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Sildenafil citrate on nitrergic transmission in anococcygeus muscles from the urogenital system of male and female mice

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Abstract

The effects of sildenafil on nitrergic relaxations were compared in anococcygeus muscles from male and female mice. In muscles from both sexes, sildenafil (10–300 nM) produced a weak, direct relaxation of carbachol-induced tone, and increased both the amplitude and duration of nitrergic relaxations. The most marked effect was on nitrergic duration (300–400% increase with 300 nM sildenafil); no differences in potency were observed between male (EC_{50}, 30 nM) and female (EC_{50}, 25 nM). The rate of onset for potentiation of nitrergic duration was similar in both sexes; but, on washout, the effects of sildenafil declined more slowly in the male muscle. Relaxations to both nitric oxide (NO) and sodium nitroprusside were also increased in amplitude and duration by 50 nM sildenafil, while those to forskolin and papaverine were unaffected. The results demonstrate that sildenafil causes a similar, potent and selective potentiation of nitrergic transmission in urogenital smooth muscle from both male and female mice.

Keywords: Anococcygeus, mouse; Forskolin; Nitrergic; Nitric oxide (NO); Papaverine; Sildenafil; Sodium nitroprusside

1. Introduction

The drug treatment of male impotence has been revolutionised by the introduction of sildenafil citrate (Viagra™) as an effective oral therapy (Marshall, 1998; Langtry and Markham, 1999; Moreland et al., 1999). The drug is a selective inhibitor of phosphodiesterase 5 and, therefore, enhances and prolongs the cellular effects of the second messenger substance cyclic GMP, the specific substrate for this enzyme (Jeremy et al., 1997; Naylor, 1998). Since the guanylyl cyclase/cyclic GMP pathway is the signal transduction mechanism activated by nitric oxide (NO) released from either neurons or endothelial cells within the penis, sildenafil potently potentiates the smooth muscle relaxant effects of this NO, thereby explaining the enhancement of penile erection (Naylor, 1998; Moreland et al., 1999). To date, most experiments with sildenafil have been carried out on the corpus cavernosum in which it has been shown to be a potent and selective inhibitor of phosphodiesterase 5 and to enhance relaxations induced by nitrergic nerve stimulation or by NO-donor drugs (Jeremy et al., 1997; Ballard et al., 1998; Carter et al., 1998; Chuang et al., 1998; Moreland et al., 1998; Stief et al., 1998). However, there have been relatively few reports of its effects on other nitrergically innervated tissues, including the anococcygeus muscle, the first tissue in which a neurotransmitter role for NO was firmly established (Gillespie et al., 1989; Li and Rand, 1989; Ramagopal and Leighton, 1989; Gibson et al., 1990). Anococcygeus muscles are found in the urogenital systems of both male and female rats and mice (although they are considerably larger in the male) and, therefore, they can be used to compare the effects of drugs on urogenital smooth muscle between the sexes (Gibson and Gillespie, 1973; Fukazawa et al., 1997; O'Kane and Gibson, 1999). This is of particular relevance for sildenafil, since there is increasing interest in the possible use of phosphodiesterase 5 inhibitors in female sexual dysfunction (Kaplan et al., 1999; Numberg et al., 1999; Shen et al., 1999). Given this, the object of the present study was to determine and compare the effects of sildenafil on nitrergic transmission in anococcygeus muscles from male

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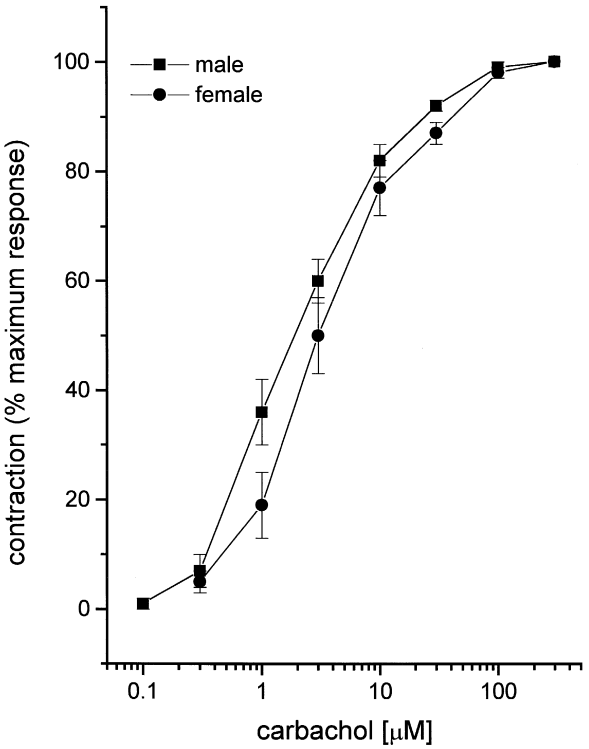

