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Characterization of the non-nitrergic NANC relaxation responses in the rabbit vaginal wall

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1 Electrical field stimulation (EFS)-induced non-adrenergic non-cholinergic (NANC) relaxation responses in the rabbit vaginal wall were investigated.

2 These NANC responses were partially inhibited with the nitric oxide synthase (NOS) inhibitors N^G-nitro-L-arginine methyl ester (L-NAME; 500 μM), N^G-nitro-L-arginine (300 μM) or N-iminoethyl-L-ornithine (500 μM) or the selective soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 10 μM). Application of L-NAME and ODQ concomitantly did not increase the degree of inhibition.

3 L-NAME or ODQ were observed to be more effective at low frequencies. The resistant part of the responses was more pronounced at higher frequencies and was completely inhibited by tetrodotoxin (1 μM).

4 Exogenous application of the peptides vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP-27 and PACAP-38), peptide histidine methionine (PHM), peptide histidine valine (PHV), helospectin-I or -II induced a relaxation response. Calcitonin gene-related peptide or substance P did not cause any relaxation.

5 The peptidase α-chymotrypsin (type II; 2 units ml^{-1}) did not affect non-nitrergic NANC responses, although it did inhibit relaxation responses elicited by exogenous VIP, PACAP-27, PACAP-38, PHM, PHV, helospectin-I or -II.

6 K^+ channel inhibitors apamin (1 μM) or charybdotoxin (100 nM) when used alone or in conjunction did not affect non-nitrergic NANC responses.

7 The non-nitrergic NANC responses were not associated with any increase in intracellular cyclic adenosine-3′, 5′-monophosphate (cyclic AMP) or cyclic guanosine-3′, 5′-monophosphate (cyclic GMP) concentrations. The peptide-induced relaxations were all associated with increases in cyclic AMP concentrations.

8 These results suggest that a neuronal factor elicits non-nitrergic NANC responses in the rabbit vaginal wall. The identity of this factor remains to be established.
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Keywords: Nitric oxide; non-adrenergic; non-cholinergic; rabbit; vagina; vasoactive intestinal peptide

Abbreviations: ATP, adenosine triphosphate; cyclic AMP, cyclic adenosine-3′, 5′-monophosphate; cyclic GMP, cyclic guanosine-3′, 5′-monophosphate; CGRP, calcitonin gene-related peptide; DETA NONOate, (2,2′-hydroxynitrosohydrazino) bis-ethanamine; EFS, electrical field stimulation; L-NA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; L-NIO, N-iminoethyl-L-ornithine; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; PACAP, pituitary adenylate cyclase activating peptide; PHM, peptide histidine methionine; PHV, peptide histidine valine; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide

Introduction

Vascular and non-vascular smooth muscle relaxation in the vaginal wall is important in sexual function, since it allows increased blood flow into the tissue, and enlargement of the vaginal canal (Park *et al*., 1997). The tone of vaginal smooth muscle is regulated by autonomic neurotransmitters. When noradrenergic and cholinergic pathways are blocked *in vitro* and the tissue tone is raised, electrical field stimulation (EFS) reveals an inhibitory non-adrenergic, non-cholinergic (NANC) relaxation response (Gillespie, 1972; Cellek *et al*., 1999).

Nitric oxide (NO) is a well-characterized neurotransmitter in the central and peripheral nervous system and mediates the NANC relaxation responses in many tissues in the genitourinary system including those of the clitoral and penile corpora cavernosae (Gillespie *et al*., 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Ignarro *et al*., 1990; Cellek & Moncada, 1997; 1998). Nerves that release NO are now known as nitrergic (Moncada *et al*., 1997). NO is generated in these nerves by activation of the neuronal nitric oxide synthase (nNOS) and diffuses into the smooth muscle and activates soluble guanylate cyclase, producing an increase in the intracellular cyclic guanosine-3′, 5′-monophosphate (cyclic GMP) concentration, leading to relaxation (for review, see Moncada *et al*., 1991).

Previous studies have shown the presence of nNOS (Grozdanovic *et al*., 1994), and the peptides vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), peptide histidine methionine (PHM), peptide histidine valine (PHV), calcitonin gene-related

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peptide (CGRP) and helospectin-I and -II in nerve fibres innervating the vagina (Graf *et al*., 1995; Hoyle *et al*., 1996). However functional evidence for the role of these neuropeptides has been limited to observations following exogenous application (Ottesen *et al*., 1987); to our knowledge there is no evidence for the endogenous release of any neuropeptide to elicit NANC responses in the vagina. We previously reported that nitrergic neurotransmission is partially responsible for the NANC relaxation in the rabbit vaginal wall (Cellek *et al*., 1999). We have therefore decided to investigate the NANC responses in detail in this tissue.

