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NANC neurotransmission in lamina propria of the rabbit urethra: regulation by different subsets of calcium channels

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1 Electrical field stimulation (EFS) of the rabbit urethral lamina propria elicited a frequency-dependent non-adrenergic, non-cholinergic (NANC) relaxation, which was abolished by N^{ω}-nitro-L-arginine (L-NOARG).

2 ω-Conotoxin GVIA, a selective blocker of N-type voltage-operated calcium channels (VOCCs), and ω-conotoxin MVIIC (blocker of N- and Q-type VOCCs) inhibited the NANC relaxation, and the inhibition was inversely related to the frequency of stimulation. Combined, the two toxins were more effective than ω-conotoxin GVIA alone.

3 The relaxation induced by the nitric oxide (NO) donor, 3-morpholino-sydnonimine (SIN-1) was not affected by ω-conotoxin MVIIC.

4 ω-Agatoxin IVA (blocker of P-type VOCCs) did not attenuate the NANC relaxation.

5 Reduction of the calcium concentration from 1.5 to 0.5 mM reduced the NANC relaxation at low but not at high frequencies of stimulation; the relaxation induced by SIN-1 was not affected.

6 EFS (20 Hz, 30 s) increased the cyclic GMP level 3 fold in normal Krebs solutions, but was unable to enhance significantly the cyclic GMP level after calcium omission. L-NOARG reduced the cyclic GMP content in 'calcium-free' medium, indicating an ongoing NO synthesis that was independent of extracellular calcium.

7 Caffeine, ryanodine and thapsigargin (inhibitors of sarcoplasmic calcium release), and CGP 37157 (inhibitor of mitochondrial sodium/calcium exchange) had no effect on the NANC relaxation.

8 It is suggested that nitrergic nerve activation in the rabbit urethral lamina propria is mediated in part by N-type (ω-conotoxin GVIA-sensitive) and in part by Q-type (ω-conotoxin MVIIC-sensitive) VOCCs. With high frequences of stimulation, another mechanism, possibly calcium-independent, appears to become operational.

Keywords: Calcium; calcium channels; NANC neurotransmission; nitric oxide; ω-conotoxins; rabbit urethra

Introduction

Nitric oxide (NO) has been suggested as the neuronal mediator of non-adrenergic, non-cholinergic (NANC) relaxation in several tissues, including the lower urinary tract (Sneddon & Graham, 1992; Andersson, 1993). Electrical field stimulation (EFS) of the rabbit urethral lamina propria elicits a tetrodotoxin-sensitive NANC relaxation (Zygmunt *et al.*, 1993a), which can be abolished by the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NOARG), suggesting that NO formation is a crucial step in the response (Zygmunt *et al.*, 1993a,b). Furthermore, NADPH diaphorase staining, presumably identifying NO synthase, has been localized to nerve fibres in rabbit urethral lamina propria, implying that activation of NO producing (nitrergic) nerves is responsible for the NANC relaxation (Zygmunt *et al.*, 1993a).

We have recently shown that ω-conotoxin GVIA (ω-CgTx GVIA), a blocker of N-type voltage-operated calcium channels (VOCCs), inhibits NANC relaxation in the rabbit urethra at low frequencies of stimulation (Zygmunt *et al.*, 1993b). However, a significant ω-conotoxin-resistant component was observed at stimulation frequencies higher than 2 Hz. The NANC relaxation was unaffected by inhibitors of L-type (nimodipine) and T-type (tetramethrin, Ni^{+}) VOCCs, which raised the possibility that the ω-conotoxin-resistant component was mediated by VOCCs distinct from the L- and T-type, or even by a calcium influx-independent mechanism. Release of VIP from the rat enteric nerves, and noradrenaline and GABA from rat brain slices have been shown to persist in 'calcium-free' medium, which could indicate that release of calcium from intracellular stores is sufficient to uphold transmitter release in some nerves (Minchin, 1980; Sandoval, 1980; Schoffelmeer & Mulder, 1983; Belai *et al.*, 1987).

To clarify further the role of calcium in NANC nerve activation, we examined the effects of ω-conotoxin MVIIC (ω-CgTx MVIIC), a neuronal VOCC blocker with a calcium channel selectivity distinct from that of ω-CgTx GVIA (Hillyard *et al.*, 1992; Randall *et al.*, 1993; Wheeler *et al.*, 1994), and the selective P channel blocker ω-agatoxin IVA (Mintz *et al.*, 1992) on NANC relaxation in the rabbit urethral lamina propria. The effects of lowering the extracellular calcium concentration and drugs interfering with endoplasmic and mitochondrial calcium transport were also studied.

