	58.3 ± 22.4	54.9 ± 24.1	9
Methylene blue (10^{-4} M)	75.0 ± 12.5	8.3 ± 1.5 [a]	5

Results are expressed as the percentage relaxation of the noradrenaline-precontracted preparations (mean ± S.E.M. to 5 consecutive electrical field stimulations); *n* denotes the number of preparations. Electrical field stimulation: single square pulses, pulse width 0.5 ms, 8 Hz at supramaximal voltage, 5 s train duration, stimulation interval 3 min.
Abbreviations: L-NAME: N^G-nitro-L-arginine-methyl-ester; L-Arg: L-arginine.
[a] $P<0.01$ (Student's paired two-tailed *t*-test).

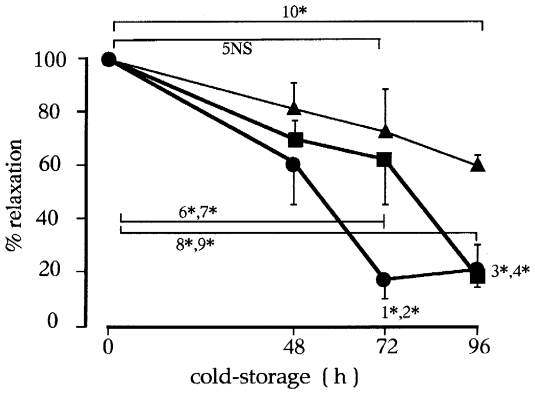

Fig. 5. Effects of cold-storage (4°C) on the relaxations induced by sodium nitroprusside (10^{-5} M; ▲), electrical field stimulation (■) and capsaicin (5×10^{-6} M; ●) in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M). The control (without cold-storage; 0 h) relaxation is shown as 100% and each point represents the mean ($n = 8$–12) with S.E.M. shown by the vertical bar. 1 * $P < 0.05$ (capsaicin vs. electrical field stimulation), 2 * $P < 0.05$ (capsaicin vs. sodium nitroprusside), 3 * $P < 0.01$ (capsaicin vs. sodium nitroprusside), 4 * $P < 0.01$ (electrical field stimulation vs. sodium nitroprusside), 5 NS: not significantly different (sodium nitroprusside: control vs. 72 h), 6 * $P < 0.05$ (electrical field stimulation: control vs. 72 h), 7 * $P < 0.01$ (capsaicin: control vs. 72 h), 8 * $P < 0.01$ (electrical field stimulation: control vs. 96 h), 9 * $P < 0.01$ (capsaicin: control vs. 96 h), 10 * $P < 0.05$ (sodium nitroprusside: control vs. 96 h) (ANOVA followed by Fisher's PLSD).

M) in noradrenaline precontracted preparations ($n = 18$, data not shown). Substance P (10^{-7} M) produced a small contraction (0.12 ± 0.02 g) over and above the basal tone ($n = 9$), but had no effect on noradrenaline-precontracted preparations ($n = 8$, data not shown). Neurokinin A (10^{-7} M) had no effect on either noradrenaline-precontracted ($n = 5$) or non-precontracted ($n = 4$) preparations (data not shown).

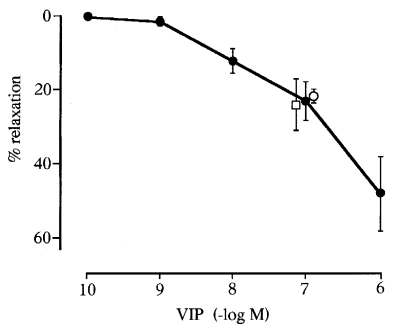

Fig. 6. Concentration–relaxation relationship for vasoactive intestinal polypeptide (VIP; ●, $n = 5$ for each concentration) in isolated dog urethral preparations precontracted by noradrenaline (10^{-5} M). Also shown is VIP-induced relaxation (10^{-7} M) following pretreatment with either VIP-(10–28) (10^{-8} M; ○, $n = 4$) or N^G-nitro-L-arginine-methyl-ester (10^{-4} M; □, $n = 6$). Each point shows the percentage relaxation of the noradrenaline-precontracted preparations and represents the mean with S.E.M. shown by the vertical bar.

