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ORIGINAL PAPER

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Investigation of the mechanism of nicotine induced relaxation in rabbit corpus cavernosum in vitro

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Abstract In order to determine whether nicotine acts on corporal smooth muscle, the mechanism of its effect on strips of rabbit corpus cavernosum was studied in vitro. Rabbit corpus cavernosum muscle strips were mounted in an organ bath with modified Krebs-Henseleit solution and aerated with 95% O_2 and 5% CO_2. Tension was measured with isometric force transducers, and muscle relaxation was expressed as the percent decrease of precontraction induced by phenylephrine. Nicotine produced concentration dependent relaxation when preparations were precontracted by phenylephrine (10^{-5} M). The maximum nicotine-induced relaxation was $60.4 \pm 4.2\%$ of the phenylephrine contraction and was not affected by indomethacin (10^{-5} M), N^{w}-nitro L-arginine methyl ester (3×10^{-5} M), methylene blue (10^{-5} M), glibenclamide (10^{-5} M), clotrimazole (10^{-6} M), tetraethylammonium (3×10^{-4} M), or 4-aminopyridine (10^{-3} M). Nicotine did not exhibit a calcium antagonizing effect. From these results, we conclude that nicotine-induced relaxation of the rabbit corpus cavernosum is not mediated by the release of nitric oxide, prostaglandins or a related substance, by the activation of potassium channels, or by the stimulation of nicotinic cholinoceptors. Further work is needed to determine the cellular mechanism(s) of the action by which nicotine acts on corporal smooth muscle.

Keywords Nicotine · Corpus cavernosum · Relaxation

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Introduction

The initiation and maintenance of penile erection requires the integration of thoracolumbar and sacral afferent signals, intact autonomic nerves to the penis, normal erectile tissue and adequate blood supply to the penis. The relaxation of corporal smooth muscle allows the expansion of lacunar spaces and the compression of subtunical venules, with entrapment of blood in the lacunar spaces lined with endothelium [5, 13]. Over the last decade, there have been significant advances in our understanding of the physiology of penile erection. The relaxation responses to electric field stimulation that persist after adrenergic and cholinergic blockade are thought to be mediated by the nonandrenergic-noncholinergic (NANC) mechanism [12, 20]; however, endothelium-dependent cholinergic neurotransmission may also mediate penile erection [23]. Many studies have shown that nitric oxide (NO) generated in response to NANC transmission is the main event leading to corporal smooth muscle relaxation, through the activation of soluble guanylyl cyclase [3, 7].

Nicotine has been reported to cause relaxation of the lower esophageal sphincter, duodenum, anococcygeus, and human cerebral artery through the stimulation of nicotinic receptors located on NANC inhibitory nerves. This relaxation is mediated largely by the release of NO or a related substance [8, 13, 19, 21].

The aim of the present study was to investigate the mechanism of nicotine-induced relaxation in rabbit corpus cavernosum smooth muscle using isolated strips from rabbit penis.

Materials and methods

Eight male New Zealand white rabbits weighing 2.5–3 kg were used. Total penectomy was performed just after exsanguination following an intravenous pentobarbital injection (40 mg/kg i.v.). The whole penis was sharply dissected free in Krebs' solution aerated with 95% O_2 and 5% CO_2. The corporal tissues were