Methods

Tissue preparation

Female rabbits (New Zealand White) with an average weight of 3 kg were stunned by a blow on the head and exsanguinated. The abdomen was opened and the bladder and urethra were removed and placed in ice-cold Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5, and glucose 11. The bladder and urethra were opened longitudinally and separated by a transverse cut at the level of the bladder neck. The lamina propria of the urethra was separated from the smooth muscle layers under a microscope, and strip preparations (1 × 2 × 5 mm) were prepared as described previously (Zygmunt *et al.*, 1993a). The strips were suspended between two

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metal pins by means of silk ligatures in organ baths (5 ml), containing Krebs solution. Vessel segments of the rabbit ear artery (REA) were suspended in the same organ baths (Zygmunt & Högestätt, 1993). The Krebs solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C, resulting in a pH of 7.4. One of the metal pins was connected to a movable unit allowing adjustment of tension, and the other to a Grass Instruments FT O3C force-displacement transducer. The 'isometric' tension was displayed on a Grass Instruments model 7 D polygraph. The resting tension of the preparations was adjusted to 4 mN during an equilibration period of 1 h.

Experimental procedure

EFS was achieved by two platinum wire electrodes placed parallel to the preparations in the organ bath. Square wave pulses with a duration of 0.3 ms were delivered at frequencies of 0.5–40 Hz. The polarity was changed after each pulse to reduce oxidation of the electrodes. In order to study relaxant responses, the preparations were contracted with 1 nM arginine vasopressin (AVP), which produced a long-lasting and stable contraction (Zygmunt *et al.*, 1993b). Phentolamine (1 μM) and propranolol (1 μM) were present in all these experiments. Initially in each experiment the preparations were stimulated electrically for 5 s (urethral lamina propria) or 10 s (REA) every second min with a stimulation frequency of 12 Hz (urethral lamina propria) or 10 Hz (REA). Optimum voltage was determined by increasing the voltage until a maximum response was obtained (this voltage was then used in all subsequent stimulations). This 'maximum response' served as a reference, and is hereafter referred to as the reference relaxation (urethral lamina propria). The reference relaxation in the urethral lamina propria was 68±4% (72 strips from 21 rabbits) of the AVP-induced contraction. Frequency-response relationships were studied by stepwise increasing the frequency of stimulation from 0.5 to 40 Hz, allowing a two-min interval between the frequency steps. The effects of the intracellular calcium modulators thapsigargin, ryanodine, caffeine and CGP 37157 on the NANC relaxation were studied at a single stimulation frequency of 12 Hz. The preparations were stimulated 'continuously' at each frequency until the response stabilized (usually 1–2 min) to obtain an estimate of the maximum response at that frequency. Each preparation was subjected to only one series of stimulation frequencies. Thus, no more than one concentration of VOCC blocker or extracellular calcium was tested in each preparation. A control group subjected to the same stimulation protocol was always run in parallel. The choice of incubation time for the ω-CgTxs was based on a series of initial experiments, where the effect of 0.1 μM ω-CgTx GVIA ($n=7$) or 1 μM ω-CgTx MVIIC ($n=2$) was studied on urethral lamina propria stimulated with 5 s trains of pulses (10 Hz) every second min. These experiments showed that a maximum inhibition was obtained after about 10 min. Thus, to assure a maximum blockade of the ω-CgTx-sensitive calcium channels, an incubation period of 20 min was used in the experiments. The preincubation period with the other drugs and in low calcium medium was also 20 min.

Cyclic GMP and cyclic AMP measurements

Lamina propria preparations were suspended in organ bath as described above. Cyclic nucleotides were measured in unstimulated preparations and in preparations subjected to 20 Hz EFS for 30 s in Krebs solution with and without calcium ($CaCl_2$ replaced by 0.1 mM EGTA). AVP (1 nM), phentolamine (1 μM) and propranol (1 μM) were present throughout. The preincubation period in 'calcium-free' Krebs solution was 10 min. The preparations were subsequently frozen in liquid nitrogen. The tissue was homogenized in 10% trichloroacetic acid (TCA) in water with a glass-glass homogenizer and centrifuged at 3000 r.p.m. (4°C) for 10 min. Protein content in the pellets was determined by the Coomassie Plus Protein Assay Reagent based on the Bradford method (Pierce, Rockford, ILL, U.S.A.). The supernatants were extracted 5 times 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 amounts of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and cyclic AMP were quantified by use of [^{125}I]-cyclic GMP and [^{125}I]-cyclic AMP RIA kit (RIANEN, Du Pont, Wilmington, DE, U.S.A.). [^{3}H]-cyclic AMP recovery marker was added to the TCA tissue homogenate. Recovery was 77%.

Drugs

(±)-Noradrenaline hydrochloride, (±)-propranolol hydrochloride, N^{ω}-nitro-L-arginine, arginine vasopressin acetate, tetrodotoxin (TTX), ω-conotoxin GVIA, thapsigargin, ryanodine (all from Sigma, St. Louis, MO, U.S.A.), caffeine (ACO, Stockholm, Sweden), phentolamine methane sulphonate (Ciba-Geigy, Basel, Switzerland), 3-morpholino-sydnonimine hydrochloride (SIN-1; Casella AG, Frankfurt am Main, Germany), ω-conotoxin MVIIC and ω-agatoxin IVA (Alomone labs, Jerusalem, Israel) were dissolved in distilled water. (±)-Nimodipine (Bayer, Leverkusen, Germany) and CGP 37157 (Ciba-Geigy AG, Basel, Switzerland) were dissolved in absolute ethanol.

