Involvement of Nitric Oxide and Vasoactive Intestinal Peptide in the Nonadrenergic-Noncholinergic Relaxation of the Porcine Retractor Penis Muscle

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ABSTRACT—Neurotransmitters mediating nonadrenergic-noncholinergic (NANC) relaxation were investigated in strips of porcine retractor penis muscle (RPM). Muscle tone was raised by phenylephrine (1 \textmu M) in the presence of atropine (1 \textmu M) and guanethidine (50 \textmu M). Upon electrical field stimulation (1 ms, 80 V, 1 – 32 Hz for 10 s), the initial fast relaxation was followed by the slow relaxation. Although the fast and the slow relaxation were completely abolished by tetrodotoxin (1 \textmu M), they showed different pharmacological sensitivities to the nitric oxide (NO) synthase inhibitor N\textsuperscript{\textdagger} -nitro-L-arginine methyl ester (L-NAME, 0.1 mM). The fast relaxation was markedly inhibited by L-NAME in a L-arginine reversible manner and by oxyhemoglobin (50 \textmu M), while the slow relaxation was hardly blocked by L-NAME. L-NAME and \alpha-chymotrypsin (\alpha-CT, 3 U/ml) selectively inhibited the fast and the slow relaxation, respectively. \alpha-CT abolished L-NAME-resistant slow relaxation, and L-NAME completely abolished the \alpha-CT-resistant fast relaxation. \alpha-CT-resistant relaxation was not significantly different from the digitally calculated L-NAME-sensitive component, and L-NAME-resistant relaxation was similar to the digitally calculated \alpha-CT-sensitive component. Vasoactive intestinal peptide (VIP, 0.003 – 0.1 \mu M) relaxed porcine RPM in a concentration-dependent manner. The effect of a VIP was partially inhibited by a VIP receptor antagonist, VIP(10 – 28) (1 and 3 \textmu M). L-NAME-resistant relaxation was also reduced by VIP(10 – 28) (3 \mu M) and by another putative antagonist, VIP(6 – 28) (1 \textmu M), although the effects of the two antagonists were somewhat inconsistent. From the histochemical staining, it was verified that nerve bundles that showed VIP-like immunoreactivities were also positive for the NADPH diaphorase reaction. These results suggest that NO and peptide neurotransmitter(s) including VIP mediate the NANC relaxation in porcine RPM.

Keywords: Boar, Retractor penis muscle, Nitric oxide, Vasoactive intestinal peptide

Animals such as bulls and boars have a fibro-elastic penis that can be pulled backwards by specialized smooth muscle called the retractor penis muscle (RPM) to form a sigmoid flexure. The relaxation of RPM stretches the sigmoid flexure and results in the protrusion of the penis, which is a pivotal step for erection in these domestic animals. It is now well established that nitric oxide (NO), one of the inhibitory nonadrenergic-noncholinergic (NANC) neurotransmitters, plays an important role in the relaxation of bovine RPM (1 – 3). In contrast, there is limited available information dealing with the mechanism of NANC relaxation in porcine RPM. The findings in impotent boar, which exhibits lower number of vasoactive intestinal peptide (VIP)-immunoreactive nerve fiber in the penis, suggest VIP as an important inhibitory NANC neurotransmitter (4). In addition, recent data suggest that NO mediates the NANC-nerve stimulated relaxation in porcine RPM (5). However, it is still unclear that VIP and NO mediate the NANC relaxation in porcine RPM because the previous study on VIP focused only on the penis itself, and that on NO obtained its results by using electrical stimulation only at single frequency (2 Hz). It is known that classical small molecule neurotransmitters and peptide neurotransmitters are released at different frequencies of stimulation, and that peptides are not released by single stimulus but only by trains of high frequency stimuli (6). Therefore, with special respect to both VIP and NO, the present study was aimed to investigate the neurotransmitters mediating the NANC relaxation caused by wide

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range of electrical stimulation in porcine RPM.

