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A pharmacological and histochemical study of hamster urethra and the role of urothelium

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1 Electrical field stimulation (EFS) of circular strips of hamster proximal urethra caused frequency-dependent relaxations at raised tone. Phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M) were present throughout the experiment. Neurogenic relaxation was attenuated by L-N^G-nitroarginine methyl ester (L-NAME) (10^{-4} M), was restored by L-arginine (3×10^{-3} M) but not by D-arginine (3×10^{-3} M) and completely blocked by tetrodotoxin (10^{-6} M). Neurogenic relaxation was also reduced by suramin (10^{-4} M) and totally blocked by suramin together with L-NAME. Strips of hamster urethra devoid of urothelium showed little, if any, relaxant response to EFS.

2 An immunohistochemical study showed nitric oxide synthase-immunoreactive nerves in the smooth muscle layers and in the *lamina propria*, just beneath the urothelium, but no nitric oxide synthase (NOS) staining in the urothelial layer.

3 Noradrenaline elicited a significantly greater contraction in strips without urothelium than in control strips. L-NAME (10^{-4} M) did not affect noradrenaline-induced contraction in both control and urothelium-free strips. The contractile response to acetylcholine was not dependent on the presence or absence of urothelium. Nevertheless the response induced by exogenous acetylcholine (10^{-3} M) was increased by L-NAME (10^{-4} M), both in intact and in urothelium-free strips.

4 Prostaglandin E_2 ($10^{-8}-5 \times 10^{-6}$ M) and 2-methyl-thio-ATP ($10^{-9}-10^{-5}$ M) relaxed proximal urethra. Suramin (10^{-4} M) significantly inhibited the relaxation induced by 2-methyl-thio-ATP. The amplitude of these responses was not significantly different between intact and urothelium-free strips and was not blocked by L-NAME (10^{-4} M).

5 These results suggest that nitric oxide (NO) is the principal transmitter involved in the non-adrenergic, non-cholinergic (NANC) relaxation of hamster proximal urethra possibly together with another inhibitory transmitter released from nerves. NO can be released from nerves located in the circular smooth muscle layer and in the *lamina propria* rather than in the urothelium. The reduced neurogenic relaxation in urothelium-free preparations suggests that a NO-dependent inhibitory factor is released from the urothelium. In addition, ATP and prostaglandin E_2 may be involved, together with NO, in the urethra during micturition.

Keywords: Hamster urethra; urothelium; non-adreneregic, non-cholinergic inhibitory system; NADPH diaphorase; nitric oxide synthase

Introduction

Pharmacological evidence indicates that nitric oxide (NO) is involved in the non-adrenergic, non-cholinergic (NANC) inhibitory response of the urethra, bladder neck and trigone from various species including: man (Klarskov *et al.*, 1983); pig (Klarskov, 1987a; Persson & Andersson, 1992; Persson *et al.*, 1993), rabbit (Dokita *et al.*, 1994; Persson & Andersson, 1994), rat (Persson *et al.*, 1992), sheep (Garcia-Pascual *et al.*, 1991), cat (Levin *et al.*, 1992) and dog (Hashimoto *et al.*, 1993; Takeda & Lepor, 1995). The possible involvement of NO in hamster urethra has not yet been investigated. In detrusor smooth muscle the role of NO is still unclear (Elliot & Castleden, 1993; Persson *et al.*, 1993); nevertheless, Klarskov (1987b) and James *et al.* (1993) described relaxation of precontracted human detrusor muscle in response to electrical field stimulation (EFS), partially mediated by NO which was thought to be released not from nerves, but from the smooth muscle. NO can also be released from epithelial cells of the guinea-pig trachea (Matera *et al.*, 1995), in the rabbit urethral lamina propria (Zygmunt *et al.*, 1993; 1995), both in response to EFS and after administration of acetylcholine, or from contracted urethral muscle (Hashimoto *et al.*, 1993). In addition, Saffrey *et al.* (1994) found nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity in mucosal epithelial cells of the guinea-pig urinary bladder. Several NANC inhibitory systems may be involved in the relaxation of the components of the outlet tract so as to enable micturition (Hoyle *et al.*, 1994), and functional disturbances in the outflow region in man may involve an alteration of these inhibitory relaxant systems in the urethra (Kulseng-Hanssen, 1987; Low *et al.*, 1989). Neuropeptides released from sensory-motor nerves and purines co-released with acetylcholine from post-ganglionic parasympathetic nerves can contribute to the relaxation of the proximal urethra. In addition, prostaglandins are generated in bladder smooth muscle and in epithelial cells (Pinna *et al.*, 1992) as a result of neurotransmitter activity (which can induce prostaglandin E_2 release) and show a dual effect, contractile on the detrusor and relaxant on the urethra (Morita *et al.*, 1994), suggesting its involvement in bladder emptying. The aim of the present work was to study these inhibitory systems in strips of hamster proximal urethra and to evaluate the possible modulatory role that urothelial cells can play in the smooth muscle response, as already demonstrated for epithelium in guinea-pig trachea (Matera *et al.*, 1995). Functional *in vitro* studies were performed in order to evaluate the presence of the NANC inhibitory systems in this tissue. Histochemical and immunohistochemical studies, using NADPH diaphorase and antibodies to nitric oxide synthase (NOS) were also carried out to establish the presence of NO-generating systems.

