Published in final edited form as:

Clin Exp Pharmacol Physiol. 2009 April ; 36(4): 358–366. doi:10.1111/j.1440-1681.2008.05071.x.

EFFECT OF THE PHOSPHODIESTERASE 5 INHIBITORS SILDENAFIL, TADALAFIL AND VARDENAFIL ON RAT ANOCOCCYGEUS MUSCLE: FUNCTIONAL AND BIOCHEMICAL ASPECTS

Haroldo A Toque^{*}, Fernanda BM Priviero^{*}, Saiprasad M Zemse[†], Edson Antunes^{*}, Cleber E Teixeira^{*,†}, and R Clinton Webb[†]

^{*} Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, Campinas, São Paulo, Brazil

[†] Department of Physiology, Medical College of Georgia, Augusta, Georgia, USA

SUMMARY

1. The anococcygeus muscle is part of the erectile machinery in male rodents. Phosphodiesterase (PDE) 5 inhibitors enhance and prolong the effects of cGMP, which has a key role in penile erection. The aim of the present study was to provide a functional and biochemical comparison of the three PDE5 inhibitors, namely sildenafil, tadalafil and vardenafil, in the rat anococcygeus muscle.
2. Muscle strips were mounted in 4 mL organ baths and isometric force recorded. Levels of cGMP were measured using an enzyme immunoassay kit. Western blots were used to determine PDE5 protein expression.
3. The PDE5 inhibitors concentration-dependently relaxed carbachol-precontracted anococcygeus muscle; however, vardenafil was more potent ($pEC_{50} = 8.11 \pm 0.05$) than sildenafil (7.72 ± 0.06) or tadalafil (7.69 ± 0.05). Addition of *N*^G-nitro-L-arginine methyl ester (100 μ mol/L) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 μ mol/L) to the organ baths caused significant rightward shifts in concentration–response curves for all PDE5 inhibitors.
4. Sildenafil, tadalafil and vardenafil (all at 0.1 μ mol/L) caused leftward shifts in the glyceryl trinitrate (GTN) concentration–response curves (by 4.0-, 3.7- and 5.5-fold, respectively). In addition, all three PDE5 inhibitors significantly potentiated relaxation responses to both GTN (0.01–10 μ mol/L) and electrical field stimulation (EFS; 1–32 Hz), with vardenafil having more pronounced effects.
5. All three PDE5 inhibitors reduced EFS-evoked contractions in a concentration-dependent manner over the concentration range 0.001–1 μ mol/L. There were no significant differences between the effects of the three PDE5 inhibitors.
6. Vardenafil (0.01–0.1 μ mol/L) was more potent in preventing cGMP degradation *in vitro* than sildenafil (0.01–0.1 μ mol/L) and tadalafil (0.01–0.1 μ mol/L).
7. Under control conditions, the expression of PDE5 was higher in the anococcygeus muscle than in the corpus cavernosum.

8. In conclusion, PDE5 inhibitors enhance exogenous and endogenous nitric oxide-mediated relaxation in the rat anococcygeus muscle. The potency of vardenafil was greater than that of either sildenafil or tadalafil.

Keywords

anococcygeus muscle; cGMP; nitric oxide; phosphodiesterase 5

INTRODUCTION

Substantial evidence has shown that nitric oxide (NO) is an important neurotransmitter in the autonomic nervous system.^{1–3} In penile tissue, NO represents the major neurotransmitter, responsible for smooth muscle relaxation during the erectile response.^{4,5} Nitric oxide is synthesised from the guanidino residue of the amino acid L-arginine in a reaction in which O₂ is required.⁶ In addition to NO, L-citrulline is formed; the reaction is catalysed by NO synthase (NOS).

Intracellular cGMP is rapidly inactivated to GMP by the activity of cyclic nucleotide phosphodiesterases (PDEs). Therefore, the concentration of cGMP in smooth muscle cells is strictly dependent on the balance between production by soluble guanylyl cyclase (sGC) and breakdown by PDEs, which represent the unique degradation pathway for this second messenger.^{7,8} To date, several PDE isoforms have been identified with variable selectivity for the second messengers cAMP and cGMP. In addition, selective inhibitors have been described.⁹ Several different cyclic nucleotide PDEs can hydrolyse cGMP and control the duration and amplitude of the cGMP signal.¹⁰ A major cGMP-hydrolysing enzyme expressed in all types of smooth muscle is PDE5 and selective inhibitors of PDE5 can cause smooth muscle relaxation. Three commercially available PDE5 inhibitors, namely sildenafil (Viagra; Pfizer, New York, NY, USA), tadalafil (Cialis; Eli Lilly, Basingstoke, England) and vardenafil (Levitra; Bayer, Leverkusen, Germany), are currently approved for the treatment of male erectile dysfunction because of their ability to potentiate the relaxant effect of NO through enhancement of cGMP accumulation in the corpus cavernosum.^{11–13} Indeed, clinical efficacy from sildenafil, tadalafil and vardenafil as potent PDE5 inhibitors in the management of erectile dysfunction has been documented recently.^{14–16}