MATERIALS AND METHODS

Preparation of smooth muscle strips
Porcine RPMs were collected at a local slaughter and transported to the laboratory in cold Krebs solution. Surrounding connective tissues were carefully removed and the proximal part of porcine RPM was dissected into strips (7 × 2 mm). The strips were suspended in the tissue holder and placed between two platinum plates, which were located in 20-ml chambers filled with warmed (37°C) and oxygenated (95% O2 and 5% CO2) Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 2.5 mM CaCl2 and 11.7 mM glucose). The upper end of the strip was connected to the isometric force transducer (FT-03; Grass-Telefactor, West Warwick, RI, USA). The output of transducer was processed through a digital interface (AD Instruments, Castlehill, Australia) and recorded on a Macintosh LC III computer. Two platinum plates were connected to the electrical stimulator (S88, Grass).

Recording of muscle tension
An initial tension of 1 g was loaded on each muscle strip during the 60-min equilibration period with rinsing every 15 min. The muscarinic antagonist atropine (1 μM) and adrenergic transmission blocker guanethidine (50 μM) were applied to set the NANC condition. Muscle tone was raised with phenylephrine (1 μM). Each muscle strip obtained from the same animal was subjected to different sets of experiments.

Electrical field stimulation (EFS; 1-ms pulse duration, 80 V, 10-s train duration) was applied via paired platinum plates in a stepwise-increment of frequency (1–32 Hz). Muscle strips were stimulated at 8 Hz at 5-min intervals before beginning experiment. Only muscle strips showing reproducible responses to EFS were used. To evaluate the effect of various drugs on EFS-induced relaxation, the muscle strip was washed out after the 1st set of EFS application and incubated with drug at least 30 min prior to and throughout the 2nd set of EFS application. Time-control experiments were routinely carried out in the absence of drugs to certify that responses did not vary over the time course of the experiment.

Magnitudes of the relaxation were measured from the difference between the pre-stimulus tension and the nadir tension of the relaxation, and were expressed as percentage of pre-contracted tension level.

Histochemical staining
The proximal part of porcine RPM was fixed for 4 h in room temperature with Lana’s fixative (4% paraformaldehyde + 0.2% picric acid) and dehydrated with 30% sucrose solution. The tissue sample was cut into transverse sections at 30-μm thickness in a cryostat. The sections were incubated with 1% normal donkey serum for 1 h at room temperature, subsequently reacted with guinea pig anti-VIP antisera (1:600; Peninsula Lab, San Carlos, CA, USA) and FITC-conjugated secondary antibody (1:200, donkey anti-guinea pig IgG; Jackson ImmunoResearch Lab, West Grove, PA, USA) at 4°C overnight. Finally, sections were mounted on gelatin/chrome-alum-coated glass slides and coverslipped with p-phenylenediamine/glycerol mixture. Repeated rinsing in 0.1% PBS were performed between all reaction steps. The specimen was examined under a fluorescent-microscope (Axioskop; Zeiss, Frankfurt, Germany) and digitaly photographed (Micromax cooled-CCD; Princeton Instrument, Trenton, NJ, USA).

For the detection of nitricergic nerves, NADPH-diaphorase (ND) histochemistry was applied because it was proved to be a specific marker of neuronal NO synthase (NOS) in the central and peripheral nervous system (7). After VIP-like immunoreactivities (VIP-LI) were photographed, the coverglass was removed, and the specimen was incubated in a solution of nitroblue tetrazolium (0.4 mg/ml) and β-NADPH (2 mg/ml). The specimen was re-coverslipped with Permount (Sigma, St. Louis, MO, USA) and examined under a light-field microscope (Axioskop, Zeiss). The same region previously examined for VIP-LI was photographed again.

