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Effects of ω-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor

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1 The effects of ω-conotoxin GVIA (an inhibitor of N-type voltage-operated calcium channels; VOCCs) were compared on adrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC) responses induced by electrical field stimulation (EFS) in the rabbit urethra and detrusor.

2 EFS induced a relaxation in urethral smooth muscle and lamina propria precontracted by arginine vasopressin (AVP). The relaxation was abolished by tetrodotoxin (TTX) or the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine. ω-Conotoxin inhibited the relaxation induced by EFS, but not that elicited by the NO donor 3-morpholino-sydnonimin. The inhibition, however, decreased with increasing frequencies of stimulation. Nimodipine, tetramethrin and nickel did not affect the ω-conotoxin-resistant relaxation in lamina propria, suggesting that neuronal L or T VOCCs were not involved in the response.

3 EFS contracted urethral smooth muscle at resting tension. The contractions were virtually abolished by TTX or prazosin. ω-Conotoxin effectively inhibited the contractile responses to EFS, but not those to exogenous noradrenaline. An ω-conotoxin-resistant contraction was, however, observed at high frequencies of stimulation.

4 The detrusor responded with frequency-dependent contractions upon EFS. A TTX-resistant contraction less than 10% of controls remained at 30 Hz stimulation. At a stimulation frequency of 10 Hz, scopolamine reduced the EFS-induced contraction by 71%. ω-Conotoxin inhibited the responses in both the absence and presence of scopolamine. The inhibition decreased with increasing frequencies of stimulation (examined in the absence of scopolamine). ω-Conotoxin did not affect the contractile responses to carbachol or adenosine 5′-triphosphate.

5 The adrenergic contraction (25 Hz) and NANC relaxation (10 Hz) in the urethra, and cholinergic and NANC contractions (10 Hz) in the detrusor were inhibited concentration-dependently by ω-conotoxin. The adrenergic contraction in the urethra was 10 times and the cholinergic contraction in the detrusor was three times more sensitive to ω-conotoxin than the NANC responses.

6 These results suggest that NANC neurotransmission is less inhibited by ω-conotoxin than transmission mediated by adrenergic and cholinergic nerves in the rabbit lower urinary tract. In the urethra a marked ω-conotoxin-resistant component of the NANC relaxation was observed which increased with increasing stimulation frequencies and was unaffected by inhibitors of L and T type VOCCs. This raises the question whether VOCCs of a type other than L, T, and N is involved in the mediation of this response.

Keywords: ω-Conotoxin; calcium channels; adrenergic neurotransmission; cholinergic neurotransmission; NANC neurotransmission; nitric oxide

Introduction

It is well known that non-adrenergic, non-cholinergic (NANC) nerves use transmitters like adenosine 5′-triphosphate (ATP) and various peptides, e.g., calcitonin gene-related peptide and substance P. NANC transmission closely resembles transmission by classical non-peptide nerves in that the transmitters are stored in vesicles and released by a calcium-activated exocytotic process (Dockray, 1992). Although the exact mechanism for vesicular release is unknown, influx of calcium through voltage-operated calcium channels (VOCCs) seems to be a crucial event. Recently it has been suggested that nitric oxide (NO) may be the transmitter substance responsible for NANC relaxation in several tissues (Persson & Andersson, 1992; Sneddon & Graham, 1992), including rabbit urethral smooth muscle and lamina propria (Andersson *et al.*, 1991; 1992; Zygmunt *et al.*, 1993). NO is formed from L-arginine by the enzyme NO synthase (NOS), which exists in different isoforms (Förstermann *et al.*, 1991). NOS can be competitively inhibited by several N^{ω}-substituted L-arginine analogues, e.g., N^{ω}-nitro-L-arginine (L-NOARG; Mülsch & Busse, 1990). The NOS found in neuronal tissues is a constitutive and Ca^{2+}/calmodulin-dependent enzyme (Bredt *et al.*, 1991; Murad *et al.*, 1992). An important distinction between NO and other neurotransmitters is that exocytosis of preformed NO-containing membrane vesicles is not obligatory for NO release. NO is rather thought to be produced on demand and to diffuse from the site of synthesis to adjacent effector cells (Snyder, 1992). Formation of more stable compounds has been considered, including dinitrosyl iron complexes (Vanin, 1991; Vedernikov *et al.*, 1992) and nitrosothiols (Myers *et al.*, 1990); the latter may subsequently be stored in acidic membrane vesicles (Ignarro, 1989).