Fig. 1. Concentration–response curves for carbachol-induced contractions of anococcygeus muscles isolated from male and female mice. Each point is the mean ± S.E.M. from six to eight individual muscle preparations.

and female mice, and to investigate the selectivity of its actions.

2. Methods

2.1. Tissue preparation

Male and female mice (LACA; 25–35 g; Tuck, Essex, UK) were killed by stunning and exsanguination. The two anococcygeus muscles (wet weight 1.2 ± 0.2 mg in the male, 0.7 ± 0.1 mg in the female) were dissected out and set up in 1 ml glass organ baths containing Krebs-bicarbonate buffer (composition in 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 continuously with 95% O_2: 5% CO_2. A resting tension of 200–400 mg 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 including 1 μM phentolamine in the Krebs bathing medium and by preincubation of each tissue with 30 μM guanethidine for 10 min during the equilibrium period.

2.2. Protocols

The concentration–response relationship for carbachol was obtained by cumulative addition of carbachol to the organ bath; the contact time for each concentration was 3 min or until the increase in tension had reached a plateau (whichever was sooner). To observe relaxations to field stimulation and to drugs, muscle tone was raised with 50 μM carbachol (see Results) and the relaxant stimuli applied when a stable increase in tone had been achieved (usually within 3 min of adding carbachol to the bath).

To determine the concentration–response relationship for sildenafil, the nitrergic nerves were stimulated with repeated trains of field stimulation at 4 Hz (10 s trains every 100 s) and increasing concentrations of sildenafil added after each fourth train of stimulation. Measurements were taken of direct relaxation (% reduction of initial carbachol-induced tone), amplitude of nitrergic relaxation (peak % reduction in tone compared with the level immediately before each train of stimulation), and duration of response (time from 50% relaxation to 50% recovery of tone).

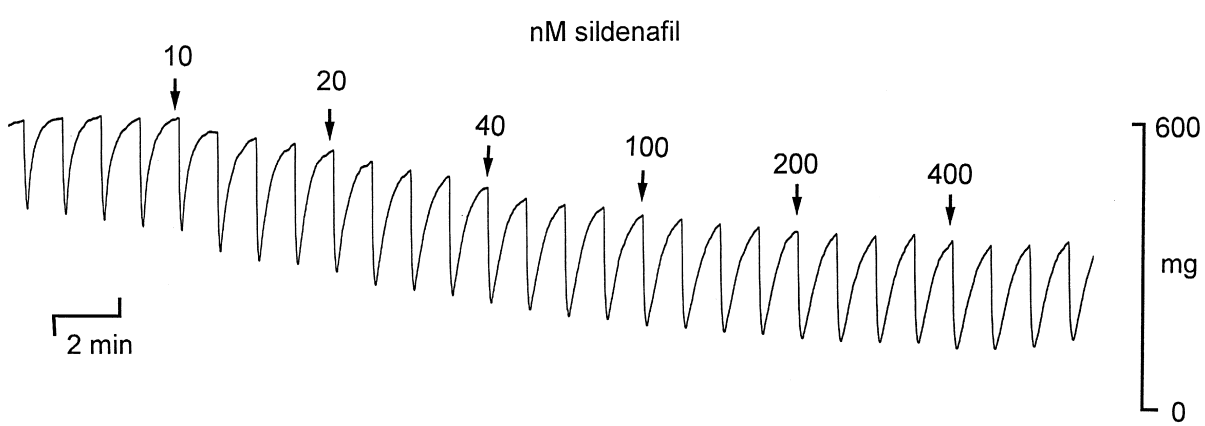

Fig. 2. Trace showing the effects of increasing concentrations of sildenafil on nitrergic relaxations of the male mouse anococcygeus (4 Hz; 10 s train every 100 s). Muscle tone was raised with 50 μM carbachol.

