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Functional role of inhibitory and excitatory nerves in the porcine lower urinary tract

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Abstract

In the trigone (three portions) and proximal urethra isolated from castrated male pigs, transmural electrical stimulation (0.5–10 Hz) induced no or slight contractions followed by frequency-related relaxations. Atropine suppressed the contraction and potentiated the relaxation. N^G-nitro-L-arginine methylester (L-NAME), a nitric oxide (NO) synthase inhibitor, depressed or abolished the relaxation induced by low frequency stimulation, but only slightly attenuated the response to high frequency stimulation. L-Arginine reversed the inhibitory effect. L-NAME-sensitive relaxation by 1 Hz stimulation was abolished by 1*H*-(1,2,4)oxadiazolo-(4,3-*a*)quinoxalin-1-one (ODQ), a guanylate cyclase inhibitor. Release of NO by nerve stimulation to trigonal strips was determined by increased formation of cyclic GMP in the incubation media containing guanylate cyclase and GTP. L-NAME-resistant relaxation by 10 Hz stimulation was not impaired by ODQ, capsaicin, chymotrypsin, K^+ channel inhibitors and β-adrenoceptor antagonists. Similar results were obtained in the trigone and urethra from normal male and female pigs. Detrusor muscle responded to nerve stimulation with contraction followed by slight relaxation. Relaxations at 1 and 10 Hz stimulation under treatment with atropine and α,β-methylene ATP were partially attenuated by L-NAME. It is concluded that there is no significant difference in the inhibitory responses, sensitive and resistant to L-NAME, to nerve stimulation in the trigone and proximal urethra from castrated and non-castrated male and female pigs. Relaxations to stimulation at 1 Hz seem to be mediated exclusively by neurogenic NO and cyclic GMP generation, whereas those to 10 Hz stimulation is mainly associated with non-NO relaxing factor(s), peptides, K^+ channel openers and β-adrenoceptor agonist being unlikely involved.

Keywords: Nitric oxide (NO); Sphincter; Nitrergic nerve; Trigone; Urethra

1. Introduction

Urinary filling/storage and micturition are controlled by excitatory and inhibitory responses of the urinary detrusor muscle and the urethral and bladder neck sphincter muscle. Efferent autonomic nerves play crucial roles in the regulation of the smooth muscle tone. In addition to classical adrenergic and cholinergic nerves, there are various nerves of nonadrenergic, noncholinergic (NANC) nature, which also contribute importantly to the control of lower urinary tract function (Andersson, 1993). Attention has long been directed to identification of NANC inhibitory and excitatory nerves. On the basis of hypothesis that NANC inhibitory responses are mediated by nitric oxide (NO) in anococcygeus (Gillespie et al., 1989; Li and Rand, 1989), cerebral arterial (Toda and Okamura, 1990a,b) and intestinal muscle (Bult et al., 1990; Toda et al., 1990), evidence for the important role of NO as a neurotransmitter is also reported in other smooth muscle, including the lower urinary tract (Andersson et al., 1991; Dokita et al., 1991; Persson and Andersson, 1992). Literatures so far presented indicate that the inhibitory response to NANC nerve stimulation is mediated by NO in many tissues, such as the detrusor, trigone and urethra, but some other molecules are also involved in the inhibitory response (Hashimoto et al., 1992; Bridgewater et al., 1993). Although regional differences in the functional role of nitrergic, noradrenergic and cholinergic nerves in the lower urinary tract have been observed (Andersson, 1993), information is not available concerning the transition from the proximal urethra and bladder neck to the detrusor muscle in autonomic nerve function.

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In the present study, we aimed to compare the functional role of inhibitory and excitatory nerves in the trigone (three regions), proximal urethra and detrusor muscle isolated from male pigs, to determine mechanisms underlying the inhibitory and excitatory responses, and to biochemically determine the involvement of NO–cyclic GMP system in the neurogenic relaxation.

2. Materials and methods

2.1. Animals

The urinary bladder and urethra from castrated male pigs (7–8 months of age; castrated about 1 month after birth) obtained at the slaughterhouse were mainly used. For comparison, the lower urinary tract of female pigs ($n=5$) from the slaughterhouse and of the non-castrated male pigs ($n=5$) obtained at KAC biological science lab (KAC, Ritto, Shiga, Japan) were also used. Isolated bladder and urethra were opened longitudinally. The trigone and urethra were cut into transverse strips of approximately 3 mm wide, 6–7 mm long and 1 mm thick. Longitudinal strips of detrusor muscle (approximately $4\times4-5\times1$ mm) were also obtained. The trigone was separated into three portions, proximal (T1), intermediate (T2) and distal (T3), as shown in Fig. 1. The strips were placed in the modified Krebs–Ringer bicarbonate solution (pH 7.4) of following composition (mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and D-glucose, 11.1. Then, the urothelium, fat and connective tissue were removed.

2.2. Experimental protocol

2.2.1. Isometric tension recording

The strips were fixed vertically between hooks by means of silk ligature in a organ bath (20-ml capacity) containing a modified Krebs–Ringer solution, which was maintained at 37 ± 0.3 °C and aerated with a mixture of 95% O_2 and 5% CO_2. One end of the strips was fixed to the bottom of the organ bath. The other hook anchoring the upper end of strips was connected to an isometric force-displacement transducer and carrier amplifier, and isometric mechanical responses were displayed on an ink-writing oscillograph (Nihon-Kohden Kogyo, Tokyo, Japan). The resting tension was adjusted to 2.0 g, which was determined optimal for inducing the maximal contraction.