Based on electrophysiological findings, VOCCs may be divided into low (LVA) and high (HVA) voltage-activated calcium channels (Sher *et al.*, 1991). The LVA calcium channel is also designated T (transient) due to its rapid inactivation kinetics (Fox *et al.*, 1987; Hagiwara *et al.*, 1988), whereas the HVA calcium channels may be further subdivided into L, N and P subtypes (Snutch & Reiner, 1992). The N-type VOCC antagonist ω-conotoxin GVIA has been shown to be a potent inhibitor of adrenergic and cholinergic neurotransmission in the peripheral nervous system (Keith *et al.*, 1989; Pruneau & Angus, 1990; Zygmunt & Högestätt, 1993). However, there are indications that the susceptibility of ω-conotoxin may differ between NANC nerves on one hand and

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adrenergic and cholinergic nerves on the other hand. In the rat bladder, the cholinergic component of the biphasic contractile response to electrical field stimulation (EFS) was more inhibited by ω-conotoxin than was the NANC response (Maggi, 1991). However, in the guinea-pig electrically-stimulated vas deferens, the ATP-mediated component of the biphasic contraction was found to be more susceptible to ω-conotoxin than was the adrenergic component (Hata et al., 1992). In neither of these studies was a full concentration-response curve for ω-conotoxin obtained.

The rabbit urethra exhibits both an adrenergic contraction and a NANC relaxation in response to EFS (Andersson et al., 1983). Such responses can also be elicited in preparations of the rabbit urethral lamina propria (Mattiasson et al., 1985; Zygmunt et al., 1993). In rabbit detrusor muscle, EFS elicits a contraction consisting of both a cholinergic and a NANC component (Ambache & Zar, 1970). The aim of the present study was to investigate whether ω-conotoxin GVIA in the rabbit lower urinary tract affects differently neurotransmission mediated by NANC nerves on one hand, and adrenergic and cholinergic nerves on the other. Therefore, we compared the effects of ω-conotoxin GVIA and some other subtype selective calcium channel blockers on NANC relaxation and adrenergic contractions in the urethra, and cholinergic and NANC contractile responses in the detrusor of the rabbit.

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 (for composition, see below). The bladder and urethra were opened longitudinally and separated by a transverse cut at the level of the bladder neck. The vesical mucosa was removed and the detrusor muscle tissue was cut into longitudinally-oriented strip preparations (1 × 2 × 5 mm). The lamina propria of the urethra was separated from the smooth muscle layers under the microscope, and the strip preparations (1 × 2 × 5 mm) were prepared as described previously (Zygmunt et al., 1993). Urethral circular muscle strips were prepared from the middle and proximal parts of the urethra (Andersson et al., 1983). The strips were transferred to organ baths (5 ml), containing 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 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. The preparations were suspended between two L-formed hooks by means of silk ligatures. One of the hooks was connected to a movable unit allowing adjustment of tension, and the other to a Grass Instruments FTO3C force 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 a frequency of 1 to 30 Hz in 5 s trains at 2 min intervals. Optimum voltage was determined individually for each preparation by increasing the voltage until a maximum response at 10 Hz (relaxations of urethral smooth muscle and lamina propria, and contractions of the detrusor) or 25 Hz (contractions of urethral smooth muscle) was obtained. The polarity was changed after each pulse to minimize oxidation of the electrodes.

In order to study relaxant responses, the preparations were precontracted with 1 nM arginine vasopressin (AVP). This concentration, which produced approximately 50% of the maximal response in lamina propria, was chosen because it produced stable contractions. Phentolamine (1 μM) and propranolol (1 μM) were present in all these experiments. Contractile responses in urethral smooth muscle were examined in the presence of 0.3 mM L-NOARG. Frequency-response relationships were studied by stepwise increasing the frequency of stimulation from 1 to 30 Hz. The same preparation was then treated with ω-conotoxin (0.1 μM) for 20 min, and the stimulation procedure repeated with the same frequencies. The effects of increasing concentrations of ω-conotoxin on electrically-induced responses were studied at a frequency of 10 Hz (relaxations of urethral smooth muscle and lamina propria, and contractions of the detrusor) or 25 Hz (contractions of urethral smooth muscle). The drug was applied cumulatively when reproducible responses were obtained. Experiments run in parallel in the absence of ω-conotoxin served as controls. TTX (1 μM) was added at the end of each experiment to obtain an estimate of the direct muscle stimulation. Responses to cumulatively added noradrenaline (urethral smooth muscle), 3-morpholino-sydnonimin (SIN-1, urethral smooth muscle and lamina propria), carbachol (detrusor), and a single concentration (1 mM) of ATP (detrusor) were examined before and 20 min after exposure to ω-conotoxin.

Drugs

(−)-Noradrenaline hydrochloride (Sigma, St Louis, U.S.A.) was dissolved in 0.9% NaCl, containing 1 mM ascorbic acid. Prazosin hydrochloride (Pfizer, N.Y., U.S.A.) was dissolved in 10% lactic acid. (±)-Propranolol hydrochloride, (−)-scopolamine hydrochloride, carbamylcholine chloride (carbachol), adenosine 5'-triphosphate (ATP), N^{ω}-nitro-L-arginine (L-NOARG), arginine vasopressin acetate (AVP), tetrodotoxin (TTX), ω-conotoxin GVIA, nickel chloride (Sigma, St Louis, U.S.A.), phentolamine methane sulphonate (Ciba-Geigy, Basel, Switzerland) and 3-morpholino-sydnonimin hydrochloride (SIN-1; Casella AG, Germany) were dissolved in distilled water. (±)-Nimodipine (Bayer, Leverkusen, Germany) and (±)-tetramethrin (Nanogen, Watsonville, U.S.A.) were dissolved in absolute ethanol.

