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Factors involved in the relaxation of female pig urethra evoked by electrical field stimulation

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1 Non-adrenergic, non-cholinergic (NANC) relaxations induced by electrical field stimulation (EFS) were studied in pig isolated urethra. The mechanism for relaxation was characterized by measurement of cyclic nucleotides and by study of involvement of different subsets of voltage-operated calcium channels (VOCCs).

2 EFS evoked frequency-dependent and tetrodotoxin-sensitive relaxations in the presence of propranolol (1 μM), phentolamine (1 μM) and scopolamine (1 μM). At low frequencies (<12 Hz), relaxations were rapid, whereas at high (>12 Hz) frequencies distinct biphasic relaxations were evoked. The latter consisted of a rapidly developing first phase followed by a more long-lasting second phase.

3 Treatment with the NO-synthesis inhibitor N^G-nitro-L-arginine (L-NOARG; 0.3 mM) inhibited relaxations at low frequencies of stimulation. At high frequencies (>12 Hz) only the first relaxation phase was affected.

4 Measurement of cyclic nucleotides in preparations subjected to continuous nerve-stimulation, revealed an increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels from 1.3 ± 0.3 to 3.0 ± 0.4 pmol mg^{-1} protein ($P<0.01$). In the presence of L-NOARG, there was a significant decrease in cyclic GMP content to control. However, there was no increase in cyclic GMP content in response to EFS. Levels of cyclic AMP remained unchanged following EFS.

5 Treatment with the N-type VOCC-inhibitor, ω-conotoxin GVIA (0.1 μM) reduced NO-dependent relaxations, the effect being most pronounced at low frequencies (1-4 Hz) of stimulation. The NO-independent second phase of the relaxation, studied in the presence of L-NOARG (0.3 mM) at 16-30 Hz, was however markedly reduced or abolished by ω-conotoxin GVIA. ω-Conotoxin MVIIC (1 μM) or ω-agatoxin IVA (30 nM) had no effect on electrically evoked relaxations.

6 These results suggest that NANC-nerve derived urethral relaxation in the pig consists of two apparently independent components. One is mediated by NO and associated with an increase in cyclic GMP content. The other mediator is unknown and produces relaxations not associated with changes in levels of cyclic nucleotides. The release of this mediator seems to involve the N-type VOCC, since the relaxation was markedly reduced or abolished by ω-conotoxin GVIA.

Keywords: NANC neurotransmission; nitric oxide; ω-conotoxin; smooth muscle relaxation; cyclic nucleotides; calcium channels; urethra

Introduction

Urethral smooth muscle tone is considered to be regulated by adrenergic, cholinergic as well as non-adrenergic, non-cholinergic (NANC) excitatory and inhibitory components (Williams & Brading, 1992; Andersson, 1993). Recently it was suggested that nitric oxide (NO) mediated, at least in part, the relaxation of the smooth muscle of the bladder outflow region in several species, including pig (Andersson & Persson, 1994). However, in the pig urethra, relaxation seems to involve more than one transmitter (Bridgewater *et al.*, 1993), since only part of the urethral relaxation was abolished by the NO-synthesis inhibitor, N^G-nitro-L-arginine (L-NOARG). The nature and identity of the presumed mediator is still unknown (Bridgewater *et al.*, 1993). A similar NO-dependent and -independent relaxation pattern has been demonstrated in the dog isolated urethra (Hashimoto *et al.*, 1993). NO is believed to amplify the relaxant effect of vasoactive intestinal peptide (VIP) in the gastric muscle cells (Makhlouf & Grider, 1993). Whether NO is able to act as a modulator of other transmitters also in the lower urinary tract remains to be established.

After release, NO is believed to activate soluble guanylyl cyclase that forms guanosine 3':5'-cyclic monophosphate (cyclic GMP) in adjacent target cells. An increase in cyclic GMP content in response to electrical field stimulation (EFS) and exogenous NO has been shown in rabbit and sheep urethra, suggesting that NO-dependent relaxation in the urethra, at least, is mediated partly via the activation of guanylyl cyclase (Dokita *et al.*, 1994; Garcia-Pasqual & Triguero, 1994; Persson & Andersson, 1994).