The strips placed between a pair of platinum electrodes were transmurally stimulated by 0.2 ms electrical pulses of supramaximal intensity at frequencies of 0.5, 1, 5 and 10 Hz for 5 s at an interval of 2 min. Before the start of experiments, all of the strips were allowed to equilibrate for 60–90 min in the bathing media, during which time the fluid was replaced every 10–15 min. The contractile response to 100 mM K^+ was first obtained, and the strips were washed several times with fresh media and equilibrated. For the measurement of relaxation, the strips were partially contracted with prostaglandin (PG)$F_{2\alpha}$ (3×10^{-6} to 10^{-5} M) in a range of 20–50% of contraction caused by 100 mM K^+. At the end of each series of experiment, papaverine (10^{-4} M) was applied to attain the maximal relaxation. Relaxant and contractile responses induced by test drugs and electrical stimulation were expressed as relative values to the papaverine-induced relaxation and K^+ (100 mM)-induced contraction, respectively. After the responses to electrical stimulation were found to be stabilized, blocking agents were applied.

2.2.2. Measurement of NO release by electrical nerve stimulation

The NO released from porcine trigonal strips was estimated from cyclic GMP accumulation in the extracellular fluids after addition of GTP, 3-isobutyl-1-methylxanthine (IBMX) and soluble fraction of rat cerebellum, as described previously (Oka et al., 2000a,b). Briefly, pieces of the

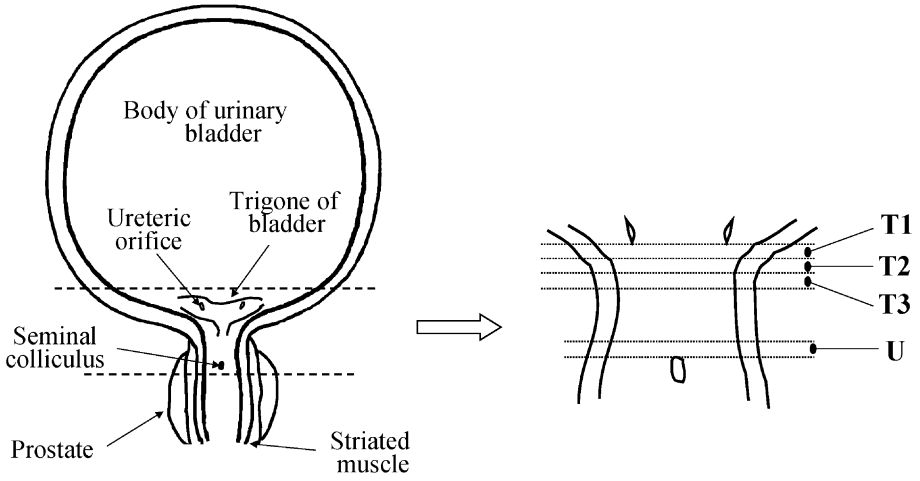

Fig. 1. Scheme of portions of the male porcine urethra (U) and trigone (T1, T2 and T3) used in the present study.

trigone (approximately 3 mm long and wide) were transferred to 24-well plastic plates containing 0.2 ml of Krebs–Ringer bicarbonate solution aerated with a mixture of 95% O_2 and 5% CO_2 and pre-incubated at 37 °C for 10 min. The supernatant fraction of rat cerebellum was prepared immediately before use, as follows: the rat cerebellum was dissected and homogenized with 10 volumes of ice-cold buffer-A (50 mM Tris–HCl containing 1 mM EDTA, 1 mM dithiothreitol and 200 mM phenylmethyl-sulfonyl fluoride) using Polytron, then the homogenate was centrifuged at 30,000 × *g* for 30 min and the resultant supernatant fraction was used as the source of soluble guanylate cyclase. After pre-incubation, the incubation media was replaced by freshly prepared modified Krebs–Ringer bicarbonate solution, to which 0.5 mM GTP, 100 μM IBMX and 15-μl aliquot of the supernatant fraction of rat cerebellum (containing approximately 10 μg protein) were added. After that, electrical stimulation (supramaximal intensity, 0.2 ms pulse duration, 1 Hz for 5 s, every 30 s) was applied to the strips for 30 min in the absence or presence of test drugs. Cyclic GMP produced in the incubation medium was measured using cyclic GMP enzyme immunoassay kit. Similar procedure was performed in the presence of 10^{-4} M N^G-nitro-L-arginine methylester (L-NAME), 10^{-4} M L-NAME plus 10^{-3} M L-arginine, or 10^{-7} M tetrodotoxin. At the end of experiments, tissues were dissolved in 1 ml of 1 N NaOH, and protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

2.3. Statistical analysis

All values shown in the text and figures are expressed as mean ± S.E. Statistical analyses were made using the Student's paired and unpaired *t*-tests for two groups and the Tukey's test after one-way analysis of variance for three or more groups (SAS, Ver. 6.12, SAS Institute, Cary, NC, USA).

2.4. Chemicals

Drugs used were N^G-nitro-L-arginine methylester (L-NAME), L-arginine, α-chymotrypsin (Nacalai Tesque, Kyoto, Japan), prostaglandin (PG)$F_{2\alpha}$ (Funakoshi, Tokyo, Japan), (±)-propranolol, yohimbine hydrochloride, acetylcholine chloride, (−)-norepinephrine hydrochloride, atropine methyl nitrate, glibenclamide, (−)-isoproterenol bitartrate, 3-isobutyl-1-methylxanthine (IBMX), α,β-methylene ATP, capsaicin, 1*H*-(1,2,4) oxadiazolo-(4,3-*a*) quinoxalin-1-one (ODQ), L-phenylephrine hydrochloride, prazosin hydrochloride, sodium nitroprusside dihydrate, [D-*p*-Cl-Phe^6,Leu^{17}]-vasoactive intestinal peptide, (Sigma, St. Louis, MO), papaverine hydrochloride (Tokyo Kasei, Tokyo), apamin, charybdotoxin, ω-conotoxin, iberiotoxin, vasoactive intestinal peptide (VIP; Peptide Institute, Osaka, Japan), cyanopindolol hemifumarate, tetrodotoxin citrate (Tocris Cookson, Missouri, USA) and cyclic GMP enzyme immunoassay system (Amersham, Buckinghamshire, UK). Other chemicals were all of guaranteed grade.