Chemicals
All the following chemicals were purchased from Sigma; atropine sulfate, guanethidine sulfate, phenylephrine hydrochloride, tetrodotoxin (TTX), α-chymotrypsin (α-CT) type I-S from bovine pancreas, Nω-nitro-L-arginine methyl ester (L-NAME), L-arginine hydrochloride, D-arginine hydrochloride, hemoglobin, VIP fragment 6–28, VIP fragment 10–28, nitroblue tetrazolium and β-NADPH. Oxyhemoglobin (OxyHb) was prepared according to the method by Feelisch and Kubitzek (8). The peptides were dissolved in a medium containing 0.1% bovine serum albumin (BSA) to avoid their absorption to the glass and plastic apparatus. When the peptides in 0.1% BSA and α-CT were used, the tissue baths were coated with Sigma-cote because of excessive foaming.

Statistical analyses
Data were expressed as the mean ± S.E.M. with n, the number of strips. Statistical comparisons were made by the paired or unpaired Student’s t-test according to the cases. Data were considered significantly different from control values when P<0.05.
RESULTS

NANC nerve-mediated relaxation

The fast and the slow relaxation: Muscle strips relaxed in a frequency-dependent manner by EFS in the NANC condition (Fig. 1). This relaxation appeared to have two components. At lower frequencies of stimulation (1 – 2 Hz), porcine RPM rapidly relaxed during the 10-s train duration and slowly recovered to the pre-stimulus tension level after the cessation of EFS. As the frequencies rose above 4 Hz, another discrete nadir became apparent after the cessation of EFS. At 32-Hz stimulus, the first nadir appeared at 10-s and the second at 50.4 ± 2.0 s (n = 17) following the start of EFS. Based on their courses of activation time, we designated the first and the second components generated at high frequencies of EFS as the fast and the slow relaxation, respectively. Both relaxations were completely abolished in the presence of 1 μM TTX, suggesting that they were mediated by neurotransmitters released from the intrinsic NANC nerves (Fig. 1A).

Involvement of NO in the fast relaxation: L-NAME (0.1 mM), a NOS inhibitor, completely abolished the fast relaxation while leaving the residual L-NAME-resistant relaxation that was slowly developed and sustained. In the presence of L-NAME, a brief and small contraction appeared instead of the fast relaxation during EFS, implicating that EFS-induced neurogenic excitation has been concealed during the fast relaxation (Fig. 1: B). For more detailed observations, traces obtained at 32 Hz were magnified and superimposed in Fig. 1C. The L-NAME-sensitive component was digitally subtracted and denoted as dotted line. Parameters on this subtracted component are summarized in Table 1. The fast relaxation that was sensitive to L-NAME was also inhibited by the NO scavenger OxyHb (50 μM) (Fig. 2B). The inhibitory effect of L-NAME on the fast relaxation was antagonized by L-arginine (1 mM), a substrate of NOS, but not by D-arginine (1 mM), an inactive isomer of L-arginine (Fig. 2B).

![Fig. 1. NANC relaxation of porcine RPM induced by EFS. A: Representative traces showing the effect of TTX (1 μM). B: Effect of L-NAME (0.1 mM) on EFS-induced NANC relaxation. The numbers at the top of each figure indicate the frequencies of EFS (1 – 32 Hz). C: Magnified view of NANC relaxation in response to 32-Hz stimulus. L-NAME-resistant relaxation was superimposed on control relaxation and subtracted to represent the L-NAME-sensitive component (dotted line). Duration of EFS application was indicated as an inset mark.](image-url)

| Parameter                  | Control (n = 5) | Digitally calculated L-NAME-sensitive component (n = 17) |
|----------------------------|-----------------|---------------------------------------------------------|
| Time to nadir (s)          | 10.5 ± 1.1      | 10.4 ± 0.5                                              |
| Magnitude of relaxation (%)| 41.9 ± 3.2      | 40.1 ± 4.5                                              |
| dF/dt (g/s)                | 0.33 ± 0.08     | 0.26 ± 0.04                                             |
| Recovery τ (s)             | τ₁ = 8.0 ± 2.0  | τ₁ = 8.0 ± 0.8                                          |
|                           | τ₂ = 107 ± 46   | τ₂ = 116 ± 20                                           |