The release of classical neurotransmitters is caused by the arrival of a nerve action potential, which allows influx of Ca^{2+} promoting fusion of vesicle membranes (Burks, 1994), an event not believed to be necessary for NO release (Snyder, 1992). Influx of Ca^{2+} in the nerve terminal through voltage-operated calcium channels (VOCCs) can be blocked by ω-conotoxins, which have been used as tools for defining different subtypes of VOCCs. ω-Conotoxin GVIA, a peptide toxin isolated from the venom of the marine snail *Conus geographus*, is a blocker of the N-type VOCC (Olivera *et al.*, 1985). The funnel web spider venom, ω-agatoxin IVA, has been demonstrated to be a potent inhibitor of P-type Ca^{2+} channels (Mintz *et al.*, 1992). Yet another peptide toxin, ω-conotoxin MVIIC, derived from the cone snail *Conus magus*, has been demonstrated to block P-type Ca^{2+} channels in Purkinje cells (Hillyard *et al.*, 1992).

The aim of this study was to investigate further the relaxation induced by electrical stimulation of nerves in the female pig urethra, focusing on the NO-independent part of the

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relaxation. The relaxation was investigated with respect to changes in cyclic nucleotide levels and the possibility of an interplay between NO and the mediator of the NO-independent part of the relaxation. To study the involvement of VOCCs in the release of the unknown transmitter, we investigated the effects of blockers of different subsets of VOCCs, i.e. ω-conotoxin GVIA, ω-conotoxin MVIIC, and ω-agatoxin IVA on the electrically induced relaxation.

Methods

Tissue Preparation

The bladder and urethra from young (6 month old) 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 smooth muscle specimens were taken in a transverse direction from the high pressure zone, approximately 4 cm below the ureteric orifices. Strips of circular smooth muscle were dissected, measuring approximately $1 \times 2 \times 6$ mm.

Experimental procedure

The strips were transferred into 5 ml temperature-controlled tissue baths containing Krebs solution, 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.

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 0.5–30 Hz, was used. The voltage was supramaximal and the train duration 5 s. The stimulation interval was regularly 2 min. The interval was increased if the tension did not reach resting level within the 2 min. Preparations used for measurement of cyclic nucleotides (see below) were subjected to continuous electrical stimulation at 10 Hz for 2 min.

The strips were mounted and stretched to a tension of 10 mN and 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), to establish the 'maximum' relaxation level, i.e. 'zero level'. After addition of Ca^{2+} containing Krebs solution, a stable tone was reestablished.

Propranolol (1 μM), phentolamine (1 μM) and scopolamine (1 μM) were present in the tissue baths in all experiments, and frequency-dependent relaxant responses to EFS (0.5–30 Hz) were recorded. After washout, the NO-synthesis inhibitor N^G-nitro-L-arginine (L-NOARG) 0.3 mM was added to every other bath (the rest serving as controls), 20 min before a second period of EFS ($n=11$, $N=6$).

At the end of the second stimulation period, preparations were stimulated continuously at 10 Hz for 2 min and then rapidly frozen in liquid nitrogen for determination of cyclic nucleotides. The preparations were analysed for cyclic nucleotide content in the presence and absence of L-NOARG, at resting tension or after continuous electrical stimulation (see below).

Relaxant responses to EFS at 1–12 Hz were studied, and after washout some of the preparations were treated with either ω-conotoxin GVIA (0.1 μM), ω-conotoxin MVIIC (1 μM) or ω-agatoxin IVA (30 nM). These concentrations were based on our own preliminary results and previously published data (Zygmunt *et al.*, 1995). Responses to a second period of EFS were studied after 20 min ($n=6$).

In another series of experiments, designed to study the second relaxation phase, the tissues were treated with L-NOARG (0.3 mM) for 20 min. Relaxant responses to EFS at 16 to 30 Hz were studied before and after treatment with ω-conotoxin GVIA (0.1 μM), ω-conotoxin MVIIC (1 μM), or ω-agatoxin IVA (30 nM) ($n=6$).

