Nitric Oxide-Cyclic GMP Signaling Pathway in the Regulation of Rabbit Clitoral Cavernosum Tone[1]

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We investigated the role of nitric oxide (NO)-guanosine 3′,5′-cyclic monophosphate (cGMP) signaling in the regulation of rabbit clitoral cavernosum (CC) tone. Tension measurements, reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting, and NADPH-diaphorase staining were performed in CC. In the precontracted CC strips with phenylephrine (10^{-5} *M*), acetylcholine (ACh) relaxed, dependent on dosage. Pretreatment with atropine, N^{ω} nitro-L-arginine-methyl ester (NAME) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), guanylate cyclase inhibitor abolished the ACh-induced relaxations, but tetrodotoxin (TTX) did not. Sodium nitroprusside relaxed the strips in the presence of atropine and NAME, but not in the presence of ODQ. Electrical field stimulation (EFS) relaxed the strips dependent on stimulus strength. Pretreatment with TTX, NAME, or ODQ abolished the EFS-induced relaxation, but atropine did not. L-Arginine partially restored the inhibited response to ACh and EFS. The inducible NO synthase (iNOS) and neuronal NOS (nNOS) mRNAs and iNOS and endothelial NOS (eNOS) proteins were identified in the CC. NADPH-diaphorase staining revealed the positivity on the nerve trunks and fine nerve fibers in the CC. Finally, results demonstrate that the nNOS, ENOS, and the NO-cGMP signaling pathway are involved in the regulation of clitoral tumescence. Exp Biol Med 227:1022–1030, 2002

Key words: clitoral cavernosum; nitric oxide; 3′,5′-cyclic monophosphate; electrical field stimulation; NADPH-diaphorase

The relaxation induced by stimulation of the nonadrenergic, noncholinergic (NANC) inhibitory nerves is mediated by the release of nitric oxide (NO), and it is well known in many tissues, including penile corpus cavernosum (PCC) (1–3). Nitrergic neurotransmission is the major neuronal pathway for the penile erection induced by relaxation of the PCC (4).

The clitoris is the embryological homolog of the penis (5). Therefore, it is possible to hypothesize that clitoral tumescence during the female sexual response is under NO regulatory control. However, direct evidence to support this has not yet been fully defined in clitoral cavernosum (CC).

The cavernosum is the erectile tissue of the penis and clitoris. Clitoral tumescence tissue has been shown to contain 40%–45% smooth muscle (6). Relaxation of CC is required for clitoral tumescence as for the penile erection. The excitement phase of the female sexual arousal is mediated by a combination of vasocongestive and neuromuscular events in the clitoris, clitoral tumescence (7), and this phase is the most important stage of female sexual function.

NO is a vasodilator and smooth muscle relaxant that is produced in a number of vascular and nonvascular tissues by the enzyme NO synthase (NOS) (8). Mammalian NO synthesis is mediated by at least three isoforms of NOS. The presence of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) has been demonstrated in the penis. nNOS and eNOS were identified in the clitoris (9–13). However, the specific role of each of these isoforms in the regulation of clitoral tumescence is not yet defined. The presence of the nitrergic nerves has previously been shown in the rabbit and human CC (10, 11, 14).

Recently, it was shown that NO relaxed PCC by increasing guanosine 3′,5′-cyclic monophosphate (cGMP) (3).

Therefore, in the present study, we investigated the role of NO-cGMP signaling in the regulation of tone of the rabbit CC using pharmacological probes, NADPH-

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diaphorase histochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blotting.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Chonbuk National University Hospital and Medical School. The experimental procedures were performed in accordance with the Guiding Principles in the Use and Care of Animals approved by the Council of American Physiological Society.

Materials. Clitoral erectile tissue was obtained from female New Zealand White rabbits (2–2.5 kg). The rabbits (n = 60) were anesthetized with sodium thiopental (30 mg/kg, i.v.) and were exsanguinated. The entire clitoris, including the vagina, was rapidly excised. The vaginal wall was dissected free from the clitoral body. The clitoral cavernosum tissue was then carefully dissected free from the surrounding tunica albuginea under a dissecting microscope. Two strips along the longitudinal axis (about 0.5 × 0.5 × 5 mm) were obtained and were mounted in a 2-ml organ bath. During the preparation, care was taken not to damage the endothelium or overstretch the tissue. The specimens used for morphological study were fixed in an ice-cold, freshly prepared solution of Zamboni's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4) for 4 hr at 4°C, followed by several washes in cold phosphate-buffered saline (PBS) solution. Prior to sectioning, all tissues were stored at 4°C in PBS containing 30% sucrose solution and 0.01% sodium azide for at least 24 hr. All sections were immediately processed by immersion in ornithine carbamoyltransferase (OCT) compound (Tissue-Tek, Sakura; Finetek, Torrance, CA) before snap-freezing in a liquid nitrogen solution. The frozen samples were then stored at –70°C for subsequent histochemical evaluation. The CC tissues for RT-PCR and Western blotting were frozen in a liquid nitrogen solution after being separated free from the tunica albuginea.

Isometric Tension Measurements and Recording of Mechanical Activity. A strip of rabbit CC was placed vertically in an organ chamber (2 ml), with one end connected by means of a silk suture to the prong of a force transducer (Model 79H, Grass Research Polygraph; Grass Instrument Company, West Warwick, RI), and the other end secured by a silk suture to a holder for isometric tension measurements (15). The tissue was kept in a HEPES-buffered physiological salt solution of the following composition (in millimoles): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 10.0, and HEPES 10 with NaOH, pH 7.4. The solution was maintained at 37°C and was aerated with 100% O_2.