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Methods

Tissue preparations and recording of mechanical activity

Male Golden hamsters (120–140 g) were killed by asphyxiation with CO_2. The abdomen was opened and the bladder and the urethra were quickly removed and placed in cold, modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.4, $NaHCO_3$ 16.4, $MgSO_4$ 0.6 and glucose 7.7. The bladder and the proximal urethra were then separated by a transverse cut at the level of the bladder neck. Urothelium was removed by scraping the lumen of the urethra with a needle (size: 0.5 mm); the absence of at least 80% of the urothelium (in 6 preparations) was observed by electron microscope scanning (Figure 1). The urethra was opened under a dissecting microscope and a strip (2 mm in length, 1 mm in width, 2.6 ± 0.3 mg wet weight) was prepared from each urethra. Each circular smooth muscle preparation was threaded through a pair of platinum-ring electrodes (3 mm in diameter, 1 cm apart) connected to a Grass SD 9 stimulator, with one end attached to a holder and the other to a Dynamometer UF1 isometric force transducer coupled to a four-channel Grass 79D ink-writing oscillograph. The strips were equilibrated for 1 h in 5 ml organ baths containing modified Krebs solution gassed with 95% O_2 and 5% CO_2 at 36 ± 0.5°C. The resting tension for the optimal force development was determined as 250–350 mg, and the strips were initially loaded to this resting tension. A slight reduction in this value occurred after the equilibration period.

Experimental procedure

After the equilibration period, each preparation was exposed to noradrenaline (approx. EC_{50}: 10^{-5} M) until two reproducible contractions were obtained. Relaxant responses to electrical field stimulation (EFS) and to the agonists prostaglandin E_2 and 2-methyl-thio-adenosine 5′-triphosphate (ATP) were studied in strips precontracted with an approximate EC_{50} concentration of arginine vasopressin (10^{-8} M) (95% confidence limits: 8.8×10^{-9} to 3×10^{-8}), which produced a long-lasting and stable contraction. Non-cumulative concentration-response curves to noradrenaline and acet-

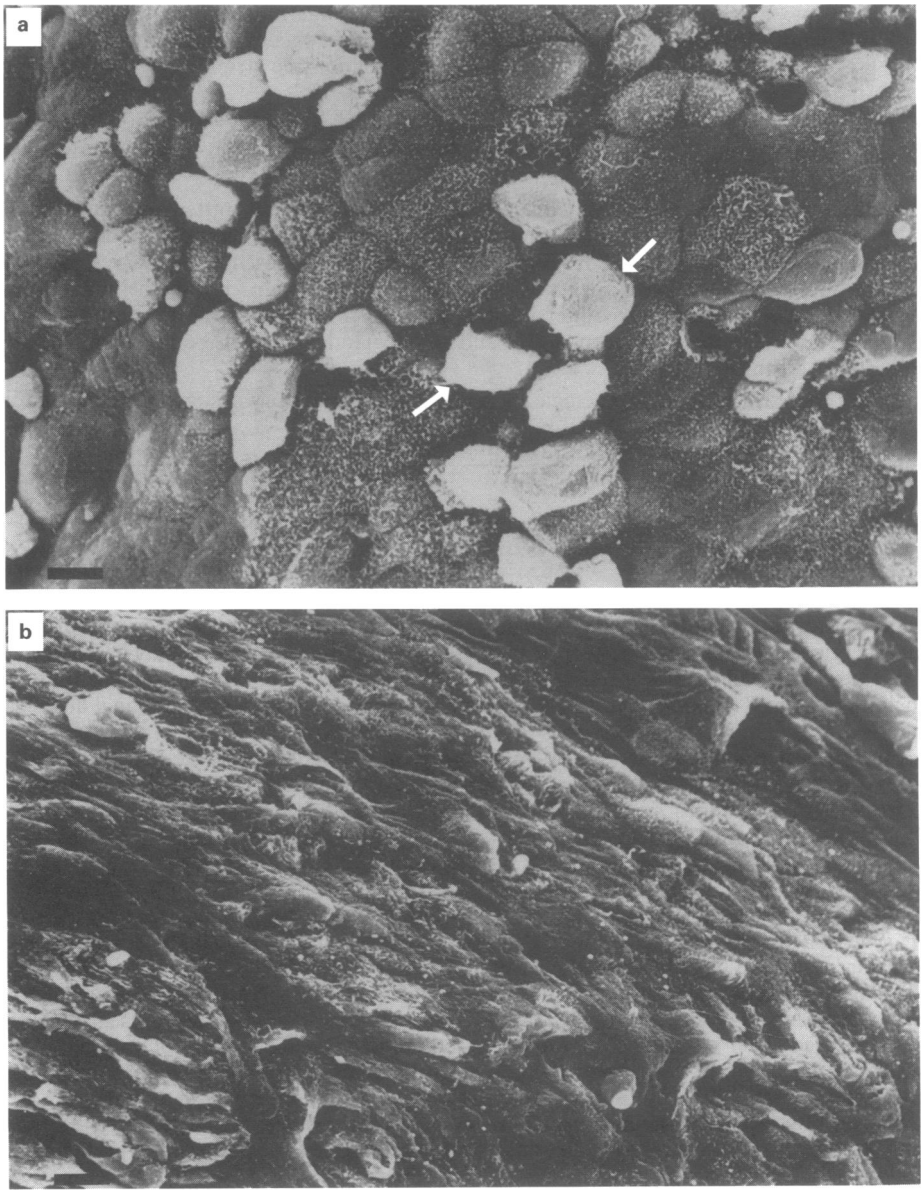

Figure 1 Scanning electron micrographs showing (a) the urothelium of an intact preparation of hamster proximal urethra and (b) an urothelium-free preparation in which the sub-epithelial layer is visible. Note white cells (indicated by arrows), are dead epithelial cells. Scale bar = 10 μm.

