Characterisation of nitrergic transmission in the isolated anococcygeus muscle of the female mouse

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Abstract

Field stimulation of anococcygeus muscles from female mice produced frequency-dependent relaxations of carbachol-induced tone, which were independent of the oestrus cycle but were abolished by the nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine (L-NOARG; 100 μM) and the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; 5 μM); L-NOARG inhibition was reversed by L-, but not D-arginine. The selective phosphodiesterase V inhibitor zaprinast (1–130 μM) directly relaxed tone and enhanced both the amplitude and duration of field stimulation-induced relaxations; the effect on amplitude was greater at lower frequencies of stimulation, while increased duration dominated at higher frequencies. The duration, but not the amplitude, of relaxations to exogenous nitric oxide (NO; 15 μM) was also increased by zaprinast. The mouse anococcygeus provides a useful model for pharmacological investigation of nitrergic neurotransmission in female urogenital smooth muscle. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Anococcygeus muscle, female mouse; Nitrergic neurotransmission; Nitroarginine; ODQ; Urogenital smooth muscle, female mouse; Zaprinast

1. Introduction

There is now clear and convincing evidence that the non-adrenergic, non-cholinergic (NANC) relaxations observed in several organs of the male urogenital tract (including the corpus cavernosum, retractor penis, anococcygeus and penile artery) are mediated by so-called nitrergic nerves, and that nitric oxide (NO) is intimately involved in the process of penile erection (for reviews, see Rand and Li, 1995a,b). The therapeutic importance of this nervous system has been highlighted by the recent introduction of the selective phosphodiesterase V inhibitor sildenafil as an effective treatment for male impotence (Ballard et al., 1998; Derry et al., 1998; Moreland et al., 1998). While the innervation of the male urogenital tract has been widely investigated, much less attention has been paid to that of females. However, there has been growing interest in possible therapeutic applications of phosphodiesterase inhibitors in female sexual dysfunction and, recently, both histological and functional evidence has been presented that relaxations of the clitoral corpus cavernosum in humans and rabbits may be nitrergic (Burnett et al., 1997; Cellek and Moncada, 1998). Such research would be aided greatly by the availability of a reliable model of nitrergic transmission in the female urogenital system; a model in the female mouse would be particularly useful given the possibilities of modifying gene function in this species. The anococcygeus muscle is found in female mice and, as in the rat (Gibson and Gillespie, 1973), muscles from female animals are much smaller than those from males (Fukazawa et al., 1997). In addition, the female mouse anococcygeus is hormone-sensitive, the cross-sectional area being increased by testosterone treatment and reduced by oestrogens (Fukazawa et al., 1997). While it is known that anococcygeus muscles from female rats display NANC relaxations (Gibson and Gillespie, 1973), it has not yet been demonstrated that the same is true in anococcygeus muscles from female mice and, if it is, whether these relaxations are nitrergic. The object of the present study, therefore, was to investigate the nitrergic nature of NANC relaxations in the female mouse anococcygeus and, in particular, to examine the effects of zaprinast, a selective inhibitor of phosphodiesterase V.

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2. Materials and methods

2.1. Tension studies

Female mice (LACA, 25–35 g; Tuck, Essex, UK) were killed by stunning and exsanguination. The two anococcygeus muscles (wet weight of single muscles, 0.73 ± 0.05 mg, $n = 8$) were dissected out separately (Fukazawa et al., 1997) and set up in 2 ml glass organ baths containing Krebs-bicarbonate buffer (composition, mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) which was maintained at 37°C and gassed with 95% O_2:5% CO_2. A resting tension of 1.96–3.92 mN was placed on the tissue and changes in tension recorded with a Biegestab K30 force–displacement transducer attached to a pen-recorder (Graphtec WR3101). Muscles were allowed to equilibrate for 30 min before beginning experimental procedures. Field stimulation was applied by two parallel platinum electrodes (6 mm apart) running down either side of the tissue. These were attached to square wave pulse generators (Grass S48; 1 ms pulse width; 70 V). Sympathetic function was inhibited by preincubation of each tissue with 30 μM guanethidine for 10 min during the equilibration period; in addition, the Krebs solution contained phentolamine (1 μM).