Determination of cyclic AMP and cyclic GMP levels

The tissue, frozen in liquid nitrogen, was homogenized at 4°C in 2 ml of 10% trichloroacetic acid with a hand glass homogenizer, and centrifuged at 1500 *g* (4°C) for 10 min. The pellet was reconstituted in 2 M NaOH and assayed for protein content (Bradford, 1976), with bovine serum albumin used as a standard. The trichloroacetic acid in the supernatant was removed by five successive extractions with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate and the amount of cyclic GMP or cyclic AMP was quantitated by use of [^{125}I]-cyclic GMP and [^{125}I]-cyclic AMP radioummunoassay kits (DuPont, Wilmington, DEL, U.S.A.) according to kit instructions. All determinations of cyclic nucleotide levels were made in duplicate. Cyclic nucleotides were acetylated with acetic anhydride to increase the sensitivity of the assay. The final values of tissue cyclic AMP were corrected for trace amounts of [^{3}H]-cyclic AMP, used to determine recovery (70%).

Drugs and solutions

The following drugs were used: L-NOARG, scopolamine, phentolamine, (±)-propranolol, tetrodotoxin (Sigma Chemical Company, St Louis, MO, U.S.A.), ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA (Almone Labs LTD, Jerusalem, Israel).

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

Results have been normalized by expressing relaxant effects of EFS as percentage reduction in tension. Normalized results have been expressed as a percentage of the maximum relaxation during the first responses of each preparation, and given as mean values ± s.e.mean. Statistical analysis of data was performed by Student's two-tailed unpaired *t* test and outliers were checked for by Dixon's gap test. A probability value of $P<0.05$ was regarded as significant. *N* denotes the number of animals and *n* the number of preparations. When $n=N$, only *n* is given.

Results

Responses to electrical field stimulation

All preparations developed a spontaneous tone, reaching a maximum of 6.1 ± 0.6 mN from 'zero level'. EFS induced frequency-dependent NANC relaxations (Figure 1). At low frequencies (< 12 Hz) the relaxation was rapid (Figure 1a), and reached a maximum of 69 ± 2% at 6 Hz (Figure 2a).

At frequencies higher than 12 Hz, EFS evoked distinct biphasic relaxations, consisting of a rapid, transient first phase followed by a more long-lasting second phase (Figure 1b). A maximum amplitude for the first phase of 70 ± 2% was obtianed at 16 Hz, wheras a second phase relaxation maximum was obtained at 30 Hz and amounted to 74 ± 3% (Figure 2).

The NO-synthesis-inhibitor, L-NOARG, abolished the EFS-induced relaxations at low frequencies, as well as the first phase of the biphasic relaxation (Figure 1, 2a). The second phase was not significantly affected by treatment with L-NOARG (Figures 1b, 2b). All experiments were abolished by 1 μM tetrodotoxin (TTX; Figure 1b).