Experimental Protocols. The strip of rabbit CC was initially adjusted at 1 g of basal tension and was allowed to stabilize at 37°C for 60 min, during which the oxygenated medium was replaced every 20 min. The preparations were successively stretched and stimulated with L-phenylephrine (PhE) until maximum tension and a stable contraction were obtained. When contractile responses to the PhE had reached a plateau, acetylcholine (ACh, 10^{-7} to 10^{-5} M) was added cumulatively. Relaxations in response to electrical field stimulation (EFS) or ACh were expressed as a negative percentage to the preceding maximal tension generated by PhE (10^{-5} M), which induced submaximal contraction. The presence of the functional endothelium was checked by the presence of at least 50% relaxation in response to ACh (10^{-5} M) in PhE-contracted tissue.

Relaxation Mediated by eNOS. Experiments (n = 10) were performed to investigate cumulative dose-dependent relaxation responses to ACh in the precontracted strip with PhE (Fig. 1A). Briefly, the preparation was contracted with PhE and when a stable tone was obtained, ACh was added to the organ bath. After a first dose-response curve was obtained, the strip was washed and incubated with indomethacin (10^{-6} M) and guanethidine (10^{-5} M) for 30 min to block cyclooxygenase activity and adrenergic neurotransmission. A second dose-response curve was then made in the PhE-precontracted strip, and the strip was washed. The strip was incubated with indomethacin, guanethidine, and tetrodotoxin (TTX, 10^{-5} M) to block intrinsic neurotransmission for 30 min. A third dose-response curve was then made in the PhE-precontracted strip, and the strip was washed. The strip was incubated with indomethacin, guanethidine, and atropine (10^{-5} M) to block cholinergic neurotransmission for 30 min, and a fourth dose-response curve was made in the PhE-precontracted strip. When an ACh-induced response was obtained and the stable contraction was attained, NO donor sodium nitroprusside (NP; 10^{-7} M) was added to the bath to determine whether the NO-cGMP pathway was intact. The strip was washed and incubated with indomethacin, guanethidine, and N^{ω} nitro-L-arginine-methyl ester (NAME; 10^{-3} M) to block NOS for 30 min, and a fifth dose-response curve was made in the PhE-precontracted strip. When an ACh-induced response was obtained and the stable contraction was attained, NP was added to the bath. The strip was washed and incubated with indomethacin, guanethidine, and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10^{-6} M) to block guanylate cyclase activity for 30 min, and a sixth dose-response curve was made. When an ACh-induced response was obtained and the stable contraction was attained, NP was added to the bath to determine whether guanylate cyclase activity was inhibited. The strip was washed. To reverse the inhibition of NOS by NAME, the strip was incubated with L-arginine (10^{-3} M) for 1 hr, and a seventh dose-response curve was made in the PhE-precontracted strip.

EFS. Briefly, preparations (n = 10) were contracted with PhE (10^{-5} M), and when a stable tone was obtained, a first EFS was performed (Fig. 1B). The strip was washed and then incubated with indomethacin, guanethidine, and atropine for 30 min, and a second EFS-induced relaxation was obtained in the PhE-precontracted strip. The strip was washed again, and was then incubated with indomethacin, guanethidine, atropine, and TTX for 30 min. After this, a