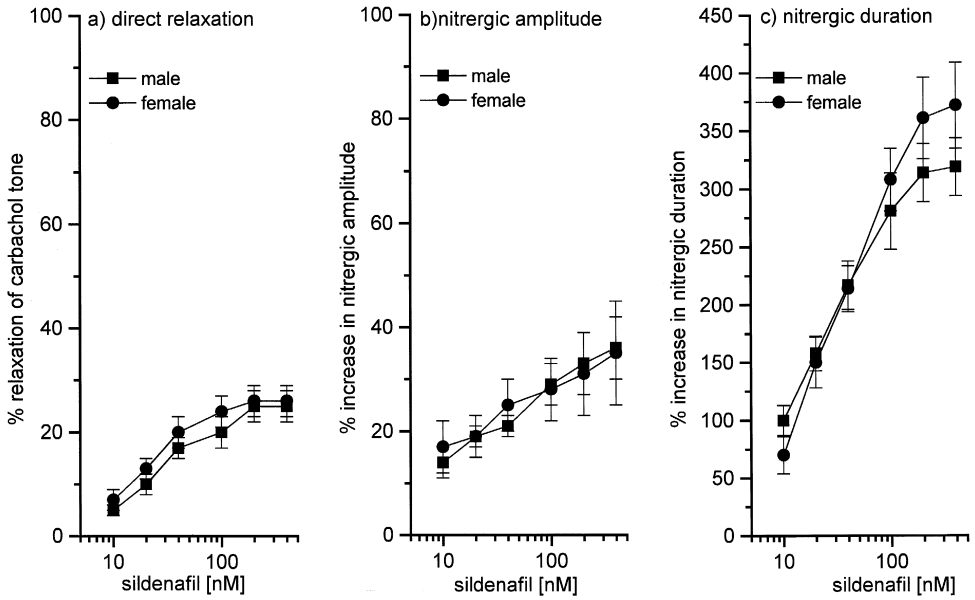

Fig. 3. Concentration–response curves for the effect of sildenafil on carbachol-induced tone (a), and on the amplitude (b) and duration (c) of nitrergic relaxations of the male and female mouse anococcygeus muscle in response to a fixed frequency of field stimulation (4 Hz, 10-s train every 100 s; see Fig. 2). Each point is the mean ± S.E.M. from six individual muscle preparations. Muscle tone was raised with 50 μM carbachol.

In another set of experiments, the effect of 50 nM sildenafil on the frequency–response curve of nitrergic relaxations to a fixed train of 100 pulses was observed. In this case, consecutive control frequency–response curves were obtained until the responses had stabilised; the muscle was then incubated in 50 nM sildenafil for 15 min before a further frequency–response curve was obtained in the presence of the drug.

The effects of 50 nM sildenafil on relaxations to authentic NO, forskolin and papaverine were also investigated. With forskolin and papaverine the concentration–response curves were obtained by cumulative addition of the drug to the bath; the contact time was 4 min or until the relaxation had peaked (whichever was sooner). Amplitude was measured as the % relaxation of carbachol tone; duration was measured as the time to half recovery of tone on washout of the relaxant drug in the continued presence of carbachol (with or without sildenafil). With NO, the relaxations to each concentration were transient, reflecting the unstable nature of NO in the presence of O_2, and so tone was allowed to recover each time (without washout) before the next dose of NO was added to the bath; in this case,

