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NANC transmitters in the female pig urethra – localization and modulation of release via α_2-adrenoceptors and potassium channels

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1 To investigate further the release, localization and identity of a non-nitrergic mediator of smooth muscle relaxation in the female pig urethra, we studied the effects of drugs acting at α_2-adrenoceptors or K^+ channels, the effects of capsaicin and chemical sympathectomy, and the actions of several transmitter candidates.

2 Electrical field stimulation (EFS; frequencies above 12 Hz) of spontaneously contracted smooth muscle strips from the female pig urethra evoked long-lasting, frequency-dependent relaxations in the presence of prazosin, scopolamine, and N^G-nitro-L-arginine. Treatment with the selective α_2-adrenoceptor agonist UK-14 304 markedly reduced the relaxations evoked by EFS at all frequencies tested (16–30 Hz). The inhibitory effect of UK-14 304 was completely antagonized by the α_2-adrenoceptor antagonist rauwolscine. The muscarinic M_1 receptor antagonist, pirenzepine, or exogenously administered carbachol, did not have any effects on the electrically evoked relaxations.

3 Inhibition of high conductance Ca^{2+} activated K^+ channels by iberiotoxin or charybdotoxin significantly enhanced the relaxations evoked by EFS at all frequencies. However, inhibition of voltage-sensitive K^+ channels with 4-aminopyridine or dendrotoxin-1, treatment with the ATP-sensitive K^+ channel blocker, glibenclamide, or treatment with the high and low conductance Ca^{2+} activated K^+ channel blockers, tetraethylammonium chloride and apamin, had no effect on the relaxations evoked by EFS.

4 Electrically evoked relaxations were not affected by adrenergic denervation with 6-hydroxydopamine (6-OHDA) at any frequency. However, treatment with 6-OHDA abolished prazosin-sensitive electrically induced contractions, and a long-lasting relaxation was revealed. Treatment with capsaicin, believed to damage selectively a subpopulation of primary afferent fibres, did not affect basal tone or relaxations evoked by EFS.

5 Exogenously applied vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP)-27, PACAP-38, adenosine, ATP and 5-hydroxy-tryptamine caused relaxations of the urethral preparations, whereas prostaglandin E_2 and calcitonin gene-related peptide had no effects. VIP 10-28, α, β-methylene-ATP, reactive blue-2, suramin or indomethacin did not reduce the electrically-evoked relaxations at any frequency. However, the relaxations were slightly reduced by trypsin or α-chymotrypsin.

6 The present results suggest that the release of the unknown mediator in the female pig urethra can be modulated via α_2-adrenoceptors and high conductance Ca^{2+} activated K^+ channels. Furthermore, the mediator does not appear to be localized to or released from adrenergic or capsaicin-sensitive sensory nerve-endings. The identity of the transmitter remains to be established.

Keywords: α-Adrenoceptors; capsaicin; neurotransmission; non-adrenergic non-cholinergic; potassium channels; presynaptic; relaxation; smooth muscle; urethra; vasoactive intestinal polypeptide

Introduction

Relaxation of urethral smooth muscle is considered to be mediated via the release of non-adrenergic, non-cholinergic (NANC) neurotransmitters (Andersson, 1993). NANC relaxation of the female pig urethra is mediated via the release of at least two nerve-derived components (Bridgewater *et al*., 1993; Werkström *et al*., 1995). One of these components has been identified as nitric oxide (NO; Persson & Andersson, 1992), whereas the identity of the non-nitrergic NANC mediator of relaxation is unknown (Bridgewater *et al*., 1993; Werkström *et al*., 1995). However, this transmitter is released at high frequencies of stimulation (i.e. >12 Hz; Werkström *et al*., 1995).

The release of noradrenaline and acetylcholine is regulated by presynaptic receptors, responding either to the neurones own transmitter, or to transmitters released from adjacent neurones or cells (Westfall & Martin, 1991). In the rabbit urethra, stimulation of muscarinic receptors by carbachol on the adrenergic nerve endings marked reduced the release of [^{3}H]-noradrenaline (Mattiasson *et al*., 1989). Stimulation of α_2-adrenoceptors on cholinergic neurones by clonidine was demonstrated to inhibit the release of acetylcholine (Mattiasson *et al*., 1988). The concept of presynaptic receptor modulation has been demonstrated not only for 'classical' neurotransmission, but also for NANC neurotransmission in for example the rat gastric fundus, where the release of NO and vasoactive intestinal polypeptide (VIP) are inhibited by α_2-adrenoceptor activation (Lefebvre & Smits, 1992).

Neurotransmitters are also known to regulate neuronal K^+ channels, the activation of which leads to a reduction in Ca^{2+} influx and transmitter release (Miller, 1990). Subsequently, blockade of various types of K^+ channels, leads to depolarization of the presynaptic membrane and enhanced transmitter release. Inhibition of K^+ channels has in fact been demonstrated to potentiate the release of NO, or a NO-related compound, in the canine ileocolic junction (De Man *et al*., 1993).

The localization of the non-nitrergic mediator has not yet been established. Loss of relaxation after 'chemical sympathectomy' by treatment with 6-hydroxydopamine (6-OHDA)

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would suggest an origin in adrenergic nerves, whereas treatment with capsaicin, a neurotoxin that has been used as a tool to damage selectively a subpopulation of primary afferent fibres and to deplete sensory neuropeptides (Bevan & Szolcsányi, 1990), may reveal sensory nerves as the transmitter source. By use of 6-OHDA- and capsaicin-treatment, NO was demonstrated not to originate from sympathetic postganglionic nerve-endings in the sheep and rat urethra (Garcia-Pasqual *et al*., 1991; Persson *et al*., 1997), or from capsaicin-sensitive sensory primary afferents in the rat urethra (Persson *et al*., 1997).