ylcholine were carried out at the resting tone before and after incubation with L-N^G-nitroarginine methyl ester (L-NAME) (10^{-4} M) in urothelium-free and intact preparations to study whether urothelial cells can effect smooth muscle response by a non-neurogenic release of nitric oxide. In order to display NANC relaxant responses, the tissues were preincubated with phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M) for 30 min and were exposed to arginine vasopressin (10^{-8} M). Frequency-response curves were then constructed: square wave pulses with a duration of 0.3 ms and a supramaximum voltage (80 V) were delivered for 30 s, at 1, 2, 4, 8, 16 Hz in this sequence, leaving a four-min interval between each frequency step. L-NAME (10^{-4} M) was added (together with phentolamine, propranolol and atropine) 30 min before the strips were once again precontracted with arginine vasopressin and subjected to electrical stimulation. Then, in the continued presence of L-NAME, the EFS was repeated 10 min after the administration of L-arginine (3×10^{-3} M) or D-arginine (3×10^{-3} M). In some experiments, the frequency-response curve to electrical stimulation was repeated three times, leaving a 30 min interval between the curves as a time control: no significant difference was observed between the first, the second and the third frequency-response curve. In some experiments, the frequency-response curve was repeated after the strips were incubated for 1 h with indomethacin (10^{-6} M) or for 30 min with suramin (10^{-4} M) in the presence or absence of L-NAME. At the end of the experiment, tetrodotoxin (10^{-6} M) was added to the organ bath and EFS repeated. Relaxant responses induced by prostaglandin E_2 and 2-methyl-thio-ATP were studied by cumulative additions to the organ bath. The tissues were incubated for 30 min with suramin (10^{-4} M) and the concentration-response curve induced by 2-methyl-thio-ATP was repeated. Contractile responses evoked by α,β-methylene ATP were studied at the resting tone, with 20–30 min intervals between each dose to avoid desensitization of P_{2X}-purinoceptors.

Nitric oxide synthase immunohistochemistry

For immunohistochemical and histochemical studies, tissues from five hamsters were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. The fixed tissues were then washed in 7% sucrose in PBS containing 0.01% sodium azide four times for 10 min each time, and stored for at least 24 h in the same solution at 4°C. Tissues were then frozen in Tissue-Tek (OCT embedding compound) and stored in liquid nitrogen. Frozen 10 μm sections were then cut in a cryostat and thawed onto gelatine coated slides and air dried for at least 30 min.

Sections processed for immunostaining were incubated with rabbit polyclonal antibody for nitric oxide synthase (NOS) type I (neuronal) or type III (endothelial) at a dilution of 1 : 1000 for 18–20 h. Tissues were then washed four times in PBS for 10 min each time, before incubation with biotinylated donkey antirabbit immunoglobulin G at a dilution of 1 : 250 for 1 h at room temperature. Tissues were again washed four times in PBS for 10 min each time before the sites of antibody-antigen reactions were revealed by incubation for 1 h at room temperature with streptavidin-conjugated fluorescein isothiocyanate at a dilution of 1 : 100. Tissues were again washed four times in PBS for 10 min each time, and mounted in Cityfluor and coverslipped. Sections were viewed under a Zeiss photomicroscope fitted for epifluorescence with ultraviolet filters.

NADPH diaphorase histochemistry

Sections stained for NADPH were incubated with 1.2 mM β-NADPH, 0.24 M nitroblue tetrazolium, 15.2 mM L-malic acid and 0.1% Triton X-100 in 0.1 M Tris HCl (pH 7.4) for 100 min at 37°C (Belai *et al.*, 1995). Slides were then washed four times in PBS for 10 min each time, mounted in Aquamount and coverslipped. Sections were viewed under a Zeiss light photomicroscope.

Drugs and solution

Acetylcholine hydrochloride, L-arginine, D-arginine, arginine vasopressin, atropine sulphate, indomethacin, L-malic acid disodium salt, α,β-methylene ATP lithium salt, nitroblue tetrazolium salt, noradrenaline (L(−)-arterenol bitartrate), L-N^G-nitroarginine methyl ester (L-NAME), nicotinamide adenine dinucleotide phosphate (β-NADPH) tetrasodium salt, prostaglandins E_2 and $F_{2\alpha}$, tetrodotoxin (TTX), Tris HCl and Triton X-100 were all purchased from Sigma; 2-methyl-thio-ATP tetrasodium salt was from RBI, phentolamine mesylate (Regitine) from Ciba, propranolol hydrochloride (Inderal) from Zeneca, rabbit polyclonal antibody for NOS type I from Diagnostica, type III from Transduction Laboratories, anti-rabbit immunoglobulin G and streptavidin-conjugated fluorescein isothiocyanate were from Amersham, Citifluor from UKC, Aquamount from BDH and suramin (Germanin) from Bayer. For pharmacological experiments, stock solutions were usually prepared in distilled water. Subsequent dilutions of the drug were prepared in 0.9% NaCl; dilutions of noradrenaline were made with 0.1 mM ascorbic acid to prevent oxidation. Incubating solutions for immunohistochemistry were diluted to the required concentrations in antibody diluting medium (sodium azide: w/v 0.1% bovine serum albumin: w/v 0.01%, lysine: w/v 0.1% and Triton X-100: v/v 0.1% in PBS).