Muscle tone was raised with carbachol (50 μM), and field stimulation applied when a stable elevation of tone had been achieved (usually within 3 min of adding carbachol to the bath). Carbachol was used to raise tone because, as in the male, muscarinic receptors in the female mouse anococcygeus are motor; in preliminary experiments, tone raised with thapsigargin (100 nM) which activates capacitative calcium entry in the anococcygeus (Gibson et al., 1998) and field stimulation-induced (10 Hz) relaxations were found to be unaffected by atropine (500 nM), confirming the lack of cholinergic contribution. In some experiments, relaxations to authentic NO and to vasoactive intestinal peptide (VIP) were determined and, again, the relaxant drugs were added when carbachol had produced a steady rise in tone; NO solutions were prepared as described previously (Gibson and Mirzazadeh, 1989). Relaxation amplitude was calculated as the peak percentage reduction in carbachol-induced tone compared with the level immediately before each train of stimulation, or addition of either NO or VIP to the bath. The duration of relaxation was determined as the time interval between the onset of relaxation to nerve stimulation, NO or VIP and recovery of tone to 50% of the pre-stimulation level (following cessation of field stimulation, degradation of the NO, or washout of VIP in the continuing presence of carbachol). The contact time for the nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine (L-NOARG), L-NOARG with L-arginine, L-NOARG with D-arginine, and the soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxodiazolo[4,3-*a*]quinoxalin-1-one (ODQ; Garthwaite et al., 1995) was 15 min in each case.

2.2. Determination of stage of oestrus

A vaginal smear was obtained by squeezing a small amount of Krebs solution in and out of the vagina three times via a pasteur pipette and then spreading the solution onto a glass microscope slide. The stage of oestrus was determined by viewing the types of cells predominating in the smear under a microscope: pre-oestrus, mainly epithelial cells; oestrus, cornified cells only; post-oestrus, leukocytes and cornified cells (Short and Woodnott, 1963).

2.3. Statistics

Results are expressed as mean $\pm$ SEM and statistical significance determined by Students' *t*-test ($P < 0.05$ taken as significant). *n* represents the number of muscle strips studied.

2.4. Drugs used

All drugs used were obtained from Sigma (UK) except carbachol (BDH), NO (BDH) and ODQ (Tocris). All drugs
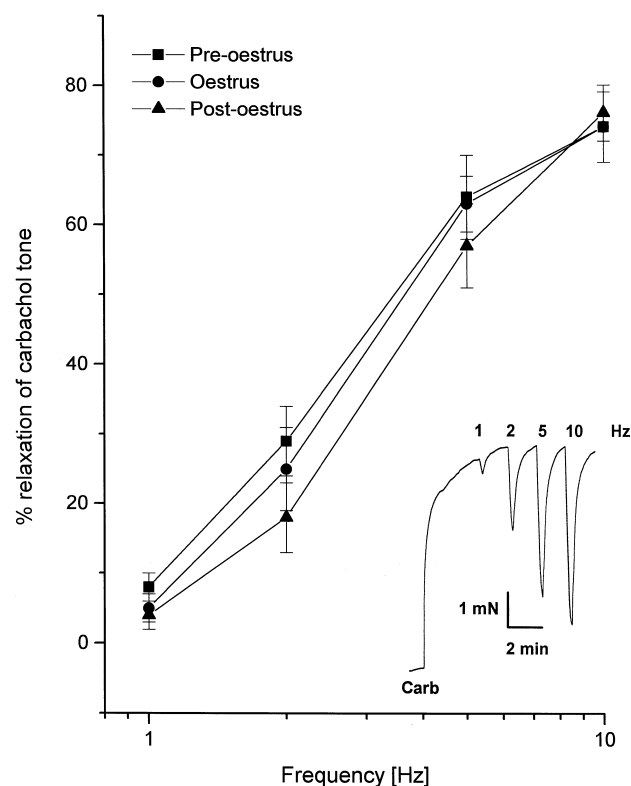