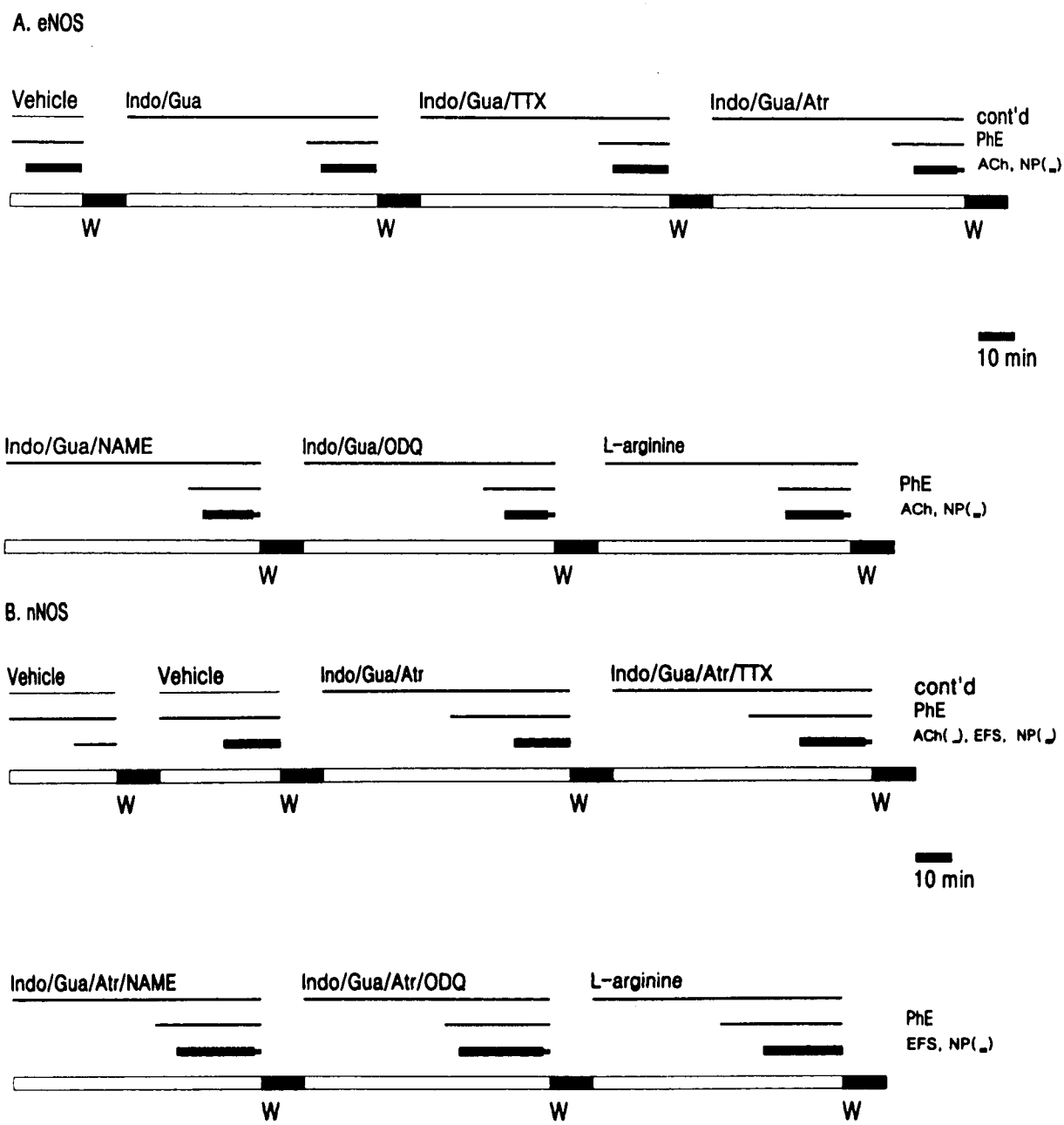

Figure 1. Protocol for present experiments. (A) Pharmacological evaluation of eNOS. (B) Pharmacological evaluation of nNOS using EFS. Atr, atropine hydrochloride; Gua, guanethidine; Indo, Indomethacin; NP, sodium nitroprusside; W, washout.

third EFS-induced response was made in the PhE-precontracted strip. After the attainment of an EFS-induced response and the stable contraction, NP (10^{-7} *M*) was added to the bath and the response was recorded. The strip was washed, and indomethacin, guanethidine, atropine, and NAME were added for 30 min, and a fourth EFS-induced response was obtained in the PhE-precontracted strip. After the attainment of the EFS-induced response and the stable contraction, NP was added to the bath and the response was recorded. The strip was incubated with indomethacin, guanethidine, atropine, and ODQ for 30 min, and a fifth EFS was performed. After the attainment of the EFS-induced response and the stable contraction, NP was added to the bath to determine whether guanylate cyclase activity was inhibited or not. The strip was washed and incubated with L-arginine (10^{-3} *M*) for 1 hr, and a sixth EFS was performed on the PhE-precontracted strip.

For the EFS of the CC strip, the preparation was mounted between two parallel platinum electrodes in the organ bath. The intrinsic nerves were stimulated electrically for 10 sec with trains of rectangular pulses of a 3-msec pulse duration, 30 V, and frequencies ranging from 0.5 to 32 Hz delivered by Grass S88 stimulator (Grass Instrument Company) every 120 sec.

ACh-Induced Relaxation Responses by iNOS. Experiments (n = 8) were performed to investigate

cumulative dose-dependent relaxation responses to ACh in a strip precontracted with PhE. Briefly, the preparation was contracted with PhE, and when a stable tone was obtained, ACh was added to the organ bath. After a first dose-response curve was obtained, the strip was washed and incubated with indomethacin (10^{-6} *M*) and guanethidine (10^{-5} *M*) for 30 min. A second dose-response curve was then made in the PhE-precontracted strip. The strip was washed and incubated with indomethacin, guanethidine, and S-ethylisothiourea HBr (10^{-4} *M*) to block iNOS for 30 min. A third dose-response curve was then made in the PhE-precontracted strip.

EFS-Induced Relaxation Responses by iNOS. Briefly, preparations (n = 10) were contracted with PhE (10^{-5} *M*), and when a stable tone was obtained, a first EFS was performed. The strip was washed. The strip was incubated with indomethacin, guanethidine, and atropine for 30 min, and then a second EFS-induced relaxation was obtained in the PhE-precontracted strip. The strip was washed. After the strip was incubated with indomethacin, guanethidine, atropine, and S-ethylisothiourea HBr (10^{-4} *M*) for 30 min, a third EFS-induced response was obtained in the PhE-precontracted strip.

RNA Extraction and RT-PCR. Total RNA was extracted from rabbit CC, PCC, and skeletal muscle (n = 8) for positive comparison using TRI reagent (MRC, Cincinnati, OH), according to the manufacturer's protocol. Total RNA concentration was quantitated by UV spectrophotometry at 260/280 nm. Two micrograms of total RNA was suspended in 20 μl of RT buffer containing (in millimoles) 10 Tris (pH 8.3), 50 KCl, 5 $MgCl_2$, and each of dATP, dCTP, dGTP, and dTTP, 20 U of ribonuclease inhibitor, 2.5 μ*M* random hexamers, and 150 U of Moloney leukemia virus reverse transcriptase (Perkin Elmer, Branchburg, NJ) and was reverse transcribed at room temperature for 10 min and at 42°C for 30 min (16). The reaction was stopped by heat inactivation for 5 min at 99°C and was then chilled on ice. Complementary DNA products were amplified by PCR using the following primers: nNOS sense, 5′-GCA ACG AGA AAG AGA AGC-3′ (3430–3417); nNOS antisense, 5′-TCC TCC CTG TAG ATG TGA-3′ (3947–3964); iNOS sense, 5′-CTA CCT GGG GAA CAC CTG-3′ (415–432); and iNOS antisense, 5′-CGC AAT GAT GGG AAC TCT-3′ (765–782).

