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SPECIAL REPORT

Nitrergic neurotransmission mediates the non-adrenergic non-cholinergic responses in the clitoral corpus cavernosum of the rabbit

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The corpus cavernosum is the erectile tissue in the penis and clitoris. Although nitrergic neurotransmission has been characterized in detail in the penile corpus cavernosum, functional studies on the inhibitory non-adrenergic non-cholinergic (NANC) transmission in the clitoral corpus cavernosum have been lacking. Here we demonstrate that electrical field stimulation (EFS) induces NANC relaxation responses in the clitoral corpus cavernosum of the rabbit. These responses were inhibited by N^G-nitro-L-arginine methylester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) or tetrodotoxin. The inhibitory effect of L-NAME was partially reversed by L-arginine but not by D-arginine. EFS-induced relaxations were enhanced by an inhibitor of type V cyclic GMP phosphodiesterase, zaprinast. These results suggest that nitrergic neurotransmission is responsible for the NANC relaxation responses in the clitoral corpus cavernosum of the rabbit.

Keywords: Nitrergic; nitric oxide; clitoris; corpus cavernosum; rabbit; non-adrenergic non-cholinergic

Abbreviations: EFS, electrical field stimulation; L-NAME, N^G-nitro-L-arginine methylester; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; TTX, tetrodotoxin

Introduction Nitric oxide (NO) has been well characterized as a mediator of inhibitory non-adrenergic non-cholinergic (NANC) neurotransmission in many organs including the urogenital system (for review see Rand & Li, 1995). The term 'nitrergic' has now been accepted as applying to nerves whose transmitter function depends on the release of NO or to transmission mechanisms that are brought about by NO (Moncada *et al*., 1997).

The corpus cavernosum is the erectile tissue of penis and clitoris. Relaxation of cavernosal smooth muscle leads to engorgement of blood in the corpus cavernosum which results in an increase of intracavernosal pressure and thus in tumescence. Nitrergic neurotransmission is known to be the major neuronal pathway responsible for the relaxation of the cavernosal smooth muscle in the penis (Burnett *et al*., 1992; for review see Andersson & Wagner, 1995); however, the relaxant neuronal pathway in the clitoris has not been characterized.

Nitrergic nerves have been shown by immunohistochemistry to be present in the human clitoral corpus cavernosum (Burnett *et al*., 1997). We have therefore investigated inhibitory NANC responses in the clitoral corpus cavernosum of the rabbit and have found nitrergic neurotransmission to be responsible for the NANC relaxation of this tissue.

Methods Female New Zealand white rabbits (3.5–4.0 kg, Harlan, U.K.) were sacrificed by an overdose of pentobarbitone (Expiral, Sanofi Animal Health Ltd, U.K.) injected into the ear marginal vein after local anaesthesia (Xylocaine Gel 2%, Astra Pharmaceuticals, U.K.). The vaginal canal including the clitoris was excised down to the pubic bone and transferred to modified Krebs' solution at room temperature. The modified Krebs' solution consisted of (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, glucose 11.5, dexamethasone (10 μM), indomethacin (5 μM) and was gassed with 5% CO_2 in O_2 (pH 7.4–7.6). The tissue was pinned onto a silicone base (Slygard, BDH, U.K.) using 0.0089 inch diameter stainless steel pins (Watkins & Doncaster, U.K.). The vaginal canal was carefully opened from the caudal side and was removed to allow access to the clitoral body. The tunica albuginea around the corpus cavernosum was cut open. Four pieces ($0.5-1\times3-4$ mm) of cavernous tissue from the proximal end of the clitoral body were isolated using fine scissors. The preparations were tied at either end using silk sutures and were mounted horizontally between the electrodes in superfusion chambers heated with circulating water at 37°C, as described previously for penile corpus cavernosum (Cellek & Moncada, 1997). The tissues were superfused at a constant flow of 1 ml min^{-1} by means of a peristaltic pump (Minipuls 2, Gilson, U.K.). One end of the preparation was tied to a Grass FT03C force-displacement transducer connected to a Linearcorder WR3101 (Graphtec, U.K.) for registration of isometric changes in tension. The mechanical responses were also recorded on a computer running specialized software (Axotape, U.S.A.) for measurement of the area under the curve of relaxation. The preparations were stretched (0.2–0.5 g) until they reached approximately the *in situ* length and allowed to equilibrate for 90 min. Scopolamine (10 μM) and guanethidine (10 μM), to inhibit cholinergic and noradrenergic pathways respectively, were added to the medium reservoir and the tissues were allowed to incubate with these inhibitors for another 30 min. The preparations were stimulated electrically for 5 s with trains of rectangular pulses of 50 V, 0.3 ms pulse duration and frequencies ranging from 1–25 Hz, delivered by Grass S88 stimulators every 120 s. The tone of the tissue was elevated with phenylephrine (1 μM, EC_{80}).