3. Results

3.1. Modifications by L-NAME of the response to nerve stimulation

In isolated strips of the porcine trigone (portion 1–3) and urethra contracted with $PGF_{2\alpha}$, transmural electrical stimulation (0.5, 1, 5 and 10 Hz) produced a frequency-dependent

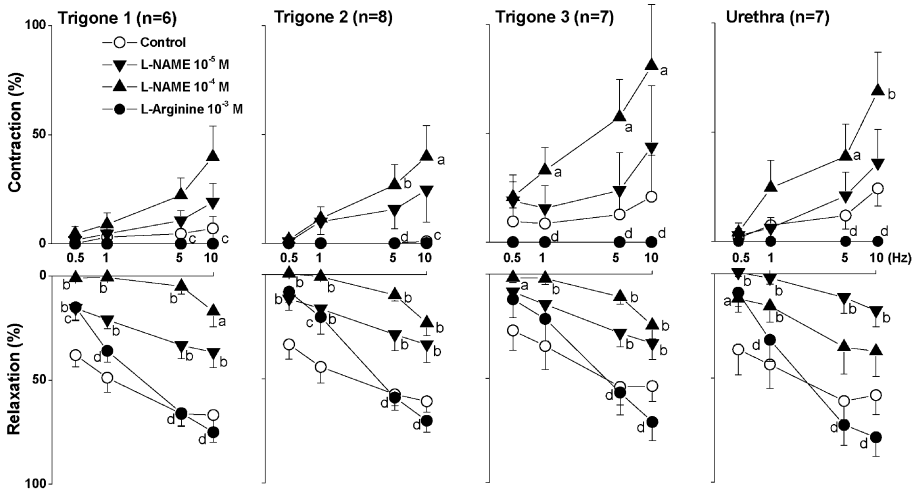

Fig. 2. Modification by L-NAME (10^{-5} and 10^{-4} M) and L-arginine (10^{-3} M) of contractile and relaxant responses to electrical stimulation at 0.5, 1, 5 and 10 Hz (abscissa) in strips of the porcine trigone (1–3) and urethra precontracted with $PGF_{2\alpha}$. Contractions induced by 100 mM K^+ were taken as 100% contraction, and relaxations to 10^{-4} M papaverine were taken as 100% relaxation (ordinate). Significantly different from control: $^aP<0.05$, $^bP<0.01$; significantly different from the value with 10^{-4} M L-NAME: $^cP<0.05$, $^dP<0.01$ (Tukey's test). *n* denotes the number of strips used. Vertical bars represent S.E.M.

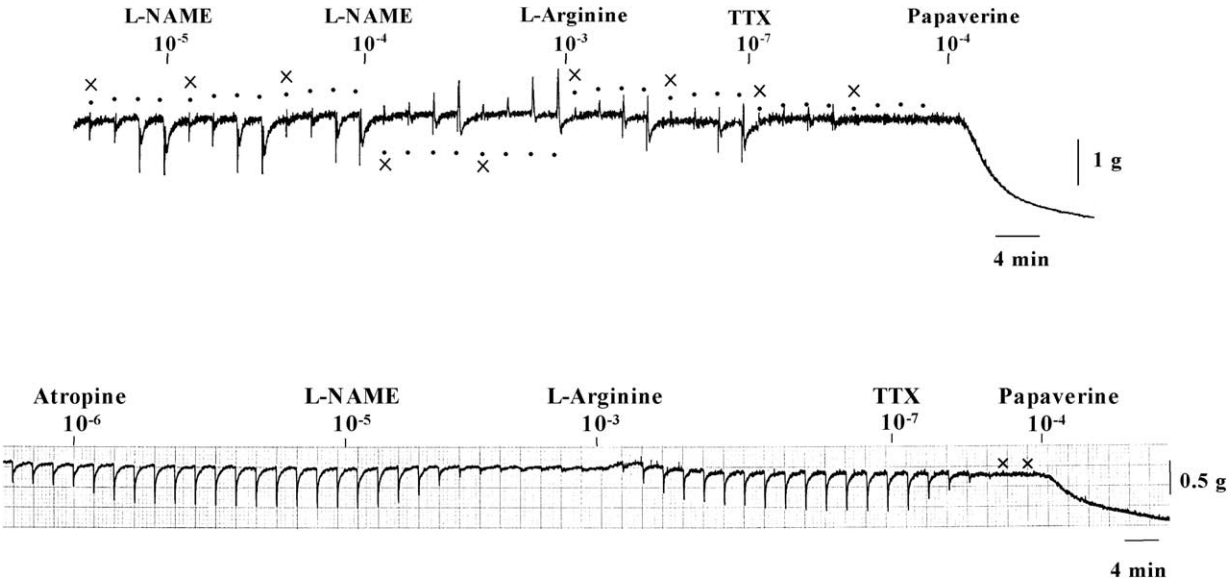

Fig. 3. Typical tracing of the response to electrical stimulation in frequencies of 0.5, 1, 5 and 10 Hz successively in this order, started from ×, at an interval of 2 min before and after L-NAME, L-arginine and tetrodotoxin (TTX) in a strip of trigone (T2) precontracted with $PGF_{2\alpha}$ (upper) and tracing of the relaxant response to 1 Hz stimulation before and after treatment with atropine, L-NAME, L-arginine and TTX (lower). In the lower tracing, atropine potentiated and L-NAME abolished the response. At the end, papaverine was applied to attain the maximal relaxation.

