Effects of Calcium Antagonists on the Nitrergic Nerve Function in Canine Corpus Cavernosum

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ABSTRACT—Effects of calcium antagonists on nitrergic nerve function were examined in the isolated canine corpus cavernosum. In the cavernous strips precontracted with phenylephrine, transmural electrical stimulation elicited frequency-dependent (2–5 Hz) relaxations that were abolished by Nω-nitro-L-arginine (10^{-5} M), a nitric oxide (NO) synthase inhibitor; 1H[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 10^{-6} M), a soluble guanylate cyclase inhibitor; and tetrodotoxin (3 × 10^{-7} M). The relaxations were not affected by treatment with nifedipine or nicardipine (10^{-8} – 10^{-6} M), L-type specific calcium channel inhibitors, but were significantly inhibited by amlodipine or cilnidipine, inhibitors of L- plus N-type calcium channels, in a concentration-related manner (10^{-7} – 10^{-6} M). All of the inhibitors used did not affect the relaxations induced by exogenous NO (acidified NaNO2). These findings suggest that N-type, but not L-type, calcium channels are responsible for increasing cytosolic free calcium, a prerequisite for the synthesis of NO, in the nitrergic dilator nerves innervating the corpus cavernosum.

Keywords: Corpus cavernosum, Nitrergic nerve, Transmural electrical stimulation, Calcium antagonist, Smooth muscle relaxation

Penile erection is induced by relaxation of the smooth muscle of penile corpus cavernosum and increased pooling of blood in the corpus (1). Autonomic nerves play an important role in the control of the function. Recently, nitric oxide (NO) released from nitrergic nerves is considered to be mainly responsible for the erection in rats (2), rabbits (3) and dogs (4, 5). Potentiation of the function by sildenafil, a selective inhibitor of cyclic GMP specific phosphodiesterase V, and its potent therapeutic effect on erectile dysfunction also demonstrate the involvement of the endogenous NO-cyclic GMP pathway in human penile erection (6).

Although importance of nitrergic nerve function in the penile erection has been recognized, the mechanism underlying the synthesis/release of neurogenic NO is not clarified in the corpus cavernosum. Our findings from experiments with cerebral arteries, which are also innervated mainly with nitrergic dilator nerves, suggest that production of neurogenic NO is calcium- and calmodulin-dependent (7, 8), and N-, but not L-, type calcium channels are involved in the response (7, 9).

Recently, several types of calcium antagonists have been clinically used for the treatment of hypertension (10): L-type specific and L- plus N-type calcium channel inhibitors (11). The latter inhibitors have been considered to possess additional inhibitory activity on the neurotransmitter release from sympathetic nerves innervating arteries and arterioles (12). However, the effects on dilator nerves have not been examined.

We, therefore, aimed to examine the effects of several calcium antagonists used clinically on the neurogenic relaxation in the isolated canine corpus cavernosum and to elucidate the calcium channels responsible for the entry of calcium which is essential for the production of NO in the nerve terminals.

MATERIALS AND METHODS

The Animal Care and Use committee at Shiga University of Medical Science approved the use of tissue from male beagles along with the experimental protocols in this study.

Preparation

Male beagles, weighing 7 to 10 kg, were anesthetized with intravenous injections of thiopental (30 mg/kg) and killed by bleeding from the carotid arteries. The penis was rapidly removed, and the corpus cavernosum was isolated.
The tunica albuginea was removed, and three or four strips (about 1 x 2 x 10 mm) were obtained. The strips were fixed vertically between hooks in a muscle bath of 10 ml capacity containing Ringer-Locke solution, which was aerated with a mixture of 95% O₂ and 5% CO₂ and maintained at 37 ± 0.3°C. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo). The resting tension was adjusted to 0.7 g, which is optimal for inducing the maximal contraction (13). Constituents of the solution were as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl₂, 1.0 mM MgCl₂, 25.0 mM NaHCO₃, and 5.6 mM dextrose. The pH of the solution was 7.36 to 7.43. Before the start of experiments, all of the strips were allowed to equilibrate in the bathing media for 60 to 90 min, during which time the solutions were replaced every 10 to 15 min.