The aim of this study was to investigate further the release, localization and identity of a non-nitrergic mediator of smooth muscle relaxation in the female pig urethra, by studying the effects of drugs acting at α_2-adrenoceptors or K^+ channels, the effects of capsaicin and 6-OHDA, and the effects of several transmitter candidates.

Methods

Preparation of strips

The bladder and urethra from young, female pigs were removed in a slaughterhouse and transported to the laboratory in ice-cold Krebs solution (for composition: see below). The urethra was opened longitudinally and circular smooth muscle strips ($1 \times 2 \times 6$ mm) were dissected in a transverse direction from an area approximately 4 cm below the ureteric orifices (Werkström *et al*., 1995).

Experimental procedure

The strips were transferred into 5 ml temperature-controlled tissue baths containing Krebs solution (for composition; see below), and bubbled with a mixture of 95% O_2 and 5% CO_2. A temperature of 37°C was maintained. The preparations were mounted between two L-shaped hooks by means of silk ligatures. One of the hooks was connected to a Grass FT 03C force transducer for registration of mechanical activity. The other hook was connected to a movable unit allowing adjustment of passive tension. Mechanical activity was recorded on a Grass Polygraph model 7E. Electrical field stimulation (EFS) was performed by means of two platinum electrodes placed in parallel to the strips in the tissue baths. A Grass S48 stimulator, delivering square wave pulses of 0.5 ms duration at a frequency of 16–30 Hz, was used. The voltage giving the optimal response was chosen. The train duration was 5 s, and the stimulation interval was regularly 2 min. The interval was increased if the tension did not reach resting level within the 2 min.

The strips were mounted, and from slack length stretched to a tension of 10 mN. They were left to equilibrate for at least 45 min, during which time a stable tension level was obtained. The preparations were then exposed to Ca^{2+}-free Krebs solution (for composition: see below), in order to establish the 'maximum' relaxation level, i.e. 'zero level' (Werkström *et al*., 1995). After addition of Ca^{2+}-containing Krebs solution, a stable tone was re-established.

Frequency-dependent NANC relaxations in response to EFS (16–30 Hz) were recorded in the presence of 1 μM prazosin, 1 μM scopolamine and 0.3 mM N^G-nitro-L-arginine (L-NOARG). When not specifically indicated, these drugs were always present in the tissue baths during recording of the electrically evoked relaxations. After washout, the preparations were treated with 1 μM capsaicin, 0.3 μM UK-14 304 (selective α_2 adrenoceptor antagonist), 0.1–1 mM 4-aminopyridine, or 1 μM dendrotoxin-1 (inhibitors of voltage-sensitive K^+ channels), 1 μM glibenclamide (inhibitor of adenosine 5′-triphosphate (ATP)-sensitive K^+ channels), 0.1 mM tetraethylammoniumchloride, 0.1 μM charybdotoxin or 0.1 μM iberiotoxin (inhibitors of high voltage Ca^{2+}-activated K^+ channels), 0.1 μM apamin (inhibitor of low voltage Ca^{2+}-activated K^+ channels), 1–10 μM VIP 10–28, 1–10 μM α, β-methylene-ATP, 1–10 μM reactive blue-2, 1–10 μM suramin, 1–10 μM indomethacin, 1 μM trypsin or α-chymotrypsin (2 u ml^{-1}), 20–30 min before a second period of EFS. In the presence of 1 μM prazosin and 0.3 mM L-NOARG, the relaxant effects of EFS (16–30 Hz) were studied before and after treatment with 1–10 μM of the M_1 receptor antagonist, pirenzepine, or 1–10 μM carbachol. The concentrations were chosen on the basis of our own preliminary experiments and previously published data (Zygmunt *et al*., 1996). Parallel experiments without treatment were run as controls.

Denervation with 6-OHDA was performed as previously described by Garcia-Pasqual *et al*. (1991). In order to avoid oxidation of 6-OHDA, an unbuffered solution containing glutathione (0.02 mM, Sigma) was bubbled with 100% N_2 and used as vehicle. The urethral strips were treated with 6-OHDA (0.45 mg ml^{-1}) for 15 min, during which time the bath solution was changed every 2 min. In control experiments, electrically-evoked contractions were evoked in the presence of 0.3 mM L-NOARG and 1 μM scopolamine. To demonstrate that these contractions were adrenergic in origin, the preparations were treated with 1 μM prazosin. To confirm the adrenergic denervation, contractions were studied before and after treatment with 6-OHDA.

After an initial control relaxation (16 Hz) without treatment, concentration-response curves to iberiotoxin (3 nM–0.3 μM), charybdotoxin (3 nM–0.3 μM) or UK 14-304 (0.01–1 μM) in the presence and absence of rauwolscine (1 μM) were performed, with 16 Hz of stimulation.