Calculations and statistics

Relaxant responses were expressed as a percentage of the response obtained before application of the test drug. When the test drug reduced the baseline tension, R/T values were calculated according to 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. The negative logarithm of the drug concentration eliciting half maximum inhibition (pIC_{50}) was determined by linear regression analysis using the values immediately above and below half maximum response. I_{max} refers to the maximum inhibition obtained. The results are expressed as mean values ± s.e.mean, and n denotes the number of experiments (animals) performed. Statistical analysis was performed by using Student's t test (two-tailed). Statistical significance was accepted when $P < 0.01$.

Results

Relaxant responses in urethral smooth muscle and lamina propria

In AVP-contracted (1 nM) urethral smooth muscle and lamina propria, EFS elicited frequency-dependent relaxations (Figure 1). Half maximum relaxation was obtained at a

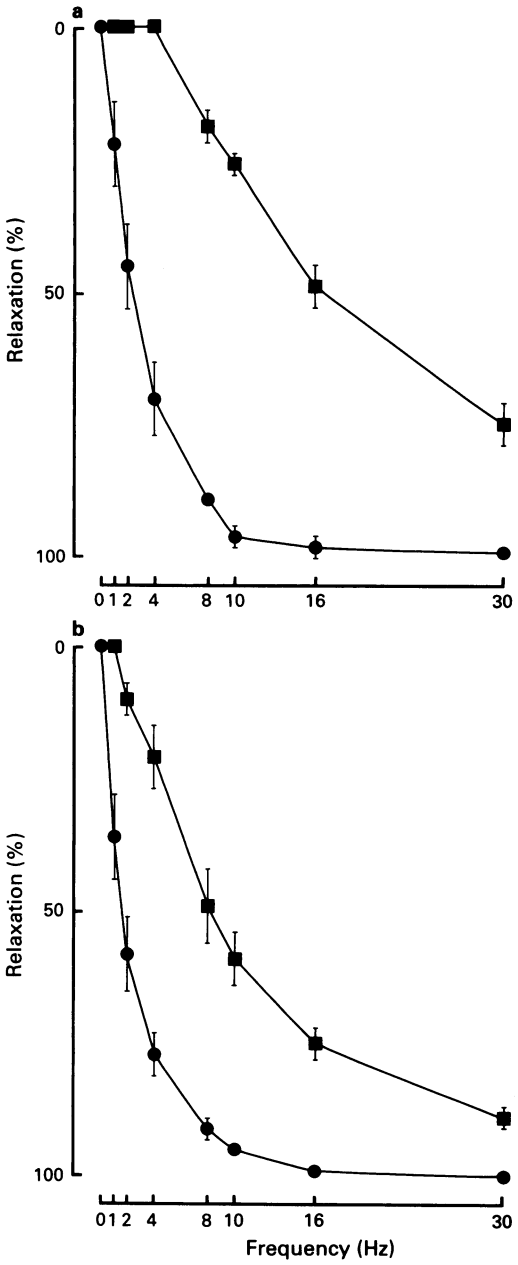

Figure 1 Frequency-dependent non-adrenergic, non-cholinergic relaxation in rabbit urethral smooth muscle (a) and lamina propria (b) precontracted with 1 nM arginine vasopressin before (●) and after (■) application of 0.1 μM ω-conotoxin. Preparations were treated with ω-conotoxin for 20 min before the second stimulation period with the different frequencies. Results are expressed as percentage of the maximum relaxation obtained during the first stimulation period, and given as mean ± s.e.mean ($n = 5$).

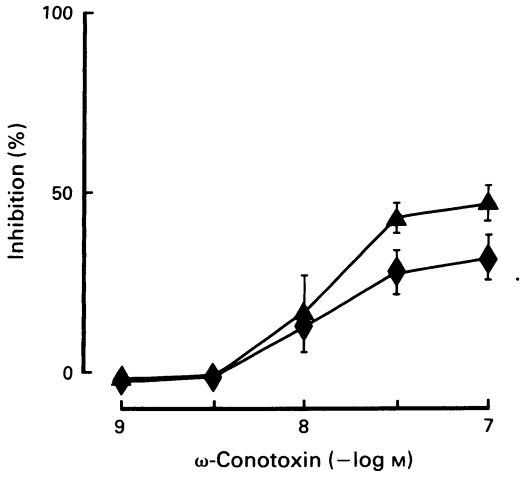

Figure 2 Inhibitory effect of cumulatively added ω-conotoxin on electrically-induced (10 Hz) non-adrenergic, non-cholinergic relaxation in rabbit urethral smooth muscle (▲) and lamina propria (◆). Preparations were precontracted with 1 nM arginine vasopressin. As shown in parallel controls, the electrically-induced relaxations were reproducible throughout the duration of the experiments (not shown). Responses are expressed as percentage of the maximum relaxation before application of ω-conotoxin, and given as mean ± s.e.mean ($n = 6$).