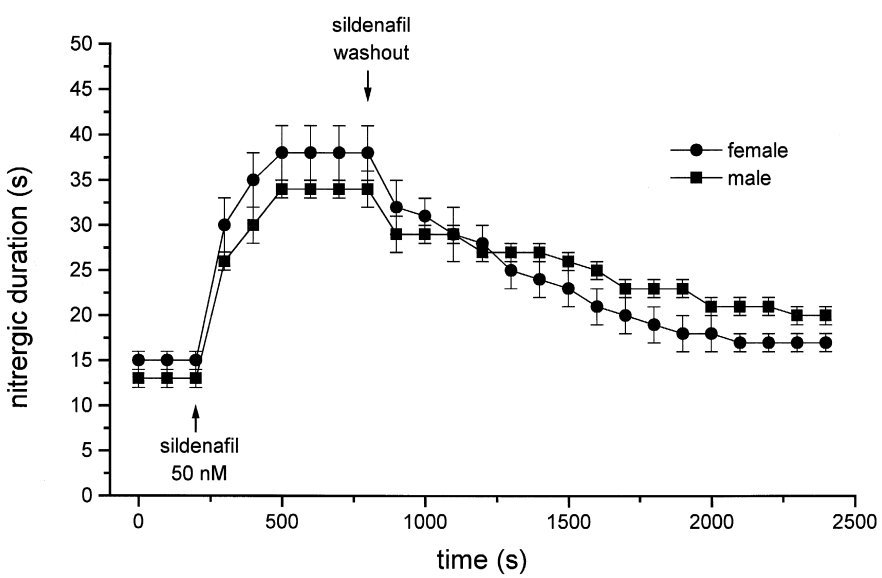

Fig. 4. Graph showing the time-course of sildenafil-induced potentiation of the duration of nitrergic relaxations (4 Hz; 10-s train every 100 s) in anococcygeus muscles from male and female mice. Each point is the mean ± S.E.M. from six individual muscle preparations.

duration was calculated as the time from 50% relaxation to 50% recovery of tone. Again, the contact time for sildenafil between the control and test curves was 15 min.

2.3. Statistics

Results are expressed as mean ± S.E.M. and statistical significance was assessed by Student's *t*-test, paired or unpaired as appropriate ($P < 0.05$ taken as significant). *n* represents the number of muscle strips.

2.4. Drugs used

All drugs were obtained from Sigma (UK) except carbachol (BDH), NO (BDH) and sildenafil (gift from Pfizer). All drugs were dissolved in water except sildenafil and forskolin, which were dissolved in dimethylsulphoxide (DMSO). The final concentrations of DMSO in the bath did not exceed 0.8% v/v and had minimal effect on tissue responses.

3. Results

3.1. Potency of carbachol as a contractile agent

Since the main object of the study was to compare the effects of sildenafil on nitrergic relaxations of carbachol-induced tone in anococcygeus muscles from male and female mice, we first examined the relative potency of