Analysis of data

Relaxant responses are expressed as % inhibition of the arginine vasopressin-induced contraction and contractile responses as mg tension mg^{-1} tissue. All data in the text are expressed as mean ± s.e.mean of six to eight experiments. Since only one strip was prepared from each urethra, the number of animals (N) used for the experiments and the number of experiments (n) made were similar and only the (n) value has been given. Student's t test (for unpaired data) was used to compare two means. When two concentration-response curves were compared, an analysis of variance (ANOVA) was used (Ludbrook, 1994) by means of the computer programme Minitab. $P<0.05$ was considered significant.

Results

Figure 1 shows the urothelium of an intact preparation of hamster proximal urethra and a sub-epithelial layer of an urothelium-free preparation. Urethral strips were loaded initially to a tension of 250 mg. A slight reduction in this value occurred during the period of equilibration in control and urothelial-free strips, so that the resting tone ranged between 200–220 mg at the start of each experiment.

Neurogenic response in precontracted tissues

Arginine vasopressin (EC_{50} 10^{-8} M) produced a sustained and long-lasting contraction: the values for intact and urothelium-free strips were 160 ± 13 and 180 ± 15 mg of tension mg^{-1} tissue (NS, $n=8$), respectively. Intact preparations, precontracted with arginine vasopressin (10^{-8} M) and incubated with phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M), showed NANC frequency-dependent relaxations in response to EFS. The relaxation developed quickly and was sustained during continuous stimulation; the recovery to baseline was biphasic: an initial fast followed by a slower phase. The response was partially inhibited by L-NAME (10^{-4} M) especially at the lower frequencies of stimulation, giving a long lasting relaxation which was completely blocked by tetrodotoxin (10^{-6} M) (Figure 2). In urethral strips devoid of urothelium, EFS caused either no relaxation or a relaxation which was significantly reduced compared with controls (Figures 2 and 3).

Effect of L-, D-arginine, indomethacin and suramin on neurogenic responses

In the presence of L-NAME, L-arginine (3×10^{-3} M) restored the frequency-dependent relaxations whereas D-arginine (3×10^{-3} M) had no such effect. Indomethacin (10^{-6} M) failed to affect the NANC-induced relaxant response and suramin (10^{-4} M) significantly reduced the amplitude of neurogenic relaxations (ANOVA, $P<0.05$; $n=6$) (Figure 3). The efficacy of suramin to inhibit the neurogenic response was higher at the lower frequencies of stimulation (47% at 0.5 Hz) than at higher frequencies (25% at 4–16 Hz).