relaxation; at 5 and 10 Hz stimulation, a slight, transient contraction preceded the relaxation. Quantitative data are summarized in Fig. 2. Contractions in trigone-3 and urethra tended to be greater than those in the other zones of trigone. Relaxations in these tissues did not appreciably differ. Even at low frequencies (0.5 and 1 Hz) of stimulation, moderate relaxations were produced. Treatment with L-NAME at 10^{-5} and 10^{-4} M dose-dependently potentiated the contraction and inhibited the relaxation. The relaxations to low frequencies (0.5 and 1 Hz) of stimulation were abolished by the higher concentration of NO synthase inhibitor, whereas those induced at high frequencies were markedly attenuated but not abolished (Fig. 2). The addition of L-arginine (10^{-3} M) suppressed the potentiated contraction by L-NAME. Relaxant responses to low frequency stimulation were only partially reversed by L-arginine, whereas those at high frequencies were completely restored. A typical tracing of the response to different frequencies of stimulation before and after treatment with L-NAME and L-arginine is illustrated in Fig. 3 (upper tracing). Relaxations induced by 1 Hz stimulation were abolished by 10^{-6} M ODQ (51.0 ± 7.7% to 2.7 ± 2.1%, $P < 0.01$, paired t-test, $n = 7$). At the end of each series of

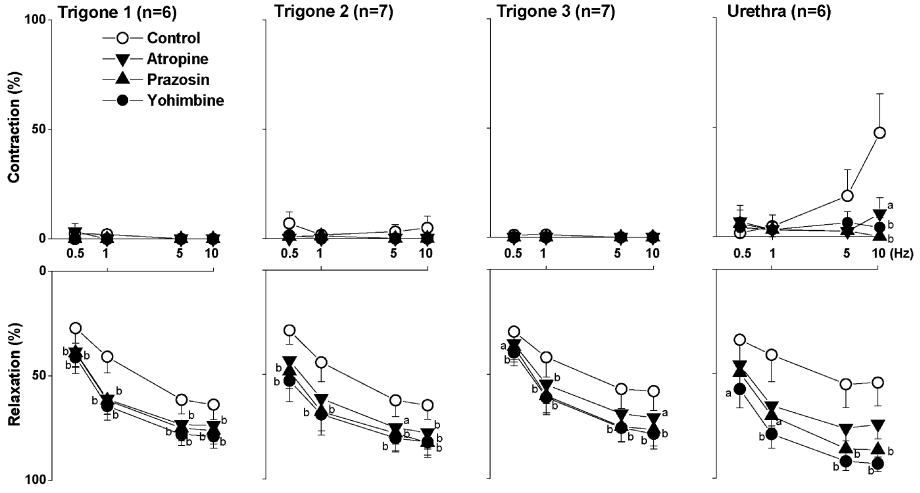

Fig. 4. Modification by atropine (10^{-6} M), prazosin (10^{-7} M) and yohimbine (10^{-7} M) of the response to electrical stimulation (0.5–10 Hz, abscissa) in strips of the porcine trigone (1–3) and urethra contracted with $PGF_{2\alpha}$. Contractions induced by 100 mM K^+ and relaxations to 10^{-4} M were taken as 100% contraction and 100% relaxation, respectively (ordinate). Significantly different from control: $^{a}P < 0.05$, $^{b}P < 0.01$ (Tukey's test). n denotes the number of strips used. Vertical bars represent S.E.M.

experiment, it was confirmed that the stimulation-induced responses were abolished by tetrodotoxin (10^{-7} M).

3.2. Modifications by L-NAME of the response to nerve stimulation

In $PGF_{2\alpha}$-contracted strips of the trigone and urethra, relaxations induced by electrical nerve stimulation were potentiated by atropine (10^{-6} M); however, additional potentiation was not elicited by treatment with 10^{-7} M prazosin and 10^{-7} M yohimbine alone (Fig. 4, lower panels). Contractions induced by stimulation at 5 and 10 Hz of the urethra were abolished and relaxations were augmented by atropine (Fig. 4, upper right panel).

In the strips treated with atropine, prazosin and yohimbine, L-NAME (10^{-5} and 10^{-4} M) dose-dependently reduced the neurogenic relaxation. The NO synthase inhibitor at 10^{-4} M abolished the relaxation associated with 0.5 and 1 Hz stimulation, partially attenuated the response to 5 Hz stimulation, and did not or only slightly inhibited the relaxation induced by 10 Hz stimulation. The data are summarized in Fig. 5. The attenuated response by 10^{-4} M L-NAME was partially reversed by 10^{-3} M L-arginine. Typical responses to 1 Hz stimulation before and after treatment with atropine, L-NAME and L-arginine are illustrated in Fig. 3 (lower panels).

3.3. Neurogenic relaxation independent of NO release

The L-NAME-resistant response to 10 Hz stimulation was not inhibited by 10^{-6} M ODQ nor by K^+ channel blockers, including glybenclamide, apamin, charybdotoxin and iberiotoxin (Table 1). The latter two rather potentiated the response. Although isoproterenol and VIP produced relaxations, β-adrenoceptor antagonist (propranolol), β_3-adrenoceptor antagonist (cyanopindolol) (McLaughlin and MacDonald, 1990; Hoey et al., 1996), VIP receptor antagonist ([D-*p*-Cl-Phe^6,Leu1^7]VIP) and capsaicin failed to depress the response to nerve stimulation. The data are summarized in Table 1. In the strips made unresponsive to VIP by repeated applications (10^{-8}–10^{-7} M VIP), the response to nerve stimulation was unaffected. α-Chymotrypsin (2 U/ml) did not inhibit the neurogenic relaxation but abolished the response to 10^{-7} M VIP ($n=4$) and suppressed it obtained at 10^{-6} M VIP from $57.4 \pm 8.2\%$ to $11.3 \pm 2.2\%$ of relaxation induced by 10^{-4} M papaverine ($n=4$). Sodium nitroprusside (10^{-7} M) sufficient to induce significant relaxation ($16.0 \pm 2.7\%$, $n=4$) failed to reduce the response to nerve stimulation. The median effective concentration (EC_{50}) of nitroprusside averaged 6.02×10^{-7} (3.4×10^{-7} to 11.8×10^{-7} M) and the maximal relaxation at 10^{-4} M was $90.4 \pm 2.1\%$.