Most of the strips were placed between stimulating electrodes. The gaps between the strip and the electrodes were wide enough to allow undisturbed contraction and relaxation and yet sufficiently narrow to stimulate intramural nerve terminals effectively. A train of 0.2-ms square pulses of supramaximal intensity (10 V) was transmurally applied at 2 and 5 Hz for 40 s. The stimulating pulses were wide enough to allow undisturbed contraction and relaxation of the strips. After the start of experiments, all of the strips were allowed to equilibrate in the bathing media for 60 to 90 min, during which time the solutions were replaced every 10 to 15 min.

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**Tension recording**

Isometric contractions and relaxations were displayed on a pen recorder. The contractile response to 30 mM K⁺ was obtained first, and the strips were washed three times with fresh medium and equilibrated for 30 to 40 min. The strips were partially contracted with phenylephrine (10⁻⁵ to 10⁻⁴ M); the contraction was in a range between 25 and 40% of the contraction induced by 30 mM K⁺. Transmural electrical stimulation was applied repeatedly at intervals of 10 min until steady responses were obtained, and then blocking agents were treated for at least 20 min. Since calcium antagonists except α-conotoxin GVIA applied during the experiments suppressed the phenylephrine-induced contractions, phenylephrine was additionally applied to maintain the level of vascular tone before the treatment with the antagonists. The mean values of the suppression of the phenylephrine-induced contractions and the concentrations of added phenylephrine did not significantly differ among the calcium antagonists used: 29.4 ± 6.3% and [2.1 ± 1.5] x 10⁻⁶ M at 10⁻⁸ M, 54.7 ± 10.5% and [2.5 ± 1.9] x 10⁻⁵ M at 10⁻⁷ M, and 70.8 ± 9.8% and [8.6 ± 2.9] x 10⁻⁵ M at 10⁻⁶ M, respectively. Each calcium antagonist was applied cumulatively to the strip. NO (acidified NaNO₂) was applied directly to the bathing media in a cumulative manner, and the strips were repeatedly washed. After the response to NO (acidified NaNO₂) was stabilized, the strips were treated for at least 20 min with blocking agents. At the end of each series of the experiments, papaverine (10⁻⁴ M) was applied to attain the maximal relaxation (100% relaxation), and relative values of the response to electrical stimulation or NO (acidified NaNO₂) were calculated.

**Statistics and drugs used**

The results shown in the text and figures are expressed as mean values ± S.E.M. Statistical analyses were made using Student’s unpaired t-test for two groups and Tukey’s test after one-way analysis of the variance for three or more groups. Drugs used were L-nitro-L-arginine (L-NA), L-arginine, α-conotoxin GVIA (Peptide Institute, Minoh); L-phenylephrine hydrochloride, nicardipine (Sigma Chemical, St. Louis, MO, USA); thiopental (Tanabe, Osaka); tetrodotoxin (Sankyo, Tokyo); nifedipine (Bayer Yakuhin, Osaka); amlopidine (Sumitomo, Osaka); cilnidipine (Fujirebio, Tokyo); and papaverine hydrochloride (Dainippon, Osaka). 1H[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ) is a generous gift from Prof. S. Moncada (Univ. of London, UK). Stock solutions of all calcium antagonists (10⁻² M) were made with 100% ethanol as a solvent. In preliminary experiments, 0.1% ethanol (a final concentration of the solvent) did not affect the basal tone or the relaxations caused by transmural electrical stimulation and NO (acidified NaNO₂). Responses to NO were obtained by adding NaNO₂ solution adjusted at pH 2 just prior to the application (14). Briefly, acidified NaNO₂ produced a potent transient relaxation, whereas neutral NaNO₂ produced a very weak persistent relaxation; therefore, relaxations induced by acidified NaNO₂ (NO) are easily distinguished from those induced by NaNO₂. The concentrations of acidified NaNO₂ solution were expressed as those of NO.