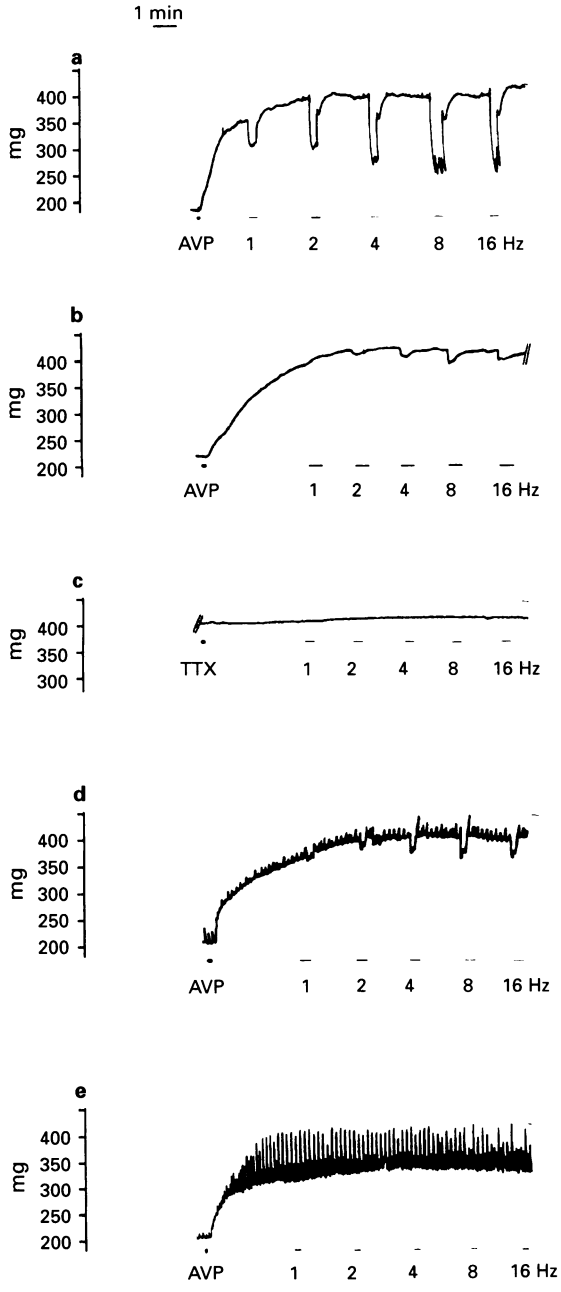

Figure 2 Recordings of responses evoked by electrical field stimulation in circular smooth muscle strips of hamster proximal urethra precontracted with arginine vasopressin (AVP: 10^{-8} M). (a) Before, (b) after incubation with L-NAME (10^{-4} M) and (c) after incubation with tetrodotoxin (TTX, 10^{-7} M); (d) and (e) show typical tracings in preparations without urothelial cells. Phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M) were present throughout the experiments. Voltage 80 V, pulse duration 0.3 ms, pulse frequency 1 to 16 Hz, train duration 30 s and stimulation interval 4 min.

Non-neurogenic relaxations in urethral strips

In circular smooth muslce urethral strips precontracted with arginine vasopressin (10^{-8} M), prostaglandin E_2 (10^{-8} M – 3×10^{-5} M) elicited a concentration-dependent relaxation (Figure 4). The relaxation induced by prostaglandin E_2 was $80 \pm 7\%$ and $76 \pm 8\%$ ($n=6$) at the highest concentration tested, in intact and urothelial-free preparations; furthermore it was not affected by L-N^G-nitroarginine methyl ester (10^{-4} M). Conversely, prostaglandin $F_{2\alpha}$ did not elicit concentration-dependent relaxation. 2-Methyl-thio-ATP (10^{-9} – 10^{-5} M) evoked concentration-dependent relaxations in intact and urothelium-free preparations (Figure 4). The relaxation induced by 2-methyl-thio-ATP at 10^{-5} M was $25.5 \pm 2.3\%$ and $23 \pm 4\%$ ($n=6$) in intact and in preparations devoid of urothelium respectively. L-NAME (10^{-4} M) did not affect these responses, whereas suramin (10^{-4} M) significantly inhibited the relaxant response induced by 2-methyl-thio-ATP (Figure 4).

Contractile responses in hamster urethra

In circular urethral strips at the resting tone, prostaglandin $F_{2\alpha}$ (10^{-8} M – 3×10^{-5} M) and 5-hydroxytryptamine (10^{-7} M – 10^{-3} M) evoked slow concentration-related contractions whereas α,β-methylene ATP elicited small contractions, but only at the highest dose tested (10^{-4} M). The contractile response evoked by α,β-methylene ATP at 10^{-4} M was 13.5 ± 4 mg of tension mg^{-1} tissue ($n=4$). Urethral strips devoid of urothelial cells exhibited an increased contraction in response to noradrenaline. The two concentration-response curves performed in urothelium-free and in intact preparations were significantly different (ANOVA, $P<0.05$; $n=6$) (Figure 5a). The response induced by acetylcholine was similar in both preparations (Figure 5b). Incubation with L-NAME did not affect the response induced by noradrenaline either or in urothelium-free strips. However, L-NAME increased the contractile response induced by acetylcholine at concentrations starting from 10^{-5} M to 10^{-3} M. The concentration-response curves to acetylcholine were significantly different (ANOVA, $P<0.05$; $n=6$), either in urothelium-free and in intact preparations, compared to control curves.

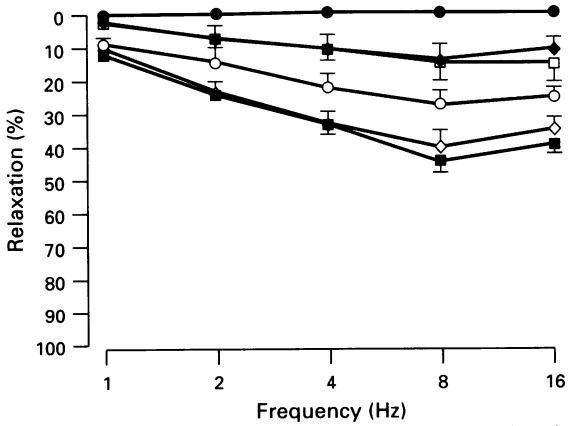

Figure 3 Neurogenic nonadrenergic, noncholinergic relaxation in circular strip of hamster proximal urethra precontracted with arginine vasopressin (10^{-8} M) and incubated with propranolol (10^{-6} M), phentolamine (10^{-6} M) and atropine (10^{-6} M), with (■) and without urothelium (□) (ANOVA, $P<0.05$, $n=10$), in the presence of L-NAME: (10^{-4} M, ◆), L-NAME and L-arginine (3×10^{-3} M, ◇), suramin (10^{-4} M, ○) and tetrodotoxin (10^{-6} M, ●). L-NAME and suramin produced significant changes in the frequency-response relationship compared with control (ANOVA, $P<0.05$). Points show mean and vertical lines s.e.mean of 6 experiments, unless occluded by symbol.