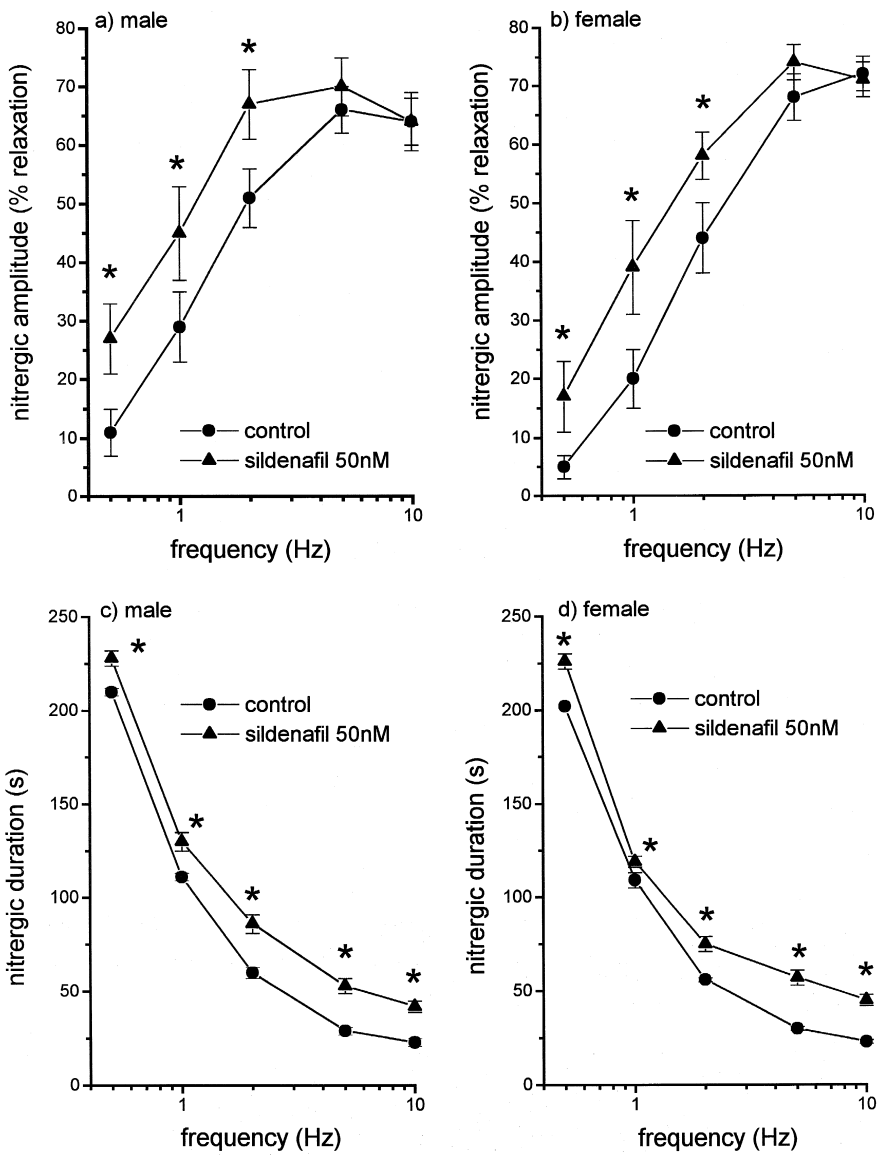

Fig. 5. Frequency–response curves showing the effect of 50 nM sildenafil on the amplitude (a, b) and duration (c, d) of nitrergic relaxations of the male and female mouse anococcygeus to 100 pulses of field stimulation at 0.5, 1, 2, 5, and 10 Hz. Each point is the mean ± S.E.M. from six individual muscle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

carbachol as a contractile agent in the two sexes. When plotted as % maximum response, the two concentration–effect curves were superimposable (Fig. 1), there being no significant difference in the pD_2 values (5.65 ± 0.10 in the male, and 5.45 ± 0.10 in the female; $n = 6$ in both cases). However, the maximum response obtained from male muscles (813 ± 107 mg tension) was greater than that from females (493 ± 86 mg tension). Based on these results, we decided to use a fixed concentration of 50 μM carbachol to raise tone in all subsequent experiments.

3.2. Effect of sildenafil on nitrergic relaxations

The potency of sildenafil on nitrergic relaxations was assessed by stimulating the nitrergic nerves with repeated trains of field stimulation (4 Hz; 10-s train every 100 s) and adding sildenafil cumulatively after each fourth train of field stimulation (Fig. 2); using these stimulation parameters, nitrergic relaxations had an amplitude (% relaxation of carbachol-induced tone) of $55 \pm 9\%$ in the male and $52 \pm 9\%$ in the female ($P > 0.05$) and a duration (time from 50% relaxation to 50% recovery) of 13 ± 1 s in muscles from either sex.

Sildenafil produced three clear effects on anococcygeus muscles from both male and female mice, with no significant differences being noted between the sexes: first, it caused a direct relaxation of carbachol-induced tone (Figs. 2 and 3a); secondly, sildenafil increased the amplitude of the nitrergic relaxations (Figs. 2 and 3b); and thirdly, the duration of nitrergic relaxations was increased (Figs. 2 and