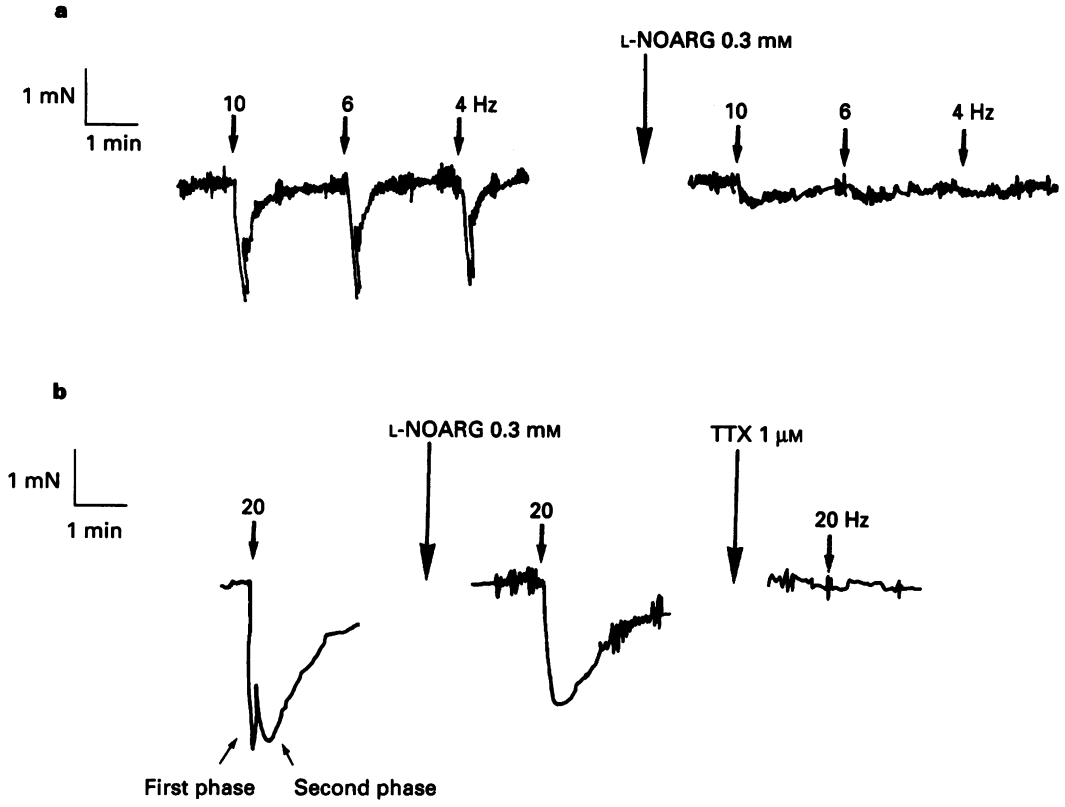

Figure 1 Tracings showing responses of pig urethral smooth muscle strips to EFS (pulse duration 0.5 ms, train duration 5 s, supramaximal voltage) before and after treatment with N^G-nitro-L-arginine (L-NOARG 0.3 mM) (a) at 4–10 Hz and (b) at 20 Hz.

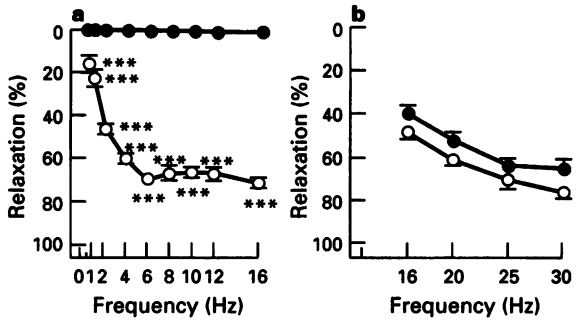

Figure 2 Effects of N^G-nitro-L-arginine (L-NOARG) on nerve-evoked pig urethral relaxation. (a) Shows the first phase of the biphasic relaxation (0.5–16 Hz), and (b) the second phase (16–30 Hz): (●) treatment with L-NOARG 0.3 mM; (○) controls ($n=6$). Each point is expressed as percentage relaxation and represents mean with s.e.mean. ***$P<0.001$.

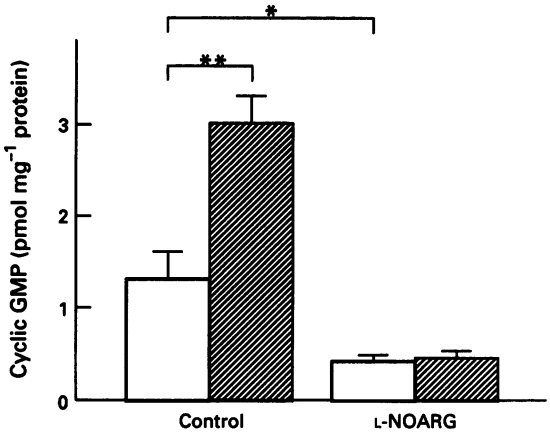

Figure 3 Effects of EFS on tissue levels of cyclic GMP before and after treatment with N^G-nitro-L-arginine (L-NOARG 0.3 mM). Open columns denote cyclic GMP content at resting tension; hatched columns denote cyclic GMP content after EFS for 2 min at 10 Hz ($n=10$). *$P<0.05$, **$P<0.01$.