Fig. 1. Frequency–response curves for NANC (10 s train; 100 s interval) relaxations of anococcygeus muscles taken from female mice at different stages of the oestrus cycle. Each point is the mean $\pm$ SEM from five to eight individual muscle preparations. Muscle tone was raised with 50 μM carbachol (Carb). Inset: trace showing a typical record from which the data for the frequency–response curves were obtained. Note that in this figure the relaxations were dependent on both increasing frequency and on increasing number of pulses per train; in subsequent experiments a fixed number of pulses was used.
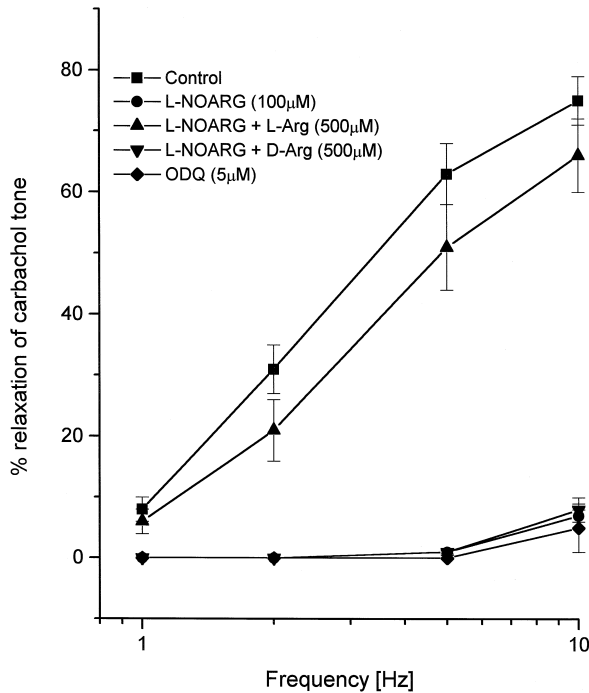
Fig. 2. The effect of the NOS inhibitor L-NOARG, alone or in combination with L-arginine or D-arginine, and the soluble guanylate cyclase inhibitor ODQ on the frequency–response curve for NANC (10 s train; 100 s interval) relaxations of the female mouse anococcygeus. Each point is the mean ± SEM from five to eight individual muscle preparations. Muscle tone was raised with 50 μM carbachol.

were dissolved in distilled water except zaprinast and ODQ which were dissolved in dimethylsulphoxide (DMSO) to give stock concentrations of 10 mM.

3. Results

Field stimulation (1, 2, 5 10 Hz; 10 s train; 100 s interval) of anococcygeus muscles from female mice produced frequency-dependent relaxations of carbachol-induced tone (Fig. 1). At least four consecutive frequency–response curves, each separated by a 15-min interval (during which the carbachol was washed from the bath) could be obtained without significant change in either contractile response to carbachol or nerve-induced relaxation (data not shown). The stage of oestrus of the mouse from which the anococcygeus muscle was removed had no effect on the contractile response to carbachol (pre-oestrus 3.97 ± 0.44 mN, $n = 14$; oestrus 3.86 ± 0.72 mN, $n = 5$; post-oestrus 5.27 ± 0.46 mN, $n = 7$) or on the frequency–response curve to NANC stimulation (Fig. 1). In subsequent experiments, therefore, the stage of oestrus was not determined before the anococcygeus was removed for investigation.