carefully dissected free from tunica albuginea and mounted in organ baths containinig Krebs' solution maintained at 37 C. One end of each corporal strip was attached to the bottom of the organ bath and the other was tied to a force transducer (FT 03, Grass Intruments, Quincy, Mass.) connected to a pen polygraph (Grass 79E). Each strip was allowed to equilibrate with a basal tension of 2 g for 1 h. Corporal tissues had an intact endothelium, as assessed by the capacity of acetylcholine (10^{-6} M) to produce relaxation. Corpus cavernosum tissue was prepared and used in organ chamber experiments as described previously [25], with relaxation assessed against a background of precontraction by phenylephrine (10^{-5} M), generating 70–80% of the maximum contraction. At the plateau of contraction, relaxation responses to cumulative concentrations of nicotine (10^{-5}–10^{-3} M) was assessed. Different antagonists were added 30 min before to the tissue bath. N^{w}-nitro L-arginine methyl ester (L-NAME; 3×10^{-5} M), methylene blue (10^{-5} M), indomethacine (10^{-5} M), 4 aminopyridin (10^{-3} M), tetraethyl ammonium (3×10^{-4} M) clotrimazole (10^{-6} M) and glibenclamide (10^{-5} M) were added to the organ bath in different experiments, in order to evaluate the possible mechanism of the action of nicotine. At the end of the experiment, papaverine (10^{-4} M) was added to the organ bath to obtain the maximal relaxation. These doses were chosen based on previous studies, indicating their ability to block nicotine-induced relaxation in precontracted tissues [8, 13, 19, 21]. In most studies, to look at the effects of antagonist drugs, tissue was exposed to the drug for 25–30 min. Therefore, the incubation times for the antagonists were chosen on the basis of the results of other investigators. Three or four antagonists were tested in each preparation. The order of the addition of drugs was randomized, and there was no observable correlation between the order of the drug addition and the measured response; approximately 30–40 min elapsed between the experiments. Buffer was replaced three to four times after each experiment, and the tissue allowed to return to baseline tension. The effects of antagonists or inhibitors on nicotine-induced relaxation were evaluated by comparing the responses before and after the addition of antagonists or inhibitors in the same preparation. To determine whether calcium antagonistic activity plays a role in the relaxation induced by nicotine, the following procedure was applied. Strips were placed in a calcium-free, high K^{+} containing (80 mM) solution. Nicotine (10^{-3} M) was added to the organ bath and 30 min later calcium (2.5 mM) was added and a contraction developed. The same procedures were repeated without nicotine.

Solution and drugs

The ionic composition of the Kreb's solution (in mM) was: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11. In high concentration K^{+} solution, NaCl was exchanged for equimolar amounts of KCl.

The following drugs were all obtained from Sigma (St. Louis, Mo., USA): phenylephrine hydrochloride, nicotine, papaverine hydrochloride, indomethacin, L-NAME, methylene blue, clotrimazole, glibenclamide, tetraethylammonium hydrochloride, and 4-aminopyridine. All drugs were dissolved in distilled water except for indomethacin which was dissolved in 1% Na_2CO_3 and glibenclamide which was dissolved in DMSO. All drugs were freshly prepared on the day of the experiments. No effect with the solvents was observed.

Data analysis

All data are expressed as mean ± SEM; the relaxant effect of agonists was expressed as a percentage of the precontraction to phenylephrine. To evaluate the effect of an agonist, the maximum response (E_{max}), the concentration for a half-maximal response (EC_{50}) and pD2 values were calculated from the concentration-response curve obtained in each experiment, as predicted from the Scatchard equation for drug-receptor interaction: response/concentration = $-1/EC_{50}\times$response + E_{max}/EC_{50}.

The pD2 value was expressed as the negative logarithm of the EC_{50}. Statistical comparisons between groups were performed using general linear models of analysis of variance (ANOVA) followed by Scheffe's F test. The P value computed was one-tailed and $P < 0.05$ was considered to be significant.

Results

In basal tonus, no effect of nicotine was observed. Nicotine (10^{-5}–10^{-3} M) produced concentration-dependent relaxation with a mean pD_2 value of 4.32 ± 0.06 and a mean maximum effect (E_{max}) at 10^{-3} M, of 60.4 ± 4.2% ($n = 8$) of the phenylephrine-induced submaximal contraction At the end of the experiment, papaverine (10^{-4} M) caused a relaxation that was 100% of the submaximal phenylephrine contraction. None of the antagonists investigated had a significant influence on basal tonus (Fig. 1 and Table 1).

The relaxation response to nicotine was observed in 3–5 min after the administration of the drug. The first relaxation occurred at a concentration of 3×10^{-5} M and a sharp response was observed at a dose of 10^{-4} M.

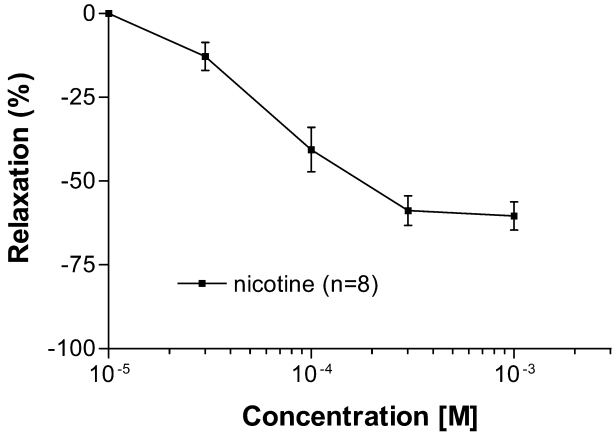

Fig. 1 Nicotine concentration-response curve in isolated rabbit corpus cavernosum strips precontracted with phenylephrine 10^{-5} M. Each point is expressed as a percentage of the contraction induced by phenylephrine and is given as the mean ± SEM from eight experiments