All parameters were not significantly different between two groups (P>0.05). τ₁: Tension recovery time kinetics were fit by a double exponential function with fast (τ₁) and slow (τ₂) time constant.
**Involvement of VIP in the L-NAME resistant, peptidergic slow relaxation: Administration of α-CT (3 U/ml) to the bath did not cause a substantial change of basal tension. As shown in Fig. 3, α-CT (3 U/ml) nearly abolished L-NAME-resistant slow relaxation, suggesting that the relaxation be mediated by peptide neurotransmitter(s) (Fig. 3: A and C). The inhibitory effect of α-CT was found to be specific to the slow relaxation because the time-matched experiments with L-NAME alone did not show any significant reduction in relaxation (P>0.7, n = 7). To prove the selectivity, we administered α-CT into the bath prior to the addition of L-NAME and tested whether L-NAME could inhibit α-CT-resistant relaxation. As expected, α-CT only abolished the slow relaxation and 0.1 mM L-NAME efficiently abolished the residual fast relaxation (Fig. 3: B and D). At 32 Hz stimulus, α-CT-resistant relaxation had characteristics (magnitudes, time to nadir, Δtension/Δtime, and recovery time course) similar to those of the digitally calculated L-NAME-sensitive component (Table 1). Furthermore, characteristics of the digitally calculated α-CT sensitive component were not greatly different from those of the L-NAME-resistant relaxation (Table 2). These results suggest that the fast and the slow relaxation are mediated by NO and inhibitory peptide neurotransmitter(s), respectively.

Considering the previous report on the decrease of VIP-containing nerves in impotent boar (4), VIP may be thought as a candidate for L-NAME-resistant peptidergic relaxation. To verify this hypothesis, we attempted to use the putative VIP-receptor antagonist, VIP(10–28). Before we determined whether VIP(10–28) could affect L-NAME-resistant relaxation, we tested its antagonizing effect on the relaxant action of exogenously applied VIP. VIP (0.003 – 0.1 μM) induced relaxation that was slowly developed and sustained. At the highest concentration we tested (0.1 μM), VIP relaxed porcine RPM to 32.6 ± 2.0% (n = 35) of pre-contracted tension. However, when it was re-administered to bath media after wash out, its relaxant effect at 0.01 and 0.03 μM was decreased to some extent, which was almost statistically significant (n = 18, P = 0.068 at both concentration). Therefore, we calculated the magnitude of relaxation in the second series of VIP application as the percentage of that induced by 0.1 μM of VIP in the first series of application (Fig. 4A). Then we compared the magnitude of relaxation by VIP under the presence of antagonist with that of the time-control as well as that of its own paired control. Compared with its paired control, 1 μM of VIP(10–28) (n = 10) significantly inhibited the relaxant action of VIP at concentrations of 0.003 to 0.03 μM. However, when the comparison was carried out with time control, the effect of 1 μM of VIP(10–28) was statistically significant only against 0.003 and 0.01 μM of VIP. Comparatively, higher concentration of VIP(10–28) (3 μM, n = 7) showed a statistically significant effect on the relaxation induced by VIP at all tested concentration regardless of its comparison partner. The VIP-induced relaxation, at 0.03 and 0.1 μM, were inhibited by 53.2 ± 10.7% (n = 7, P<0.01) and 24.9 ± 5.4% (P<0.05) by 3 μM of VIP(10–28), respectively.