One hundred microliters of PCR buffer contained (in millimoles) 10 Tris (pH 8.3), 50 KCl, 2 $MgCl_2$, with 200 μ*M* each of dATP, dCTP, dGTP, and dTTP, 2.5 U of Taq polymerase, and 100 p*M* each of sense and antisense primers. A hot-start PCR was used to increase the specificity of amplification. The temperature profile of amplification consisted of 1 min of denaturation at 95°C, 1 min of annealing at 56°C, and 1.5 min of extension at 74°C for 30 cycles. PCR products were separated in 2% agarose gels and bands were visualized by ethidium bromide staining. Photographs of gels were taken with Polaroid 665 film. PCR products were confirmed by sequence analysis.

Western Blotting. The expression of eNOS and iNOS protein in the rabbit CC (n = 6) and PCC for positive comparison was examined by Western blot analysis. The 100 mg of muscle tissues was isolated from the clitoris, homogenized in 1 ml of lysis buffer containing 150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris-HCl (pH 8.0), 1% NP-40, aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and phenyl methylsulfoxide (100 μg/ml), and left on ice for 30 min. The homogenized sample was centrifuged at 14,000*g* for 30 min (eNOS) or at 13,000*g* for 5 min (iNOS) at 4°C, and soluble fraction was moved. The protein concentration was determined by Lowry methods with bovine serum albumin as standard. Samples containing equal amounts of protein (10 μg) from lysates of the tissues were electrophoresed on a 8% SDS-polyacrylamide gel with prestained molecular mass marker, and were transferred to polyvinyl difluoride membranes in a Tris-glycine transfer buffer. The blots were incubated for 1 hr at room temperature in PBS with 0.1% Tween-20 (PBST) in 5% nonfat milk powder to block nonspecific antibody binding. After three or four washes in PBST, the blots were incubated for 1 hr at 4°C with the specific rabbit anti-human eNOS polyclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:500 in Blotto (Amersham Life Science, Arlington Heights, IL), or specific mouse anti-human iNOS monoclonal antibody (Alexis Biochemicals, San Diego, CA) diluted 1:1,000 in Blotto. After three washes in PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody diluted 1:5,000, or horseradish peroxidase-conjugated secondary anti-mouse antibody diluted 1:5,000 in Blotto for 40 min, relatively. The membranes were washed again in PBST and were processed for protein detection according to the manufacturer's protocol. Specific proteins were identified using an enhanced chemiluminescence agent (Amersham).

NADPH-Diaphorase Histochemistry. Tissues (n = 8) were cut with an Ames cryostat in 20-μm thickness. Tissue demonstration of NADPH-diaphorase activity was made by incubating tissue sections with 1 m*M* β-NADPH and 0.5 m*M* nitroblue tetrazolium dissolved in 0.1 *M* PBS containing 0.3% Triton X-100 for 30 min at 37°C (17). After three rinses in 0.1 *M* PBS of 10 min each, the sections were mounted on slides, air-dried, coverslipped using a cytoseal (Stephens Scientific, Riverdale, NJ), and viewed with a regular microscope. Photographs were taken at a magnification of 200× with film (100 ASA; Kodak, Rochester, NY).

Pharmacological Agents. ACh, atropine hydrochloride, guanethdine, indomethacin, β-NADPH, NAME, nitroblue tetrazolium, ODQ, L-phenylephrine, and NP were purchased from Sigma Chemicals (St. Louis, MO). TTX was purchased from Tocris Cookson (Langford, Bristol, UK). Anti-human eNOS polyclonal antibody was purchased from Transduction Laboratories, and anti-human iNOS

monoclonal antibody was purchased from Alexis Biochemicals (San Diego, CA). S-ethylisothiourea HBr was purchased from BIOMOL (Plymouth, PA). Horseradish peroxidase-conjugated secondary anti-rabbit antibody and horseradish peroxidase-conjugated secondary anti-mouse antibody were purchased from Amersham Life Science (Arlington Heights, IL). Zamboni's solution was purchased from Newcomer Supply (Middleton, WI). All other chemicals were purchased from standard suppliers. Drugs were dissolved in distilled water except for ODQ, which was dissolved in dimethyl sulfoxide (DMSO), and indomethacin, which was dissolved in 100% ethanol and diluted in buffer solution. (The highest DMSO or ethanol concentration in the various test system was less than 1%, v/v). The remaining reagents were dissolved and diluted in isotonic saline solution.

Statistics. The submaximal contractile responses induced for PhE were taken as the 100% values, and all subsequent responses to ACh or EFS were expressed as a percentage of this value. The results were expressed as mean ± SEM, and n represents the number of tissues in each group. Statistical significance of differences was calculated by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Dose-dependent responses before and after the treatment with blockers were compared by Student's paired t test. A probability value of less than 0.05 was considered significant.

Results

Responses to ACh. ACh resulted in relaxation in a dose-dependent manner in precontracted strips with PhE (n = 10; Fig. 2). Relaxations induced by 10^{-7}, 10^{-6}, and 10^{-5} M ACh were 10.1% ± 2.4%, 34.1% ± 4.2%, and 53.9% ± 6.7% of the tone induced by PhE, respectively. These responses were reproducible. The addition of indomethacin, guanethidine, and TTX did not change the 10^{-7}, 10^{-6}, and 10^{-5} M ACh-induced relaxation (10.5% ± 1.9%, 34.1% ± 3.5%, and 61.6% ± 5.4%, respectively, $P > 0.05$). Atropine (10^{-5} M) blocked the effect of ACh. NAME and ODQ significantly inhibited the relaxations to 10^{-7}, 10^{-6}, and 10^{-5} M ACh (NAME; 5.0% ± 1.2%, 7.5% ± 2.2%, and 11.5% ± 2.9% and ODQ; 3.5% ± 1.3%, 7.5% ± 1.5%, and 10.9% ± 4.0%, respectively, $P < 0.05$).