frequency of approximately 2 Hz. The maximum relaxation amounted to 73% and 89% of the AVP-induced contraction in the urethral smooth muscle and lamina propria, respectively. In the presence of 0.1 μM ω-conotoxin, the relaxation was significantly reduced (Figure 1). The inhibition was larger the lower the frequency of stimulation. An ω-conotoxin-resistant relaxation was observed at frequencies higher than 1 Hz (lamina propria) and 4 Hz (urethral smooth muscle), and at 30 Hz stimulation this relaxation was 89% and 75% of the maximum relaxation, respectively. TTX (1 μM) abolished the relaxation (tested at 30 Hz) both in the absence and presence of ω-conotoxin.

The relaxant responses at 10 Hz stimulation amounted to 96% (urethral smooth muscle) and 95% (lamina propria) of the maximum relaxation. These relaxations were concentration-dependently inhibited by ω-conotoxin (Figure 2, Table 1). The pIC_{50} values in the urethral smooth muscle and lamina propria did not differ significantly (Table 1). As can be seen in Figure 2, a significant portion of the relaxation remained in the presence of 0.1 μM ω-conotoxin; 53% (urethral smooth muscle) and 68% (lamina propria). TTX (1 μM, $n = 6$) abolished the ω-conotoxin-insensitive relaxant response in both types of preparation.

In the lamina propria, nimodipine (0.1 μM) and nickel (0.3 mM) reduced the AVP-induced contraction (Figure 3) by 33 ± 5% ($n = 6$) and 55 ± 6% ($n = 4$), respectively. The effects of nimodipine and nickel were therefore evaluated by comparing R/T values (see Methods). These values were not significantly different before and after application of nimodipine (81 ± 6% versus 84 ± 6%, $n = 6$) or nickel (81 ± 6% versus 106 ± 13%, $n = 4$). Tetramethrin (0.3 μM, $n = 3$) did not affect the AVP-induced contraction nor the relaxation elicited by EFS. The NO donor, SIN-1, relaxed both types of preparation ($n = 5-6$) contracted by AVP (1 nM) in a concentration-dependent manner. At the highest SIN-1 concentration tested (0.1 mM), the relaxation amounted to 81 ± 7% and 92 ± 8% of the AVP-induced contraction in the urethral smooth muscle and lamina propria, respectively. ω-Conotoxin (0.1 μM) did not affect the SIN-1-induced relaxation in either type of preparation ($n = 5-6$).

Contractile responses in urethral smooth muscle

As shown previously (Andersson *et al.*, 1983) and confirmed in the present study ($n = 5$, data not shown), EFS induced frequency-dependent contractions in urethral smooth muscle preparations at resting tension. These contractions were virtually abolished by 1 μM prazosin ($n = 3$). Since the frequency-response curve did not reach a plateau, the frequency for half maximum contraction (>14 Hz) could only be estimated. Contractions elicited by frequencies between 4–30 Hz were all significantly inhibited by 0.1 μM ω-conotoxin ($n = 5$). The relative inhibition amounted to 100% (4 Hz), 91% (8 Hz), 86% (12 Hz), 84% (16 Hz), 74% (25 Hz) and 69% (30 Hz). Thus an ω-conotoxin-resistant response was obtained with increasing frequencies of stimulation (≥ 12 Hz) and amounted at 30 Hz to 31%.

Table 1 Inhibitory effects of ω-conotoxin and tetrodotoxin (TTX) on electrically-induced adrenergic, cholinergic and NANC responses

	n	ω-Conotoxin pIC_{50}	ω-Conotoxin I_{max} (%)	TTX (1 μM) (%)
Relaxant responses				
Lamina propria (10 Hz)[a]	6	7.9 ± 0.1	32 ± 6	100 ± 0
Urethral smooth muscle (10 Hz)[a]	6	7.9 ± 0.1[b]	47 ± 5	100 ± 0
Contractile responses				
Urethral smooth muscle (25 Hz)[a]	6	8.9 ± 0.1[b]	77 ± 4	95 ± 1
Detrusor (10 Hz)[a]				
In the absence of scopolamine	7	8.7 ± 0.1[c]	88 ± 2	90 ± 2
In the presence of scopolamine (1 μM)	7	8.2 ± 0.1[c]	85 ± 2	87 ± 4

[a]Frequency of stimulation.
[b,c]Significant ($P < 0.01$).
pIC_{50} = negative logarithm of the ω-conotoxin concentration producing half maximum inhibition. I_{max} = maximum inhibition expressed as percentage reduction of the maximum response.
Results are expressed as mean values ± s.e.mean, and n indicates the number of preparations (animals) examined.