Drugs and solutions

The following drugs were used: N^G-nitro-L-arginine, (−)-scopolamine hydrochloride, prazosin hydrochloride, tetrodotoxin, 4-aminopyridine, glibenclamide, tetraethylammonium chloride, 6-hydroxydopamine, pirenzepine dihydrochloride, acetylcholine chloride, carbamylcholine chloride, α,β-methylene ATP, reactive blue-2, suramin, α-chymotrypsin, bovine pancreas trypsin, vasoactive intestinal polypeptide (VIP), VIP 10–28, ATP, adenosine, prostaglandin E_2, 5-hydroxytryptamine (Sigma Chemical Company, St Louis, MO), apamin, iberiotoxin, dendrotoxin-1 (Almone Labs LTD, Jerusalem, Israel), synthetic charybdotoxin (Latoxan, Rosans, France), UK-14 304 (5-bromo-6-[2-imidazoline-2-ylamino]quinoxaline; Reckitt & Colman, Kingston upon Hull, U.K.), indomethacin (Confortid, Dumex, Copenhagen, Denmark), pituitary adenylate cyclase-activating peptide (PACAP)-27, PACAP-38, cyclic gene-related peptide (Peninsula Laboratories Inc, St Helens, U.K.), rauwolscine hydrochloride (Rothicrom, Carl Roth, Karlsruhe, Germany).

The Krebs solution had the following composition (mM): NaCl 119, KCl 4.6, CaCl 1.5, MgCl 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. Ca^{2+} free Krebs solution was prepared by omitting $CaCl_2$ and adding EGTA (0.1 mM).

Analysis of data

The relaxant effects of electrical field stimulation have been expressed as percentage of the maximum response before drug treatment, which means that relaxations after treatment may exceed 100%. The values are given as mean ± s.e.mean. Statistical analysis of data was performed by Student's two-tailed unpaired *t* test. In case of multiple comparisons, a one-way analysis of variance, ANOVA, followed by Bonferroni/Dunn *post-hoc* test, was used. A probability value of $P<0.05$ was regarded as significant. *n* denotes the number of animals.

Results

Presynaptic modulation

Effects of EFS As described previously (Werkström *et al*., 1995), transmural nerve stimulation of spontaneously con-

tracted smooth muscle preparations of the female pig urethra evoked frequency-dependent relaxations in the presence of prazosin (1 μM), scopolamine (1 μM) and L-NOARG (0.3 mM). The relaxations were long-lasting, and sensitive to treatment with tetrodotoxin (1 μM).

α_2-Adrenoceptor modulation Treatment with UK-14 304 (0.3 μM) did not affect the spontaneously developed smooth muscle tension, but markedly reduced the relaxation evoked by EFS at all frequencies tested (16–30 Hz). At 16 Hz, the relaxation was reduced from 68 ± 6% in the control preparations, to 19 ± 7% in preparations treated with UK-14 304 ($n=5$; $P<0.001$; Figure 1a,b).

The inhibition caused by UK-14 304 was concentration-dependent, and completely antagonized by 1 μM rauwolscine ($n=5$; Figure 1c).

Muscarinic receptor modulation During EFS, acetylcholine and the non-nitrergic mediator of relaxation are released simultaneously (Bridgewater *et al*., 1995). To investigate the possibility that neurally released acetylcholine modulates the release of the non-nitrergic mediator, the preparations were treated with the M_1 receptor antagonist, pirenzepine (1–10 μM). However, neither pirenzepine nor exogenously administered carbachol (1–10 μM), had any effects on the electrically evoked relaxations (16–30 Hz; $n=4$).

Effects of K^+-channel inhibitors Treatment with iberiotoxin (0.1 μM) or charybdotoxin (0.1 μM; blockers of high conductance Ca^{2+}-activated K^+ channels) markedly enhanced the relaxations evoked by EFS at all frequencies tested (16–30 Hz). At 16 Hz the relaxation was 77 ± 5% in the control preparations, 124 ± 14% in preparations treated with charybdotoxin ($n=6$; $P<0.01$; Figures 2a,b) and 103 ± 6% in preparations treated with iberiotoxin ($n=6$; Figure 2b).

The enhancing effects of iberiotoxin and charybdotoxin were concentration-dependent and the effects of the toxins were most prominent at 0.1 μM ($n=6$; Figure 2c). Neither iberiotoxin (3 nM–0.3 μM), nor charybdotoxin (3 nM–0.3 μM), had any effects on the spontaneously developed smooth muscle tone.

Treatment with 4-aminopyridine (0.1–1 mM) or dendrotoxin-1 (1 μM; blockers of voltage-sensitive K^+ channels), glibenclamide (1 μM; ATP-sensitive K^+ channel blocker), apamin (0.1 μM) or tetraethylammonium chloride (0.1 mM; blockers of low and high conductance Ca^{2+}-activated K^+ channels) had no effects on the electrically evoked relaxations ($n=4$).

Localization

Effects of 6-OHDA and capsaicin During treatment with 6-OHDA (0.45 mg ml^{-1}) and a control vehicle all preparations