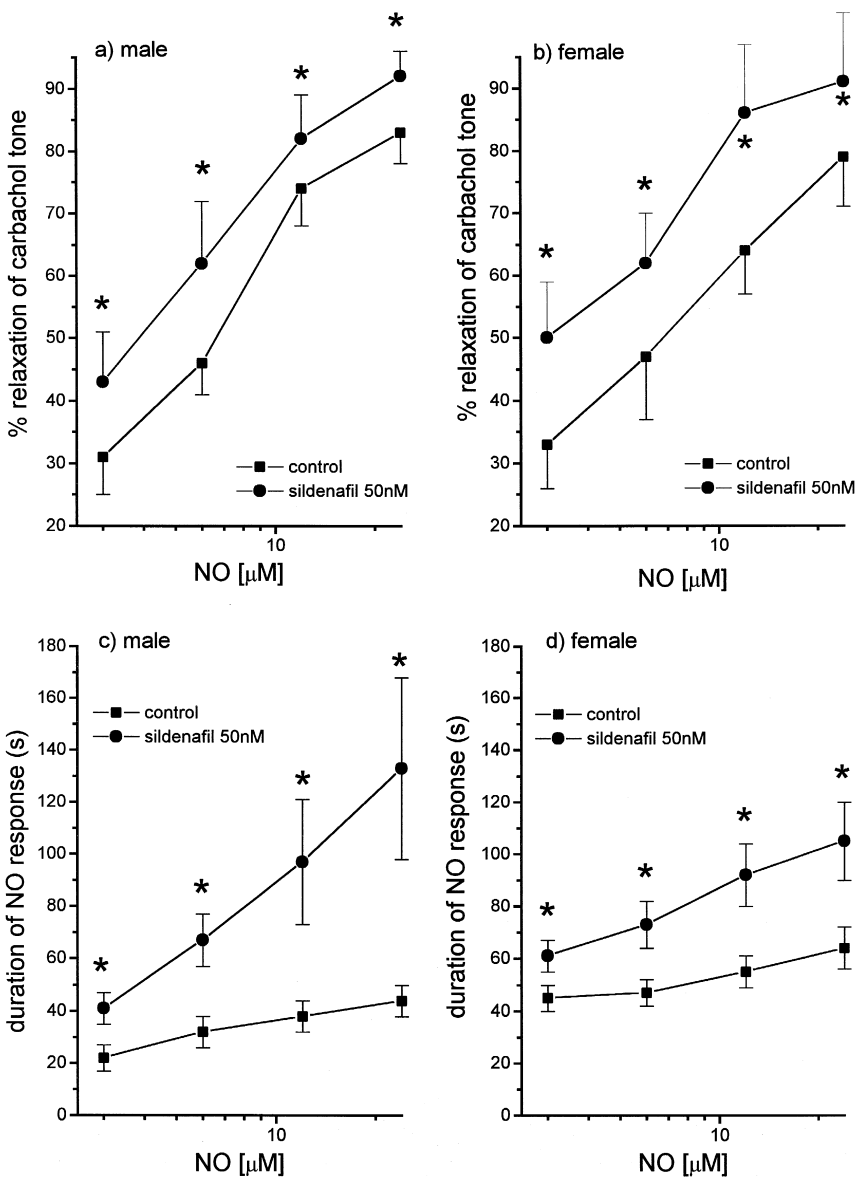

Fig. 6. Concentration–response curves showing the effect of 50 nM sildenafil on the amplitude (a, b) and duration (c, d) of relaxations of the male and female mouse anococcygeus in response to NO. Each point is the mean ± S.E.M. from six individual muscle preparations. Muscle tone was raised with 50 μM carbachol. *, Value for sildenafil significantly different from corresponding control.

3c). As can be seen from Figs. 2 and 3, the most marked effect was the 300–400% increase in duration of the nitrergic response; using this parameter, the estimated pD_2 values for sildenafil were 7.6 ± 0.05 in the male ($n = 8$) and 7.5 ± 0.5 ($n = 6$) in the female ($P > 0.05$).

The time-course of the effect of sildenafil on nitrergic duration was determined using the same parameters of field stimulation, but this time with a fixed concentration of sildenafil (50 nM; Fig. 4). In both male and female muscles, the peak increase in the duration of nitrergic relaxations was observed within 300 s of adding sildenafil to the bath. However, the half-time of recovery on washout of the drug was greater in the male (766 ± 111 s; $n = 6$) than the female (408 ± 44 s; $n = 6$; $P < 0.05$).

Having established the concentration–response relationship for sildenafil using a fixed frequency, we went on to determine the effects of 50 nM sildenafil on the frequency–response curve to trains of field stimulation using a fixed number of pulses (100 pulses at 0.5, 1, 2, 5 and 10 Hz). Again, the effect was similar in muscles from both male and female mice (Fig. 5). Sildenafil increased the amplitude of nitrergic relaxations at 0.5, 1, and 2 Hz but not at 5 and 10 Hz; nitrergic duration, however, was increased at all frequencies by sildenafil, with the relative magnitude of the sildenafil-induced increase being greater at higher frequencies (9% increase at 0.5 Hz; 88% increase at 10 Hz).

Since the transmitter of the nitrergic nerves of the anococcygeus muscle is believed to be NO, we next determined the effects of sildenafil on the concentration–response curves for relaxations to exogenous NO (1–30 μM; Fig. 6). In both the male and female anococcygeus, 50 nM sildenafil increased the amplitude of relaxations to NO and the duration of the response; the magnitude of this latter effect increased as the concentration of NO increased (101% increase at 3 μM; 150% increase at 24 μM).