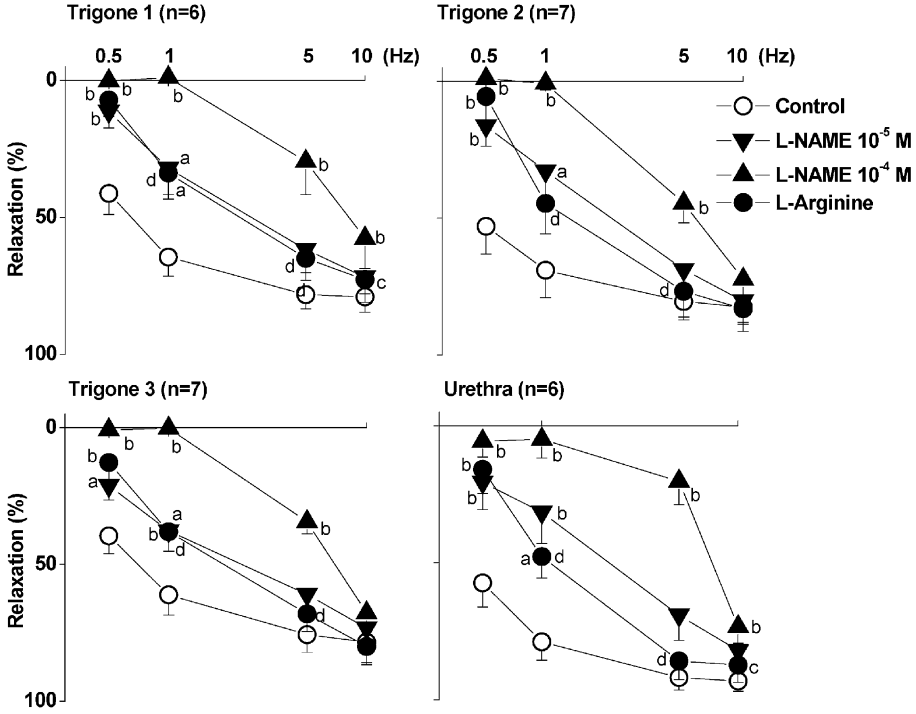

Fig. 5. Modification by L-NAME (10^{-5} and 10^{-4} M) and L-arginine (10^{-3} M) of the relaxation induced by electrical stimulation (0.5–10 Hz, abscissa) in the porcine trigone (1–3) and urethra contracted with $PGF_{2\alpha}$ and treated with atropine (10^{-6} M), prazosin (10^{-7} M) and yohimbine (10^{-7} M). Relaxations induced by 10^{-4} M papaverine were taken as 100% relaxation (ordinate). Significantly different from control: $^aP<0.05$, $^bP<0.01$; significantly different from the value with 10^{-4} M L-NAME: $^cP<0.05$, $^dP<0.01$ (Tukey's test). *n* denotes the number of strips used. Vertical bars represent S.E.M.

Table 1
Modification by blockers of the inhibitory response to 10 Hz electrical stimulation, resistant to 10^{-4} M L-NAME, in trigonal strips (T2–3) contracted with $PGF_{2\alpha}$ and treatment with atropine and α-adrenoceptor blockers

	Treatment	Concentration (M)	n	Relaxation (%)	
				Control	Experimental
K^+ channel	Glybenclamide	10^{-6}	11	54.4 ± 6.9	49.4 ± 5.9
	Iberiotoxin	10^{-7}	15	85.0 ± 2.8	89.6 ± 2.1[a]
	Charybdotoxin (ChTx)	10^{-7}	15	87.1 ± 3.5	91.0 ± 2.3[b]
	Apamin	10^{-6}	12	84.4 ± 4.5	82.8 ± 7.0
	ChTx + Apamin	10^{-7} + 10^{-6}	8	85.8 ± 2.9	92.3 ± 1.6[a]
Peptide	VIP-antagonist	10^{-6}	9	78.4 ± 5.2	78.3 ± 6.3
	VIP-tachyphylaxis	10^{-7}	8	61.4 ± 5.7	55.7 ± 4.8
	Capsaicin	10^{-6}	13	71.9 ± 5.8	73.9 ± 6.2
		10^{-5}	6	59.2 ± 5.8	54.5 ± 11.1
	α-Chymotrypsin	2U/ml	4	54.0 ± 5.9	48.9 ± 3.1
Adrenergic	Propranolol	10^{-7}	8	69.2 ± 5.6	70.9 ± 6.8
	Cyanopindolol	10^{-6}	8	63.1 ± 7.4	58.5 ± 7.5
		3×10^{-6}	8	60.0 ± 2.2	59.5 ± 2.2
NO	SNP	10^{-7}	8	63.4 ± 6.5	72.6 ± 6.0
	ODQ	10^{-6}	9	55.7 ± 7.0	51.0 ± 9.5

n = number of strips from different pigs.
Values are means ± S.E.M.
[a] $P < 0.01$: significantly different from the control (paired t-test).
[b] $P < 0.05$: significantly different from the control (paired t-test).

3.4. Neurogenic contraction by high frequency stimulation

Under treatment with 10^{-4} M L-NAME, the contractile response to 10 Hz stimulation was suppressed or abolished by 10^{-6} M atropine in trigonal strips, and the inhibition by additional application of prazosin and yohimbine was, if any, minimal (Fig. 6, upper panels). On the other hand, in the urethra, atropine inhibited the contraction by 1 and 10 Hz stimulation only partially, and additional treatment with prazosin and yohimbine almost abolished it (Fig. 6, lower left). When the order of inhibitors application was changed, similar results were also obtained (Fig. 6, lower right).

3.5. Comparison of the neurogenic responses in the trigone, urethra and detrusor

From the data obtained so far, relaxations mediated by NO and non-NO vasodilators are compared in the three different tissues (Table 2). The values were evaluated by subtraction of the value obtained with specific antagonists or inhibitors from the nontreated control. NO-mediated response to 10 Hz stimulation would be underestimated, possibly because of interactions to non-NO relaxant.