Methods

Tissue preparation

Female New Zealand white rabbits (3.6 ± 0.5 kg, range 3.0–5.2 kg, $n=120$, Harlan, U.K.) were sacrificed by an overdose of pentobarbitone (Euthatal, Rhône Merieux, U.K.) injected into the ear marginal vein. The vaginal canal including the clitoris was excised down to the pubic bone and transferred to modified Krebs' solution consisting of (mM): NaCl 136.9, KCl 2.7, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, $CaCl_2$ 1.8, glucose 12.5, dexamethasone 0.01, indomethacin 0.01. The inducible isoform of NOS (iNOS) can be induced by trace amounts of endotoxin in the buffer (Rees *et al*., 1990). Since we wanted to study nNOS in isolation, dexamethasone (10 μM) was added to the Krebs' solution to prevent induction of iNOS. We also added the cyclooxygenase inhibitor indomethacin (10 μM) to prevent synthesis of prostaglandins, since these can cause non-neurogenic relaxations (Daniel *et al*., 1979). The modified Krebs' solution was kept at room temperature and gassed with 95% O_2/5% CO_2. The vaginal canal was carefully opened, the clitoral body removed and four longitudinal strips of vaginal wall (2×8 mm) were dissected free of connective tissue. For experiments involving the clitoral corpus cavernosum, the tunica albuginea of the clitoral body was cut open to reveal the corpus cavernosum. Two pieces of cavernous tissue (2×6 mm) were isolated using fine scissors. The ends of the strips were tied with silk suture and mounted horizontally between two platinum electrodes in superfusion chambers continually perfused at 1 ml min^{-1} with modified Krebs' solution at 37°C as described previously for penile corpus cavernosum (Cellek & Moncada, 1997).

For experiments using circular strips of rat vaginal wall female, mature female Sprague-Dawley rats (290 ± 49 g, range 205–400 g, $n=4$, Charles River, U.K.), were sacrificed by stunning followed by cervical dislocation. The vaginal canal was excised and placed in modified Krebs' solution as above. Circular vaginal strips (1×7 mm) were taken from the distal region of the vagina and dissected free of connective tissue. The strips were tied and mounted in the perfusion chambers as for the rabbit tissue strips.

Measurement of responses

One end of the preparation was tied to a Grass FT03C force-displacement transducer connected to a Linearcorder WR3101 (Graphtec, U.K.) for measurement of isometric changes in tension. The mechanical responses were also recorded on a computer running specialized software (Axotape, Axon Instruments, U.S.A.). The preparations were stretched to approximately their *in situ* length by applying tension of 0.4 g and allowing to equilibrate for 90 min. The preparations were either stimulated by infusion of drugs or by EFS. The drugs were introduced either by addition to the reservoir feeding the superfusion chamber or by injection into the perfusate at a rate of 100 μl min^{-1} using a syringe pump (Harvard Apparatus Model '22', U.K.). EFS was applied as 5 s trains of rectangular pulses of 50 V, 0.3 ms pulse duration, 1–50 Hz, delivered by Grass S88 stimulators every 120 s.

Measurement of intracellular cyclic nucleotide concentrations

Modified perfusion chambers in which the tissues were accessible from above (Cellek *et al*., 1996) were used in experiments to evaluate intracellular concentrations of cyclic adenosine-3′, 5′-monophosphate (cyclic AMP) or cyclic guanosine-3′, 5′-monophosphate (cyclic GMP). The tissues were set up as above, and were incubated for 20 min with the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) before being freeze-clamped either under basal conditions or at the peak of their relaxation response to EFS or drugs. The samples were then homogenized in a stainless steel pestle and mortar on dry ice. Each sample was then incubated in 1 ml of 0.5 M perchloric acid for 1 h on ice then sonicated for 5 s at 4°C (18 microns using Soniprep-150). The samples were centrifuged (10 min, $10{,}000 \times g$, 4°C), and supernatants used for cyclic nucleotide assays and for measuring soluble protein content (BCA protein assay kit, Pierce, U.S.A.). For measurement of intracellular cyclic nucleotide concentrations, 450 μl of the supernatant was neutralized with 300 μl of 1 M K_3PO_4. The sample was centrifuged ($8000 \times g$, 5 min, 4°C); the supernatant was recovered, lyophilized and assayed for cyclic AMP and cyclic GMP content using specific enzyme immunoassay systems (Amersham Pharmacia, U.K.).

Chemicals

PACAP-27 and PACAP-38 were purchased from Calbiochem, U.K. PHM was purchased from Bachem, U.K. PHV and helospectin-I and -II were synthesized by the Scientific Support Services in the Wolfson Institute for Biomedical Research. All other chemicals were from Sigma, U.K. and were dissolved in distilled water, with the exception of indomethacin (5 mg ml^{-1} in 5% $NaHCO_3$), dexamethasone (10 mg ml^{-1} in ethanol), 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; 10 mM in DMSO), N^G-nitro-L-arginine (L-NA; 200 mM in 1 M NaOH) and isobutylmethylxanthine (500 mM in DMSO).

Analysis of the results

In experiments to establish frequency dependence of the responses, duration and magnitude of relaxation were taken into account by measuring the relaxation as the area above the trace from the start of stimulation until the tissue

returned to resting tone and expressing it as a percentage of the maximum relaxation to 50 Hz EFS-induced relaxation (calculated using Clampfit Software, Axon Instruments, U.S.A.).

Over long periods, loss of phenylephrine-induced tone was observed in the vaginal wall. To enable us to compare responses over these periods, EFS-induced relaxations were measured as a percentage of the tone at the time of the relaxation. The effects of inhibitors were expressed as percentage of control relaxation prior to addition of the inhibitor. The following equation was used:

$$\text{Per cent of relaxation unaffected by inhibitor} = 100 - ((\text{magnitude of relaxation at time t (mN)} \div \text{tone at time t (mN)}) \div (\text{magnitude of control relaxation (mN)} \div \text{control tone(mN)} \times 100) \quad (1)$$

Calculations of EC_{50} values for responses to phenylephrine and IC_{50} values for relaxation responses to peptides were performed using Origin software (OriginLab Corporation, U.S.A.). Tension changes in tissue strips were measured in mN.