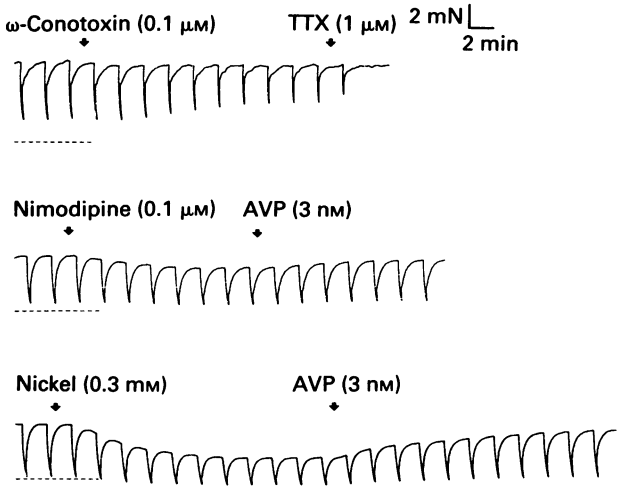

Figure 3 Original tracings showing the effects of 0.1 μM ω-conotoxin (upper panel), 0.1 μM nimodipine (middle panel) and 0.3 mM nickel (lower panel) on electrically-induced (10 Hz) non-adrenergic, non-cholinergic relaxations in rabbit urethral lamina propria. Preparations were precontracted with 1 nM arginine vasopressin (AVP). The second arrow indicates application of tetrodotoxin (TTX) or a higher concentration of AVP. Broken line denotes the baseline tension before application of AVP.

The sensitivity to ω-conotoxin was studied at a frequency of 25 Hz. At this frequency, the contraction was 82% of the response to the highest frequency tested (30 Hz). A small TTX (1 μM)-resistant contraction (5 ± 1%, $n = 5$) remained at 25 Hz; this component increased with increasing stimulation frequencies (data not shown). ω-Conotoxin concentration-dependently inhibited the contractions. The pIC_{50} value for ω-conotoxin was significantly larger for the contractile than for the relaxant responses in the urethra (Table 1). Contractile responses to noradrenaline (0.01 μM– 0.1 mM, $n = 4$) were not affected by 0.1 μM ω-conotoxin (data not shown).

Contractile responses in the detrusor

EFS induced frequency-dependent contractions in the detrusor ($n = 5$, data not shown). Half maximum contraction was obtained at a frequency of approximately 6 Hz. A small TTX (1 μM)-resistant contraction (6 ± 1%, $n = 5$) remained at the highest frequency used (30 Hz). Contractions elicited by frequencies between 4–30 Hz were all significantly reduced by 0.1 μM ω-conotoxin ($n = 5$). The relative inhibition amounted to 88% (4 Hz), 84% (8 Hz), 81% (12 Hz), 78% (16 Hz), 69% (20 Hz) and 53% (30 Hz). Thus an ω-conotoxin-resistant response was obtained with increasing frequencies of stimulation (≥ 20 Hz) and amounted at 30 Hz to 47%.

The contractions obtained at 10 Hz stimulation amounted to 70% of the maximum response. Scopolamine reduced these contractions by 71 ± 3% ($n = 7$). ω-Conotoxin inhibited the response in a concentration-dependent manner in both the absence and presence of 1 μM scopolamine (Table 1). The pIC_{50} value for ω-conotoxin was significantly larger for the 'total' contraction than for the NANC contraction. Contractile responses to carbachol (0.01 μM–0.1 mM, $n = 4$) and 1 mM ATP ($n = 4$) were unaffected by 0.1 μM ω-conotoxin (data not shown).

Discussion

The morphology of the lamina propia and smooth muscle preparations of the rabbit urethra differ in that the former is rich in connective tissue and blood vessels (Mattiasson *et al.*, 1985). NOS localized to neurones has been demonstrated by NADPH diaphorase staining around arteries and in the smooth muscle bundles in the rabbit urethral lamina propria (Zygmunt *et al.*, 1993). NOS has also been demonstrated by immunohistochemistry and/or NADPH diaphorase staining in rat and pig urethral smooth muscle (Burnett *et al.*, 1992; Larsson *et al.*, 1992; Alm *et al.*, 1993). Even though similar studies on the rabbit urethral smooth muscle have not to our knowledge been published, the observation that the electrically-induced NANC relaxation in rabbit urethral smooth muscle and lamina propria was sensitive to L-NOARG and TTX supports the existence of NO releasing nerves in both these preparations (Andersson *et al.*, 1991; 1992; Zygmunt *et al.*, 1993). In the present study, ω-conotoxin inhibited the relaxation with similar potency in both types of preparation. The inhibitory effect was not due to an interaction with the NO effector system, since ω-conotoxin did not affect the relaxation induced by SIN-1. The inhibition, however, decreased with increasing frequencies of stimulation, and at 10 Hz stimulation, a large ω-conotoxin-resistant component remained in the urethral smooth muscle (53%) and lamina propria (68%).