NP relaxed CC in the presence of NAME (93.7% ± 6.9%), but did not do so in the presence of ODQ (Fig. 2). L-Arginine partially restored relaxant responses inhibited by NAME, although there was no statistical significance (Fig. 2; $P > 0.05$).

Response to EFS. EFS, 0.5 to 16 Hz, but not EFS, 32 Hz, resulted in stimulus strength-dependent relaxation in precontracted CC strips with PhE (Figs. 3 and 4 and Table I, $P < 0.01$). The responses were reproducible. TTX significantly blocked the relaxant response to EFS. Atropine did not modulate the relaxant responses. Treatment with NAME also significantly inhibited the relaxant responses to EFS with statistical significance (Fig. 3 and Table II; $P < 0.05$). However, NAME or TTX failed to change NP-induced relaxant responses (Fig. 3). ODQ significantly inhibited the relaxant responses to EFS and NP (Fig. 3 and Table I; $P < 0.05$). L-Arginine partially restored the effect of EFS in high frequency (Fig. 3 and Table II; $P < 0.05$).

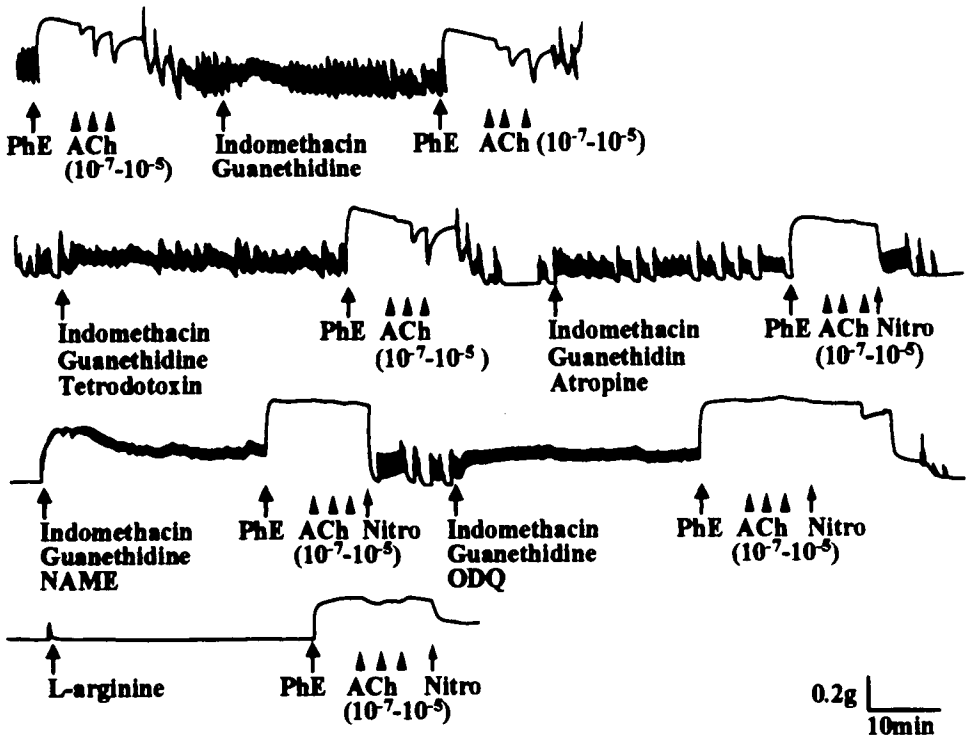

Figure 2. Representative figure of effect of atropine, NAME, and ODQ on ACh-induced relaxation of the CC tissue (n = 10). Nitro, sodium nitroprusside.