However, VIP(10–28) proved to be an inconsistent antagonist in the experiment that examined its effect on L-NAME-resistant relaxation. At 1 μM, VIP(10–28) reduced the magnitude of L-NAME-resistant relaxation only in 6 of 17 strips, and statistical significance was not detected at any frequency (Fig. 4B). At 3 μM, only 6 of 11 strips...
J. La et al. showed the inhibitory action of VIP(10–28). Due to this inconsistency, we attempted to use another putative VIP receptor antagonist, VIP(6–28), which was reported to be more potent than VIP(10–28) (9). The effect of VIP(6–28) (1 μM) was also inconsistent. It inhibited L-NAME-resistant relaxation in 6 of 9 strips. In the 6 strips from each group, where VIP-receptor antagonists attenuated L-NAME-resistant relaxation, the degree of inhibition by those 3 treatments did not differ from each others. At 16 and 32 Hz, each treatment inhibited L-NAME-resistant relaxation by

### Table 2. A comparison of L-NAME-resistant relaxation with digitally calculated α-CT-sensitive component at 32-Hz stimulus

|                      | L-NAME-resistant relaxation (n = 17) | Digitally calculated α-CT-sensitive component (n = 5) |
|----------------------|-------------------------------------|------------------------------------------------------|
| Time to nadir (s)    | 64.1 ± 3.2                          | 61.7 ± 5.0                                           |
| Magnitude of relaxation (%) | 13.7 ± 2.1                          | 17.4 ± 5.2                                           |
| dF/dt (g/s)          | 0.020 ± 0.003                       | 0.017 ± 0.006                                        |
| Half recovery time (s) | 181 ± 12                            | 151 ± 15                                             |

All parameters were not significantly different between two groups (P>0.05).
about 50% and 40%, respectively (Fig. 4C).

**Histochemical staining**

VIP-LI and ND activity were detected only from the nerve bundles rather than from smooth muscle cells. In the transverse sections, large-sized nerve bundles (diameter = 104.7 ± 8.3 μm, detected number of bundles = 8.2 ± 0.8, n = 32 slides from 12 animals) that showed ND activity were found in a large portion of the interfascicular space (Fig. 5A). The small-sized ND-positive nerve bundles (diameter = 25.1 ± 2.5 μm, detected number of bundles = 12.5 ± 0.3, n = 32 slides from 12 animals) were also detected in the perivascular region of the interfascicular space (Fig. 5B). Every ND-positive nerve bundle also had VIP-LI (Fig. 5: B and C). However, individual axon fiber positive to both VIP-LI and ND activity was hard to distinguish due to the dense reactivity.

**DISCUSSION**

In the present study, we observed the biphasic relaxation...
of porcine RPM in response to the stimulation of NANC nerves: an initial fast relaxation followed by a slow relaxation. The fast relaxation was blocked by L-NAME in a L-arginine-reversible manner and was nearly abolished by OxyHb, suggesting that it is mediated by NO. Furthermore, the presence of numerous ND-positive nerve bundles in RPM tissue confirmed that the fast relaxation is indeed mediated by NO. A number of studies indicate that NO is a principal neurotransmitter for erection, not only in animals with fibro-elastic penis but also in those with a muscular-cavernosal type (1–3, 10). In addition to NO, the participation of other inhibitory NANC neurotransmitters in the process of penile erection has been suggested (11). In the present study, NANC relaxation of porcine RPM showed different pharmacological characterististics from those of bovine RPM. The same concentration of NOS inhibitor L-NAME (0.1 mM) completely blocked NANC relaxation in bovine RPM (3) while slowly occurring relaxation still remained in porcine RPM (Fig. 1). The L-NAME-resistant relaxation of porcine RPM could be completely abolished by peptide cleaving agent α-CT (Fig. 3), indicating the involvement of peptide neurotransmitter(s).