Calculations and statistics

Responses are expressed as a percentage of the initial reference response (see above). Nimodipine, caffeine and low calcium medium reduced the AVP-induced contraction. The AVP concentration was therefore increased in these cases to regain the initial tension. When the preparation failed to return to the initial tension, R/T values were used to calculate drug effects (Garcia-Pascual *et al.*, 1991). R/T values express the relaxation (R) as a percentage of the tension (T) recorded immediately before each train of stimulation. Results are expressed as mean values ± s.e.mean, and n denotes the number of experiments (animals) performed. Student's t test (two-tailed) for unpaired observations was used in the statistical analyses. Where appropriate, analysis of variance (ANOVA) followed by Bonferroni/Dunn's *post hoc* test (Statview 4.0) was used (Ludbrook, 1994). The results from the cyclic GMP measurements were subjected to logarithmic transformation before statistical analysis to adjust for intergroup differences in variance. Statistical significance was accepted when $P<0.05$.

Results

Effect of L-NOARG

EFS of the AVP-contracted rabbit urethral lamina propria elicited a frequency-dependent relaxation, which was abolished by 1 μM TTX (Figure 1). At high frequencies of stimulation (≥12 Hz), the response generally became 'biphasic' with an initial transient relaxation that stabilized at a lower level after 1–2 min (Figure 2). At 20 Hz stimulation the transient and stable component amounted to 108±4% and 93±5% ($n=10$) of the initial reference relaxation (elicited by 12 Hz stimulation), respectively. The NO synthase inhibitor L-NOARG (0.3 mM) abolished both components of the relaxation, and reversed the response to a contraction at stimulation frequencies ≥12 Hz (Figure 1). The EFS-induced contraction was partially TTX-sensitive at 40 Hz stimulation (Figure 1).

Effects of ω-CgTx MVIIC and ω-CgTx GVIA

ω-CgTx MVIIC (1 μM) and ω-CgTx GVIA (0.1 μM) both inhibited the NANC relaxation, and the effect was larger at low than at high frequencies of stimulation (Figure 3, Table 1). A

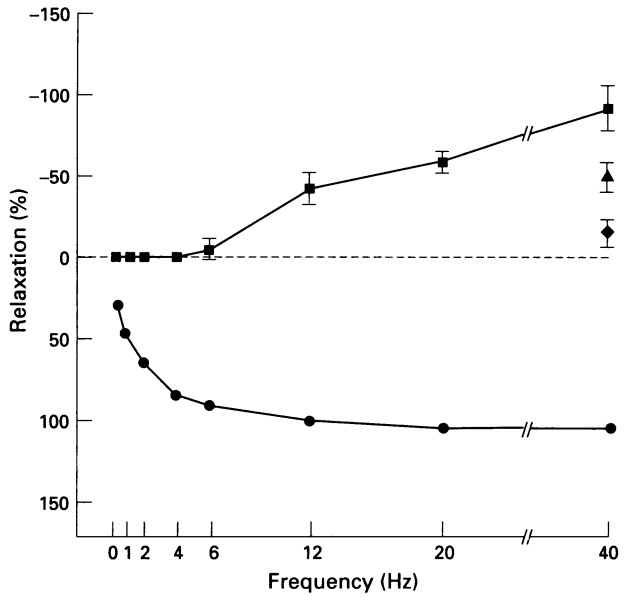

Figure 1 Responses to electrical field stimulation (frequency 0.5–40 Hz, pulse duration 0.3 ms, train duration 1–2 min, optimum voltage) in the urethral lamina propria incubated with 0.3 mM N^{ω}-nitro-L-arginine (■) or vehicle (●). The effects of 1 μM tetrodotoxin at 40 Hz stimulation in the absence (◆) and presence (▲) of N^{ω}-nitro-L-arginine are also shown. The preparations were contracted by arginine vasopressin (1 nM) before stimulation. Results are expressed as a percentage of the initial reference relaxation (see Methods), and are given as mean values ± s.e.mean ($n=4$).

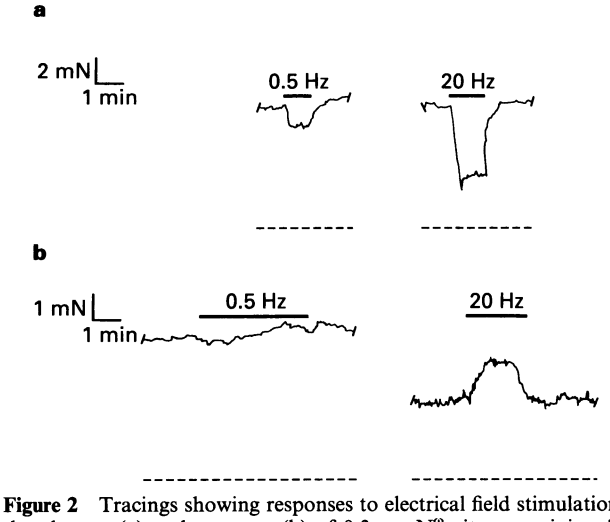

Figure 2 Tracings showing responses to electrical field stimulation in the absence (a) and presence (b) of 0.3 mM N^{ω}-nitro-L-arginine. The tracings in (a) and (b) were obtained from two separate urethral lamina propria preparations. Solid bars indicate the periods of electrical field stimulation. The preparations were contracted by arginine vasopressin (1 nM) before electrical field stimulation, and broken lines indicate the baseline tension before the contraction.