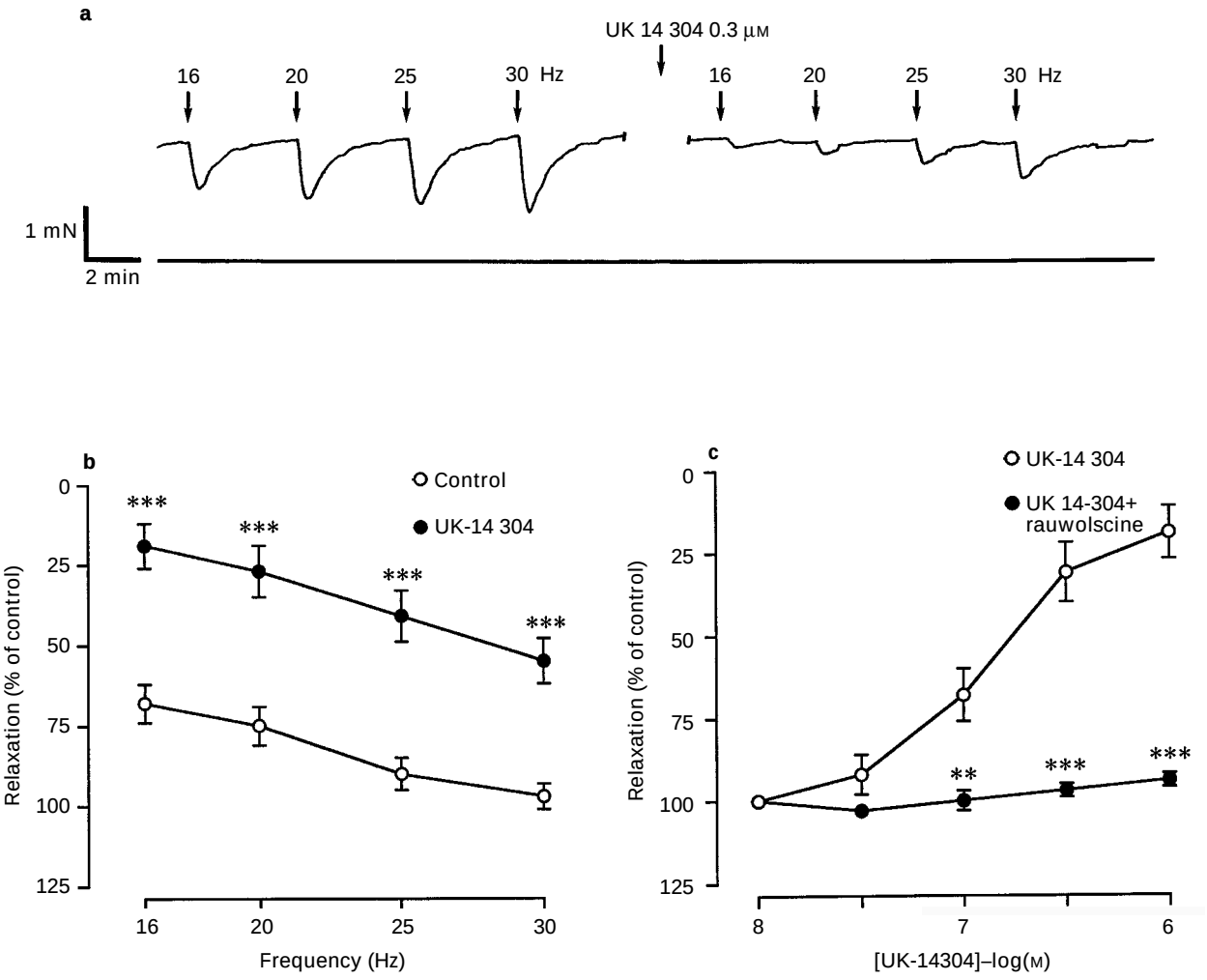

Figure 1 Effects of UK-14 304 on electrically evoked relaxations (16–30 Hz) in the female pig urethra in the presence of N^G-nitro-L-arginine 0.3 mM, prazosin 1 μM and scopolamine 1 μM. (a) Original tracing from an isolated smooth muscle strip. Line indicates the maximum relaxation level ('zero level'). (b) Relaxations evoked by electrical field stimulation (16–30 Hz) in the presence of UK-14 304 0.3 μM. (c) Concentration-response curve to UK-14 304 (16 Hz) in the absence and presence of rauwolscine 1 μM. values represent mean and vertical lines show s.e.mean ($n=6$; $^{**}P<0.01$; $^{***}P<0.001$).