Levels of cyclic nucleotides

Measurement of cyclic nucleotides in preparations subjected to continuous nerve-stimulation, revealed an increase in cyclic GMP levels from 1.3 ± 0.3 to 3.0 ± 0.4 pmol mg^{-1} protein ($n=10$; $P<0.01$). Treatment with L-NOARG significantly decreased the cyclic GMP content at resting tension from 1.3 ± 0.3 to 0.4 ± 0.1 pmol mg^{-1} protein ($n=10$; $P<0.05$). There was no increase in cyclic GMP content in response to EFS (Figure 3).

Levels of cyclic AMP remained unchanged following nerve stimulation. In the absence of L-NOARG the value was 20 ± 2 ($n=10$) pmol mg^{-1} protein before and after continuous EFS; corresponding values in the presence of L-NOARG were 24 ± 3 ($n=10$) and 24 ± 1 ($n=10$) pmol mg^{-1} protein.

Effects of ω-conotoxins on electrically-induced relaxations

In order to characterize further the EFS-induced NANC relaxations, ω-conotoxin GVIA (0.1 μM) was added to the baths (Figure 4). The toxin reduced NO-dependent relaxations, and the effect was most pronounced at low frequencies of stimulation (1–4 Hz).

The NO-independent second phase of the relaxation was studied in the presence of L-NOARG (0.3 mM). EFS (16–30 Hz) evoked a slow, long-lasting relaxation, which was

markedly reduced or abolished by ω-conotoxin GVIA (0.1 μM) ($n=6$; Figures 5a, 6). ω-Conotoxin GVIA-treatment of strips not exposed to L-NOARG markedly decreased the duration of the relaxation evoked by EFS at high frequencies (>16 Hz; Figure 5b).

ω-Conotoxin MVIIC (1 μM) or ω-agatoxin IVA (30 nM) had no effect on the EFS-induced relaxations, neither in the presence ($n=3$), nor in the absence ($n=3$) of L-NOARG, at any frequency.

Discussion

In the female pig urethra, a high pressure zone at approximately 3 cm below the bladder neck, has been identified by urethral pressure profilometry (Bridgewater *et al.*, 1993). It was also demonstrated that strips dissected from this area developed a spontaneous, myogenic tone. We used circular smooth muscle strips from the mid-urethra, approximately 4 cm below the ureteric orifices, and were able to confirm the occurrence of

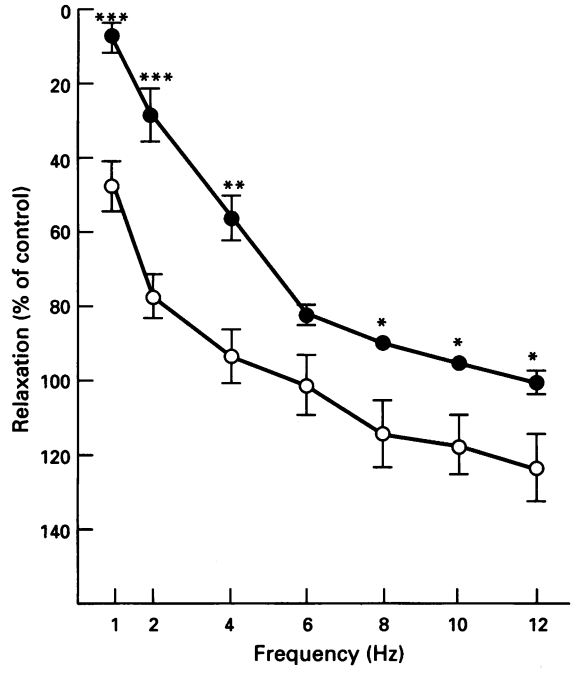

Figure 4 Effect of ω-conotoxin GVIA on the NO-dependent first phase of the relaxation, expressed as percentage of the maximal response before treatment, and given as mean with s.e.mean: (●) treatment with ω-conotoxin GVIA 0.1 μM; (○) controls ($n=6$). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