Chemicals

1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, Novo Nordisk, Denmark) was dissolved in dimethylsulfoxide (10 mM) and diluted in isotonic saline. Dexamethasone and zaprinast (Sigma, U.K.) were dissolved in 100% ethanol and

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indomethacin (Sigma, U.K.) was dissolved in 5% $NaHCO_3$. All other chemicals (Sigma, U.K.) were dissolved and diluted in isotonic saline.

Statistics

Results are expressed as means ± s.e.mean of relaxation measured as area under the curve. Statistical analysis was performed using Student's *t*-test for paired samples. A probability value (*P*) less than 0.05 was taken as significant. Results were presented as a point plot where each point represents the mean of the experiments in that series. Vertical bars represent the standard error of the mean. *n* denotes the number of animals used in that series of experiments.

Results EFS of the phenylephrine-contracted clitoral corpus cavernosum resulted in reproducible relaxation responses (Figure 1). These responses were frequency-dependent. The optimum frequency for this tissue was found to be 5 Hz (not shown). Addition of N^G-nitro-L-arginine methylester (L-NAME, 1–300 μM) to the medium caused a concentration-dependent inhibition of the responses (Figures 1 and 2). The IC_{50} for L-NAME was calculated as 23.8 ± 0.9 μM ($n=8$). Addition of 1 mM L-arginine to the medium after total inhibition of the responses restored 49.7 ± 5.4% of the response (Figure 1, $n=8$). Addition of D-arginine (1 mM) did not cause any reversal (not shown). Further addition of tetrodotoxin (1 μM) elicited complete inhibition of the responses (Figure 1).

In another set of experiments the effect of ODQ, an inhibitor of soluble guanylate cyclase (Garthwaite *et al*., 1995; Cellek *et al*., 1996), was studied on EFS-induced relaxation of the tissue. ODQ (1 nM–3 μM) inhibited the responses in a concentration-dependent manner (Figure 2) with an IC_{50} of 165 ± 10 nM ($n=8$).

Zaprinast (0.3–10 μM) enhanced the magnitude and duration of EFS-induced relaxations in a concentration-dependent manner (Figure 3, $n=4$). Concentrations of zaprinast above 10 μM were not evaluated in this study as they caused significant loss of tone in the tissue (not shown).

Discussion The human clitoris plays a functional role during sexual arousal (Gillan & Brindley, 1979; Levin, 1980, 1992). Sexual excitement causes engorgement of blood in the corpus cavernosum of the clitoris (Levin, 1980, 1992; Toesca *et al*., 1996), and physical stimulation of the tumescent clitoris provokes the contraction of pelvic floor muscles which triggers the female orgasm (Gillan & Brindley, 1979). Immunohistochemical study of the clitoral corpus cavernosum has revealed it to be very similar to

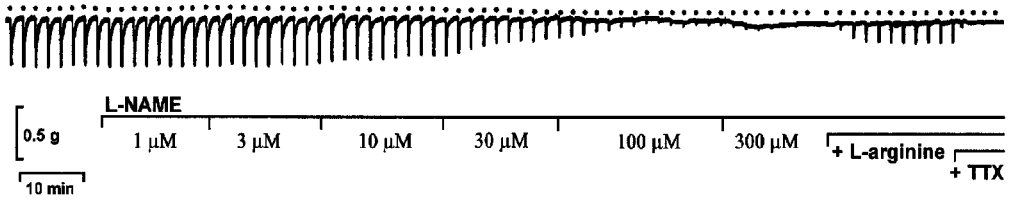

Figure 1 Response of the rabbit clitoral corpus cavernosum elicited by EFS (50 V, 0.3 ms pulse duration, 5 Hz, for 5 s, every 2 min, indicated by dots) in the absence and presence of L-NAME. Increasing concentrations of L-NAME (1–300 μM) caused progressive inhibition of EFS-induced relaxation, an effect that was reversed by addition of 1 mM L-arginine. Addition of tetrodotoxin (TTX, 1 μM) completely abolished the response to EFS. The mechanogram is the original recording of the responses of one preparation and is representative for all experiments in this series ($n=8$).