Statistics

Results are expressed as mean values ± standard error of mean from a number (n) of tissue strips. Small n denotes the number of tissue strips used, and capital N denotes the number of animals used for each set of experiments. Statistical analyses were performed using Prism v3.0 software (GraphPad Software Inc, U.S.A.). Data were compared as appropriate by Student's unpaired t-test or one-way analysis of variance (ANOVA) followed by Dunnett test. P values of less than 0.05 were considered significant.

Results

Effect of NOS inhibitors and ODQ on EFS-induced relaxations in the rabbit vaginal wall

In the rabbit vaginal wall in which cholinergic and noradrenergic pathways were blocked with scopolamine (10 μM) and guanethidine (10 μM) respectively, the tone was raised with 1 μM phenylephrine ($EC_{50} = 673 \pm 54$ nM, $n/N = 5/4$, data not shown). EFS of phenylephrine-contracted vaginal wall elicited relaxations that were frequency-dependent. EFS at 1, 2.5, 5, 10, 25 and 50 Hz produced 32.1 ± 3.0%, 38.7 ± 0.7%, 52.4 ± 1.7% 75.9 ± 1.8%, 91.4 ± 1.0% and 100% relaxations respectively (Figure 1, $n/N = 6/6$). In all further experiments 5 Hz was used unless otherwise stated, as this frequency was producing 50% of the maximum response.

NOS inhibitors N^G-nitro-L-arginine (L-NA; 1 – 300 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 1 – 500 μM) and N-iminoethyl-L-ornithine (L-NIO; 1 – 500 μM) or the soluble guanylate cyclase inhibitor ODQ (0.1 – 10 μM) caused partial inhibition of these relaxations in a concentration-dependent manner (Figures 2 and 3). 72.02 ± 1.8%, 81.1 ± 2.1%, 74.4 ± 6.7% and 68.7 ± 1.8% of the relaxation responses were unaffected by 500 μM L-NAME ($n/N = 15/15$), 300 μM L-NA ($n/N = 4/4$), 500 μM L-NIO ($n/N = 4/4$) and 10 μM ODQ ($n/N = 5/4$) respectively.

NOS inhibitors or ODQ were observed to be more effective at low frequencies. After L-NAME (500 μM) or ODQ (10 μM)

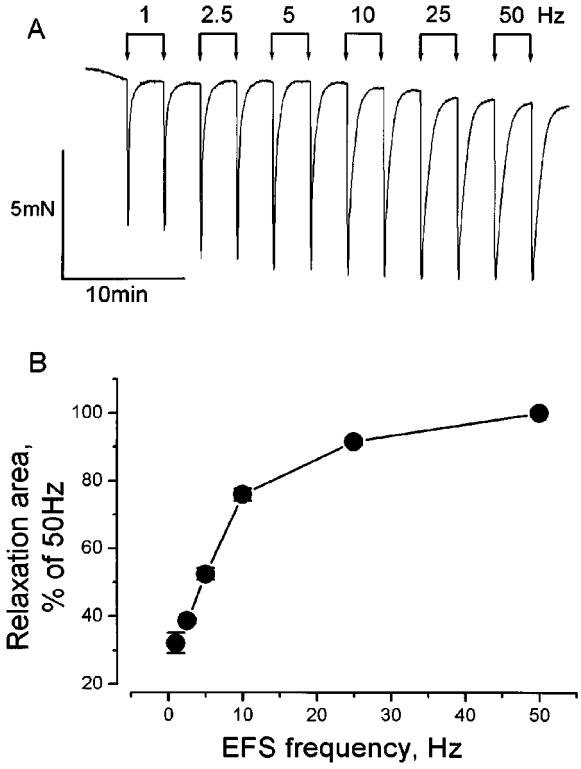

Figure 1 NANC relaxation responses elicited by EFS (indicated by arrows) were frequency-dependent. The mechanogram (A) is an original recording of a single tissue preparation and is representative of all the experiments in this series ($n/N = 6/6$). The frequency-dependence of the relaxation magnitude and duration is expressed as the area of the relaxation as a percentage of relaxation elicited by the maximum frequency used (50 Hz). Data points represent mean ± s.e.mean (B).

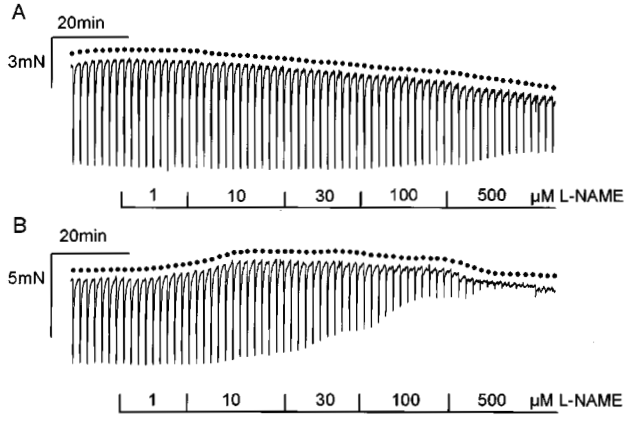

Figure 2 Vaginal wall and clitoral corpus cavernosum strips from the same animals were treated simultaneously for comparison. NANC relaxation responses elicited by EFS (5 Hz, every 2 min, indicated by dots) were partially inhibited by L-NAME (1 – 500 μM) in the vaginal wall (A) but completely abolished in the clitoral corpus cavernosum (B). The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series ($n/N = 4/4$).