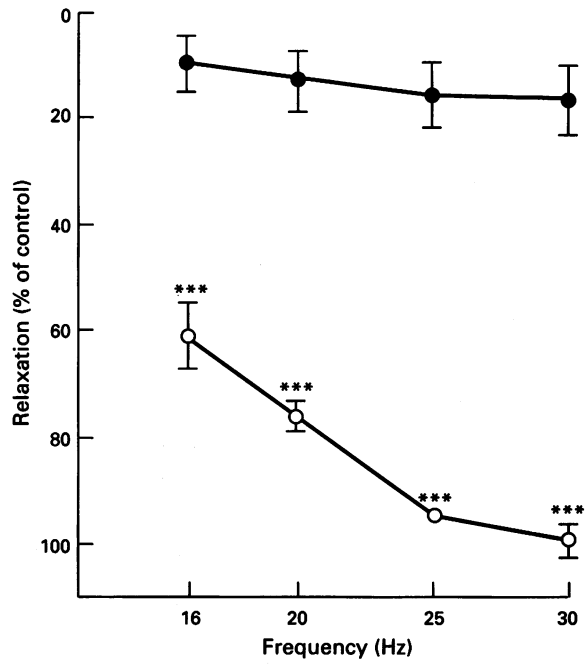

Figure 6 Effect of ω-conotoxin GVIA on the NO-independent second phase of the relaxation evoked by EFS, expressed as percentage of the maximal response before treatment, and given as mean with s.e.mean: (●) treatment with ω-conotoxin GVIA 0.1 μM; (○) controls ($n=5-6$). ***$P<0.001$.

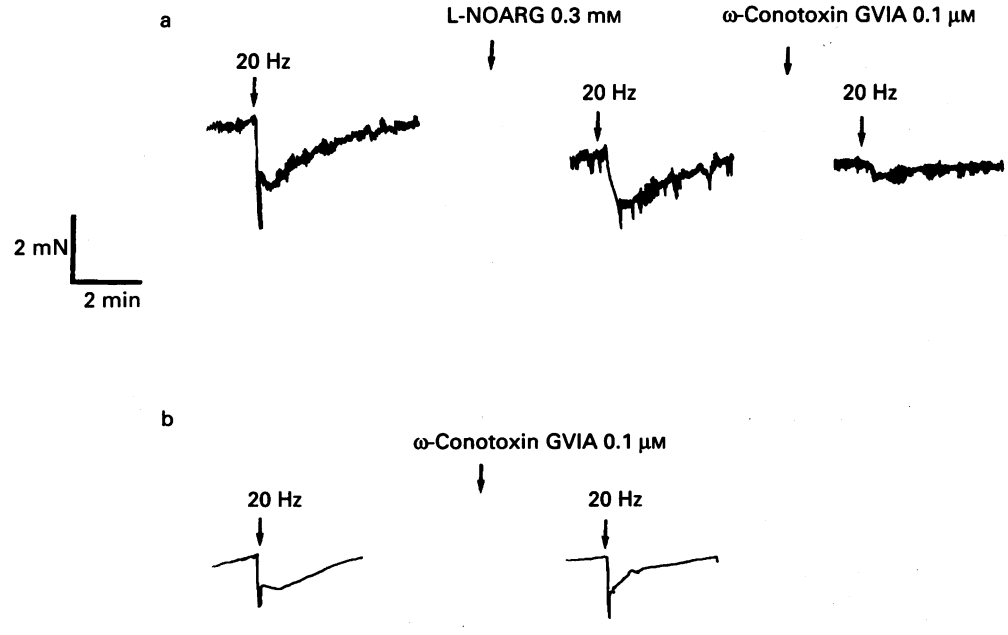

Figure 5 Tracings showing the effects of ω-conotoxin GVIA 0.1 μM on the NO-independent second phase of the electrically evoked relaxation at 20 Hz (a) in the presence of N^G-nitro-L-arginine (L-NOARG 0.3 mM) and (b) in the absence of L-NOARG.

a spontaneous myogenic tone. Such a tone does not seem to be present in, for example, female rabbit or human urethra (Andersson, 1993).