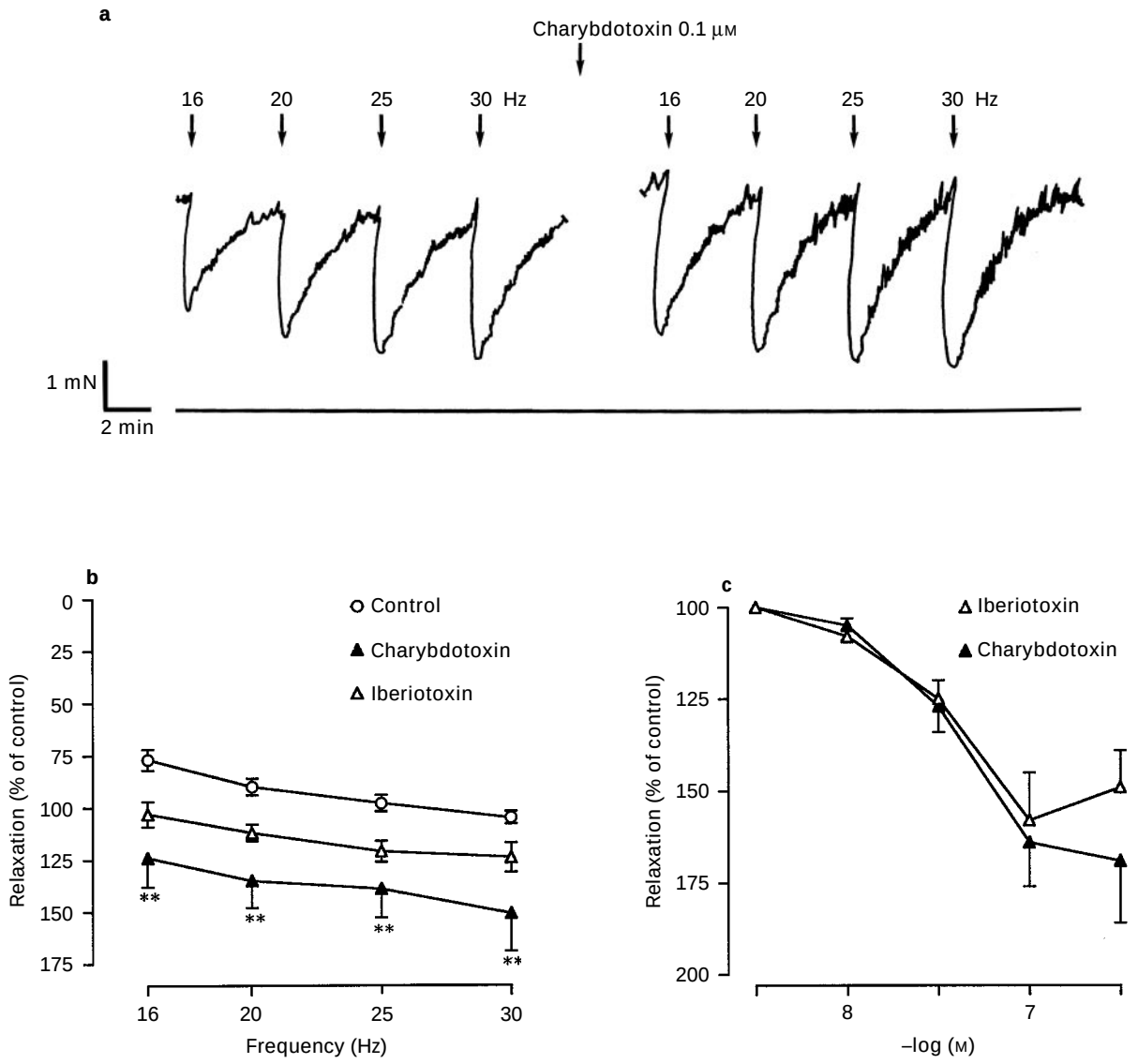

Figure 2 In (a) an original tracing and in (b) a graph displaying enhancing effects of iberiotoxin 0.1 μM and charybdotoxin 0.1 μM on electrically evoked relaxations (16–30 Hz) in the female pig urethra in the presence of N^G-nitro-L-arginine 0.3 mM, prazosin 1 μM and scopolamine 1 μM. (c) Concentration-response curves to iberiotoxin and charybdotoxin at 16 Hz. Values represent mean and vertical lines show s.e.mean ($n=6$; $^{**}P<0.01$).

lost tension, but the tone was immediately re-established when Krebs solution was added to the baths. To confirm that adrenergic denervation had been achieved by treatment with 6-OHDA, electrical stimulation (25 Hz) was performed in the presence of L-NOARG (0.3 mM) and scopolamine (1 μM). Treatment with 6-OHDA abolished electrically evoked prazosin-sensitive contractions, and a long-lasting relaxation was revealed (Figure 3a,b). No effects on the electrically evoked contractions were observed in preparations exposed to a control vehicle. Relaxations evoked by EFS were not affected by treatment with 6-OHDA at any frequency tested (16–30 Hz; $n=5$; Figure 3c),

Treatment with capsaicin (0.1–10 μM) did not affect basal tone or relaxations evoked by EFS (16–30 Hz; $n=5$).

Transmitter candidates

Exogenously applied agents Exogenously applied VIP, PACAP-27 and PACAP-38 (1 nM–1 μM) caused concentration-dependent relaxations of the smooth muscle tone, reaching a maximum of 71±8%, 27±9% and 34±10%, respectively ($n=4-5$; Figure 4a,c). Adenosine and ATP (0.1–300 μM) caused relaxations, reaching a maximum of 62±6% and 55±4% at 300 μM, respectively ($n=4-6$, Figure 4b,d), whereas the relaxation evoked by 5-HT (0.01–10 μM) reached a maximum of 94±4% at 10 μM ($n=5$; Figure 4d). PGE_2 (0.01–100 μM) or CGRP (0.01–0.1 μM) did not affect the spontaneously developed smooth muscle tone ($n=4$).

Antagonists The proposed VIP antagonist, VIP 10–28 (1–10 μM), did not affect relaxations evoked by EFS (16–30 Hz; $n=4$), or relaxations evoked by exogenously applied VIP. However, treatment with α-chymotrypsin (2 u ml^{-1}), or trypsin (1 μM), slightly reduced the relaxations evoked by EFS ($n=7$; Figure 5), and abolished the relaxations evoked by exogenously applied VIP.

α,β-methylene-ATP (1–10 μM), reactive blue-2 (1–10 μM) or suramin (1–10 μM) did not reduce electrically evoked relaxations. Indomethacin (1–10 μM) did not affect the relaxations evoked by EFS ($n=4$).