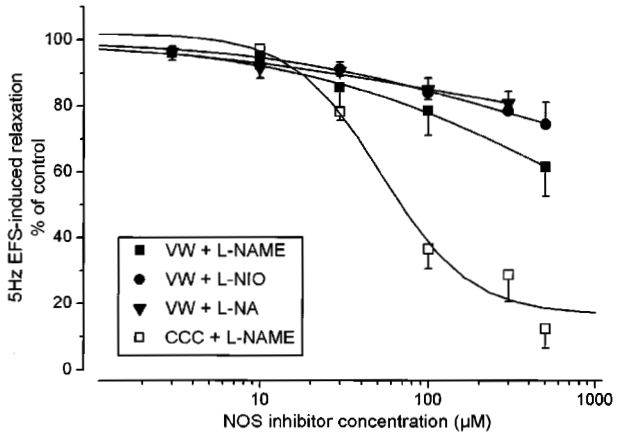

Figure 3 Concentration-response curves showing the effect of the NOS inhibitors L-NAME, L-NA and L-NIO on 5 Hz EFS-induced relaxations in the vaginal wall (VW), and the effect of L-NAME on 5 Hz EFS-induced relaxation responses in the clitoral corpus cavernosum (CCC). Data points represent mean ± s.e.mean ($n/N = 4/4$ for each NOS inhibitor).

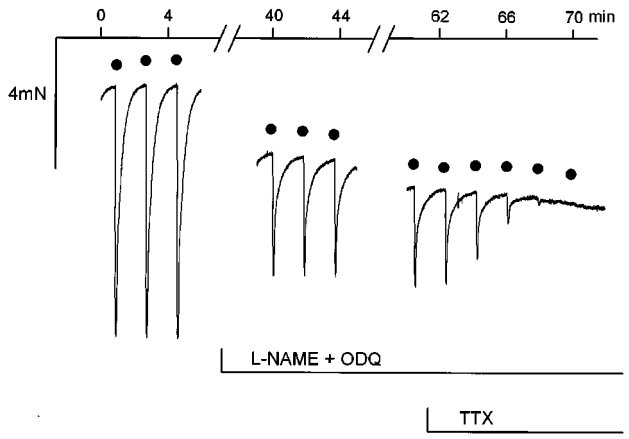

Figure 4 NANC responses elicited by EFS (5 Hz, every 2 min, indicated by dots) were partially inhibited by a combination of L-NAME (500 μM) and ODQ (1 μM). Addition of the sodium channel blocker TTX (1 μM) completely abolished the responses. The mechanogram is an original recording of a single tissue preparation and is representative of all experiments in this series ($n/N = 4/4$).

43.0 ± 10.8% or 24.6 ± 6.3% of relaxation were unaffected respectively at 1 Hz, and at 10 Hz 81.3 ± 6.8% or 65.4 ± 7.6% were unaffected respectively ($n/N = 4/4$ for both L-NAME and ODQ; $P < 0.05$ L-NAME 1 Hz *vs* 10 Hz; $P < 0.01$ ODQ 1 Hz *vs* 10 Hz; $P > 0.05$ L-NAME *vs* ODQ at any frequency).

As a control for the efficacy of the NOS inhibitors, the compounds were used simultaneously to inhibit NANC relaxation responses of the vaginal wall and clitoral corpus cavernosum from the same animal. Responses of the clitoral cavernosum were completely abolished by L-NAME (1–500 μM), but those of the vaginal wall were only partially inhibited ($n/N = 4/4$; Figures 2 and 3).

Effect of tetrodotoxin on L-NAME and ODQ resistant EFS-induced relaxations in the rabbit vaginal wall

When L-NAME and ODQ were used in combination, the resultant inhibition was no greater than the inhibition with either agent alone. After combination of L-NAME (500 μM) and ODQ (1 μM, $n/N = 9/4$), L-NAME alone (500 μM, $n/N = 15/15$) or ODQ alone (10 μM, $n/N = 5/4$), 70.3 ± 4.0%, 72.0 ± 1.8%, and 68.7 ± 1.8% of the relaxation responses were unaffected respectively ($P > 0.05$). Treatment of the tissue with tetrodotoxin (TTX, 1 μM) completely abolished EFS-induced non-nitrergic relaxation responses (Figure 4; $n/N = 4/4$ for TTX after L-NAME + ODQ; $n/N = 8/4$ for TTX after L-NAME alone).

Effect of α-chymotrypsin on EFS-induced, and exogenous peptide-induced relaxation in the rabbit vaginal wall

Addition of VIP (0.001 – 3 μM, $n/N = 4/4$), PACAP-27 (0.001 – 5 μM, $n/N = 5/4$), PACAP-38 (0.001 – 3 μM, $n/N = 4/4$), PHM (0.001 – 5 μM, $n/N = 5/4$), PHV (0.001 – 3 μM, $n/N = 4/4$), Helospectin I (0.001 – 1 μM, $n/N = 6/4$) and Helospectin II (0.001 – 3 μM, $n/N = 4/4$) in cumulative concentrations induced relaxation responses in a concentration-dependent manner (Figures 5 and 6A). The efficacy and potency of the different peptides (efficacy was measured as the relaxation induced by the highest concentration of peptide compared to that induced by 5 Hz EFS-induced relaxation and potency was represented by IC_{50} values) are compared in Table 1.