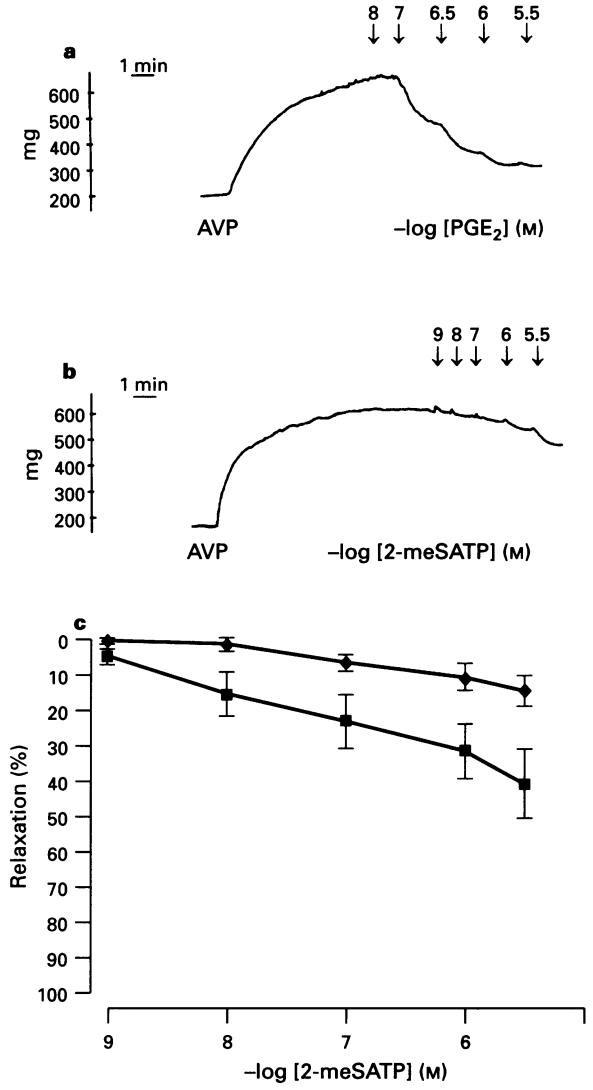

Figure 4 Original tracing showing the effects of (a) prostaglandin E_2 (PGE_2, 10^{-8} M–3×10^{-6} M), (b) 2-methyl-thio-ATP (2-meSATP, 10^{-9} M–3×10^{-6} M). (c) The concentration-response curve to 2-methyl-thio-ATP before (■) and after (◆) suramin (3×10^{-4} M) on circular strips of hamster proximal urethra precontracted with arginine vasopressin (AVP, 10^{-8} M). Phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M) were present throughout the experiment (ANOVA, $P<0.05$). Points show mean and vertical lines s.e.mean of 6 experiments.

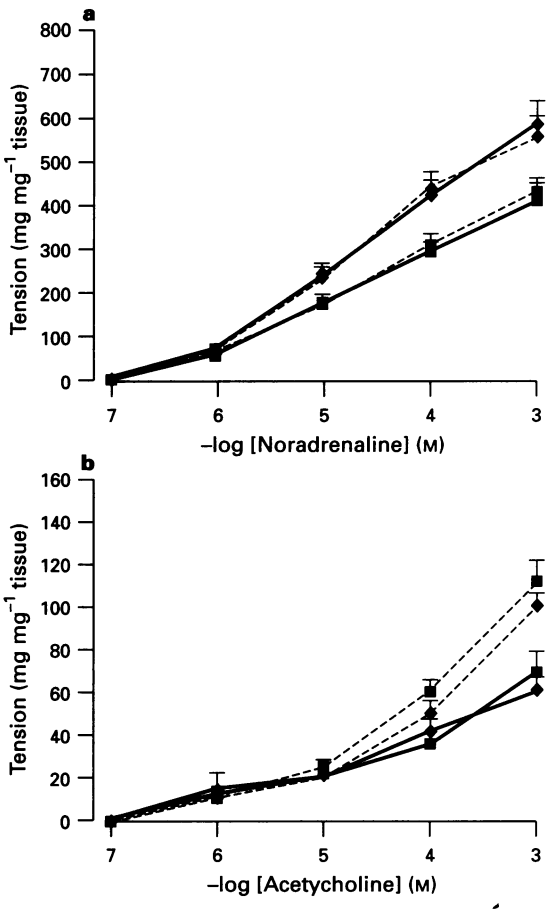

Figure 5 Non cumulative concentration-response curves for (a) noradrenaline and (b) acetylcholine, before (continuous line) and after (dashed line) L-NAME (10^{-4} M), in intact (■) and in urothelium-free (◆) preparations of hamster proximal urethra. Strips without urothelium caused a significantly increased contraction in response to noradrenaline as compared to intact preparations (ANOVA, $P<0.05$). Points show mean and vertical lines s.e.mean of 6 experiments, unless occluded by symbol.

NOS immunohistochemistry

Immunohistochemistry with antibodies to both the neuronal and epithelial isoforms of NOS yielded similar immunohistochemical profiles in sections of the hamster urethra. NOS-immunoreactive nerve fibres were present in the *lamina propria* just beneath the urothelium and the inner circular smooth muscle layer of proximal urethra sections (Figure 6a). No NOS-immunoreactive nerve fibres were seen in the outer smooth muscle layer. Immunolabelling was not observed in the urothelium cell layer, but rather at the base of these urothelial cells.