The nitrergic nature of the NANC relaxations was investigated using the NOS inhibitor L-NOARG and the soluble guanylyl cyclase inhibitor ODQ. NANC relaxations were almost abolished in the presence of 100 μM L-NOARG, with only a small residual response remaining at the highest frequency used (10 Hz). This inhibition was totally reversed when 500 μM L-arginine was added to the L-NOARG, while D-arginine was ineffective under the same conditions (Fig. 2). Like L-NOARG, ODQ (5 μM) completely inhibited the NANC relaxations. The above results demonstrate that NANC relaxations of the female mouse anococcygeus are indeed nitrergic.

We next compared the effects of the selective phosphodiesterase V inhibitor zaprinast with those of the non-
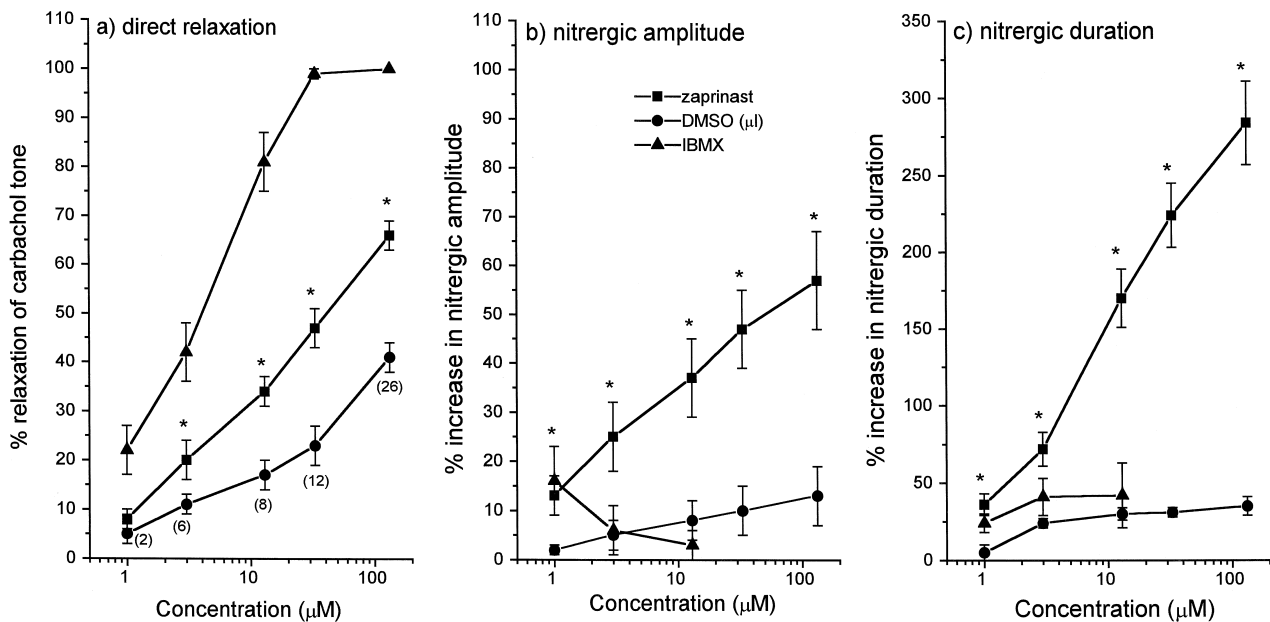