We tried to examine whether VIP is involved in the generation of peptidergic relaxation by using a VIP antagonist that partially but effectively inhibited the relaxation induced by exogenously administered VIP (Fig. 4A). However, the putative VIP antagonists showed an inconsistent effect on L-NAME-resistant relaxation induced by EFS. Other investigators also reported the inconsistent effects of VIP antagonists in the opossum lower esophageal sphincter where all commercially available VIP antagonists failed to reliably inhibit either VIP-induced or EFS-induced relaxation (12). Though the result obtained from our experiment using the VIP antagonist was somewhat confusing, the possibility that VIP may be involved in L-NAME-resistant peptidergic relaxation cannot be ruled out because statistically significant reduction of this relaxation was observed in about one half of the investigated muscle strips. In the muscle strips that responded to VIP antagonists, there was not a significant difference of inhibitory effect among the three antagonists treatments; i.e., VIP antagonist did not inhibit L-NAME-resistant relaxation in a concentration-dependent or potency-dependent manner. This raises a question about whether VIP mediates only a portion of the L-NAME-resistant relaxation and at least one other peptide neurotransmitter may be responsible for the remaining relaxation. We are planning to investigate this issue in further experiments by using other agents that specifically interfere with peptidergic neurotransmission.

VIP is considered as an important NANC inhibitory neurotransmitter in smooth muscles of penile tissue and suggested to be a cotransmitter with NO (13). In histological study, we found that all VIP-like immunoreactive nerve bundles contained ND-positive nerve fibers in porcine RPM. Although we did not precisely determine the colocalization of VIP with NOS, it was previously reported in other penile tissues such as the cavernous nerve of rats (14) and humans (15). In the latter, 50% of the perivascular nerve fibers and more than 90% of the trabecular nerve fibers within the corpus cavernosum showed positive staining for both NOS and VIP. In addition to these histological investigations, there have been several studies reporting the functional positive interaction between NO and VIP. NO stimulates the presynaptic release of VIP in isolated guinea pig intestine ganglia (16) and in synaptosomal fraction of rat intestine (17). On the other hand, VIP stimulates the postsynaptic generation of NO in guinea pig stomach (18), ileum (19) and cecum (20). In rabbit corpus cavernosum, VIP induces relaxation partially by NO synthesis (21). However, such positive interaction between NO and VIP seems unlikely in our experiment. If NO stimulates the release of VIP, L-NAME-sensitive component should consist of NO and VIP; therefore, it must be larger in magnitude or in duration than α-CT-resistant relaxation in which all peptide neurotransmitter(s) were removed. Similarly, if VIP stimulated the synthesis or release of NO, both VIP and NO should be included in the calculated α-CT-sensitive component, and consequently the relaxation in the presence of NOS inhibitor would be smaller. However, none of these suppositions was confirmed in our studies. All characteristics of α-CT-resistant relaxation were not significantly different from those of the calculated L-NAME-sensitive component (Table 1). Furthermore, L-NAME-resistant relaxation had similar characteristics to those of the calculated α-CT-sensitive component (Table 2). Therefore, we suggest that, at least in our experimental condition, NO and peptide neurotransmitter(s) including VIP independently relax the porcine RPM. The biphasic pattern of relaxation is likely to be due to the two overlapping relaxations mediated by NO and peptide neurotransmitter(s) with different time kinetics.

Among the reports that suggest the possible involvement of VIP in neurogenic NANC relaxation, there are several negative reports suggesting that VIP does not participate in the relaxation of some penile tissue. In canine corpus cavernosum, the relaxation induced by nerve stimulation is mediated solely by NO, although VIP-containing nerve fibers are immunohistochimically detected (22). In human (23) and monkey (24) cavernosal smooth muscle, VIP was also reported not to be involved in neurogenic relaxation because α-CT or VIP receptor desensitization was ineffective. These reports imply there are differences in penile neuromuscular transmission among species.

In conclusion, we found that NANC relaxation of porcine RPM consisted of two components: NO-mediated fast relaxation and slow peptidergic relaxation. Our results
also implicate VIP involvement in a peptidergic component, although the possible participation of other peptide neurotransmitters remains as an issue for further investigation.

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