Table 1 E_{max} (percent of 10^{-5} M phenylephrine) and pD_2 values (−log EC_{50}) for nicotine in the absence (control) or presence of antagonists or inhibitors in rabbit corpus cavernosum strips

	E_{max}	pD_2	n
Control (10^{-5}–10^{-3} M)	60.4 ± 4.2	4.32 ± 0.06	8
L-NAME (3×10^{-5} M)	58.2 ± 3.9	4.26 ± 0.09	8
Methylene blue (10^{-5} M)	61.2 ± 2.8	4.26 ± 0.06	8
Indomethacine (10^{-5} M)	58.8 ± 4.1	4.30 ± 0.05	8
Glibenclamide (10^{-5} M)	56.8 ± 4.4	4.30 ± 0.08	8
Clotrimazole (10^{-6} M)	57.1 ± 5.2	4.27 ± 0.06	8
4-Aminopyridine (10^{-3} M)	55.7 ± 3.9	4.32 ± 0.05	8
Tetraethylammonium (3×10^{-4} M)	56.8 ± 5.0	4.36 ± 0.08	8

After washing out the nicotine, the loss was reversible in 10–15 min (Fig. 2).

NO synthase inhibitor L-NAME and NO related substance inhibitor methylene blue were added to the tissue bath to inhibit the NO release and NO pathway. The concentration-response curve of nicotine in L-NAME and methylene blue pretreated corpus cavernosum showed no significant difference. The E_{max} values of nicotine treated strips (control) were not changed by adding L-NAME or methylene blue. The pD_2 value of the control strips was not different from the L-NAME and methylene blue treated strips (Table 1).

Indomethacin was added to the tissue bath to inhibit the cyclooxygenase pathway. It did not cause any significant change in the concentration-response curve of nicotine. The E_{max} values of nicotine treated strips (control) were not changed by adding indomethacin. The pD_2 value of the control strips was not different from the indomethacin treated strips (Table 1).

Tetraethylammonium and clotrimazole (Ca^{+2}-activated K^+ channel blockers), 4-aminopiridine (voltage dependent K^+ channel blocker), and glibenclamide (ATP sensitive K^+channel blocker) were added to the tissue bath in a different experiment. They did not affect the concentration-response curve of nicotine and there were no significant differences from nicotine treated strips. There was no significant difference in pD_2 values between control and K^+ channel blocker treated strips (Table 1).

The contraction induced by the addition of calcium was not changed by nicotine.

Discussion

In this study, we demonstrated for the first time that nicotine causes concentration-dependent relaxation in isolated rabbit corpus cavernosum precontracted by phenylephrine. In contrast to the vasodilatation effect, the corpus cavernosum smooth muscle relaxation effect of nicotine was not related to cyclooxygenase products and NO which is synthesized and released either from postganglionic parasympathetic nerves or from the endothelium of the corpus cavernosum. To investigate whether relaxation induced by nicotine was due to an interaction with the cyclooxygenase, nitric oxide pathways or NO release, tissues were pretreated with indomethacin, methylene blue and L-NAME, respectively. The treatment of cavernosal tissue strips with these inhibitors did not significantly alter the relaxant activity of nicotine. These findings indicate that the relaxant action of nicotine on the rabbit corpus cavernosum is not mediated by the release of NO or a related substance from the L-arginine/NO pathway. However, in bovine penile retractory muscle, the relaxation induced by nicotine is inhibited by methylene blue [11]. In our study, methylene blue did not inhibit the nicotine-induced relaxation. Therefore, any role for the guanylate cyclase/cGMP pathway can be excluded. The relaxation induced by nicotine was not impaired by tetraethylammonium, clotrimazole, 4-aminopiridine or glibenclamide. K^+ channels openers such as nicorandil, pinacidil, and cromakalim relax various types of smooth muscle cells, opening the rubidium permeable K^+ channels leading to hyperpolarization of the cells. To investigate whether relaxation induced by nicotine involved the Ca^{+2}-activated, voltage dependent, ATP sensitive K^+ channel blockers, the effects of tetraethylammonium, clotrimazole, 4-aminopiridine and glibenclamide were investigate. Neither tetraethylammonium, clotrimazole nor 4-aminopiridine, glibenclamide affected the relaxant effects or the pD_2 or the E_{max} values of the nicotine. These results indicate that K^+ channel blockers do not play an important role in the relaxation responses to nicotine. In this study, nicotine did not exhibit any Ca^{2+} antagonizing effect. The addition of nicotine previous to the administration of high doses of K^+ and Ca^{2+} did not alter the contractile effect of calcium. However, a very well-known Ca^{2+} antagonist, verapamil, relaxed the corpus cavernosum contracted by Ca^{2+} [13].