In the lamina propria, nimodipine and nickel reduced the AVP-induced contraction. The effects of these substances on EFS-induced relaxation were therefore evaluated by comparing R/T values before and after their application. A positive correlation has been demonstrated between the amplitude of the electrically-induced relaxation (R) and the agonist-induced tension (T), i.e. the higher the contraction amplitude

the greater the relaxant response (Garcia-Pascual *et al.*, 1991). As shown in the present study, it was possible to regain the initial tension and relaxation recorded before addition of nimodipine or nickel by increasing the AVP concentration. Thus, the effects of nickel and nimodipine on the relaxation were considered to be attributable to the reduction of the AVP-induced tension rather than to inhibition of neurotransmitter release. Nickel is known for its dual action on L and T channels, although it is claimed to have some selectivity towards the T channel (Fox *et al.*, 1987; Hagiwara *et al.*, 1988). However, it is not probable that the effect of nickel on the AVP-induced contraction was caused by T channel blockade in the present study, since the T channel blocker, tetramethrin, had no effect. The tetramethrin concentration used in this study was three times higher than that previously shown to block T channel currents in the rabbit sinoatrial node (Hagiwara *et al.*, 1988). Our results suggest that neither L nor T channels are involved in the NO release process. Furthermore, it appears that influx of calcium through ω-conotoxin-sensitive VOCCs is only partially responsible for NANC relaxation in urethral smooth muscle and lamina propria at stimulation frequencies higher than 1 Hz (lamina propria) and 4 Hz (urethral smooth muscle).

The ω-conotoxin-resistant relaxation may have been caused by calcium influx through yet another type of VOCC (i.e. a non-L, N and T channel). A third HVA calcium channel, insensitive to ω-conotoxin and dihydropyridines, has been demonstrated in cerebellar Purkinje cells and therefore denoted P (Llinas *et al.*, 1989). Lundy *et al.* (1992) recently suggested that P channels were responsible for calcium influx in mammalian brain synaptosomes, since the influx was only slightly reduced by ω-conotoxin and dihydropyridines, but blocked to a greater extent by the venom from the spider *Hololena curta*. Another venom from the spider *Grammostola spatula* blocked calcium influx in rat synaptosomes, [^{3}H]-D-aspartate release from rat hippocampal brain slices and the whole cell calcium current of guinea-pig hippocampal CA1 neurones to a greater extent than did ω-conotoxin or dihydropyridine calcium antagonists (Keith *et al.*, 1992). It has also been suggested that P channels mediate calcium influx into mouse motor nerve terminals, since neuromuscular transmission was blocked by the funnel-web spider venom FTX, but not by ω-conotoxin and dihydropyridine calcium antagonists (Uchitel *et al.*, 1992). There is also evidence of a VOCC-independent release of VIP from rat enteric nerve and noradrenaline and GABA from rat brain slices (Minchin, 1980; Sandoval, 1980; Schoffelmeer & Mulder, 1983; Belai *et al.*, 1987). It was proposed that an intracellular Na^+ accumulation in the nerve endings may increase the cytoplasmic calcium level through a Na^+/Ca^{2+} exchange mechanism with intracellular calcium stores, e.g., in mitochondria (Minchin, 1980; Sandoval, 1980; Schoffelmeer & Mulder, 1983).

In accordance with a previous study by Andersson *et al.* (1983), electrically-induced contractile responses in the urethral smooth muscle were virtually abolished by TTX or α-adrenoceptor blockade, suggesting that the contractions were caused by neuronal release of mainly noradrenaline. ω-Conotoxin inhibited the contractions in a concentration-dependent manner without affecting the exogenous response to noradrenaline. The adrenergic contraction was approximately 10 times more sensitive to ω-conotoxin than was the NANC relaxation in the same preparation. The responses also differed with regard to the frequency-response relationship and the magnitude of the ω-conotoxin-resistant component, which appeared to be less prominent for the adrenergic than for the NANC responses. This may indicate that calcium influx through N-type VOCCs is more important for transmitter release in adrenergic than in NANC nerves in the rabbit urethra. Only approximately 20% of the EFS-induced contraction remained in the presence of ω-conotoxin at 25 Hz stimulation. Since TTX and prazosin virtually abolished this response, the ω-conotoxin-resistant contraction could not be attributed to a direct electrical stimulation of the smooth muscle. As shown by Larsson *et al.* (1984), the electrically-induced [^{3}H]noradrenaline release in the rabbit urethra was insensitive to the L channel blocker, nifedipine. This does not favour the view that influx of calcium through neuronal L channels was involved in the ω-conotoxin-resistant adrenergic response.

The electrically-induced contractile response in the rabbit detrusor consists of an atropine-sensitive and an atropine-resistant component, which have been attributed to neuronal release of acetylcholine and ATP, respectively (Ambache & Zar, 1970; Igawa *et al.*, 1993). A relatively small scopolamine-resistant component was found in the present study at 10 Hz stimulation. ω-Conotoxin concentration-dependently inhibited and eventually abolished the cholinergic and NANC contractile responses at this frequency. The effect of ω-conotoxin was considered to be prejunctional, since the contractile responses to carbachol and ATP were unaffected. A small but significant difference in the sensitivity to ω-conotoxin was found between the cholinergic and NANC contractile responses; the 'total' contraction was approximately three times more sensitive to ω-conotoxin than the scopolamine-resistant component. In the rat bladder, the cholinergic component was also inhibited more by ω-conotoxin than was the atropine-resistant response (Maggi, 1991).