In the detrusor muscle contracted with $PGF_{2\alpha}$, transmural electrical stimulation elicited a marked contraction followed by slight or moderate relaxation. The contraction was unaffected by prazosin (10^{-7} M, $n = 6$) and yohimbine (10^{-7} M, $n = 6$), but was partially attenuated by atropine (10^{-6} M) and depressed by additional treatment with α,β-methylene ATP (10^{-5} M) (from 45.9 ± 28.0% to 31.8 ± 21.1% at 1 Hz, 36.8 ± 3.1% inhibition, $P < 0.01$ by paired t-test, and from 96.6 ± 51.3% to 53.0 ± 35.8% at 10 Hz, 57.9 ± 5.6% inhibition, $P < 0.01$). In the strips thus treated, the stimulation-induced relaxation was potentiated or unaffected; average relaxations at 1 Hz stimulation

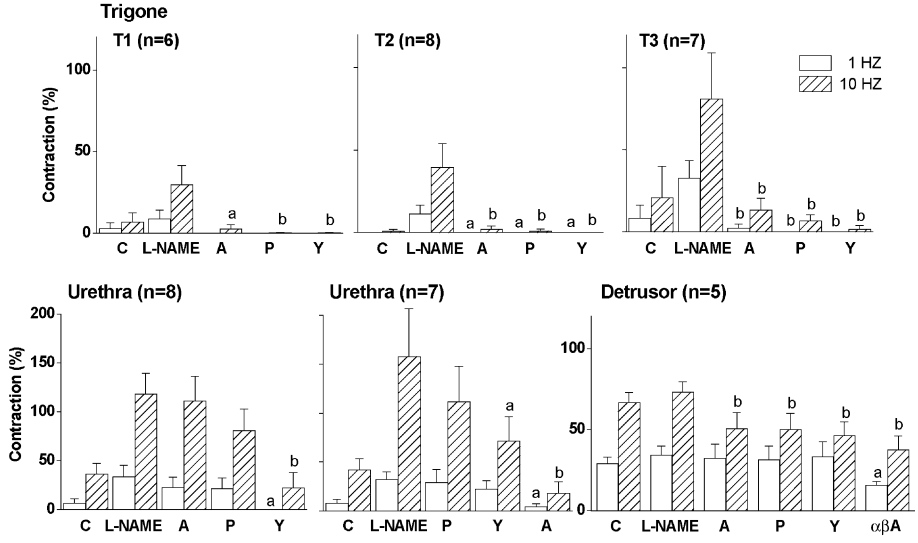

Fig. 6. Modification by L-NAME (10^{-4} M), atropine (A, 10^{-6} M), prazosin (P, 10^{-7} M), yohimbine (Y, 10^{-7} M) and α,β-methylene ATP (αβA, 10^{-5} M) of the contractile response to 1 and 10 Hz electrical stimulation in the porcine trigone (1–3, upper figures), and urethra (lower left two figures) and detrusor (lower right figure) contracted with $PGF_{2\alpha}$. C, control. Contractions induced by 100 mM K^+ were taken as 100% in the ordinate. In the lower figures, the order of applications of blocking agents differed. Significantly different from the value with L-NAME: [a] $P < 0.05$, [b] $P < 0.01$ (paired t-test). n denotes the number of strips used. Vertical bars represent S.E.M.

Table 2
Comparison of components (L-NAME-sensitive and -resistant) of the relaxation induced by 1 and 10 Hz electrical stimulation in the urethra (U), trigone (T1–3) and detrusor (D)

	Relaxation (%)									
	U ($n=6$)		T1 ($n=6$)		T2 ($n=7$)		T3 ($n=7$)		D ($n=6$)	
	1 Hz	10 Hz	1 Hz	10 Hz	1 Hz	10 Hz	1 Hz	10 Hz	1 Hz	10 Hz
Total	78.9 ± 6.1	93.1 ± 3.5	64.4 ± 6.3	78.9 ± 5.1	69.0 ± 9.3	82.3 ± 5.8	61.1 ± 6.8	78.5 ± 6.8	44.9 ± 12.7	53.0 ± 13.3
NO	72.4 ± 9.8	19.7 ± 3.7	64.4 ± 6.3	21.3 ± 6.2	67.1 ± 10.9	10.0 ± 3.6	60.0 ± 7.5	10.7 ± 3.8	15.0 ± 4.5	15.0 ± 5.8
Non-NO	6.5 ± 5.8	73.4 ± 5.6	0.0 ± 0.0	57.6 ± 10.0	1.88 ± 1.9	72.3 ± 8.5	1.11 ± 1.1	67.8 ± 7.3	30.2 ± 11.3	37.9 ± 14.4

NO: neurogenic relaxation sensitive to 10^{-4} M L-NAME under treatment with atropine and α-adrenoceptor blockers in U and T and additional treatment with α,β-methylene ATP in D.
Non-NO: neurogenic relaxation resistant to 10^{-4} M L-NAME under treatment with atropine and α-adrenoceptor blockers in U and T and additional treatment with α,β-methylene ATP in D.

before and after treatment were 46.4 ± 7.8% and 42.5 ± 11.0% (1.4 ± 8.1% decrease, $P>0.05$), respectively, and those at 10 Hz were 47.5 ± 9.9% and 62.1 ± 12.9% (25.8 ± 9.0% increase, $P<0.05$), respectively. The relaxation was partially inhibited by 10^{-4} M L-NAME, and the remaining relaxation was abolished by 10^{-7} M tetrodotoxin ($n=6$). Mean values of the relaxant response before and after L-NAME are shown in columns of the right end of Table 2.