As demonstrated in several species, urethral relaxation is mediated by a NANC mechanism (Williams & Brading, 1992; Andersson, 1993). Nitric oxide has been shown to be the transmitter partly responsible for this NANC relaxation, as was demonstrated in rabbit, pig, rat, sheep, dog and human urethral smooth muscle (Andersson & Persson, 1994). There is, however, evidence suggesting that NO might not be the only transmitter responsible for urethral relaxation. Part of the relaxations in pig (Bridgewater *et al.*, 1993) and dog (Hashimoto *et al.*, 1993), could be abolished by L-NOARG, but a NO-independent mechanism was also demonstrated. When subjected to electrical stimulation at high frequencies, the preparations in this series of experiments responded in a biphasic manner; a rapid first phase, followed by a second more long-lasting phase. Treatment with L-NOARG abolished the first, but not the second phase, suggesting that the second phase was not mediated by NO. However, the second phase was sensitive to TTX, and therefore presumably nerve-derived.

Before the identification of NO as a mediator of NANC-relaxation in the urethra and bladder neck, several other transmitter candidates were investigated. Although 5-hydroxytryptamine (5-HT), vasoactive intestinal polypeptide (VIP), adenosine and adenosine-5'-triphosphate (ATP) produced relaxation in the bladder neck, the electrically evoked relaxation was not blocked by antagonists to these substances (Hills *et al.*, 1984). The application of prostaglandins, VIP, 5-HT or ATP did not mimic the relaxant response evoked by EFS in the pig lower urinary tract (Klarskov, 1988). Similarly, relaxation in the dog urethra was not affected by the presence of 5-hydroxytryptaminergic, histaminergic or purinergic blocking drugs (Hashimoto *et al.*, 1992). Recently, the prolonged NO-independent relaxation in the pig urethra was investigated by using different 5-HT H_1, H_2 or $GABA_B$ receptor antagonists (Bridgewater & Brading, 1993). Certainly, 5-HT relaxes the pig urethra, but the 5-HT uptake blocker, zimelidine, did not enhance the prolonged relaxation, and it was not abolished by the 5-HT neurotoxin, 5,7-dihydroxtryptamine. The presence of prostaglandin synthesis inhibitors did not block the response evoked by EFS, excluding the involvement of prostanoids (Bridgewater & Brading, 1993). VIP produces relaxation in the pig urethra (Klarskov, 1988). However, the prolonged EFS-evoked relaxation was not abolished by chymotrypsin (Bridgewater & Brading, 1993), or by [4-Cl-D-Phe^6,Leu^{17}]VIP, a competitive VIP antagonist (Hashimoto *et al.*, 1993). The fact that [4-Cl-D-Phe^6,Leu^{17}]VIP did not block the relaxant response to exogenously applied VIP in the dog urethra (Hashimoto *et al.*, 1993) suggests that the antagonist is not an appropriate tool. Chymotrypsin, on the other hand, has been demonstrated to block VIP-induced relaxations, whereas relaxations evoked by EFS were not affected (Bridgewater & Brading, 1993), possibly because the molecule is too large to enter the synaptic cleft.

The release of the putative transmitter mediating the second phase in this series of experiments was frequency-dependent, and the relaxation was more pronounced at high frequencies of stimulation. In rat gastric fundus, it was suggested that NO is released in response to EFS at low frequencies, whereas at high frequencies (16 Hz), NO and a peptide, probably VIP, is released (Boeckxstaens *et al.*, 1992). If this is also the case in our preparation, the mediator of the second relaxation phase, appearing at 16–30 Hz, may be a peptide.