3.3. Selectivity of sildenafil

The selectivity of sildenafil was assessed by comparing its effects on relaxations to the NO-donor sodium nitroprusside, the adenylyl cyclase activator forskolin, and the general smooth muscle relaxant drug papaverine (Fig. 7). Only the results obtained from muscles from male mice are shown here, but similar results were obtained with muscles from female mice. As expected from the results detailed above, 50 nM sildenafil increased the amplitude of relaxations to sodium nitroprusside (0.01–3 μM); further, the duration of the response to 300 nM sodium nitroprusside (measured as the time taken to half-recovery of tone

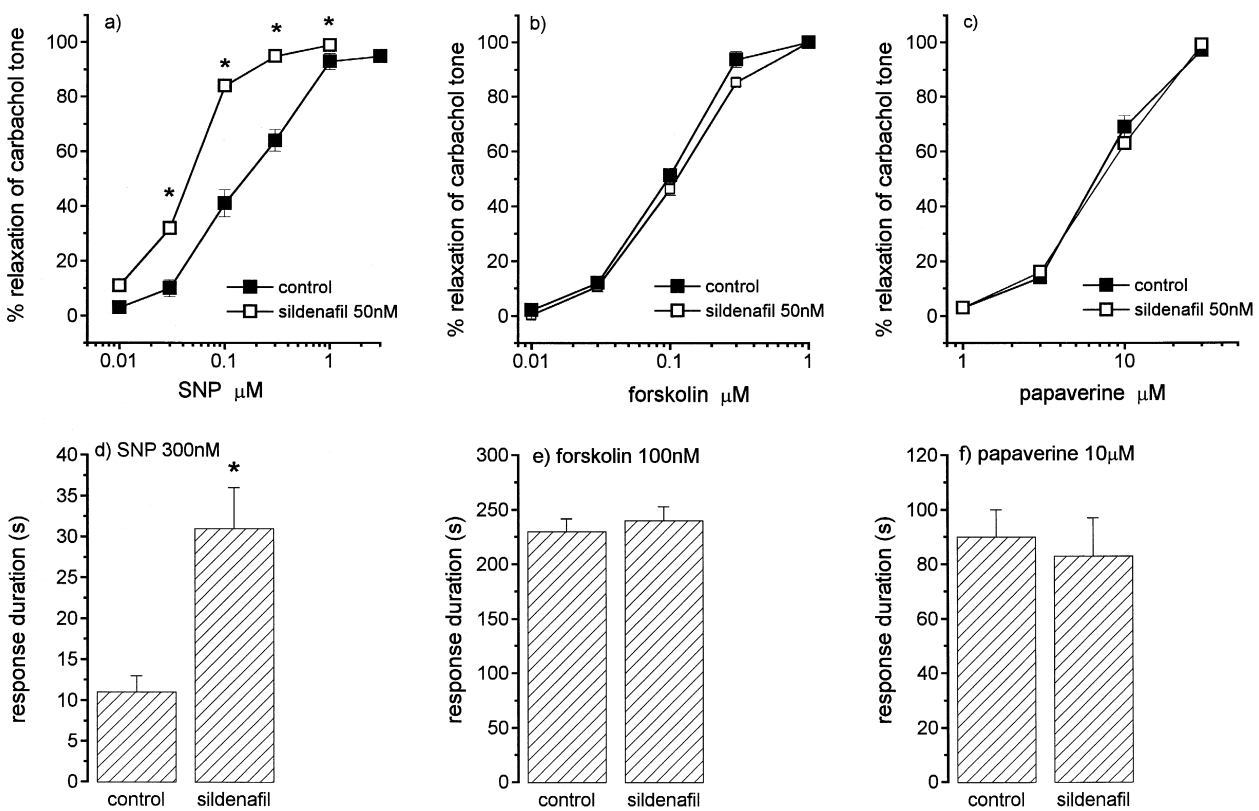

Fig. 7. The effect of 50 nM sildenafil on the concentration response curves for relaxations of the male mouse anococcygeus muscle to SNP (a), forskolin (b) and papaverine (c), and on the duration of relaxations to 300 nM SNP (d), 100 nM forskolin (e) and 10 μM papaverine (f). Each point is the mean ± S.E.M. from five to six individual muscle preparations. Muscle tone was raised with 50 μM carbachol. *, Value for sildenafil significantly different from corresponding control.

following washout of sodium nitroprusside) was increased threefold. Conversely, neither the amplitude nor duration of responses to forskolin or papaverine were significantly altered by 50 nM sildenafil.