3.6. Data obtained in trigone-2 from non-castrated (normal) male and female pigs

Relaxations induced by 1 and 10 Hz electrical stimulation in trigonal strips treated with atropine, prazosin and yohimbine were 76.0 ± 5.7% and 90.8 ± 4.1% ($n=5$), respectively, in normal male pigs, and 58.2 ± 8.2% and 74.1 ± 7.1% ($n=5$), respectively, in female pigs, as compared with those of 60.1 or 69.0 ± 9.3% and 82.3 ± 5.8% ($n=7$), respectively, in castrated male pigs (Table 2). The response to 1 Hz stimulation was abolished by 10^{-4} M L-NAME in the normal male and female pigs, as seen in castrated male pigs. Under treatment with the NO synthase inhibitor, mean values of relaxation induced at 10 Hz in normal male, castrated male and female pigs were 82.9 ± 6.2% ($n=5$), 72.3 ± 8.5% ($n=7$) and 52.4 ± 8.4% ($n=5$), respectively (statistically insignificant, Tukey's test).

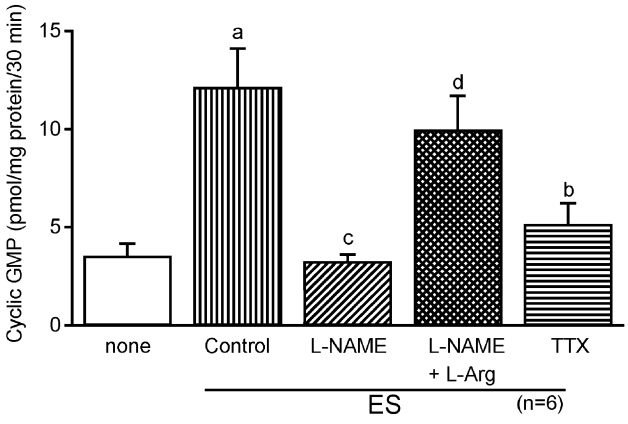

Fig. 7. Cyclic GMP levels in the incubation media, containing soluble guanylate cyclase, GTP and IBMX before (none) and after nerve stimulation with and without L-NAME (10^{-4} M), L-arginine (L-Arg; 10^{-3} M) and tetrodotoxin (TTX, 10^{-6} M) in trigonal strips. Significantly different from none: $^{a}P<0.01$ (unpaired t-test); significantly different from control under electrical stimulation (ES): $^{b}P<0.05$, $^{c}P<0.01$; significantly different from the value with L-NAME: $^{d}P<0.05$ (Tukey's test). n denotes the number of strips used. Vertical bars represent S.E.M.

3.7. Measurement of cyclic GMP in the tissue and external media

Release of NO from the trigone with or without electrical stimulation (1 Hz) was assayed by measurement of cyclic GMP in the external fluids, which contained the crude extract of soluble guanylate cyclase obtained from the rat cerebellum, GTP and IBMX. The amounts of cyclic GMP that accumulated in the incubation medium under control and stimulated conditions were 3.57 ± 0.61 and 12.2 ± 1.94 pmol/mg protein, respectively ($n=6$, $P<0.01$, unpaired t-test). The nucleotide content under stimulation was decreased to the prestimulation level by treatment with 10^{-4} M L-NAME, and the inhibitory effect was reversed by the addition of L-arginine (10^{-3} M) (Fig. 7). Tetrodotoxin (10^{-6} M) also depressed the production of cyclic GMP in the electrically stimulated strips.

4. Discussion

Transmural electrical stimulation elicited a contraction followed by relaxation in the isolated porcine urethra, trigone (three portions) and detrusor muscle. The responses were abolished by treatment with tetrodotoxin, suggesting the release of neurotransmitters due to nerve action potentials generated by electrical stimulation. Contracting responses to 10 Hz stimulation were in the order of detrusor>urethra>trigone. Contractions of the trigonal strip did not differ in the region proximal to ureter orifices to the distal region. The responses were almost abolished by atropine, suggesting

that neurogenic acetylcholine and muscarinic receptors are mainly involved. In the human trigone, muscarinic, α-adrenergic and NAMC mechanism are reportedly involved in the neurogenic contraction (Speakman et al., 1988). Detrusor muscle contractions were moderately inhibited by atropine and α,β-methylene ATP but were not abolished. α_1- and α_2-Adrenoceptor antagonists failed to alter the response. Therefore, cholinergic and purinergic nerves would be involved in the response, but there is still other unidentified mechanism. Atropine-sensitive component of the contraction (Fig. 6) was smaller than in other published work (Sibley, 1984). Whether the difference is due to different experimental conditions is not determined. On the other hand, contractions of the urethral strip by nerve stimulation at 10 Hz were attenuated by atropine, prazosin and yohimbine, indicating that muscarinic receptors and α_1- and α_2-adrenoceptors are stimulated by nerve-derived acetylcholine and norepinephrine, respectively, resulting in the contraction. In the isolated dog urethra, α_1-adrenergic, but not cholinergic, receptors are involved in the neurogenic contraction (Hashimoto et al., 1992), and in the human and rabbit urethra, muscarinic receptors as well as α_1-, and to a lesser extent α_2-, adrenoceptors are involved (Levin et al., 1988).