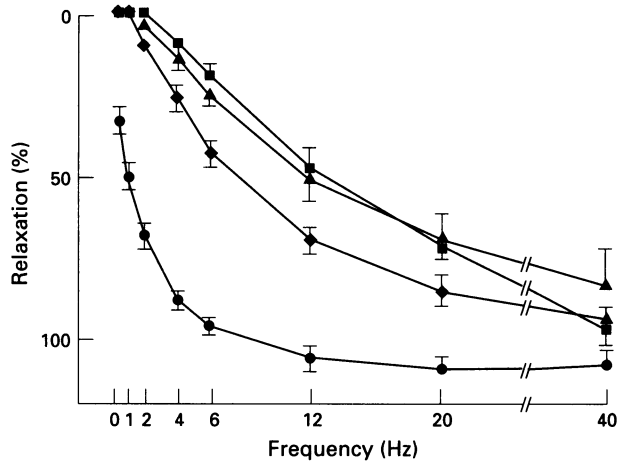

Figure 3 Frequency-response curves for the NANC relaxation in urethral lamina propria incubated with 0.1 μM ω-conotoxin GVIA (◆), 1 μM ω-conotoxin MVIIC (▲), 0.1 μM ω-conotoxin GVIA + 1 μM ω-conotoxin MVIIC (■) or vehicle (●). The preparations were contracted by arginine vasopressin (1 nM) before electrical field stimulation (stimulation parameters as in Figure 1). Results are expressed as a percentage of the initial reference relaxation (see Methods), and are given as mean values ± s.e.mean ($n=5-9$).

higher concentration of ω-CgTx MVIIC (3 μM, $n=2$) or ω-CgTx GVIA (1 μM, $n=7$) did not produce a further inhibition (tested only at 10 Hz stimulation). The NANC relaxation at 6 Hz stimulation was significantly smaller after incubation with ω-CgTx MVIIC than after incubation with ω-CgTx GVIA (Figure 3). The inhibition obtained by ω-CgTx MVIIC (1 μM) plus ω-CgTx GVIA (0.1 μM) was significantly larger than that obtained by ω-CgTx GVIA (0.1 μM) alone at 4, 6 and 12 Hz stimulation, but almost identical with that seen with ω-CgTx MVIIC (1 μM) alone (Figure 3, Table 1). ω-CgTx MVIIC (1 μM) did not affect the AVP-induced contraction or the relaxation induced by SIN-1. The pIC_{50} and I_{max} values for SIN-1 were 4.9 ± 0.1 and 77 ± 6% ($n=5$), and 5.0 ± 0.1 and 98 ± 8% ($n=5$) in the absence and presence of ω-CgTx MVIIC, respectively.

In order to mimic a physiological impulse pattern, i.e. fairly regular pulse-synchronous burst of impulses often occurring in short sequences separated by periods of neural silence (Wallin, 1981), some of the lamina propria preparations were subjected to 10 Hz EFS in 1 s trains at 10 s or 5 s intervals. Using these stimulation parameters, ω-conotoxin GVIA (0.1 μM) reduced the maximum relaxant response from 72 ± 6% to 30 ± 2% (10 s intervals, $n=4$) and from 88 ± 5% to 41 ± 2% (5 s intervals, $n=4$) of the initial reference relaxation.

The effects of ω-CgTx MVIIC and ω-CgTx GVIA on adrenergic contractions in the REA were also examined. As shown in Figure 4, the EFS-induced contractile responses were abolished by ω-CgTx MVIIC (1 μM) as well as by 0.1 μM

Table 1 Inhibitory effects of ω-conotoxins on electrically-induced non-adrenergic, non-cholinergic relaxation in the urethral lamina propria

Treatment	EF_{50}	E_{max}(%)	n
Control	$1.3 \pm 0.2^{a,b,c}$	110 ± 4	9
ω-Conotoxin GVIA 0.1 μM	$7.0 \pm 0.5^{a,d}$	99 ± 5	9
ω-Conotoxin MVIIC 1 μM	10 ± 1.3^{b}	83 ± 11	5
ω-Conotoxin GVIA 0.1 μM + ω-conotoxin MVIIC 1 μM	$13 \pm 1.3^{c,d}$	97 ± 5	7

The preparations were contracted by arginine vasopressin (1 nM) before electrical field stimulation. ^{a,b,c,d}Significant differences ($P<0.05$) between groups with identical letters according to analysis of variance (ANOVA) followed by Bonferroni/Dunn's *post hoc* test. EF_{50} denotes the frequency producing half maximum relaxation. E_{max} indicates the maximal relaxation obtained within the frequency interval tested (0.5–40 Hz), and is expressed as a percentage of the initial reference relaxation (see Methods). Results are expressed as mean values ± s.e.mean, where *n* indicates the number of preparations (animals) examined.