The results of the present study demonstrate differences between adrenergic, cholinergic and NANC nerves with regard to the frequency-response relationship and the susceptibility to inhibition by ω-conotoxin in the rabbit lower urinary tract. The stimulation frequency for half maximum response was significantly smaller for the NANC relaxation (2 Hz) than for the contractile responses in the urethra (>14 Hz) and detrusor (6 Hz). ω-Conotoxin concentration-dependently inhibited the adrenergic contraction and NANC relaxation in the urethra, and the cholinergic and NANC contractions in the detrusor. The NANC responses in the urethra and detrusor appeared to be less sensitive to ω-conotoxin than the adrenergic and cholinergic contractions, respectively. An ω-conotoxin-resistant response was observed in all preparations. This response increased with increasing frequencies of stimulation, and was more prominent for the NANC relaxation than for the adrenergic and cholinergic contractions. Calcium influx through neuronal L or T channels did not seem to be involved in the ω-conotoxin-resistant relaxation in the lamina propria. This component may have been caused by calcium influx through another type of neuronal VOCC, e.g., a P channel, or by calcium mobilized via routes independent of VOCCs.

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References

ALM, P., LARSSON, B., EKBLAD, E., SUNDLER, F. & ANDERSSON, K.-E. (1993). Immunohistochemical localization of peripheral nitric oxide synthase-containing nerves using antibodies raised against synthesized C- and N-terminal fragments of a cloned enzyme from rat brain. *Acta Physiol. Scand.*, **148**, 421–429.

AMBACHE, N. & ZAR, M.A. (1970). Non-cholinergic transmission by post-ganglionic motor neurones in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

ANDERSSON, K.-E., MATTIASSON, A. & SJÖRGREN, C. (1983). Electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. *J. Urol.*, **129**, 63–67.

ANDERSSON, K.-E., GARCIA PASCUAL, A., FORMAN, A. & TÖTTRUP, A. (1991). Non-adrenergic, non-cholinergic nerve-mediated relaxation of rabbit urethra is caused by nitric oxide. *Acta Physiol. Scand.*, **141**, 133–134.

ANDERSSON, K.-E., GARCIA PASCUAL, A., PERSSON, K., FORMAN, A. & TÖTTRUP, A. (1992). Electrically-induced, nerve-mediated relaxation of rabbit urethra involves nitric oxide. *J. Urol.*, **147**, 253–259.

BELAI, A., RALEVIC, A. & BURNSTOCK, G. (1987) VIP release from enteric nerves is independent of extracellular calcium. *Regul. Pept.*, **19**, 79–89.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351**, 714–718.

BURNETT, A.L., LOWENSTEIN, C.J., BREDT, D.S., CHANG, T.S.K. & SNYDER, S.H. (1992). Nitric oxide: a physiologic mediator of penile erection. *Science*, **257**, 401–403.

DOCKRAY, G.J. (1992). Transmission: Peptides. In *Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V., pp. 409–464. Chur, Switzerland: Harwood Academic Publishers.

FÖRSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849–1857.

FOX, A.P., NOWYCKY, M.C. & TSIEN, R.W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol.*, **394**, 149–172.

GARCIA-PASCUAL, A., COSTA, G., GARCIA-SACRISTAN, A. & ANDERSSON, K.-E. (1991). Relaxation of sheep urethral muscle induced by electrical stimulation of nerves: involvement of nitric oxide. *Acta Physiol. Scand.*, **141**, 531–539.

HAGIWARA, N., IRISAWA, H. & KAMEYAMA, M. (1988). Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J. Physiol.*, **395**, 233–253.

HATA, F., FUJITA, A., SAEKI, K., KISHI, I., TAKEUCHI, T. & YAGASAKI, O. (1992). Selective inhibitory effects of calcium channel antagonists on the two components of the neurogenic response of guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.*, **263**, 214–220.

IGAWA, Y., MATTIASSON, A. & ANDERSSON, K.-E. (1993). Functional importance of cholinergic and purinergic neurotransmission for micturition contraction in the normal, unanaesthetized rat. *Br. J. Pharmacol.*, **198**, 473–479.

IGNARRO, L.J. (1989). Endothelium-derived nitric-oxide: actions and properties. *FASEB J.*, **3**, 31–36.

KEITH, R.A., MANGANO, T.J., PACHECO, M.A. & SALAMA, A.I. (1989). Characterization of the effects of ω-conotoxin GVIA on the responses of voltage-sensitive calcium channels. *J. Auton. Pharmacol.*, **9**, 243–252.