3.8. Effects of VIP-(10–28) on the capsaicin-induced relaxation

Pretreatment with VIP-(10–28) (10^{-8} M, $n = 4$) did not affect the capsaicin-induced relaxation (data not shown).

4. Discussion

In the present study, we have demonstrated that capsaicin causes a NANC relaxation of the isolated dog urethral preparation. The relaxing mechanism is dependent on the release of endogenous NO, since the capsaicin-induced relaxation was inhibited by L-NAME, a NO synthase inhibitor, and by methylene blue, which inhibits the activation of guanylate cyclase. The reversal of the L-NAME-induced inhibitory effect by L-arginine further supports the involvement of the L-arginine/NO system in the capsaicin-induced relaxation. Although the magnitude of the capsaicin-induced relaxation was smaller than that of the NO mediated relaxation caused by electrical stimulation in our dog urethral preparations, to the best of our knowledge, the finding that an NO-mediated relaxation occurs in response to capsaicin stimulation in the urethral smooth muscle has not been reported so far.

What mechanism might be involved in the capsaicin-induced relaxation, and where is endogenous NO released from during the capsaicin-induced relaxation? NO has been implicated recently as the main mediator of NANC inhibitory neurotransmission in urethral preparations from several species, including the dog (Hashimoto et al., 1993; Takeda and Lepor, 1995). The present results with electrical field stimulation-induced relaxation are compatible with this idea. Recently, Pinna et al. (1996) reported that an NO-dependent inhibitory factor is released from the hamster urethral urothelium, and that adenosine triphosphate and prostaglandin E_2 may be involved. In our study of the dog urethra, however, the urothelium seemed not to play a modulatory role in either the capsaicin-induced relaxation or the electrical field stimulation-induced relaxation. Moreover, endogenous prostaglandins do not seem to be involved in mediating the capsaicin-induced relaxation, since pretreatment with indomethacin had no significant effect in our preparations and the concentration of indomethacin used has been shown to be sufficient to inhibit cyclooxygenase (Vane, 1971; Eckenfels and Vane, 1972). Vascular smooth muscle cells produce NO via an increase in the expression of inducible NO synthase on exposure to endotoxin or cytokines (Busse and Mulsch, 1990; Fleming et al., 1991). Aminoguanidine is a selective inhibitor of inducible NO synthase (Misko et al., 1993; Griffiths et al., 1993). In our study, aminoguanidine, at a concentration sufficient to inhibit inducible NO synthase (Griffiths et al., 1993), had no effect on the capsaicin-induced relaxation, so that inducible NO synthase seems unlikely to contribute to this relaxation.

It has been reported that cold storage for 2 days abolishes cholinergic-nerve mediated contractions to electrical field stimulation in the rat oesophageal tunica muscularis mucosae, even though the muscle remains mechanically viable (Akbarali et al., 1987). In that study, cold storage (4°C) of the tissue was used to denervate the preparation to enable assessment of the role of intramural nerves. However, Jurkiewicz et al. (1992) later put forward the view that the primary changes associated with nerve degeneration are followed by secondary changes in smooth muscle tissue activity during prolonged cold storage. In the present study, preparations stored at 4°C for 72 h showed significantly weaker responses to electrical field stimulation, but not to sodium nitroprusside, than controls that had not undergone cold storage. These results suggest that after 72 h cold storage, the urethral smooth muscle itself is still able to relax, although the intramural nerves in the urethra have suffered significant functional deterioration. Under these conditions, the relaxation induced by capsaicin was also significantly diminished. Moreover, the inhibitory effect of 72 h cold storage on the capsaicin-induced relaxation was more marked than its effect on the electrical field stimulation- or sodium nitroprusside-induced relaxations. Consequently, it may be assumed that, of the female dog, capsaicin acts in the urethra on capsaicin-sensitive primary afferent nerves, rather than on efferent nerves or on the urethral smooth muscle itself.

Both capsaicin and its ultrapotent analog, resiniferatoxin, have homovanillic acid as the key structural motif and consequently the capsaicin receptor is termed the vanilloid receptor (Szallasi, 1994). It is unlikely that vanilloid receptors tonically regulate NANC-nerve-mediated inhibitory neurotransmission, since the desensitization of vanilloid receptors (produced by either repeated or continuous exposure to capsaicin) did not affect the electrical field stimulation-induced relaxation. However, the possibility cannot be excluded that NO mediated neurotransmission is involved in the capsaicin-induced relaxation in our preparations.