**RESULTS**

The strips of canine corpus cavernosum partially contracted with phenylephrine, transmural electrical stimulation at 2 and 5 Hz produced frequency-related relaxations; mean values of the responses were 28.9 ± 4.0% (n = 10) and 42.0 ± 3.9% (n = 10), respectively, relative to those induced by 10⁻⁴ M papaverine. The relaxations were abolished by 10⁻⁵ M L-NA (n = 5), 10⁻⁴ M ODQ (n = 5) and 3 x 10⁻⁷ M tetrodotoxin (n = 5). The relaxations caused by electrical stimulation were not affected by treatment with nifedipine (10⁻⁸ – 10⁻⁶ M), an L-type specific calcium channel inhibitor, but were inhibited by amlopidine (10⁻⁸ – 10⁻⁶ M), an L- plus N-type calcium channel inhibitor, in a concentration-related manner (Fig. 1). Similar results were obtained with another L-type specific calcium channel inhibitor nicardipine and another L- plus N-type specific calcium channel inhibitor cilnidipine. Quantitative
Fig. 1. Real recordings of the response to transmural electrical stimulation (TES) at 2 and 5 Hz in canine cavernous strips in the absence and presence of nifedipine, N\textsuperscript{G}-nitro-L-arginine (L-NA), amlodipine and 1H[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ). The strips were partially contracted with phenylephrine. PA represents 10\textsuperscript{-4}M papaverine that produced the maximal relaxation.

Fig. 2. Concentration-inhibition curves of nifedipine, amlodipine, cilnidipine and nicardipine on the relaxations induced by transmural electrical stimulation at 2 Hz (left) and 5 Hz (right) in canine cavernous strips partially contracted with phenylephrine. The ordinate denotes inhibition (%) calculated by comparison of the relaxations in the presence and absence of calcium antagonist. Numbers in parentheses indicate the number of strips from individual dogs. Significantly different from the values with nifedipine, \textasciitilde P<0.05 and \textasciitilde\textasciitilde P<0.01 (Tukey’s test). Significantly different from zero, *P<0.05 and **P<0.01 (Tukey’s test). Vertical bars represent S.E.M.
data are summarized in Fig. 2; the mean values of the relaxations relative to those induced by $10^{-4}$ M papaverine in $10^{-7}$ M nifedipine-, nicardipine-, amlodipine- and cilnidipine-containing media were 29.4 ± 5.7% (P<0.05, Tukey’s test, n = 8), 26.1 ± 5.3% (P<0.05, Tukey’s test, n = 7), 13.7 ± 2.8% (P<0.05, Tukey’s test, n = 12), and 17.3 ± 3.5% (P<0.05, Tukey’s test, n = 10), respectively. Relaxations induced by electrical stimulation at 2 Hz were significantly inhibited by treatment with $\alpha$-conotoxin GVIA, a specific N type calcium channel inhibitor (15), in a concentration-related manner (50.9 ± 8.4% inhibition at $10^{-8}$ M, P<0.05; 85.4 ± 9.6% inhibition at $10^{-7}$ M, P<0.01, unpaired t-test, n = 4). $\alpha$-Conotoxin GVIA even at $10^{-7}$ M did not attenuate the precontraction induced by phenylephrine. When treated with a combination of $10^{-8}$ M $\alpha$-conotoxin GVIA and $10^{-6}$ M amlodipine, at which each agent alone inhibited the relaxation by electrical stimulation roughly 50%, additional inhibition was not obtained (52.9 ± 10.1% inhibition, n = 4, P>0.05).

Response to exogenous NO (acidified NaNO₃)

In the cavernous strips partially contracted with phenylephrine, the addition of NO (acidified NaNO₃, $10^{-6} - 10^{-4}$ M) produced a concentration-dependent relaxations. The NO (acidified NaNO₃)-induced relaxations were not affected by $10^{-5}$ M L-NA (n = 5) but were abolished by $10^{-6}$ M ODQ (n = 5) (data not shown). Treatment with amlodipine ($10^{-7}$ and $10^{-6}$ M) did not affect the relaxations caused by NO (acidified NaNO₃) (Fig. 3). Either cilnidipine, nifedipine or nicardipine did not affect the NO (acidified NaNO₃)-induced relaxations. Quantitative data at $10^{-6}$ M are summarized in Fig. 4 (P>0.05, Tukey’s test).