Discussion

Inhibition of NANC neurotransmission by UK-14 304 has previously been demonstrated in studies of the canine ileocolic

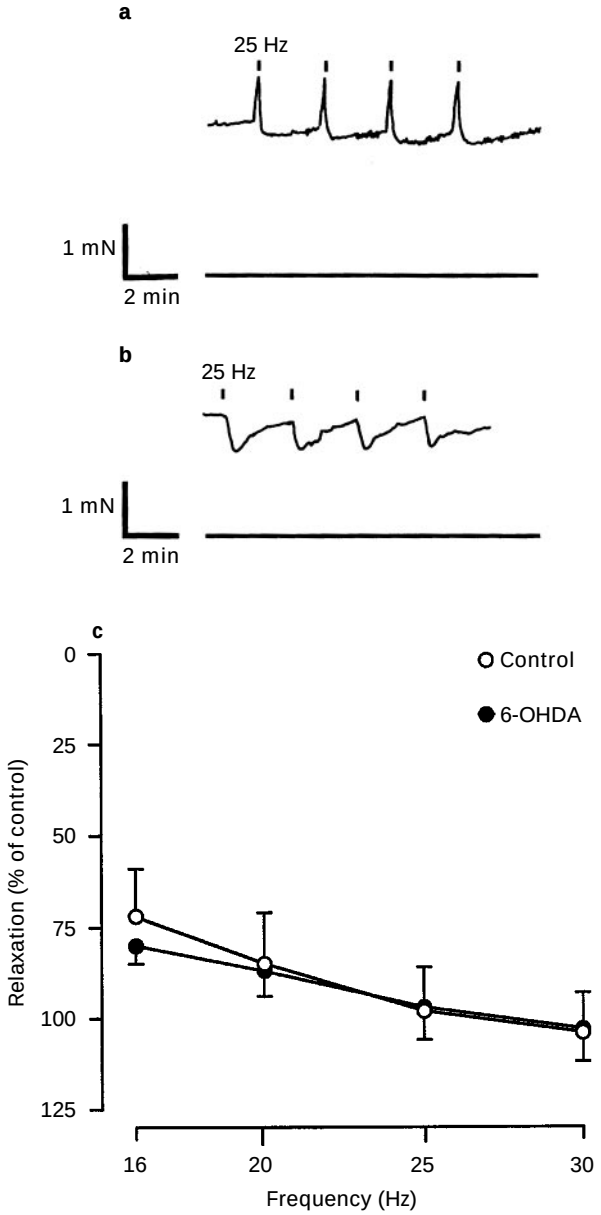

Figure 3 Tracing showing contraction of a urethral smooth muscle strip evoked by electrical field stimulation (EFS; 25 Hz) in the presence of N^G-nitro-L-arginine 0.3 mM and scopolamine 1 μM (a) before and (b) after 15 min treatment with 6-hydroxydopamine (6-OHDA; 0.45 mg ml^{-1}). Lines indicate the maximum relaxation level ('zero level'). (c) Frequency-dependent relaxations evoked by EFS (16–30 Hz) in urethral preparations in the presence of N^G-nitro-L-arginine 0.3 mM, prazosin 1 μM and scopolamine 1 μM before and after treatment with 6-OHDA. Values represent mean and vertical lines show s.e.mean ($n=5$).

junction (Boeckxstaens *et al*., 1993; De Man *et al*., 1994). The inhibitory effects in the present investigation were clearly prejunctional, since the selective α_2-adrenoceptor agonist, UK-14 304, had no effects *per se* on the preparations but the effects were antagonized by the α_2-adrenoceptor antagonist rauwolscine. This implies the presence of α_2-adrenoceptors on the neurones containing the unknown mediator of relaxation. On stimulation, these α_2-adrenoceptors inhibit the release of the mediator, which suggests that the neurones are susceptible to modulation by endogenous noradrenaline.

There is a variety of muscarinic receptor subtypes, localized both pre- and postjunctionally (Caulfield, 1993), which may modulate the responses to acetylcholine released by EFS in the pig urethra (Bridgewater *et al*., 1995). However, receptor stimulation with the non-subtype-selective muscarinic receptor agonist carbachol, did not affect the relaxations evoked by EFS. M_1 muscarinic receptors have been demonstrated to mediate facilitation of acetylcholine release in parasympathetic postganglionic nerve terminals in the rat urinary bladder (Somogyi *et al*., 1994). In the present study, inhibition of M_1 muscarinic receptors with pirenzepine did not affect the relaxations. Presynaptic muscarinic modulation cannot be excluded in this region, but the present data suggest that the presynaptic α_2-adrenoceptors are the more important in regulating the release of the non-nitrergic mediator.

The mechanism behind receptor-activated inhibition of neurotransmitter release might be inhibition of Ca^{2+} channels, or activation of K^+ channels, on the presynaptic membrane (Miller, 1990). We have previously demonstrated the release of the non-nitrergic mediator to be inhibited by ω-conotoxin GVIA (Werkström *et al*., 1995) and, thus, dependent on influx of extracellular Ca^{2+} through N-type voltage-operated Ca^{2+} channels. In the present study, the electrically evoked relaxations were enhanced by treatment with iberiotoxin and charybdotoxin, demonstrating the involvement of high conductance Ca^{2+} activated K^+ channels in the release of the unknown mediator. Inhibition of voltage-sensitive K^+ channels has been shown to potentiate the release of NO in the gastrointestinal tract (De Man *et al*., 1993), but in the pig urethra 4-aminopyridine and dendrotoxin-1 were without effects on the release of the unknown mediator. Zygmunt *et al*. (1996) showed that inhibition of high conductance Ca^{2+} activated K^+ channels did not affect nitrergic neurotransmission in the lamina propria of the female rabbit urethra, whereas iberiotoxin and charybdotoxin enhanced adrenergic transmission. The unknown mediator therefore appears to resemble 'classical' neurotransmitters, probably being stored in synaptic vesicles and released by a Ca^{2+}-dependent exocytotic process (Werkström *et al*., 1995; 1997b).