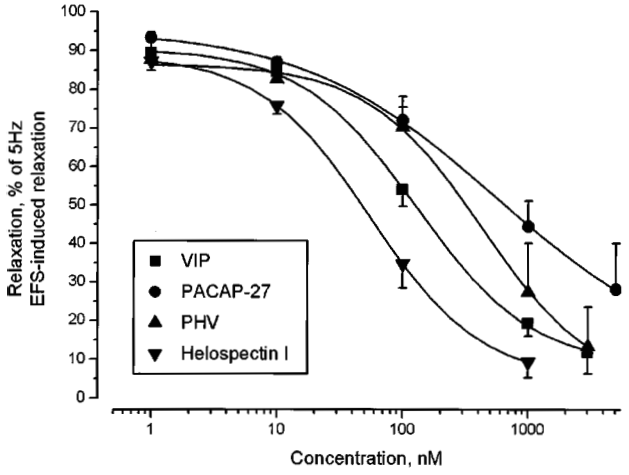

Figure 5 Concentration-response curves showing the relaxant effect of the peptides VIP ($n/N = 4/4$), PACAP-27 ($n/N = 5/4$), PHV ($n/N = 4/4$) and Helospectin I ($n/N = 6/4$). Relaxations are expressed as a percentage of 5 Hz EFS-induced relaxations in the absence of peptides. Data points represent mean ± s.e.mean.

In the presence of α-chymotrypsin, relaxation responses to cumulative concentrations of exogenous peptides were not observed (Figure 6B for helospectin-II, $n/N = 4/4$). The presence of α-chymotrypsin lowered the tone of the tissue but had no effect on EFS-induced non-nitrergic relaxations (Figure 6C, $n/N = 4/4$).

When the peptides VIP (300 nM) and PACAP-27 (400 nM) were added into the superfusion medium as a bolus injection, a marked relaxation response was observed ($n/N = 5/4$ for VIP, $n/N = 4/4$ for PACAP-27, Figure 7). These relaxations were completely abolished when the peptidase α-chymotrypsin was added into the superfusion medium (type-II; 2 units ml^{-1}; Figure

7). After removal of the peptidase from the medium, further relaxations to VIP and PACAP were obtainable (Figure 7).

Effect of substance P and CGRP on rat and rabbit genital tissue

Exogenous application of CGRP (0.1 – 1 μM) or substance P (1 μM) had no effect on rabbit vaginal wall tension in the absence or presence of L-NAME (500 μM; $n/N = 7/5$ for CGRP, $n/N = 9/5$ for substance P; Figure 8A for CGRP in the absence of L-NAME). As a positive control for the efficacy of CGRP circular strips of rat vaginal wall were used as these have previously been shown to be relaxed by CGRP (Giraldi *et al.*, 2001). In this preparation 200 nM CGRP caused marked relaxation in the absence or presence of L-NAME (500 μM; $n/N = 6/4$; Figure 8B in the absence of L-NAME). In the rabbit clitoral corpus cavernosum CGRP (100 nM, $n/N = 6/4$) and substance P (1 μM, $n/N = 4/4$) caused relaxation and contraction respectively (data not shown).

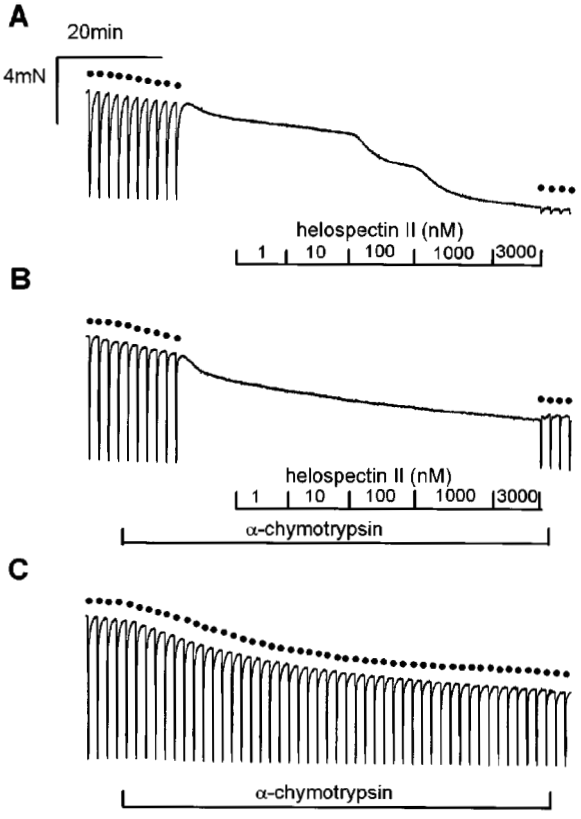

Figure 6 After treatment of the tissue with guanethidine, scopolamine, phenylephrine and L-NAME, exogenous application of VIP (A, 300 nM for 4 min, $n/N = 5/4$) or PACAP-27 (B, 400 nM for 4 min, $n/N = 4/4$) produced relaxations that were completely inhibited by α-chymotrypsin type II (2 units ml^{-1}). Removal of the peptidase restored the relaxation responses. α-chymotrypsin did not affect EFS- (5 Hz, every 2 min, indicated by dots) induced non-nitrergic NANC relaxations. The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series.

Effect of potassium channel inhibitors on non-nitrergic NANC relaxations in the rabbit vaginal wall

When the potassium channel blocker apamin (1 μM) was added into the superfusion medium, either individually or in combination with charybdotoxin (100 nM) in the presence of L-NAME (500 μM), a decrease in tone occurred. However no effect was observed on the non-nitrergic relaxations ($n/N = 6/4$; Figure 9).