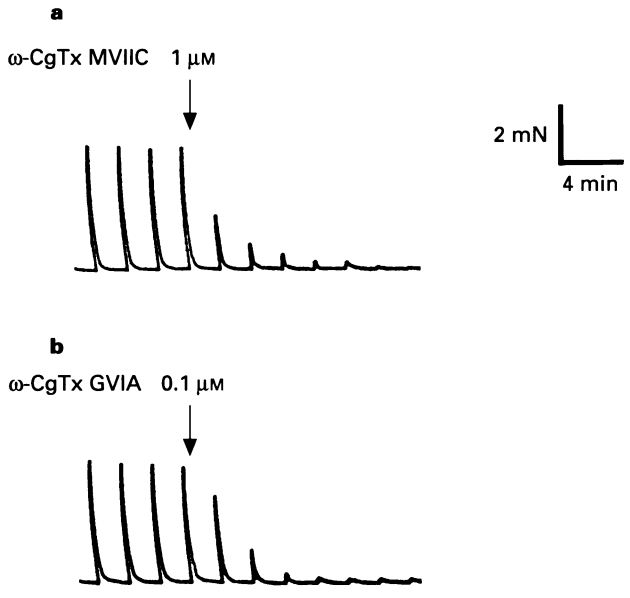

Figure 4 Tracings showing the effect of ω-conotoxin (ω-CgTx) MVIIC (a) and GVIA (b) on the contractile responses to electrical field stimulation (10 Hz for 10 s every second min, pulse duration 0.3 ms) in two segments of the rabbit ear artery.

ω-CgTx GVIA ($n=3$). The contractile response to exogenous noradrenaline (10 nM–0.1 mM) was, however, unaffected by the ω-CgTxs (data not shown).

Effects of ω-agatoxin IVA and nimodipine

ω-Agatoxin IVA (30 nM) and nimodipine (0.1 μM, $n=4$, data not shown) did not attenuate the electrically-induced NANC relaxation in the rabbit urethral lamina propria. However, at stimulation frequencies $\geqslant 4$ Hz, ω-agatoxin IVA caused a small enhancement of the response (Figure 5). The SIN-1-induced relaxation was not affected by ω-agatoxin IVA and nimodipine (data not shown).

Effects of extracellular calcium

When the extracellular calcium concentration was reduced from 1.5 mM to 0.5 mM the maximum NANC relaxation in rabbit urethral lamina propria was significantly reduced at frequencies $\leqslant 4$ Hz and increased at 40 Hz (Figure 6), whereas the relaxation induced by SIN-1 was unaffected ($n=3$). It was not possible to study the effect of nominally 'calcium-free' solution, since AVP failed to elicit a contraction in that medium.

Changes in cyclic nucleotides

In ordinary Krebs solution, EFS (20 Hz for 30 s) increased the cyclic GMP content from 1.4 ± 0.5 to 4.6 ± 1.3 pmol mg^{-1} protein ($n=6$). When calcium was omitted from the Krebs solution the basal cyclic GMP content increased 3 fold (Figure 7). EFS did not significantly enhance the cyclic GMP content in 'calcium-free' solution. However, the wide range of cyclic GMP levels measured at baseline (1.8 to 9.4 pmol mg^{-1} protein) and after EFS (1.7 to 20 pmol mg^{-1} protein) made it difficult to detect a statistically significant difference. Notably, pretreatment with L-NOARG (0.3 mM) in 'calcium-free' solution caused an 84% reduction of the cyclic GMP content after EFS (Figure 7). The cyclic AMP contents in Krebs solution were 46 ± 7 and 42 ± 7 pmol mg^{-1} protein ($n=6$) in unstimulated and stimulated preparations, respectively. The corresponding values in 'calcium-free' solution were 36 ± 2 and 36 ± 4 pmol mg^{-1} protein ($n=6$). These values did not differ significantly.

Effects of modulators of intracellular calcium release

Thapsigargin (1 μM), ryanodine (10 μM) and caffeine (1 mM), inhibitors of calcium release from different intracellular stores (Takemura *et al.*, 1989; Ehrlich *et al.*, 1994), and CGP 37157 (10 μM), which is an inhibitor of mitochondrial sodium/calcium exchange in heart (Cox *et al.*, 1993), had no effect on the NANC relaxation induced by 12 Hz stimulation in the lamina propria (Table 2).