NADPH-diaphorase histochemistry

Nerve fibres were not clearly visible following NADPH staining. However the NADPH diaphorase reaction was found to be particularly strong in the urothelium (Figure 6b).

Discussion

The normal pattern of micturition involves urethral relaxation which precedes bladder contraction (Tanagho & Miller, 1970). Urethral relaxation has been shown to be mediated by a NANC inhibitory innervation in several mammals including man (Andersson & Persson, 1993; Hoyle *et al.*, 1994). In hamster proximal urethra, the NANC relaxation induced by EFS developed quickly and was sustained during continuous stimulation. This relaxant response was attenuated by L-NAME and was sensitive to tetrodotoxin; furthermore L-arginine but not D-arginine restored this response, suggesting that nitric oxide is the principal transmitter in these NANC nerves. The results obtained in this study also support the view that stimulation of inhibitory nerves causes a relaxation which seems heavily urothelium-dependent as EFS in precontracted circular strips devoid of urothelium exhibited either a lack or a significant decrease in relaxation compared with controls. The pattern of NADPH diaphorase staining in the proximal urethra was different from that shown by NOS-immunohistochemistry. Nerve fibres in the smooth muscle layer were visualised with NOS immunolabelling. In contrast to the NADPH diaphorase technique, no labelling was observed in the urothelium with NOS-immunohistochemistry with antibodies raised against either the neuronal or epithelial isoforms of NOS. A similar observation has previously been seen in the urethra of the pig (Persson *et al.*, 1993), and strong NADPH diaphorase staining has also been shown in the urothelium of