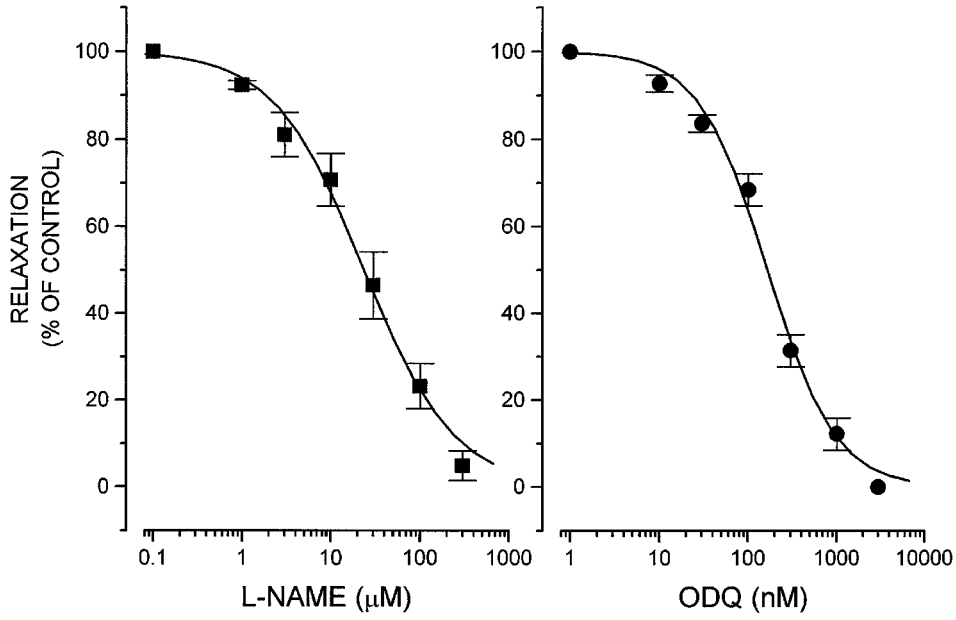

Figure 2 Effect of L-NAME (0.1–300 μM) and ODQ (1 nM–3 μM) on the relaxation elicited by EFS (50 V, 0.3 ms pulse duration, 5 Hz, for 5 s) in the rabbit clitoral corpus cavernosum ($n=8$).

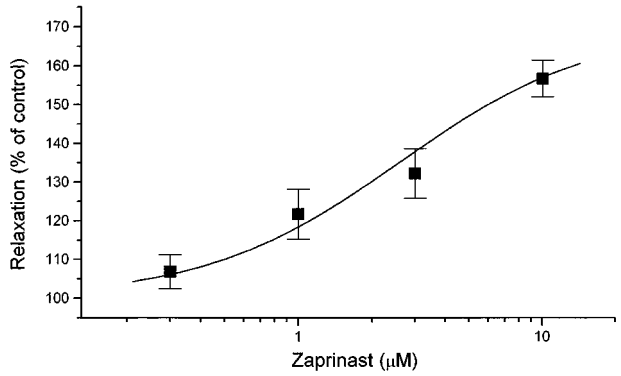

Figure 3 Effect of zaprinast (0.3–10 μM) on the relaxation elicited by EFS (50 V, 0.3 ms pulse duration, 2.5 Hz, for 5 s) in the rabbit clitoral corpus cavernosum ($n=4$).

its embryological homologue, the penile corpus cavernosum, except that the subalbugineal layer, which contains a rich venous plexus between the corpus cavernosum and the tunica albuginea, does not exist in the clitoris (Toesca *et al*., 1996). This may account for the lack of rigidity in the tumescent clitoris.

The neuronal mechanisms involved in relaxation of the clitoral corpus cavernosum have not been studied in detail. The anatomical, morphological and functional similarities between the penis and clitoris suggest that nitrergic neurotransmission may also be operating as the major erectile neuronal pathway in the clitoris. This hypothesis has been supported by Burnett *et al*. (1997) who showed that nitrergic nerves do exist in the human clitoral corpus cavernosum. However, functional studies to characterize the erectile neuronal pathways in this tissue have not been carried out.

In the present study we have shown that EFS elicits NANC relaxation responses in the clitoral corpus cavernosum of the rabbit. These responses are nitrergic in nature since they can be blocked with an inhibitor of the NO synthase, L-NAME, and this inhibition can be reversed with the precursor of NO, L-arginine. This reversal is enantiomer-specific since D-arginine is without effect. We have also shown that the relaxation responses are neuronal in origin since they were blocked by tetrodotoxin.

EFS-induced relaxation responses were also blocked by ODQ, confirming that the NO-cyclic GMP pathway underlies these responses and suggesting that the target enzyme for NO in the effector smooth muscle of this tissue is the soluble guanylate cyclase.

Although the number of studies investigating the pathophysiology of female sexual dysfunction is significantly lower than those of male erectile dysfunction (Levin, 1992), the studies that do exist suggest that a deficiency in clitoral tumescence may be important in female sexual dysfunction (Levin, 1980, Park *et al*., 1997). Sildenafil, an inhibitor of type V cyclic GMP phosphodiesterase, has been shown to be effective in the treatment of male erectile dysfunction (Boolell *et al*., 1996). Here we have shown that zaprinast, another inhibitor of the same enzyme, enhanced nitrergic relaxations in the clitoral corpus cavernosum of the rabbit. If it is true *in vivo*, the current use of selective inhibitors of type V cyclic GMP phosphodiesterase in the treatment of male erectile dysfunction may be extended to female sexual dysfunction.

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