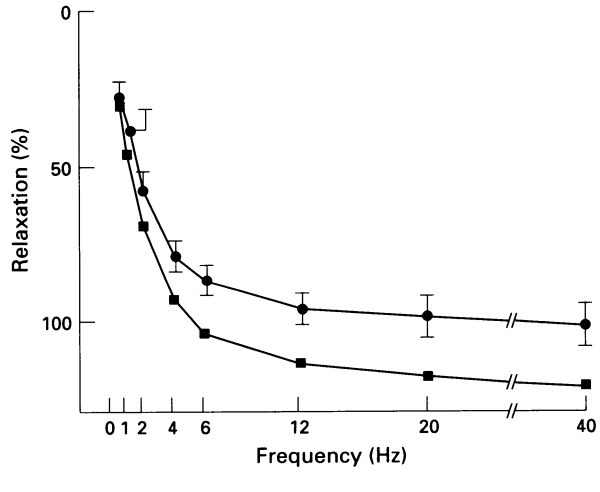

Figure 5 Frequency-response curves for the NANC relaxation in urethral lamina propria incubated with 30 nM ω-agatoxin IVA (■) or vehicle (●). The preparations were contracted by arginine vasopressin (1 nM) before electrical field stimulation (stimulation parameters as in Figure 1). Results are expressed as a percentage of the initial reference relaxation (see Methods), and are given as mean values ± s.e.mean ($n=4$).

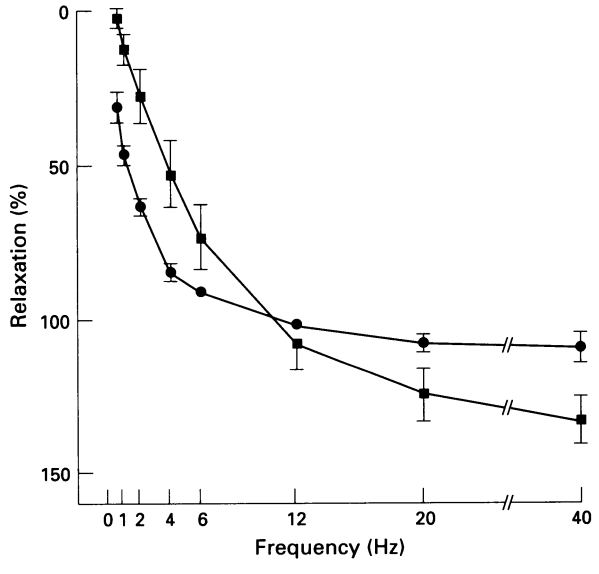

Figure 6 Effect of the extracellular calcium ion concentration on the NANC relaxation in the urethral lamina propria contracted by arginine vasopressin (1 nM) before electrical field stimulation (stimulation parameters as in Figure 1). Responses were recorded in Krebs solution, containing 1.5 mM (●) or 0.5 mM (■) calcium. Results are expressed as a percentage of the initial reference relaxation (see Methods), and are given as mean values ± s.e.mean ($n=5$).

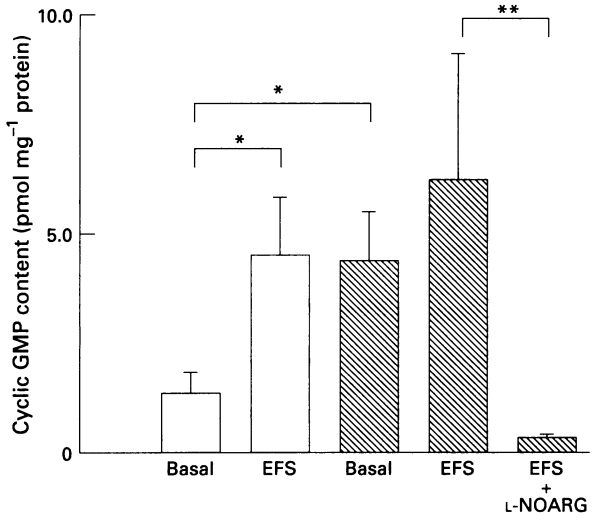

Figure 7 Effect of calcium omission on cyclic GMP accumulation after electrical field stimulation (EFS, 20 Hz for 30 s) in the urethral lamina propria. The strip preparations were suspended in organ baths and contracted by arginine vasopressin (1 nM). The cyclic GMP content was measured in 'normal' Krebs solution (1.5 mM calcium, open columns) or after a 10 min incubation period in nominally 'calcium-free' solution (0.1 mM EGTA, hatched columns). Basal refers to the cyclic GMP content in preparations not subjected to EFS. N^{ω}-nitro-L-arginine (L-NOARG, 0.3 mM) was present in the incubation medium in some experiments. The strips were quickly frozen in liquid nitrogen, and the cyclic GMP content determined by radioimmunoassay. Results are given as mean ± s.e.mean ($n = 5-6$). $^{*}P<0.05$, $^{**}P<0.01$.

Discussion

We have previously studied the frequency-dependence of the NANC relaxation in rabbit urethral lamina propria (Zygmunt *et al.*, 1993b). In that study, the stimulation period at each frequency was limited to 5 s, and hence the number of impulses delivered at each period increased the higher the frequency of stimulation (e.g. 5 pulses at 1 Hz and 100 pulses at 20 Hz). In order to obtain measurement of the maximum response at each frequency, the stimulation was continued for 1–2 min in the present study. Despite the difference in stimulation parameters the frequency-response curves turned out to be almost identical with that obtained previously. However, as evident from the individual tension tracings, a stimulation period of 30–60 s was often required to reach a maximum response at frequencies ≤2 Hz.