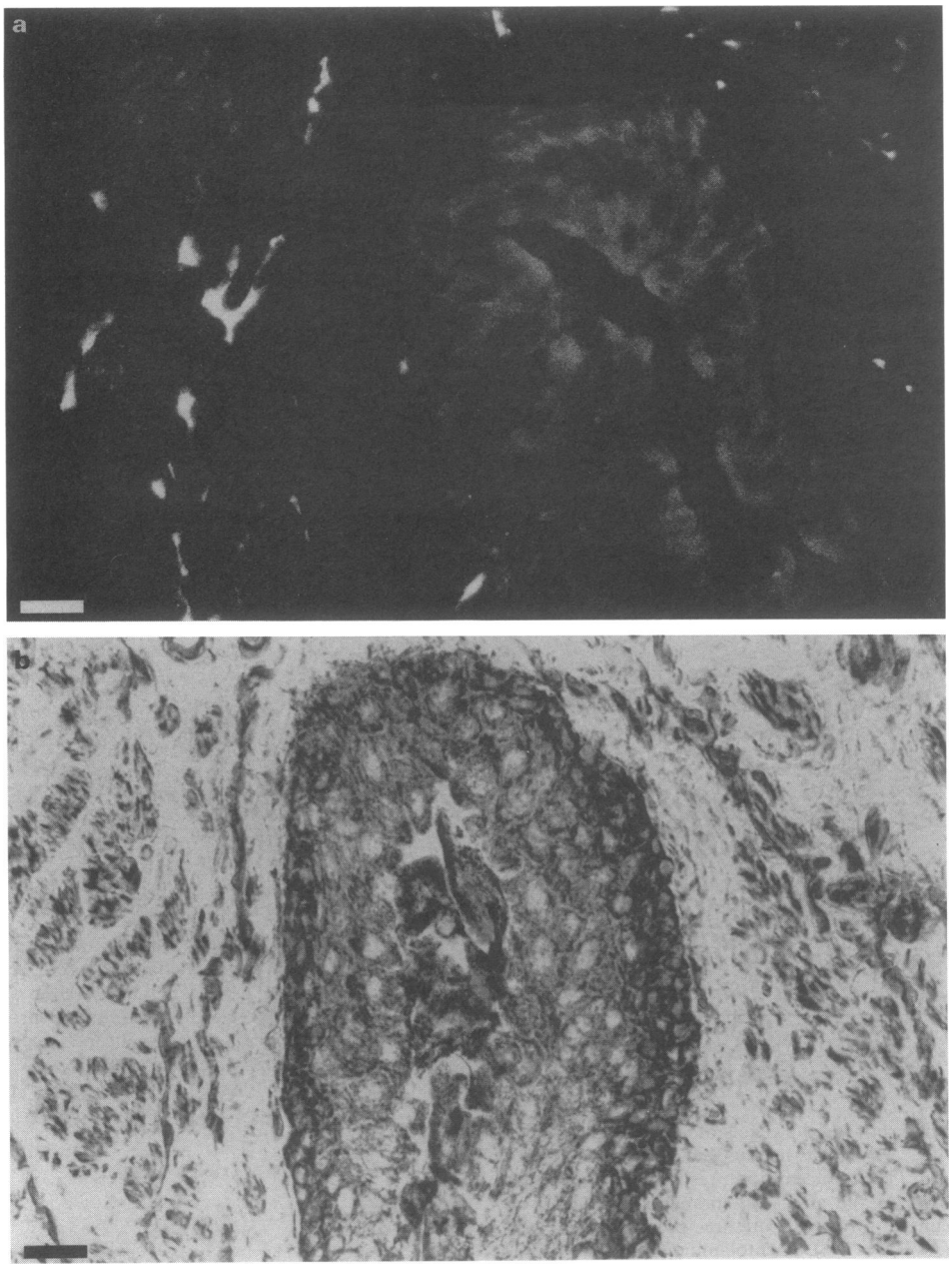

Figure 6 Light micrographs showing the distribution of neuronal nitric oxide synthase (NOS)-immunoreactivity and NADPH-diaphorase activity in sections of the hamster proximal urethra. Immunohistochemical localization of NOS (a) shows nerve fibres in the inner circular smooth muscle layer (M) and a few sparsely distributed nerve fibres in the *lamina propria* (L), just beneath the urothelium (U). In contrast, these nerve fibres could not be distinguished amongst the background muscle staining seen in sections stained with the NADPH-diaphorase technique (b). The urothelium (U) is stained positively for NADPH-diaphorase activity (b) but not for NOS-immunoreactivity (a). Scale bar = 20 μm.

the mouse (Grozdanovic *et al.*, 1992). This suggests that the NADPH diaphorase technique stains additional enzymes besides NOS, as observed by Tracey *et al.* (1993), or that NADPH diaphorase staining of the hamster urothelium represents an isoform of NOS that was not readily detectable with the antisera used in our immunohistochemical experiments. The first possibility appears more likely even though our functional organ bath experiments showed the electrically-evoked inhibition to be heavily dependent on the presence of an intact urothelium as well as being TTX-sensitive. This explanation is in agreement with our experiments with exogenously applied noradrenaline and acetylcholine. Urothelium-free strips produced a significantly greater contraction than intact preparations in response to noradrenaline. The response was insensitive to L-NAME suggesting that nitric oxide was not involved. Possible explanations are that (1) the urothelial layer may reduce the passive diffusion of the exogenously added drug, (2) the transitional urothelium normally constitutes a site for noradrenaline inactivation (Meister *et al.*, 1993) and (3) an inhibitory factor such as prostaglandin E_2 may be released from the urothelium. Such an urothelial inhibitory factor may also be released by neuronal nitric oxide following electrical nerve stimulation. The response induced by exogenous acetylcholine was similar in controls and in strips devoid of urothelium but was affected to the same extent in both preparations by L-NAME, suggesting the involvement of nitric oxide, which probably originates from nerves located in the smooth muscle. Therefore neuronally released nitric oxide may be able to act directly as well as indirectly by stimulating the release of an inhibitory factor(s) from the urothelium. An alternative explanation for the lack of an electrically-evoked inhibition observed in some urothelium-free preparations is

that the nitrergic fibres running underneath the urothelial layer were damaged or removed along with the urothelial cells. In an attempt to verify if other neurotransmitters could be involved in the NANC response, we studied the effect of purines and prostaglandins in circular strips of hamster proximal urethra, either in the presence or absence of urothelium. ATP has been proposed as an excitatory neuromuscular neurotransmitter in the bladder detrusor (Igawa *et al.*, 1993; Burnstock, 1994; Hoyle *et al.*, 1994; Theobald, 1995). However in the guinea-pig urethra (Callahan & Creed, 1981) and in pig bladder neck (Hills *et al.*, 1984) ATP even at high doses ($10^{-3}-10^{-2}$ M) does not produce contraction. In agreement, in circular strips of hamster proximal urethra at the resting tone, α,β-methylene ATP, a more selective agonist for P_{2X}-purinoceptors, only evoked slight contractions at the highest concentration used (10^{-4} M). Nevertheless, 2-methyl-thio-ATP, a selective agonist for P_{2Y}-purinoceptors, elicited a concentration-dependent relaxation with a threshold concentration of 10^{-8} M in precontracted hamster urethra; this response was significantly inhibited by suramin. Furthermore, suramin reduced significantly the neurogenic relaxations induced by electrical field stimulation: the efficacy of suramin was higher at the lower frequencies of stimulation than at higher frequencies. These data suggest that in hamster proximal urethra, ATP or a closely related compound, acting preferentially on P_{2Y}-purinoceptors, may contribute to the urethral relaxation during micturition, but has no influence in maintaining the urethral pressure during the filling phase. Similarly, prostaglandin E_2 elicited a strong and long lasting relaxation in hamster urethra when the tone was raised. In our experiments *in vitro*, indomethacin failed to affect the NANC neurogenic relaxation. Nevertheless, prostaglandin E_2 could be released from the smooth muscle layer and/or urothelium, following the first phasic relaxation of the urethra induced by nitric oxide and ATP, perhaps helping to maintain the relaxation and to reduce the urethral pressure during micturition. Further *in vivo* experiments are needed to test this hypothesis. In conclusion, the results presented in this paper show that stimulation of inhibitory nerves causes a relaxation which is partially urothelium-dependent. Nitric oxide is the principle transmitter involved in the non-adrenergic, non-cholinergic relaxation of hamster proximal urethra. The decreased neurogenic relaxation in strips devoid of urothelium suggests that nitric oxide can also activate the release of an inhibitory factor from the urothelium. The results also suggest that ATP or a closely related compound, and prostaglandin E_2, may by inhibitory factors together with nitric oxide in the hamster proximal urethra during micturition.

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