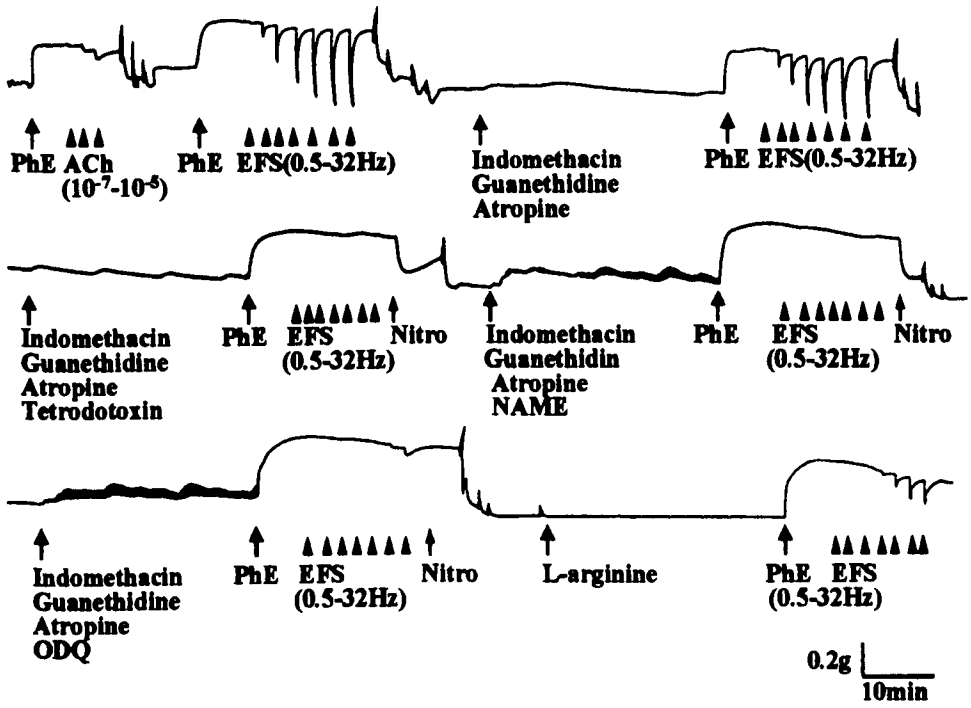

Figure 3. Representative figure of effect of EFS and blockade by TTX, NAME, or ODQ in the CC.

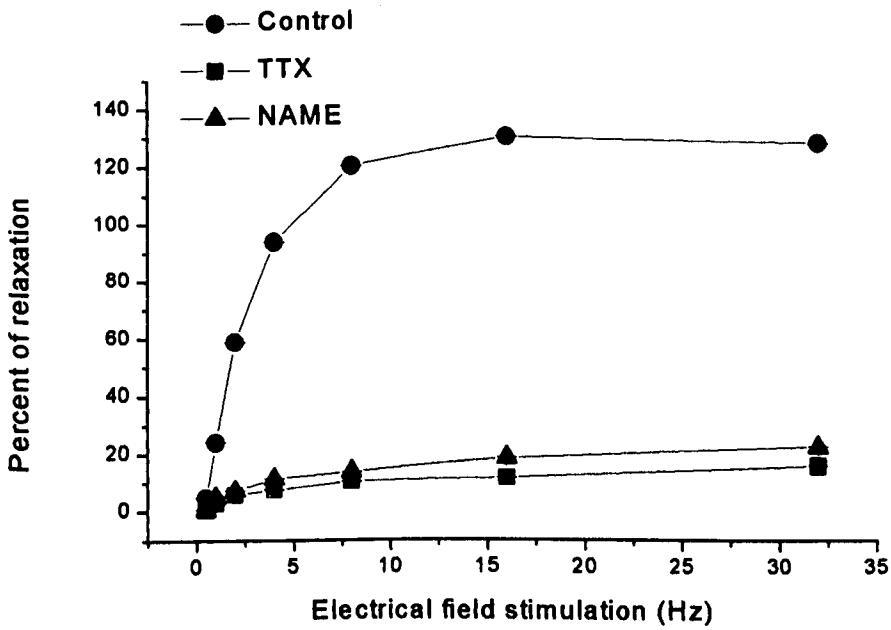

Figure 4. Blockade by TTX and NAME of the EFS-induced relaxation of the CC (n = 10). The error bars are masked by the symbol.

Responses to iNOS Inhibitor. S-ethylthiourea at 10^{-4} M inhibited the relaxations to 10^{-7}, 10^{-6}, and 10^{-5} M ACh (4.1% ± 3.3%, 6.9% ± 4.6%, and 8.3% ± 5.5%, relatively). S ethylthiourea at 10^{-4} M also inhibited the relaxations induced by EFS (Tables I and II). The effects were not different from inhibition by the NOS inhibitor NAME.

Detection of nNOS and iNOS mRNAs by RT-PCR. The presence of nNOS and iNOS transcripts was determined by RT-PCR in the rabbit CC and PCC, but not in skeletal muscle. In the CC, nNOS transcript was observed in ethidium bromide-stained gels with the expected size of 535 base pairs (bp) after 30 cycles of amplification, and iNOS transcript with 368 bp was also detected after 30 cycles of amplification (Fig. 5). The same size of RT-PCR products from PCC as positive control were observed. No transcripts were observed in RNA samples without Moloney leukemia virus reverse transcriptase.

Detection of eNOS and iNOS Proteins by Western Blotting. Western blot analysis of homogenates obtained from dispersed CC and PCC tissues, using polyclonal antibody to eNOS or monoclonal antibody to iNOS, demonstrated the presence of immunoreactive protein bands corresponding to 140 kDa eNOS and 130 kDa iNOS (Fig. 6).

Table I. ACh-Induced Relaxation

Agents \ ACh (*M*)	10^{-7} *M*	10^{-6} *M*	10^{-5} *M*
Control	10.1 ± 2.4	34.1 ± 4.2	53.9 ± 6.7
TTX (10^{-5} *M*)	10.5 ± 1.9	34.1 ± 3.5	61.6 ± 5.4
NAME (10^{-3} *M*)	5.0 ± 1.2	7.5 ± 2.2[a]	11.5 ± 2.9[a]
ODQ (10^{-6} *M*)	3.5 ± 1.3	7.5 ± 1.5[b]	10.9 ± 4.0[b]
NAME (10^{-3} *M*) + L-Arginine (10^{-3} *M*)	5.9 ± 0.8[c]	10.6 ± 2.2[c]	12.8 ± 1.1[c]
S-Ethylthiourea (10^{-4} *M*)	4.1 ± 3.3[d]	6.9 ± 4.6[d]	8.3 ± 5.5[d]

Note. Each point represents the means ± SEM (n = 13) of percentages of maximal relaxation of the preceding submaximal contractile responses generated by PhE (10^{-5} *M*).
[a] $P < 0.05$ vs control.
[b] $P < 0.05$ vs control.
[c] $P < 0.05$ vs control.
[d] $P < 0.05$ vs control.