It is not yet known whether NO and the non-nitrergic mediator of smooth muscle relaxations, demonstrated in for example female pig and dog urethra (Bridgewater *et al*., 1993; Hashimoto *et al*., 1993; Werkström *et al*., 1995), are released from the same or adjacent nerve terminals. Immunohistochemical studies indicate that nitric oxide-synthase (NOS) containing nerve fibres in the lower urinary tract belong to the parasympathetic nervous system (Vizzard *et al*., 1994; Alm *et al*., 1995). As demonstrated in the sheep and rat urethra, chemical sympathectomy with 6-OHDA did not influence the relaxation mediated by NO (Garcia-Pasqual *et al*., 1991; Persson *et al*., 1997). The fact that the relaxations in the present study were unaffected by 6-OHDA, suggests that also the non-nitrergic mediator of relaxation has its origin in non-adrenergic nerves. 6-OHDA abolished the electrically evoked prazosin-sensitive contractions in the pig urethra and revealed a long-lasting relaxation. This suggests that noradrenaline is released at the same frequencies of stimulation as the relaxant mediator. The adrenergic response might have been potentiated, or easier to evoke, if we had been able to block the synthesis and action of the non-nitrergic mediator.

Primary afferent nerve fibres may affect smooth muscle tone by release of transmitters. Capsaicin has been used as a tool to affect selectively a subpopulation of primary afferent fibres and to release sensory neuropeptides (Bevan & Szolcsányi, 1990). In for example the rat external urethral sphincter, activation of capsaicin-sensitive primary afferents has been suggested to cause a local release of CGRP, thereby causing urethral relaxation (Parlani *et al*., 1993). If the unknown transmitter is localized to capsaicin-sensitive nerves, a relaxation in response to capsaicin would have been expected. This was not found. Furthermore, EFS-evoked relaxations were unaffected by capsaicin. Taken together, the non-nitrergic mediator of relaxation does not seem to originate from adrenergic or from capsaicin-sensitive sensory nerve fibres.

Not only ATP, adenosine and 5-HT, but also VIP, and the VIP-related peptides PACAP-27 and PACAP-38, which are