Little is known of the postsynaptic mechanisms mediating this relaxation. Measurement of cyclic nucleotides in preparations subjected to electrical stimulation revealed an increase in cyclic GMP levels. In the presence of L-NOARG there was no such increase, suggesting that the increase in cyclic GMP content was dependent on the release of NO. This is in agreement with the findings of other investigators (Persson & Andersson, 1994). In the gut, receptors for the VIP family of peptides are typically linked to activation of adenylate cyclase (Dockray, 1994). Levels of cyclic AMP remained unchanged following nerve stimulation, suggesting that the long-lasting relaxation is not mediated via the activation of adenylate cyclase.

The mechanisms involved in transmitter release are also of interest. In rabbit and dog intestinal smooth muscle, release of VIP is thought to be evoked by depolarizing stimuli through a Ca^{2+}-dependent mechanism (Dockray, 1994). There is, however, evidence suggesting that release of VIP from enteric nerves of rat ileum is independent of extracellular Ca^{2+} (Belai *et al.*, 1987). Our results suggest that the release of the mediator of the long-lasting relaxation is dependent on extracellular Ca^{2+}, since the relaxation was abolished by the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA. ω-Agatoxin IVA and ω-conotoxin MVIIC had no effect on electrically induced relaxations in our preparations. Neither the first, nor the second relaxant phase was influenced. However, it is puzzling that ω-conotoxin MVIIC was without effect, since the toxin has been demonstrated to inhibit, reversibly, N-type Ca^{2+} channels in chick synaptosomes (Grantham *et al.*, 1994). ω-Conotoxin MVIIC has in fact been shown to be even more effective than ω-conotoxin GVIA in inhibiting NANC relaxation in rabbit urethral lamina propria (Zygmunt *et al.*, 1995).

In contrast to its abolition of the long-lasting inhibitory response, ω-conotoxin GVIA was less effective in inhibiting the NO-dependent relaxation. The responses to low frequencies of stimulation (1–4 Hz) were greatly reduced, but at higher frequencies the responses in the presence of ω-conotoxin GVIA were reduced by only about 20%. Other investigators have found similar results (Boeckxstaens *et al.*, 1993; Zygmunt *et al.*, 1993). Zygmunt *et al.* (1993) also showed that NANC relaxation of the urethra was less inhibited by ω-conotoxin than were the responses evoked by release of the more 'classical' transmitters such as noradrenaline and acetylcholine. The differential sensitivity of the two inhibitory transmitters to ω-conotoxin GVIA might indicate that they are localized in different nerve terminals. Histological indentification of NOS and different peptide-containing nerves in the pig urethra has been obtained by Persson *et al.* (1995). These authors showed that occasionally, peptides and NOS are co-localized within the same nerves, which may favour co-transmission, and the possibility that some modulatory interaction between them may occur. NO is believed to act as a modulator of VIP release in the gastrointestinal tract (Makhlouf & Grider, 1993), or at least be released in parallel to VIP (Keef *et al.*, 1994). Although at frequencies greater than 10 Hz, simultaneous release of NO and the second transmitter occurred in our preparations, a modulatory effect of NO was not apparent, since the second relaxant phase was not significantly affected when NO synthesis was prevented by the addition of L-NOARG.

Why should the urethral inhibitory system be composed of two components, different both with respect to release of mediators and to mechanism of action? 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). It cannot be excluded that the rapid NO-dependent relaxation is associated with the prevoiding reduction in urethral pressure, and that the long-lasting relaxation contributes to the maintainance of a low urethral pressure during micturition.

These results suggest that NANC-nerve derived pig urethral relaxation is partly NO-dependent, and partly dependent on an as yet unidentified mediator. NO-dependent relaxations are associated with an increase in cyclic GMP content, whereas relaxations produced by the unknown mediator are not associated with changes in levels of cyclic nucleotides. The release of the unknown mediator is dependent on influx of Ca^{2+} through the N-type VOCC, since the relaxation was markedly reduced or abolished by ω-conotoxin GVIA. The identity of this unknown transmitter remains to be established.

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