KEITH, R.A., DEFEO, P.A., LAMPE, R.A., SPENCE, K.T., LO, M.M.S. & FFRENCH-MULLEN, J.M.H. (1992). Inhibition of neuronal calcium channels by a fraction from *grammostola spatulata* venom. *Pharmacol. Commun.*, **1**, 19–25.

LARSSON, B., ALM, P., PERSSON, K. & ANDERSSON, K.-E. (1992). Studies on the localization of some neurotransmitters, nerve markers, and NADPH diaphorase activity in tissues from the pig lower urinary tract. *Neurourol. Urodyn.*, **11**, 444–445.

LARSSON, B., HÖGESTÄTT, E.D., MATTIASSON, A. & ANDERSSON, K.-E. (1984). Differential effects of nifedipine, verapamil, and diltiazem on noradrenaline-induced contractions, adrenergic transmitter release, and alpha-adrenoceptor binding in the female rabbit urethra. *Naunyn-Schmied. Arch. Pharmacol.*, **326**, 14–21.

LLINAS, R., SUGIMORI, M., LIN, J.-W. & CHERKSEY, B. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 1689–1693.

LUNDY, P.M., HONG, A. & FREW, R. (1992). Inhibition of a dihydropyridine, ω-conotoxin insensitive Ca^{2+} channel in rat synaptosomes by venom of the spider *Hololena curta*. *Eur. J. Pharmacol.*, **225**, 51–56.

MAGGI, C.A. (1991). Omega conotoxin and prejunctional modulation of the biphasic response of the rat isolated urinary bladder to single pulse electrical field stimulation. *J. Auton. Pharmacol.*, **11**, 295–304.

MATTIASSON, A., ANDERSSON, K.-E. & SJÖGREN, C. (1985). Contractant and relaxant properties of the female rabbit urethral submucosa. *J. Urol.*, **133**, 304–310.

MINCHIN, M.C.W. (1980). The role of Ca^{2+} in the protoveratrine-induced release of γ-aminobutyrate from rat brain slices. *Biochem. J.*, **190**, 333–339.

MÜLSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^5-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 143–147.

MURAD, F., FÖRSTERMANN, U., NAKANE, M., SCHMIDT, H., POLLOCK, J., SHENG, H., MATSUMOTO, T., WARNER, T., MITCHELL, J., TRACEY, R. & BUECHLER, W. (1992). The nitric oxide-cyclic GMP signal transduction pathway in vascular smooth muscle preparations and other tissues. *Jpn. J. Pharmacol.*, **58**, 150P–157P.

MYERS, P.R., MINOR Jr, R.L., GUERRA Jr, R., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.

PERSSON, K. & ANDERSSON, K.-E. (1992). Nitric oxide and relaxation of pig lower urinary tract. *Br. J. Pharmacol.*, **106**, 416–422.

PRUNEAU, D. & ANGUS, J.A. (1990). ω-Conotoxin GVIA is a potent inhibitor of sympathetic neurogenic responses in rat small mesenteric arteries. *Br. J. Pharmacol.*, **100**, 180–184.

SANDOVAL, M.E. (1980). Sodium-dependent efflux of [^{3}H]GABA from synaptosomes probably related to mitochondrial calcium mobilization. *J. Neurochem.*, **35**, 915–921.

SCHOFFELMEER, A.N.M. & MULDER, A.H. (1983). [^{3}H]noradrenaline release from brain slices induced by an increase in the intracellular sodium concentration: role of intracellular calcium stores. *J. Neurochem.*, **40**, 615–621.

SHER, E., BIANCARDI, E., PASSAFARO, M. & CLEMENTI, F. (1991). Physiopathology of neuronal voltage-operated calcium channels. *FASEB J.*, **5**, 2677–2683.

SNEDDON, P. & GRAHAM, A. (1992). Role of nitric oxide in the autonomic innervation of smooth muscle. *J. Auton. Pharmacol.*, **12**, 445–456.

SNUTCH, T.P. & REINER, P.B. (1992). Ca^{2+} channels: diversity of form and function. *Curr. Opinion Neurobiol.*, **2**, 247–253.

SNYDER, S.H. (1992). Nitric oxide: first in a new class of neurotransmitters? *Science*, **257**, 494–496.

UCHITEL, O.D., PROTTI, D.A., SANCHEZ, V., CHERKSEY, B.D., SUGIMORI, M. & LLINAS, R. (1992). P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3330–3333.

VANIN, A.F. (1991). Endothelium-derived relaxing factor is a nitrosyl iron complex with thiol ligands. *FEBS*, **289**, 1–3.

VEDERNIKOV, Y.P., MORDVINTCEV, P.I., MALENKOVA, I.V. & VANIN, A.F. (1992). Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **211**, 313–317.

ZYGMUNT, P.K.E., PERSSON, K., ALM, P., LARSSON, B. & ANDERSSON, K.-E. (1993). The L-arginine/nitric oxide pathway in the rabbit urethral lamina propria. *Acta Physiol. Scand.*, **148**, 431–439.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1993). Calcium channels at the adrenergic neuroeffector junction in the rabbit ear artery. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 617–623.

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