Fig. 3. Concentration–response curves for the effect of zaprinast, its vehicle DMSO, and IBMX on carbachol-induced tone, and on the amplitude and duration of nitrergic relaxations of the female mouse anococcygeus muscle in response to a fixed frequency of field stimulation (4 Hz, 10 s train, 100 s interval). Each point is the mean ± SEM from five to eight individual muscle preparations. Muscle tone was raised with 50 μM carbachol. * Value for zaprinast significantly different for corresponding DMSO control (volumes of DMSO given in parenthesis).
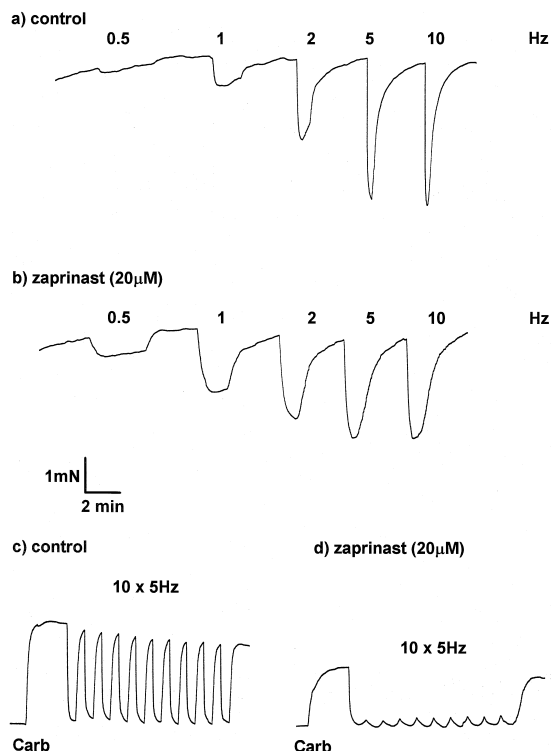
Fig. 4. Traces showing the effect of 20 μM zaprinast on nitrergic relaxations of the female mouse anococcygeus muscle to various patterns of field stimulation. (a) and (b) show the frequency response curve to 100 pulses at 0.5, 1, 2, 5, and 10 Hz (100 s interval) before (a) and in the presence (b) of zaprinast. (c) and (d) show the relaxant responses to repeated bursts of field stimulation (5 Hz for 30 s with 30 s interval) before (c) and in the presence (d) of zaprinast. Muscle tone was raised with 50 μM carbachol (Carb).

selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). In these experiments, nitrergic relaxations of carbachol-induced tone were obtained using consecutive trains of field stimulation at a fixed frequency (4 Hz; 10 s trains; 100 s interval). Increasing concentrations of either zaprinast or IBMX were added after each fourth train of stimulation. IBMX (1–130 μM) caused a marked direct relaxation of carbachol-induced tone, but had little effect on either amplitude or duration of nitrergic relaxations (Fig. 3); because of the almost complete loss of induced tone, measurement of the effects of the higher concentrations of IBMX on amplitude and duration of nitrergic relaxations was not possible. Zaprinast (1–130 μM) also produced direct relaxation of tone, but this was much less than that observed with IBMX (Fig. 3). In addition, zaprinast increased the amplitude of nitrergic relaxations and markedly enhanced the duration of the nitrergic response (Fig. 3).

Having established the concentration–effect relationship for zaprinast using a fixed frequency, we then observed the effects of 20 μM zaprinast on the frequency–response curve to trains of stimulation using a fixed number of pulses (100 pulses at 0.5, 1, 2, 5, 10 Hz). The volume of

DMSO added to the bath was kept low (2 μl) since higher volumes of the solvent were found to produce significant effects, especially on carbachol-induced tone (Fig. 3); using this volume, the direct relaxant effect of 20 μM zaprinast was 11 ± 3% ($n = 14$). As can be seen in Figs. 4 and 5, zaprinast increased the amplitude of nitrergic relaxations at each frequency; the duration of nitrergic relaxation was also increased, only slightly at 0.5 and 1 Hz but much more so at 2, 5 and 10 Hz.