Table II. EFS-Induced Relaxation

Agents \ Frequency	0.5 (Hz)	1 (Hz)	2 (Hz)	4 (Hz)	8 (Hz)	16 (Hz)	32 (Hz)	Nitroprusside
Control	4.8 ± 1.5	24.1 ± 5.1	58.8 ± 10.7	93.7 ± 14.7	120.1 ± 13.1	130.6 ± 12.1	128.2 ± 13.2	
TTX (10^{-5} *M*)	0.5 ± 0.5[a]	0.7 ± 0.7[a]	1.9 ± 0.6[a]	2.1 ± 0.6[a]	3.5 ± 0.5[a]	4.7 ± 1.6[a]	5.1 ± 1.4[a]	79.6 ± 6.4
NAME (10^{-3}) *M*)	0.5 ± 0.5[b]	1.5 ± 1.5[b]	2.2 ± 0.7[b]	3.6 ± 0.6[b]	3.9 ± 0.5[b]	5.4 ± 1.1[b]	6.1 ± 1.3[b]	80.6 ± 5
ODQ (10^{-6} *M*)	0.5 ± 0.3[c]	1.4 ± 1.2[c]	2.2 ± 0.5[c]	3.1 ± 0.7[c]	3.8 ± 1.1[c]	5.1 ± 1.6[c]	7.3 ± 1.1[c]	5.8 ± 6.6
NAME (10^{-3} *M*) + L-Arginine (10^{-3} *M*)	0.5 ± 0.5[d]	4.4 ± 1.4[d]	7.0 ± 1.5[d]	11.5 ± 1.5[d]	14.2 ± 1.4[d]	18.1 ± 1.9[d]	21.5 ± 2.1[d]	
S-Ethylthiourea (10^{-4} *M*)	8.3 ± 2.4	10.3 ± 3[e]	10.3 ± 2.2[e]	14.2 ± 2.4[e]	18.1 ± 3.1[e]	23.0 ± 4.3[e]	24.8 ± 3.6[e]	

Note. Each point represents the means ± SEM (n = 10) of percentages of maximal relaxation of the preceding submaximal contractile responses generated by PhE (10^{-5} *M*).
[a] $P < 0.05$ vs control.
[b] $P < 0.05$ vs control.
[c] $P < 0.05$ vs control.
[d] $P < 0.05$ vs control.
[e] $P < 0.05$ vs control.

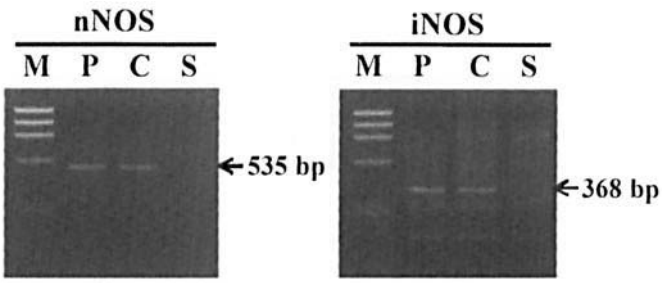

Figure 5. Detection of nNOS and iNOS mRNAs by RT-PCR in the rabbit PCC (P) and CC (C). M, DNA molecular marker.

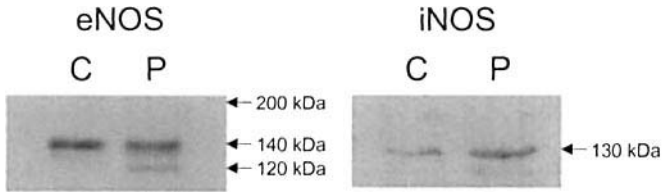

Figure 6. Western blot analysis of eNOS and iNOS in homogenates obtained from rabbit CC and PCC. After polyvinyl difluoride membrane transfer, proteins were probed with polyclonal antibody to eNOS or monoclonal antibody to iNOS. Bands were evident with antibody to eNOS (140 kDa) or to iNOS (130 kDa).

NADPH-Diaphorase Histochemistry. nNOS-containing fibers were visualized by NADPH-diaphorase histochemical staining, counterstained with H&E staining in cryostat crosssections of the CC (Fig. 7). These nerves present different sizes and coarse nerve trunks. The endothelium of the CC spaces was not stained with the NADPH-diaphorase method.

Discussion

The present study indicates that NO-cGMP signaling is involved in the regulation of CC tone. This data suggests that neuronally released NO, as endothelium-derived NO, is important in this control mechanism. The data also suggests that iNO may be involved in the signaling pathway.

Both CC relaxation and clitoral arterial smooth muscle dilation increase CC artery inflow and intraclitoral pressure, which result in clitoral tumescence (7). The clitoral tumescence is regulated by a delicate balance between the contraction and relaxation of CC (13). The contraction of CC by excitatory substances, such as noradrenaline and angiotensin II, resulted in clitoral detumescence (13). The present data indicates that an inhibitory substance, NO, should induce clitoral tumescence.