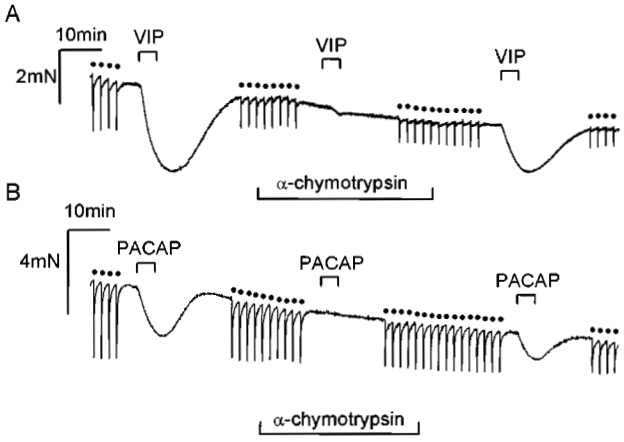

Figure 7 After treatment of the tissue with guanethidine, scopolamine, phenylephrine and L-NAME, exogenous application of cumulative concentrations of helospectin-II (1 nM – 3 μM) resulted in relaxation responses (A). These responses were inhibited in the presence of α-chymotrypsin type II (2 units ml^{-1}), B). EFS- (5 Hz, every 2 min, indicated by dots) induced relaxations were not inhibited by α-chymotrypsin (C). The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series ($n/N = 4/4$).

Table 1 Efficacy and potency of different peptides in producing relaxation responses in the the rabbit vaginal wall

Peptide	*Concentration range* (μM)	*Maximum relaxation* (% of 5 Hz EFS-induced relaxation ± s.e.)	*IC_{50} ± s.e.* (μM)	n/N
VIP	0.001 – 3	88.2 ± 5.5	0.13 ± 0.01	4/4
PACAP-27	0.001 – 5	72.0 ± 12.1	0.54 ± 0.11	5/4
PACAP-38	0.001 – 3	83.1 ± 7.3	0.21 ± 0.07	4/4
PHM	0.001 – 5	68.2 ± 11.3	3.18 ± 0.03	5/4
PHV	0.001 – 3	86.7 ± 10.2	0.42 ± 0.12	4/4
Helospectin I	0.001 – 1	90.8 ± 3.9	0.13 ± 0.07	6/4
Helospectin II	0.001 – 3	91.8 ± 5.8	0.23 ± 0.01	4/4

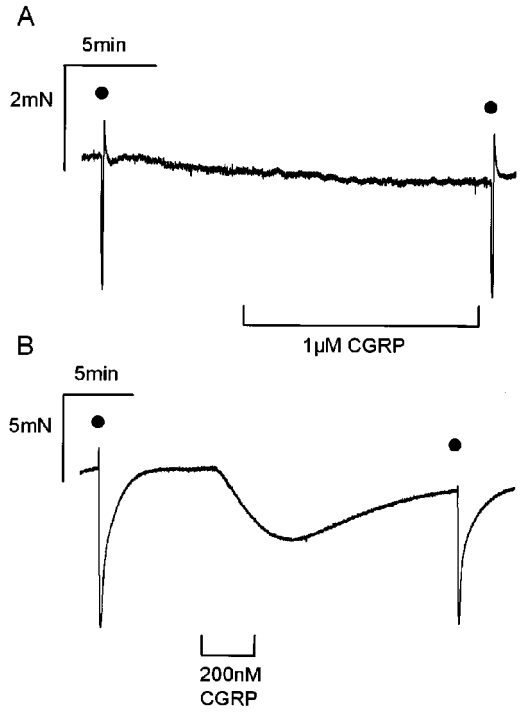

Figure 8 Exogenous application of CGRP has no effect on the rabbit vaginal wall (A, 1 μM; $n/N=7/5$). In the rat vaginal wall exogenous application of CGRP (B, 200 nM; $n/N=6/4$) induces a relaxation response. EFS (5 Hz, 5 s) is indicated by dots. The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series.

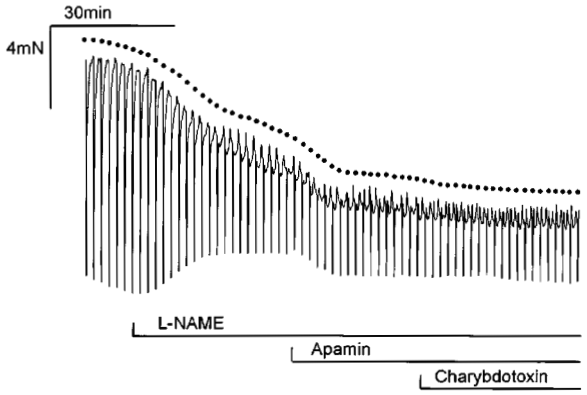

Figure 9 After treatment with L-NAME (500 μM), combination of apamin (1 μM) and charybdotoxin (100 nM) had no effect on NANC relaxations in the rabbit vaginal wall. The mechanogram is an original recording of a single tissue preparation and is representative of all experiments in this series ($n/N=6/4$).

Measurement of changes in intracellular cyclic nucleotide concentrations in the rabbit vaginal wall

Rabbit vaginal wall strips were frozen under basal conditions, when stimulated by EFS in the absence and presence of L-NAME (500 μM), in the presence of peptides (all at 400 nM), the NO donor DETA NONOate (250 μM) or the adenylate cyclase activator forskolin (10 μM). Measurement of changes in the intracellular concentrations of cyclic nucleotides showed that concentrations of cyclic GMP were unaltered except when stimulated by EFS, or in the presence of DETA NONOate. EFS-induced increases in cyclic GMP were completely abolished by L-NAME. EFS did not cause any changes in concentrations of cyclic AMP, but VIP, PACAP-27, PACAP-38, PHM, PHV, helospectin I and II and forskolin all caused some increase in cyclic AMP concentrations ($n/N=4/4$ for each treatment; Figure 10).