Repeated application of capsaicin in our preparations produced desensitization, suggesting that the relaxant effect of capsaicin depends specifically on activation of capsaicin-sensitive primary afferents (Maggi and Meli, 1988; Holzer, 1991). It is well known that capsaicin activates capsaicin-sensitive primary afferents, resulting in the release of several neuropeptides from their peripheral nerve endings (Holzer, 1988, 1991; Maggi, 1991). This being so, the possibility arises of an interaction between NO and peptides locally released from capsaicin-sensitive primary afferent neurons. Such an interaction has been reported to occur during neurogenic vasodilator responses in the rat gastric mucosa (Whittle et al., 1992), the rat oral mucosa (Fazekas et al., 1994) and rat skin (Herbert and Holzer, 1994). Merchant et al. (1994) reported that CGRP is a mediator of capsaicin-induced gastric mucosal hyperaemia in the rat and that this effect may be dependent on the production of both NO and prostaglandin E_2. It has been reported that capsaicin produces a relaxation in the isolated rat external urethral sphincter which could also be mediated by CGRP (Parlani et al., 1993a). Moreover, NO mediated NANC inhibitory transmission has also been demonstrated in this tissue (Parlani et al., 1993b).

In an attempt to establish the role of neuropeptides, we examined the effects of substance P, neurokinin A, CGRP and VIP on our urethral preparations. Among these peptides, only VIP caused a relaxation of the noradrenaline-precontracted preparation, as previously reported by Hashimoto et al. (1992). VIP relaxes the isolated pig gastric smooth muscle and rabbit corpus cavernosum and NO is involved in those VIP-mediated relaxations (Grider et al., 1992; Kim et al., 1995). However, pretreatment with L-NAME did not affect the relaxation induced by VIP in our study. Thus, VIP-induced relaxation seems to be independent of the L-Arg-NO pathway in dog urethral preparations, suggesting that VIP may relax this tissue through a mechanism different from that involved in the capsaicin-induced NO-mediated relaxation. VIP-(10–28), a VIP receptor antagonist, is a C terminal partial sequence of VIP (Grider and Rivier, 1990). It has been reported that VIP-(10–28) inhibits both VIP-induced and electrical field stimulation-induced relaxations of pig gut smooth muscle (Grider and Rivier, 1990) and electrical field stimulation-induced relaxation in the rabbit corpus cavernosum (Kim et al., 1995). In our study, however, the VIP-induced relaxation was not inhibited by the pretreatment with VIP-(10–28), nor was the capsaicin-induced relaxation.

In contrast to its effect in the rat external sphincter (Parlani et al., 1993a), we found that CGRP had no significant relaxant effect on preparations isolated from the female dog proximal urethra, although capsaicin did induce an NO-mediated relaxation and NO-mediated inhibitory transmission could be demonstrated in our preparations. Our preparations were isolated from the proximal third of the whole urethra of female dogs, in which there is no striated muscle component (Cullen et al., 1981). Thus, what we investigated in the present study was the response of smooth muscle, not of striated muscle, to capsaicin and electrical field stimulation. This may be one of the reasons why the capsaicin-induced relaxation in our urethral preparations proved not to be mediated by CGRP, in apparent disagreement with the result in the rat external urethral sphincter.

In conclusion, capsaicin produces an NO-dependent relaxation in the isolated dog urethra through activation of specific mechanisms which undergo significant deterioration after prolonged cold storage of the preparation. Of the neuropeptides that may be released from the peripheral nerve terminals of capsaicin-sensitive primary afferent neurons, CGRP, VIP, substance P and neurokinin A do not seem to be involved in the capsaicin-induced relaxation. However, the involvement in the capsaicin-induced relaxation of other neuropeptides or transmitters that may be

released from the capsaicin-sensitive primary afferents deserves further study. Further investigation will be needed to evaluate the physiological significance of the capsaicin-induced relaxation of urethral smooth muscle.

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