It is now well recognized that a NANC mechanism plays a major role in signal transduction, modulating vascular smooth muscle and PCC tone by synthesizing and metabolizing NO (18, 19). It is also believed that endothelium-dependent relaxation to ACh plays a role in signal transduction, which results in vascular and penile smooth

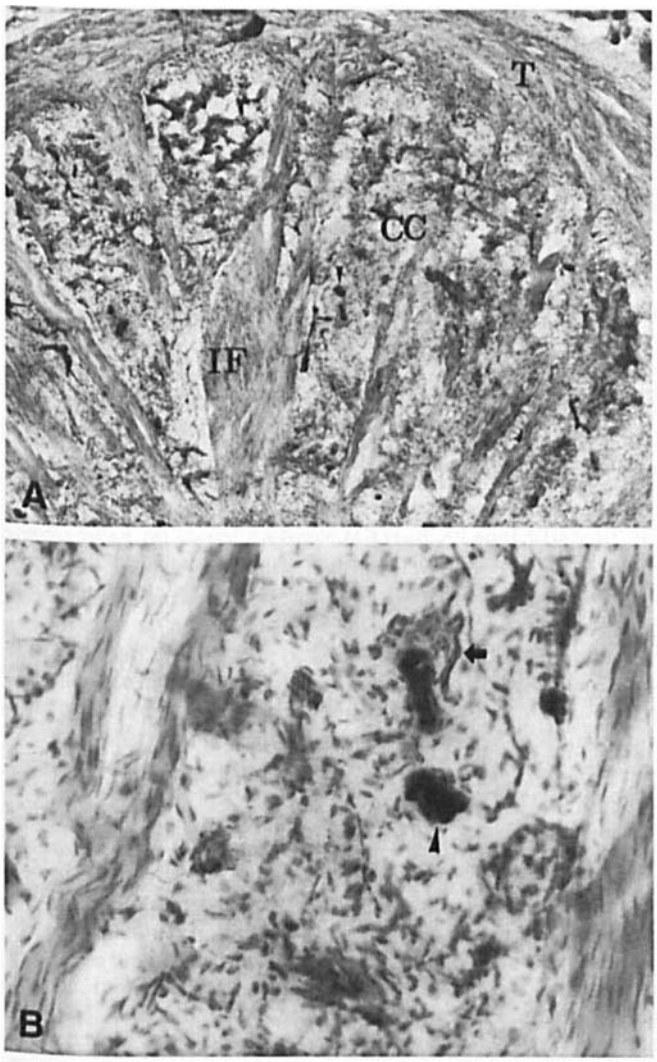

Figure 7. NADPH-diaphorase histochemical staining counterstained with H & E staining in the rabbit CC. Nerve fibers (arrow) and trunks (arrowhead) were stained. (A) ×40. (B) ×200. T, tunica albuginea; IF, intracavernosal fibrous framework.

muscle relaxation by the releasing of NO. Previously, it was shown that NO is involved in the regulation of CC tone (11, 13). In the present study, EFS-induced relaxation was significantly inhibited by the neuronal sodium channel blocker TTX or the NOS inhibitor NAME in the CC. The effect was reversible, and excess L-arginine, a substrate of NO synthesis, partially reversed the effect. ACh-induced relaxation was inhibited by NAME, but not by TTX. These results suggested that NO from nitrergic nerves and endothelium was involved in the relaxation of rabbit CC. The inhibition of EFS- or ACh-induced relaxation by NAME strongly suggests that a diffusible NO was released from the clitoral autonomic nerves, and/or CC and vascular endothelium. NADPH-diaphorase histochemistry shows positivity in the clitoral autonomic nerves and vascular endothelia, and suggests that nNOS plays an important role in the regulation of CC tone.

NO is an extremely labile, readily diffusible substance and it exerts its effect via the activation of guanylate cyclase and the elevation of intracellular cGMP concentration (20). The inhibition of EFS- and ACh-induced relaxation by ODQ, a selective guanylate cyclase inhibitor, suggests that these relaxing responses were mediated by an increase in cGMP production.

NO is synthesized from L-arginine by NOS. Constitutive calcium-dependent isoforms in eNOS and nitrergic nerves (nNOS), and the calcium-independent isoform (iNOS) inductions by bacterial lipopolysaccharide or cytokines have been well described in other tissues (9, 21). The presence of the eNOS and nNOS has been shown to be present in the CC (11, 22, 23). However, three types of NOS may be involved in clitoral tumescence.

It is known that constitutive NOS has potential role as mediator of the relaxation of CC smooth muscle in the female sexual response (11, 24), although the role of iNOS in the female sexual function is not clear. Clitoral sensation is important in arousal phase of the female sexual response. Decreased clitoral sensation and engorgement are causes of the females sexual arousal disorder. This condition may occur secondary to medical bases, such as pelvic surgery, medications, and pelvic trauma (24).

The physiological roles of eNOS and nNOS, but not iNOS, in clitoral tumescence were easily determined. It should be stressed that our study shows only the expression of the nNOS and iNOS mRNA transcripts by RT-PCR, but not the functional status, because the cDNA gene of eNOS is not known in the rabbit. However, we also found immunoreactive protein bands corresponding to eNOS and iNOS by Western blotting, and positive stainings for NOS containing nerve fibers and vascular endothelia (not in figure) by NADPH-diaphorase histochemistry in the CC. ACh and EFS induced relaxations in the strips precontracted by PhE. EFS induced relaxations similar to such as ACh-induced relaxations, but they were more steep. These results suggest that nNOS and eNOS are involved in the regulation of CC tone via the NO system. iNOS may also be involved in the regulation of clitoral tumescence under certain pathophysiological conditions, although we still have to develop a research tool for the physiological role of iNOS. The study of female sexual dysfunction is gradually evolving (24). Clarification of the physiologic role of iNOS in female sexual function is forthcoming.

In summary, it is suggested that NO-cGMP signaling is involved in the regulation of clitoral tumescence via nNOS and eNOS. It is also suggested that NO produced by iNOS may be involved in clitoral tumescence under certain clinical conditions in the CC.

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