Discussion

The female sexual response is a multifactorial event involving both psychological and physiological responses. The three physiological components of the female genital sexual response: vaginal wall engorgement, increased lubrication, and clitoral erection are all associated with changes in smooth muscle tone. Neurogenic control of smooth muscle tone is mediated by cholinergic, noradrenergic and NANC neurotransmission. In the clitoral corpus cavernosum NANC neurotransmission leads to relaxation that can be abolished by NOS inhibitors or a soluble guanylate cyclase inhibitor indicating that NO is the sole neurotransmitter responsible for the relaxations that lead to clitoral erection *in vitro* (Cellek & Moncada, 1998). In our current study we have shown that neurogenic relaxation of the rabbit vaginal wall is partially mediated by NO, since partial inhibition of the response was achieved using either the NOS inhibitors or a selective inhibitor of soluble guanylate cyclase or a combination of the two. The degree of inhibition attained with any of the NO-cyclic GMP pathway inhibitors was no greater than 30%, suggesting that the non-nitrergic component is the major part of NANC relaxation responses in the rabbit vagina.

The existence of a non-nitrergic NANC neurotransmitter in the smooth muscle of another urogenital organ, the urethra, has been demonstrated (Bridgewater *et al*., 1993; 1995; Werkström *et al*., 1995) in the female pig. In this tissue high frequency stimulation (>12 Hz) elicited non-nitrergic NANC relaxation responses (Werkström *et al*., 1995). Similar responses have been reported in the guinea-pig trachea (Moffatt *et al*., 1999). This suggested that a neuropeptide might mediate these responses since neuropeptides are known to be released following high frequency stimulation (Lundberg, 1996). Indeed, the peptidase α-chymotrypsin has been shown to attenuate the responses in the guinea-pig trachea (Moffatt *et al*., 1999) and in the female pig urethra (Werkström *et al*., 1997). In the current study non-nitrergic NANC responses in the rabbit vaginal wall were inhibited with TTX and were greater in magnitude and duration at high frequencies, as in the pig urethra and guinea-pig trachea, thus we hypothesized that they were mediated by a neuropeptide. We therefore aimed to investigate possible candidate neuropeptides which may be responsible for the non-nitrergic NANC responses.

Previous studies have demonstrated VIP, PACAP, PHM, PHV, CGRP, substance P and helospectin-I and -II immunoreactive nerve fibres in the human vagina (Graf *et al*., 1995; Hoyle *et al*., 1996) and human genital tract (Steenstrup *et al*., 1995), and co-localization of VIP with

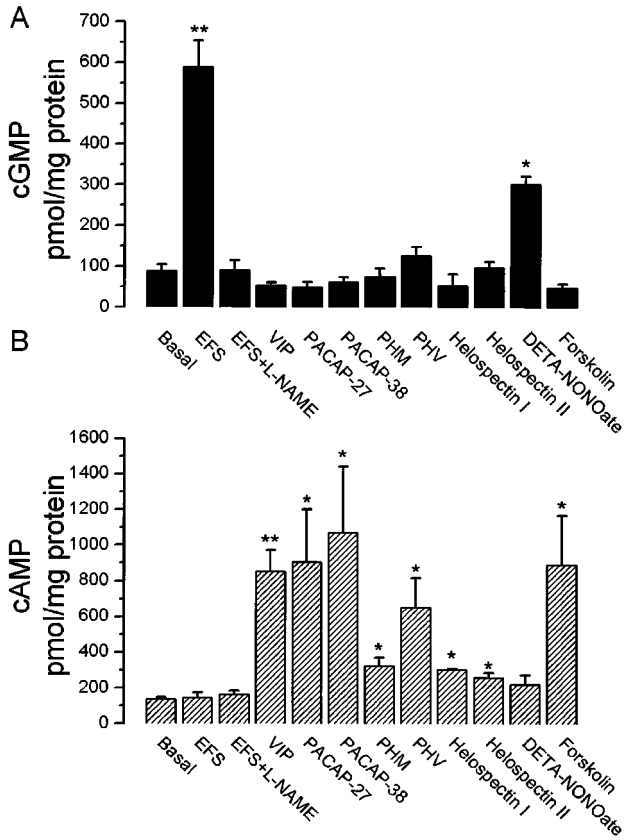

Figure 10 Intracellular concentrations of cyclic AMP (A) and cyclic GMP (B) in the rabbit vaginal wall. Tissues frozen during EFS (5 Hz) showed a marked increase in cyclic GMP concentrations that was completely inhibited in the presence of L-NAME (500 μM). EFS did not cause an increase in cyclic AMP concentrations. Exogenous application of peptides (all 400 nM) caused no increase in cyclic GMP, but caused marked increase in cyclic AMP. DETA NONOate (250 μM) and forskolin (10 μM) produced significant increase in cyclic GMP and cyclic AMP concentrations respectively. *Significantly different from basal, $P<0.05$. **Significantly different from basal, $P<0.001$; $(n/N=4/4)$.

nNOS in nerve fibres in the porcine and bovine vagina (Majewski *et al*., 1995). Furthermore, it has been shown that exogenously applied VIP inhibited spontaneous contractions in cat and rabbit myometrial strips and caused increased vaginal blood flow and increased vaginal lubrication in humans (Ottesen *et al*., 1981; 1987).