Since it has been suggested that parasympathetic fibres may fire in repetitive bursts, rather than continuously, under physiological conditions (Tobin et al., 1990), we determined the effect of zaprinast on bursts of nitrergic stimulations (5 Hz for 30 s every 30 s). In control tissues, this pattern of stimulation produced discrete peaks of relaxation followed by complete recovery of induced tone between each train (Fig. 4c); in the presence of 20 μM zaprinast the individual relaxations became fused, with only small undulations of peak relaxation (Fig. 4d). The area of relaxation, measured over a period of 10 trains of
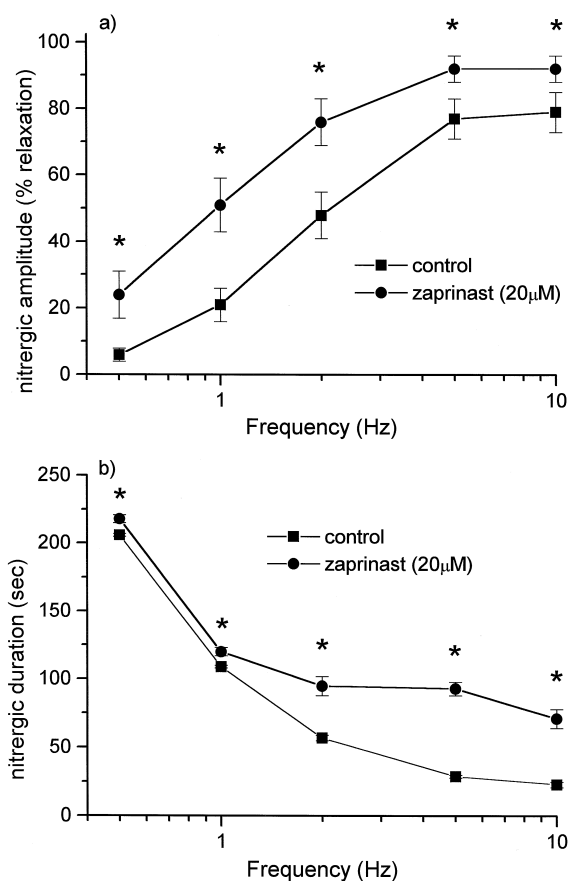

Fig. 5. Frequency–response curves showing the effect of 20 μM zaprinast on the amplitude (a) and duration (b) of nitrergic relaxations of the female mouse anococcygeus to 100 pulses of field stimulation at 0.5, 1, 2, 5, and 10 Hz. Each point is the mean ± SEM from five to eight individual muscle preparations. Muscle tone was raised with 50 μM carbachol. * Value for zaprinast significantly different from corresponding control.

stimulation, was increased by $97 \pm 7\%$ ($n = 5$) in the presence of zaprinast.

Finally, the interactions of zaprinast with authentic NO and VIP were determined. 15 μM NO relaxed carbachol-induced tone by $44 \pm 7\%$, with a response duration of 42 ± 2 s. In the presence of 20 μM zaprinast, the relaxation amplitude was unchanged ($45 \pm 5\%$) but the duration was clearly enhanced (104 ± 13 s, $P < 0.05$, $n = 8$ in each case). 20 nM VIP produced relaxations of $32 \pm 6\%$ with a duration of 177 ± 8 s ($n = 7$); neither the amplitude ($40 \pm 8\%$) nor the duration (170 ± 8 s; $n = 7$) of relaxations to VIP were significantly altered in the presence of 20 μM zaprinast.

4. Discussion

The anococcygeus muscle in male rodents is part of the erectile machinery and is closely associated with the retractor penis (Gillespie, 1997); both tissues have played important roles in identifying the vital contribution of nitrergic nerves to penile erection (Rand and Li, 1995a,b). The anococcygeus muscle is also found in female rats and mice (Gibson and Gillespie, 1973; Fukazawa et al., 1997) although, as might be expected, the muscle is smaller than that in male animals. The main object of the present study was to determine whether the female mouse anococcygeus displayed NANC relaxations and, if so, whether these were nitrergic. Clearly this was the case. Field stimulation produced frequency-dependent relaxations which were virtually abolished by the NOS inhibitor L-NOARG, and this blocking effect of L-NOARG was reversed in the presence of L-arginine, but not D-arginine. Such results have become the standard fingerprint for the identification of nitrergic transmission (Rand and Li, 1995a,b). In anococcygeus muscles from male animals, nitrergic relaxations are mediated via activation of guanylyl cyclase in the smooth muscle cell (Mirzazadeh et al., 1991; Cellek et al., 1996; Fonseca et al., 1998). It seems the same is true in the female mouse anococcygeus since nitrergic relaxations were abolished by the soluble guanylyl cyclase inhibitor ODQ (Garthwaite et al., 1995).