Relaxing responses to nerve stimulation at frequencies of 0.5–10 Hz of the trigonal and urethral strips under control conditions were almost abolished by high concentrations of L-NAME (Fig. 2). The attenuated response was reversed by L-arginine. On the other hand, in the preparations treated with atropine, prazosin and yohimbine, relaxations induced by stimulation at low frequencies (0.5 and 1 Hz) were abolished by NO synthase inhibition, but those to high frequency stimulation were partially attenuated or not influenced (Fig. 5). It is possible that the rapid phase of relaxation associated with high frequency stimulation was depressed by the NO synthase inhibitor. However, rapid and slow phases could not always be separated in the present study. Concentration (10^{-8}–10^{-5} M)-related relaxations by sodium nitroprusside, a NO donor, were observed in porcine urethral strips (Bridgewater et al., 1993). Neurons containing NADPH diaphorase have histochemically demonstrated in the lower urinary tract (Persson et al., 1993; Levin et al., 1988). These findings strongly suggest that the response to low frequency stimulation is mediated exclusively by NO released from nerves, while the relaxation induced by high frequency stimulation is mediated only partially by neurogenic NO and also by inhibitory substance(s) other than NO that appears to mostly compensate the NO-mediated response even though the rate of relaxation declines. Under nontreated conditions, this relaxation resistant to the NO synthase inhibitor would be counteracted by the contracting response to nerve-derived acetylcholine and norepinephrine and also by prejunctional inhibition by acetylcholine of the neurogenic relaxation at 10 Hz (K. Noda and N. Toda., unpublished data). ODQ, an inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995), in the concentration used (10^{-6} M) sufficient to suppress the response mediated by cyclic GMP (Toda et al., 1999) abolished the relaxation induced by 1 Hz stimulation but did not alter the response to 10 Hz stimulation under treatment with cholinergic and adrenergic blockade. NO-mediated neurogenic relaxation appears to be induced by intracellular cyclic GMP, as already reported (Garthwaite et al., 1995). Human lower urinary tract responds to nerve stimulation with relaxations that are almost abolished by L-NAME (Ehren et al., 1994). Whether the inability to demonstrate the L-NAME-resistant relaxation is due to a lack of muscarinic receptor blockade remains to be determined.

Biochemical study on increased cyclic GMP production in extracellular fluids in incubated trigonal strips that were electrically stimulated support the idea that NO released from nerves stimulates guanylate cyclase and produces cyclic GMP in the tissue. Release of NO from the vascular endothelium has been detected by the same method (Noda et al., 2001). NO is produced from L-arginine via a catalysis of NO synthase in the rat and human lower urinary tract (Burnett et al., 1992; Ehren et al., 1994). On the other hand, in detrusor muscle, relaxations elicited by nerve stimulation at 1 and 10 Hz were resistant to L-NAME, thus being considered that the response is mainly mediated by non-NO inhibitory substance(s). Similar results have also been obtained in in vivo studies on female minipig (Turner, 1994).

Moderate relaxations induced by nerve stimulation at 1 Hz, mediated by NO, did not differ in the urethra and three regions of the trigone, whereas the stimulation-induced, NO-mediated relaxation in the detrusor was, if any, slight. Mean values of relaxations resistant to L-NAME were similar in the urethra, trigone and detrusor at 10 Hz. Only in the detrusor, electrical stimulation at 1 Hz induced relaxations partially resistant to L-NAME. Sodium nitroprusside, a NO donor, did not inhibit the response to 10 Hz stimulation in L-NAME-treated trigonal strips, suggesting that simultaneously liberated NO does not seem to interfere with the synthesis and release of non-NO substances. On the other hand, endothelium-derived NO is suggested to depress the synthesis of endothelium-derived hyperpolarizing factor (Bauersachs et al., 1996; McCulloch et al., 1997). We could not determine whether non-NO substance(s) inhibits the NO synthesis, because this substance was not identified.

Neurogenic relaxations resistant to NO synthase inhibitors are observed in female dog urethra (Hashimoto et al., 1993) and female pig urethra (Bridgewater et al., 1993; Werkstrom et al., 1995, 1997). The mechanism has not been clarified. In the present study, inhibitors of ATP-dependent K^+ channels, glibenclamide, and of small conductance Ca^{2+}-sensitive K^+-channels, apamin, did not inhibit the response, whereas high and intermediate conductance Ca^{2+}-activated K^+ channel inhibitors, charybdotoxin and iberiotoxin, respectively, potentiated the response. Similar findings were also obtained in the female pig urethra by Werkstrom et al (1997). Therefore, K^+-channel opening substance(s) would be excluded from the candidates of

inhibitory neurotransmitter in the porcine trigone and urethra. On the other hand, relaxations induced by NANC nerve stimulation in isolated monkey corpus cavernosum are reportedly mediated by NO and inhibitory substance(s) that opens K^+ channels (Okamura et al., 1998).

Involvement of VIP was also excluded from the results with pharmacological and biochemical inhibitors, including a receptor antagonist, capsaicin and chymotrypsin, and with preparations, in which tachyphylaxis to VIP developed. Igawa et al. (1999) have shown that β_3-adrenoceptors play a major role in the relaxation of human detrusor muscle. However, in the present study, propranolol and cyanopindolol, an inhibitor of β_3-adrenoceptors, were ineffective in the response to nerve stimulation, suggesting that β-adrenoceptors are not involved in the response of the porcine trigone.

There was no significant difference in the relaxations induced at 1 and 10 Hz stimulation in trigones and urethra from normal and castrated male pigs and female pigs. Modifications by L-NAME of the response were also similar. The effect of castration and gender difference were not observed in functioning of inhibitory nerves that liberate NO and non-NO molecule(s).

As far as our investigations on porcine urethra, trigone and detrusor are concerned, excitatory (adrenergic and cholinergic) and inhibitory (nitrergic and non-nitrergic) nerves contribute to the control of smooth muscle tone. The extent of nerve functions involved differs in these tissues and stimulation frequencies. Neurotransmitter of the non-nitrergic inhibitory nerve was not identified, but evidence for the involvement of VIP, K^+ channel opening substances and β-adrenoceptors in the neurogenic relaxation was not obtained. According to Andersson and Alm (2000), normal voiding in humans is characterized by an initial drop of urethral pressure followed by an increase in intravesicular pressure via a urethrovesical reflex. It is quite interesting to see if the efferent parasympathetic nerve excitation at low frequencies (0.5–1 Hz) and NO release participate in a drop of urethral pressure triggering the urethrovesical reflex that leads cholinergic and NANC neurogenic contractions of the detrusor muscle, together with intense, persistent relaxation of sphincters due to activation of nitrergic and non-nitrergic inhibitory nerves at high frequencies (5–10 Hz) for normal voiding.

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