The NANC relaxation was completely inhibited by L-NOARG and accompanied by an increase of the cyclic GMP level, indicating that the response was mediated by NO. In the presence of L-NOARG, a contractile response was observed at high frequencies of stimulation (≥12 Hz). The contraction was partially sensitive to TTX (tested at 40 Hz stimulation), suggesting that the response was mediated in part by non-adrenergic nerves (resistant to phentolamine and propranolol) and in part by direct muscle stimulation. The NANC relaxation generally became 'biphasic' at high frequencies of stimulation (>12 Hz); after an initial peak the relaxation decreased before the tension stabilized. This response occurred at the same frequencies as did the L-NOARG-resistant contraction, suggesting that the reduction of the relaxation reflected the development of a contractile response superimposed on the NANC relaxation.

A 'biphasic' response to high frequency stimulation has also been demonstrated in pig and dog urethra (Hashimoto *et al.*, 1992; 1993; Bridgewater *et al.*, 1993). In pig urethra, EFS induced an initial transient relaxation, which at high frequencies (40–80 Hz) was followed by a prolonged second relaxation after cessation of stimulation (Bridgewater *et al.*, 1993). Both relaxation components were of NANC origin, but only the initial component was inhibited by L-NOARG. A similar 'biphasic' relaxant response was observed at stimulation frequencies ≥5 Hz in dog urethra (Hashimoto *et al.*, 1993). Again, the initial transient component was markedly reduced by L-NOARG, whereas the second slow component was unaffected. The neurotransmitter mediating the second relaxation component could not be identified in these studies. We found no evidence of a second component of relaxation produced by an additional neurotransmitter in rabbit urethral lamina propria.

In line with our previous study (Zygmunt *et al.*, 1993b), ω-CgTx GVIA inhibited the NANC relaxation in rabbit urethral lamina propria in a frequency-dependent manner, implying that activation of N-type VOCCs is an important step linking EFS to smooth muscle relaxation particularly at low frequencies of stimulation. The NANC relaxation does not seem to depend on L- and T-type VOCCs, since nimodipine (L channel blocker; present study), nickel and tetramethrin (T channel blockers) have been shown not to affect the response (Zygmunt *et al.*, 1993b). P-type VOCCs also do not seem to be involved, since the selective P channel blocker ω-agatoxin IVA (Mintz *et al.*, 1992) was unable to attenuate the NANC relaxation, but rather enhanced the response at high stimulation frequencies. We have no explanation for this enhancement, but the failure of ω-agatoxin IVA to affect the SIN-1-induced relaxation makes an interaction with the NO effector system unlikely.

ω-CgTx MVIIC, another Conus peptide isolated from the cone snail *Conus magus* (Hillyard *et al.*, 1992), induced a frequency-dependent inhibition of the NANC relaxation similar to that obtained with ω-CgTx GVIA. The ω-CgTx MVIIC concentration used in these experiments (1 μM) has been shown to inhibit $^{45}Ca^{2+}$ influx by approximately 70% in rat brain synaptosomes (Hillyard *et al.*, 1992). Incubation with both ω-CgTx GVIA and ω-CgTx MVIIC was more effective than treatment with ω-CgTx GVIA alone. The concentration of ω-CgTx GVIA used in these experiments was sufficient to elicit a maximum inhibition, since a 10 times higher con-

Table 2 Effects of modulators of intracellular calcium-release on the maximum NANC relaxation induced by electrical field stimulation (12 Hz for 1–2 min) in the urethral lamina propria

	Relaxation (%)		
Treatment	*Control*	*Treatment*	n
Thapsigargin 1 μM	101 ± 3	97 ± 3	4
Ryanodine 10 μM	102 ± 3	122 ± 12	6
Caffeine 1 μM	99 ± 3	93 ± 4	4
CGP 37157 10 μM	99 ± 3	102 ± 9	4

The preparations were contracted by arginine vasopressin (1 nM) before electrical field stimulation. Results are expressed as a percentage of the initial reference relaxation (see Methods), and are expressed as mean values ± s.e.mean, where *n* indicates the number of preparations (animals) examined.

centration had no further effect. These results suggest that ω-CgTx MVIIC has an additional effect, which is not shared with ω-CgTx GVIA. Clearly, this effect of ω-CgTx MVIIC was not postjuntional, since the relaxant response to the NO donor SIN-1 was unaffected. Hence, it is possible that yet another subtype of VOCC is involved in NANC neurotransmission in the rabbit urethral lamina propria.

ω-CgTx MVIIC has been shown to block P channel-mediated (ω-CgTx GVIA-resistant) synaptic transmission (Hillyard *et al.*, 1992). However, the effect of ω-CgTx MVIIC observed in the present study does not appear to be mediated by inhibition of P channels, since ω-agatoxin IVA was found to have no inhibitory effect. ω-CgTx MVIIC has recently been suggested to inhibit another type of neuronal VOCC termed Q channel (Hillyard *et al.*, 1992; Randall *et al.*, 1993; Wheeler *et al.*, 1994). This channel type may in addition to N channels mediate calcium influx in NANC nerves in the rabbit urethral lamina propria.