NO is widely accepted as playing an important role in the relaxation of corporal smooth muscle and of the vasculature. The endothelium and/or the nerves innervating the corpus cavernosum may be the source of NO and probably more than one isoform of NOS may be involved. It has been shown that NO is present in the

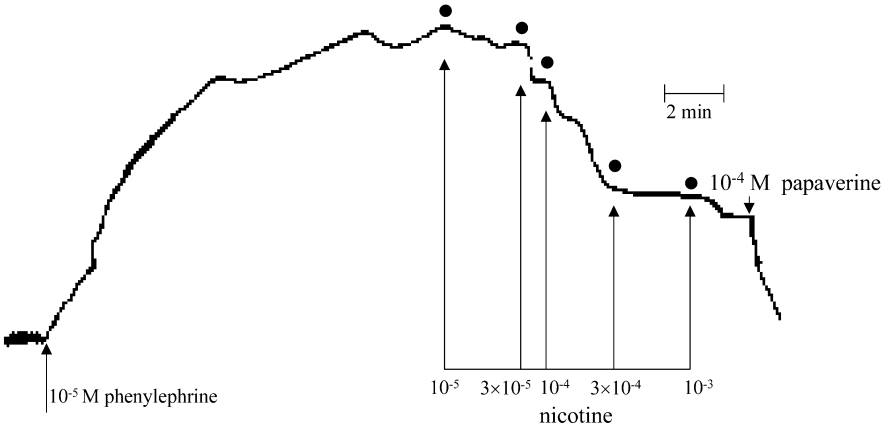

Fig. 2 Original tracing showing the effect of nicotine (10^{-5}–10^{-3} M) om rabbit corpus cavernosum smooth muscle

cavernosal nerves and their terminal endings in the corpora cavernosa, and in the branches of the dorsal penile nerves and neural plexus in the adventitia of the deep cavernosal arteries [1, 4]. PGE_1 effectively relaxed human trabecular tissue and cavernosal artery segments contracted by noradrenaline and $PGF_{2\alpha}$. PGE_1 inhibits the release of noradrenaline from penile adrenergic nerves and increases intracellular cAMP levels in corporal smooth muscle [16]. Purinergic neurotransmitter agonists have a potent relaxant activity on the corpus cavernosum, acting through a mechanism different from the NO pathway [15, 22]. Adenosine increases cyclic adenosine monophosphate levels by activating adenylate cyclase in smooth muscle cells [9]. Recently, it was shown that the relaxation of various smooth muscles is mediated by the activation of different types of potassium channels [17]. The ATP sensitive K^+ channel subtype is likely to play an important role in the relaxation of isolated corporeal tissue strips and, in addition, these are the molecular target for the K^+ channel modulators/openers levcromakalim and pinacidil. ATP-sensitive potassium channels in human penile arteries and corporal smooth muscle have been demonstrated [18, 24].

Previous studies have shown that peptides and ATP are the two other candidates, as NO is the main inhibitory neurotransmitter in corporal smooth muscle, which does not necessarily mean that it is released from NANC nerves [2, 6]. As neither peptides (VIP, adrenomedullin) nor ATP antagonists were used in the present study, we cannot draw any conclusions on their involvement.

In conclusion, nicotine produced relaxation of corpus cavernosum strips from the rabbits and this relaxation was not mediated by the release of NO, prostaglandins, or any related substances, by the activation of potassium channels, or by the stimulation of nicotinic cholinoceptors. Further experiments are required to establish whether peptides or ATP contributes to nicotine induced relaxation.

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