Previous studies have shown that the anococcygeus muscle is sensitive to sex hormones. The contractility of the male rat anococcygeus is increased following administration of testosterone (Gibson, 1977) and the cross-sectional area of the female mouse anococcygeus is increased by testosterone and reduced by oestrogen (Fukazawa et al., 1997). In the present study, however, nitrergic relaxations of the female mouse anococcygeus were similar in muscles taken from mice at different stages of the oestrus cycle. This suggests that the physiological changes in sex hormones which occur during the oestrus cycle are not sufficient to influence nitrergic potency, although it cannot be ruled out that administration of large doses of hormones to animals may have an effect. Nevertheless, the stability of nitrergic responses throughout the oestrus cycle enhances the potential usefulness of the female mouse anococcygeus for pharmacological investigation of nitrergic function.

One group of drugs which interacts with the nitrergic system and which is the subject of great current interest is the phosphodiesterase V inhibitors, especially in terms of their effects on urogenital smooth muscle (Ballard et al., 1998; Moreland et al., 1998; Derry et al., 1998). Zaprinast is one of the original drugs of this type and has been widely investigated. However, its effects on nitrergic neurotransmission have varied depending on the experimental conditions and tissues used, having little or no effect on the amplitude of nitrergic relaxations in some circumstances (Barbier and Lefebvre, 1992; McMahon et al., 1993; Fernandes et al., 1994) but producing a marked potentiation in others (Ahlner et al., 1991; Liu et al., 1992; Barbier and Lefebvre, 1995; Ellis and Conanan, 1995). The present results show that zaprinast produces a clear potentiation of nitrergic relaxations in the female mouse anococcygeus, measured not only as an increase in the amplitude of the relaxant response but also in its duration, as identified in other tissues (McMahon et al., 1993; Lefebvre et al., 1995). In the case of relaxations to authentic NO, zaprinast produced a marked increase in duration while having no effect on amplitude of response. It seems that the relative effect of zaprinast on amplitude and duration of the nitrergic response may vary with the rate at which the NO is delivered to the smooth muscle. Thus, at a frequency of 0.5 Hz the ratio of the percentage increases in amplitude compared with duration was 300:6, while at 5 Hz this was reversed to 19:225; increased amplitude appears to be the main effect at low frequencies of stimulation, whereas increased duration predominates at higher frequencies. When the nitrergic nerves were stimulated in repeated bursts of 5 Hz, zaprinast converted discrete relaxations into sustained relaxation; since it has been suggested that parasympathetic nerves, including those to blood vessels, may fire in such bursts (Tobin et al., 1990) this observation may be of relevance to the therapeutic actions of phosphodiesterase V inhibitors, both in vascular and non-vascular smooth muscle. The non-selective phosphodiesterase inhibitor IBMX produced a much greater direct relaxation of tone compared with zaprinast, making examination of its interaction with the nitrergic system more difficult; this presumably reflects the more widespread enzyme inhibition produced by IBMX with consequent increase in other second messengers in addition to cyclic GMP. The selectivity of zaprinast was confirmed by its lack of effect on relaxations to VIP.

In conclusion, this study has shown that anococcygeus muscles from female mice provide a stable and convenient model for the study of nitrergic transmission in smooth muscle from the female urogenital system and may be useful for the investigation of the interactions between nitrergic nerves and inhibitors of phosphodiesterase V.

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