**DISCUSSION**

Involvement of neurogenic NO in the relaxation of penile corpus cavernosum has been reported in a variety of mammals including humans (2 – 4, 16). In the anesthetized dogs, pelvic nerve stimulation produced a penile erection with an elevation of intracavernous pressures that was not affected by phentolamine or atropine, but was abolished by a ganglion-blocking agent, hexamethonium, and NO synthase inhibitors, L-NA and $N^\alpha$, $N^\alpha$-dimethylarginine (5). VIP, a possible neurotransmitter in the corpus cavernosum,
is not considered to be involved in this neurotransmission even if VIP-containing nerves are present in canine corpus cavernosum (13). One week after surgical denervation of bilateral pelvic nerve plexuses running to the penis, relaxations of isolated canine cavernous strips in response to transmural electrical stimulation and NADPH (nicotinamide adenine dinucleotide phosphate) diaphorase-positive nerve fibers in the trabecula of corpus cavernosum were abolished (16, 17). These previous findings of ours suggest that neurogenic relaxations of canine corpus cavernosum are mediated solely by NO synthesized from L-arginine in nerve terminals, and these postganglionic nitrergic nerves participate importantly in the control of penile erection.

In the present study, canine cavernous strips contracted with phenylephrine responded to transmural electrical stimulation with a frequency-related relaxation that was abolished by a NO synthase inhibitor, a soluble guanylate cyclase inhibitor and a Na+ channel inhibitor. These results confirm that NO liberated from the nerves is responsible for the relaxation of corpus cavernosum by elevation of intracellular cyclic GMP through activation of soluble guanylate cyclase (13). Treatment with L-type specific calcium channel inhibitors such as nifedipine and nicardipine did not influence the neurogenic relaxation, whereas amlodipine and cilnidipine, L- plus N-type calcium channel inhibitors, significantly inhibited the relaxation in a concentration-related manner. On the other hand, relaxations induced by NO applied exogenously were not affected by amlodipine and cilnidipine at the same concentrations that inhibited the relaxations caused by electrical stimulation. These results suggest that N-type, but not L-type, calcium channels participate in the process of the synthesis or release of NO from the nerve terminals. Inhibition of the neurogenic relaxation by ω-conotoxin GVIA and failure to obtain an additional inhibition by combined treatment with amlodipine and ω-conotoxin GVIA supports this idea. Activity of constitutive NO synthase such as the endothelial type or neuronal type is dependent on the calcium concentration (18), and endothelium-dependent and neurogenic relaxations mediated by NO are suppressed by removal of calcium or non-specific calcium antagonists (7, 19). Therefore, production of NO is probably stimulated by increasing calcium introduced into nitrergic nerve terminals through N-type calcium channels. Similar finding has been reported in canine cerebral arteries which are mostly innervated with nitrergic nerves as well (9).

Recently, not only L-type specific calcium antagonists but also L- plus N-type calcium antagonists became available for clinical treatment for cardiovascular disorders, especially for hypertension (10). Since peripheral arteries are mainly innervated with noradrenergic vasoconstrictor nerves, suppression of noradrenaline release by inhibition of N-type calcium channel leads to vasodilation and reduction of blood pressure. On the other hand, these compounds may raise the tone of smooth muscles, which are mainly innervated with dilator nerves, such as penile corpus cavernosum. A recent report about quality of life in the hypertensive patients treated chronically with calcium antagonists suggests that the amlodipine-treated group has more sexual symptom distress compare to the nifedipine-treated group (20). However, this does not directly mean that L- plus N-type calcium antagonists have an inhibitory effect on penile erection, because the penile artery supplying blood to the corpus cavernosum is mainly innervated with noradrenergic vasoconstrictor nerves (21).

In summary, calcium responsible for NO synthase activation would be introduced into nerve terminals of nitrergic dilator nerves via N-type calcium channels in canine corpus cavernosum. L- plus N-type calcium channel inhibitors may suppress the neurogenic relaxation of the cavernosum. Further study is required to clarify whether this is the case under the in vivo condition.

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