A combination of ω-CgTx MVIIC and ω-CgTx GVIA did not produce a larger inhibition of the NANC relaxation than did ω-CgTx MVIIC alone. This would indicate that ω-CgTx MVIIC also inhibits ω-CgTx GVIA-sensitive N-type VOCCs, a possibility previously suggested by Hillyard *et al.* (1992) and Grantham *et al.* (1994). To address this question further we studied the effect of ω-CgTx MVIIC on adrenergic contractions in the REA. Adrenergic neurotransmission in this artery has been suggested to depend on N-type VOCCs (Zygmunt & Högestätt, 1993). ω-CgTx MVIIC was able to inhibit this response completely. Since the contractile response to exogenous noradrenaline was unaffected by the ω-CgTxs, these findings provide additional evidence that ω-CgTx MVIIC can inhibit N-type VOCCs.

A significant ω-CgTx-resistant component of the NANC relaxation was observed at frequencies $\geqslant 2$ Hz. This component increased the higher the frequency of stimulation, and at 40 Hz stimulation the relaxation was almost completely resistant to the ω-CgTxs. It may be argued that the regular high frequency impulse pattern used in these experiments does not occur in intact animals, and consequently the ω-CgTx-resistant component may be physiologically irrelevant. To the best of our knowledge, the impulse pattern in NANC nerves has not been determined. However, impulse activity in sympathetic nerves has been recorded in man (Wallin, 1981). The activity consisted of high frequency bursts of impulses separated by silent intervals. In order to mimic this impulse pattern, the lamina propria was stimulated with 1 s trains of pulses (10 Hz) at 10 s or 5 s intervals. Again, ω-CgTx GVIA produced only a partial inhibition of the response, inferring that the mechanisms responsible for the ωCgTx-resistant component of the NANC relaxation may be of physiological importance.

To study whether the NANC relaxation is dependent on extracellular calcium the response to EFS was determined in a solution containing 0.5 mM calcium. At low stimulation frequencies ($\leqslant 4$ Hz) the relaxation was significantly reduced, but at 40 Hz the relaxation was significantly increased. Unfortunately, the effect of a nominally calcium-free solution could not be examined, because the preparation failed to contract in that medium. Instead, we measured the cyclic GMP level as an indirect measure of NANC nerve activation. If the ω-CgTx-resistant component of the NANC relaxation is independent of extracellular calcium, EFS should be able to increase the formation of cyclic GMP in 'calcium-free' medium. However, EFS at 20 Hz (mainly ω-CgTx-resistant) failed to increase the cyclic GMP level significantly in the absence of extracellular calcium, although the large variability of the data made it almost impossible to demonstrate a difference between the groups. Notably, L-NOARG significantly reduced the cyclic GMP level in 'calcium-free' medium. This would indicate that NO can be formed in the absence of extracellular calcium, indicating that activation of NO synthase may in part be independent of calcium influx in rabbit urethral lamina propria. However, the results do not allow us to speculate on the type or origin of the NO forming enzyme.

We have previously speculated that the ω-CgTx GVIA-resistant component of the NANC relaxation in the rabbit urethral lamina propria may be independent of calcium influx (Zygmunt *et al.*, 1993b). To study whether release of calcium from intracellular stores was involved in the NANC relaxation, the effects of drugs affecting this process were investigated. Caffeine has been shown to inhibit IP_3-induced calcium release, but it is also capable of activating the ryanodine receptor-calcium release channel (Ehrlich *et al.*, 1994). Ryanodine can inhibit stimulation-induced intracellular calcium release by binding to the ryanodine receptor on the sarcoplasmic reticulum (Ehrlich *et al.*, 1994). However, neither caffeine nor ryanodine had an effect on the NANC relaxation in the present study. Thapsigargin, which acts by depleting IP_3-sensitive intracellular calcium stores (Takemura *et al.*, 1989), also had no effect on the NANC relaxation. Finally, the NANC relaxation was unaffected by CGP 37157, which has been suggested to antagonize selectively mitochondrial sodium/calcium exchange (Cox *et al.*, 1993). It therefore seems unlikely that release of calcium from intracellular stores is involved in NANC neurotransmission in the rabbit urethral lamina propria. This raises the question whether neuronal NO synthase may in part be regulated in a calcium-indpendent manner, a possibility previously suggested by Kostka *et al.* (1993). An important distinction between NO and other neurotransmitters is that NO does not seem to be stored in vesicles, and therefore is not released by a calcium-activated exocytotic process. NO is rather thought to be produced on demand and to reach adjacent effector cells by passive diffusion (Snyder, 1992). Although the constitutive NO synthase present in neurones is a calcium/calmodulin-dependent enzyme (Bredt *et al.*, 1991; Murad *et al.*, 1992), the mechanisms regulating enzyme activity in the intact cell have not been delineated. Thus, the 'calcium hypothesis' for neurotransmitter release (Katz, 1969) may not be applicable to NO release from NANC nerves.

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