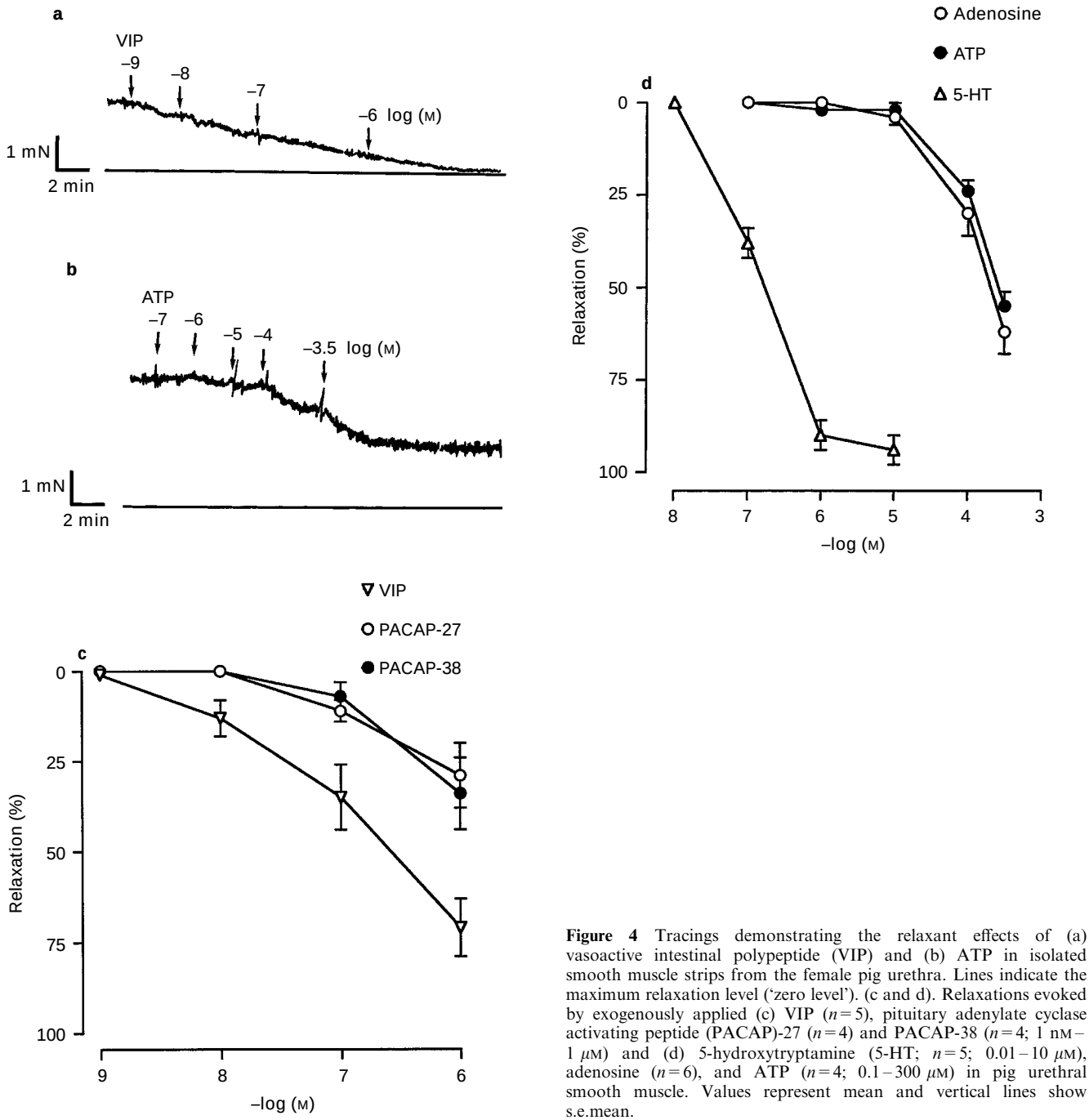

Figure 4 Tracings demonstrating the relaxant effects of (a) vasoactive intestinal polypeptide (VIP) and (b) ATP in isolated smooth muscle strips from the female pig urethra. Lines indicate the maximum relaxation level ('zero level'). (c and d). Relaxations evoked by exogenously applied (c) VIP ($n=5$), pituitary adenylate cyclase activating peptide (PACAP)-27 ($n=4$) and PACAP-38 ($n=4$; 1 nM–1 μM) and (d) 5-hydroxytryptamine (5-HT; $n=5$; 0.01–10 μM), adenosine ($n=6$), and ATP ($n=4$; 0.1–300 μM) in pig urethral smooth muscle. Values represent mean and vertical lines show s.e.mean.

believed to cause relaxation by activating the adenylate cyclase/cyclic AMP-system, induced relaxation of the smooth muscle. Neither the non-selective P_2-purinoceptor blocker suramin, or reactive blue-2, believed to inhibit relaxation mediated by P_{2Y}-purinoceptors (Dalziel & Westfall, 1994) had any effects, making it less likely that the unknown mediator is an ATP-related substance. Although 5-HT induced the most prominent relaxation, the non-nitrergic relaxation is not blocked by a 5-HT neurotoxin or enhanced by a 5-HT uptake blocker (Bridgewater & Brading, 1993). PGE_2 and indomethacin were without effects, probably also excluding one of the prostaglandins as the unknown mediator. These findings are in agreement with those of previous investigations (Hills *et al*., 1984; Klarskov, 1988; Bridgewater & Brading, 1993; Hashimoto *et al*., 1993). A slight inhibition of the electrically induced relaxations was achieved by the unspecific proteases, trypsin and α-chymotrypsin. However, more specific tools are desirable to investigate whether VIP, or a VIP-related peptide, is involved in regulation of pig urethral smooth muscle tone. Another suggested mediator of smooth muscle relaxation is carbon monoxide (CO), synthesized by the enzyme haem oxygenase. CO causes relaxation of pig urethral smooth muscle, but is probably not identical to the nerve-derived unknown transmitter (Werkström *et al*., 1997a).

The common pattern of voiding is an initial drop in urethral pressure, followed by a rise in intravesical pressure as well as a maintained reduction in urethral pressure (Tanagho & Miller, 1970). The rapid relaxation, possibly mediated by NO, may be responsible for the initial drop in urethral pressure, whereas the non-nitrergic mediator may contribute to the maintained decrease in urethral pressure. Speculatively, since the relaxation evoked by NO is short-lasting and NO is rapidly inactivated, presynaptic inhibition of NO-release might be of minor importance. However, after micturition has occurred, excitatory adrenergic nerves are likely to be activated to contract the urethra. It might be that the

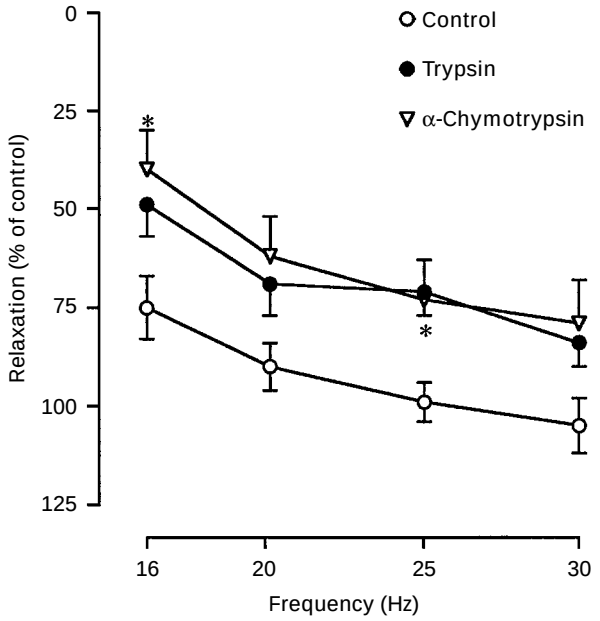

Figure 5 Effects of trypsin (1 μM) and α-chymotrypsin (2 u ml^{-1}) on relaxations evoked by electrical field stimulation (16–30 Hz) in the female pig urethra in the presence of N^{G}-nitro-L-arginine 0.3 mM, prazosin 1 μM and scopolamine 1 μM. Values represent mean and vertical lines show s.e.mean ($n=7$; $^{*}P<0.05$).

noradrenaline released both contracts the urethral smooth directly and activates α_2-adrenoceptors on the neurones containing the unknown mediator of relaxation. Bearing in mind the fact that inhibitory α_2-adrenoceptors are also present on the adrenergic nerves, the muscle response to α_2-adrenoceptor-mediated modulation of transmitter release in the pig urethra is likely to be complex. However, the mechanism of modulating the release of the non-nitrergic mediator might be of physiological significance in order to close the urethra after micturition and, in consequence, to maintain urinary continence.

The present results thus suggest that the release of the unknown mediator in the female pig urethra can be modulated by α_2-adrenoceptor stimulation and by inhibition of high conductance Ca^{2+} activated K^{+} channels. Furthermore, the mediator does not appear to be localized to or released from sympathetic or capsaicin-sensitive sensory nerve-endings. The identity of the mediator remains to be established.

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