4. Discussion

Qualitatively, the results obtained with sildenafil in the present study are similar to those recently reported with zaprinast in the mouse anococcygeus (O'Kane and Gibson, 1999), reflecting the common property of the two drugs of inhibiting phosphodiesterase 5. Thus, both drugs produce direct relaxation of carbachol-induced tone and increase the amplitude and duration of nitrergic relaxations. The most marked effect in each case is the increase in nitrergic duration, an action that is greater at higher frequencies of field stimulation. Quantitatively, however, sildenafil is much more potent than zaprinast. Comparison of the concentrations of sildenafil (40 nM; present study) and zaprinast (20 μM; O'Kane and Gibson, 1999) required to increase nitrergic duration by 200% reveals that sildenafil is some 500 times the more potent, consistent with the relative potencies of the two drugs as inhibitors of the phosphodiesterase 5 isoenzyme (Ballard et al., 1998). The EC_{50} for sildenafil in the anococcygeus is around 30 nM, which is similar to its potency in the human corpus cavernosum (Ballard et al., 1998).

Sildenafil enhanced nitrergic relaxations in anococcygeus muscles from both male and female mice, there being no significant difference in the potency of the drug between the sexes. Indeed, the only difference observed in the actions of sildenafil was the slower rate of recovery on washout in the male muscle; at present, we have no explanation for this, although it may simply be due to the greater size of the muscle in the male reducing the rate of diffusion of the drug out of the tissue. The observed effectiveness of sildenafil on nitrergic transmission in the female is important since there is growing interest in the possible use of phosphodiesterase 5 inhibitors in the treatment of sexual dysfunction in women. Early clinical results have, however, been equivocal. Clear beneficial effects of sildenafil have been demonstrated in women whose sexual dysfunction is associated with antidepressant therapy (Numberg et al., 1999; Shen et al., 1999). In premenopausal women with sexual dysfunction, sildenafil did not enhance overall sexual function, although there were significant improvements in vaginal lubrication and clitoral sensitivity (Kaplan et al., 1999). Certainly, the clitoris has been shown to contain neuronal NO synthase and relaxations of the clitoris in response to field stimulation are nitrergic (Burnett et al., 1997; Cellek and Moncada, 1998). In addition, sildenafil has been found to enhance cyclic GMP production by sodium nitroprusside in the human clitoris (Park et al., 1998). The present results with the anococcygeus confirm the scientific rationale for the investigation of phosphodiesterase 5 inhibitors in female sexual dysfunction, since the drug was as effective at enhancing nitrergic transmission in female urogenital smooth muscle as it was in the male.

The selectivity of sildenafil was determined by investigating its effects on authentic NO, the NO-donor drug sodium nitroprusside, the direct adenylyl cyclase activator forskolin, and the general smooth muscle relaxant papaverine. As expected, sildenafil produced changes in the responses to NO and sodium nitroprusside, which were similar to its effects on nitrergic field stimulation, enhancing both amplitude and duration of the relaxations. However, relaxations to either forskolin or papaverine were unaffected. Forskolin acts on the regulatory site of adenylyl cyclase to activate the enzyme and has been shown to increase cyclic AMP, but not cyclic GMP, in the rat anococcygeus (Mirzazadeh et al., 1991). The cellular mechanisms associated with the relaxant effects of papaverine are less clear, and may involve nonselective PDE inhibition or interference with calcium flux in smooth muscle (Huddart et al., 1984; Beavo and Reifsnyder, 1990; Miller et al., 1994). Nevertheless, the lack of effect of sildenafil on responses to forskolin and papaverine demonstrates the marked selectivity of the phosphodiesterase 5 inhibitor for enhancing relaxations mediated via the cyclic GMP signalling pathway.

In conclusion, the results of this study have shown that sildenafil produces a similar, potent, and selective potentiation of nitrergic transmission in urogenital smooth muscle from both male and female mice.

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