We have shown in this study that exogenous application of VIP, PACAP-27, PACAP-38, PHM, PHV, helospectin-I and helospectin-II caused relaxation in the rabbit vaginal wall, which could be inhibited with the protease α-chymotrypsin. EFS-induced relaxations however were not affected by α-chymotrypsin. It has previously been reported that α-chymotrypsin is able to block non-nitrergic EFS-induced relaxations in the guinea-pig trachea (Moffatt *et al*., 1999) suggesting that the peptidase is capable of inhibiting responses to endogenously-released neuropeptides as well as inhibiting responses to exogenously-applied peptides. Since the peptidase had no effect on EFS-induced relaxations in our experiments it is unlikely that any of these peptides are involved in the non-nitrergic component of the NANC relaxation in the rabbit vaginal wall.

VIP and PACAP-27 induce relaxations by binding to cell surface receptors which activates adenylate cyclase *via* the G protein G_s leading to an increase in intracellular cyclic AMP concentrations (Hirata *et al*., 1985; Warren *et al*., 1991). In our study all the peptides that were capable of inducing relaxation when applied exogenously caused an increase in intracellular cyclic AMP concentrations. However EFS did not cause any increase in cyclic AMP concentrations in the absence or presence of L-NAME, suggesting that the mechanism leading to non-nitrergic NANC relaxation is different from that induced by VIP, PACAP-27, PACAP-38, PHM, PHV, and helospectin-I and -II.

Kishi *et al*. (2000) have shown that VIP-induced relaxations were charybdotoxin-sensitive in rat colon, suggesting that VIP can elicit its function *via* a cyclic AMP-independent mechanism involving activation of K^+ channels. In our current study however we have shown that EFS-induced non-nitrergic relaxations are charybdotoxin and apamin-insensitive. Thus, whichever pathway is involved in responses evoked by exogenous VIP, our results strongly suggest that this is not involved in EFS-induced non-nitrergic relaxations.

It has previously been reported that the human vagina stains positively for the peptides CGRP and substance P (Hoyle *et al*., 1996). In this study we have demonstrated that CGRP and substance P did not cause any relaxation responses in the rabbit vaginal wall at concentrations at which they produced relaxation and contraction respectively in the clitoral corpus cavernosum. This suggests that they do not have a functional role as a NANC neurotransmitter in the rabbit vaginal relaxation.

CGRP has recently been shown to cause relaxation in the rat vagina (Giraldi *et al*., 2001 and this study), but in our study we have found CGRP to be unable to cause relaxations in the rabbit vagina. This is probably due to the absence of CGRP receptors in the rabbit vagina, which requires further investigation.

VIP causes relaxation in the rabbit vagina (this study and Berman *et al*., 1999) however in the rat it has been reported not to induce relaxations (Giraldi *et al*., 2001). Surprisingly we have observed VIP-induced relaxations in the rat vagina (unpublished observations). This discrepancy may be due to degradation of VIP in the study of Giraldi and colleagues, or due to differences in the responsiveness between our experimental models or the sensitivity between our experimental apparatus. Thus, there seems to be a species-difference in the vaginal smooth muscle between rat and rabbit in respect to responses to CGRP, and possibly also VIP. It should be noted that intravenous infusion of VIP in (pre-menopausal) humans has been shown to increase vaginal blood flow and lubrication (Ottesen *et al*., 1987), which is likely to occur due to VIP-induced relaxation of the smooth muscle in the vascular and non-vascular structures of the vagina. However as yet no *in vitro* pharmacological studies have shown that VIP or CGRP induce a relaxation response in human vaginal smooth muscle. Further studies with human vaginal tissue should clarify which species most resembles human tissue.

In our experiments we used strips from both upper and lower parts of the vaginal wall, and achieved similar results in tissues from both regions. This may suggest that non-nitrergic

NANC neurotransmission is not limited to one region in the rabbit vaginal canal. Stimulation of the pelvic nerves causes lengthening of the vaginal canal, as well as decreasing the luminal pressure suggesting that longitudinal muscle is important in the responses (Park *et al*., 1997). Therefore we used longitudinal strips in this study. We have also investigated NANC responses in circular strips from the rabbit vaginal wall, and have found them to respond in a similar way to longitudinal strips (unpublished observations).

We have used three different NOS inhibitors in this study with a rank of potency L-NA > = L-NAME > L-NIO. L-NA and L-NAME are derivatives of L-arginine and L-NIO is a derivative of L-ornithine. L-NA has been reported to have the greatest potency against nNOS (R.G. Knowles personal communication; Hobbs & Gibson, 1990) however its solubility in water is limited. Since this may effect the concentration of drug reaching its target, we have also used L-NAME, which is slightly less potent than L-NA (R.G. Knowles personal communication; Hobbs & Gibson, 1990) but more soluble, which may account for its greater efficacy *in vitro*. We also used L-NIO, which is less potent than L-NAME, in order to confirm our results with a non-arginine derivative NOS inhibitor.

ATP and adenosine have also been proposed as candidates for NANC neurotransmitters (for review, see Burnstock, 1997), and it is possible that these may be involved in non-nitrergic NANC relaxations in the rabbit vagina. Other neuropeptides are also candidates as neurotransmitters; alternatively a novel neurotransmitter may be involved.

Female sexual dysfunction is common and estimated to occur in 22–43% of women (Goldstein, 2000). FSD consists of disorders of desire, arousal, orgasm and sexual pain (Goldstein, 2000). Disorders of female sexual arousal include diminished vaginal lubrication, decreased clitoral engorgement and lack of vaginal wall relaxation (Goldstein & Berman, 1998; Goldstein, 2000). Identification of the neurotransmitters and their signalling mechanisms involved in the regulation of vaginal wall tone may provide possible targets for treatment of FSD.

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