The rat anococcygeus muscle is a dynamic smooth muscle preparation for experimental pharmacology owing to its rich adrenergic and cholinergic transmission, as well as non-adrenergic, non-cholinergic (NANC) innervation.^{17,18} It is reported that 60–70% of fibres supplying the rat anococcygeus muscle are noradrenergic and that approximately 5% of all nerve terminals having cholinergic characteristics.¹⁹ The NANC terminals comprise approximately 40% of fibres. Nitric oxide has been reported as an important inhibitory NANC transmitter in the rat anococcygeus muscle.^{18,20,21} Accordingly, this muscle contains large amounts of nitrergic neurons, identified by their high content of both neuronal (n) NOS fibres²² and nNOS activity.²³ In the anococcygeus muscle, NO evokes relaxation via stimulation of sGC, thereby increasing the cGMP content of the tissue.^{24–26} Although sildenafil has been shown previously to potentiate nitrergic transmission in the mouse anococcygeus muscle,²⁷ no detailed comparative analysis of the effects of the PDE5 inhibitors sildenafil, vardenafil and tadalafil have been investigated in the anococcygeus muscle. Taking into consideration that the anococcygeus is part of the erectile machinery in male rodents,²⁸ the purpose of the present study was to provide a functional and biochemical analysis of the effects of sildenafil, tadalafil and vardenafil in the rat anococcygeus muscle. Different procedures were applied to examine the effects of PDE5 on endogenous and exogenous NO-mediated relaxations, nerve-derived noradrenaline-evoked contractions and

intra-cellular cGMP degradation. In addition, expression of PDE5 protein was evaluated in the anococcygeus muscle and corpus cavernosum.

METHODS

Animals

All experiments were conducted in accordance with institutional guidelines and were approved by the local committee on animal experiments. Experiments were performed on adult male Sprague-Dawley rats (250–275 g) obtained from Harlan Laboratories (Indianapolis, IN, USA). Rats were housed two per cage on a 12 h light–dark cycle and were fed standard chow and water *ad libitum*.

Rat anococcygeus preparation

Rats were killed humanely by carbon dioxide inhalation, followed by decapitation. After the abdomen had been opened and the urogenital organs visualized, the paired anococcygeus muscles were seen passing from the vertebral column to the ventral side of the colon. The whole pair of muscles was removed, each carefully freed of connective tissue and tied at both ends by cotton thread ligatures.²⁹ Each muscle strip was subsequently immersed in chilled Krebs' solution (pH 7.4) of the following composition (in mmol/L): NaCl 118; NaHCO₃ 25; dextrose 5.5; KCl 4.7; KH₂PO₄ 1.18; MgSO₄·7H₂O 1.17; CaCl₂·2H₂O 2.5. Tissues were transferred to 4 mL myograph chambers (Danish Myograph Technology, Aarhus, Denmark) containing Krebs' solution (37°C and bubbled continuously with a mixture of 95% O₂ and 5% CO₂), attached horizontally between two mounting clamps and connected to an isometric force transducer. Then, strips were subjected to a resting force of 10 mN and allowed to equilibrate for 60 min. Changes in isometric force were recorded using a PowerLab 8/SP data acquisition system (software Chart 5.0; ADInstruments, Colorado Springs, CO, USA). Electrical field stimulation (EFS) was applied to muscles placed between platinum pin electrodes attached to a stimulus splitter unit (Stimu-Splitter II; Med-Lab Instruments, Loveland, CO, USA), which was connected to a Grass S88 stimulator (Astro-Med Industrial Park, West Warwick, RI, USA). Electrical field stimulation consisted of 50 V, 1 msec pulse width and trains of stimuli lasting 10 s at varying frequencies (1–32 Hz).

Experimental protocols

In order to verify the viability of preparations, a K⁺ solution (80 mmol/L) was added to the organ baths at the end of the equilibration period. Initially, cumulative concentration–response curves to sildenafil, tadalafil and vardenafil (0.0001–10 µmol/L) were constructed in muscle strips precontracted with the muscarinic agonist carbachol (10 µmol/L), in the absence and in the presence of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L) or the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 µmol/L). One concentration–response curve to sildenafil, tadalafil and vardenafil was constructed in each muscle preparation owing to the lower contractile effect of carbachol observed in the second curve.

In a second set of experiments, concentration–response curves to either the NO donor glyceryl trinitrate (GTN; 0.01–10 µmol/L) or the adenylyl cyclase stimulator forskolin (0.001–1 µmol/L) were obtained in carbachol-precontracted preparations in the absence and presence of each PDE5 inhibitor (all at concentrations of 0.1 µmol/L).

In a third set of experiments, EFS (1–32 Hz) was applied at the baseline level in order to elicit frequency dependent contractions in the absence or presence of L-NAME (100 µmol/L).

L). Then, tissues were treated with 0.001–1 $\mu\text{mol/L}$ sildenafil, tadalafil or vardenafil and the series of stimuli repeated.

In a fourth set of experiments, tissues were pretreated with bretylium tosylate (30 $\mu\text{mol/L}$) for 30 min to deplete the catecholamine stores of adrenergic fibres. Tissues were washed and then contracted with carbachol (10 $\mu\text{mol/L}$). When a stable level of contraction was attained, a series of EFS-induced relaxations was obtained. Next, the effects of the PDE5 inhibitors (0.1 $\mu\text{mol/L}$ each) sildenafil, tadalafil or vardenafil were investigated during EFS (1–32 Hz). The sodium channel blocker tetrodotoxin (TTX; 1 $\mu\text{mol/L}$) was used to explore the neurogenic stimulation evoked by EFS of rat anococcygeus muscle. Phentolamine (1 $\mu\text{mol/L}$) was used to block α -adrenoceptors. These drugs were used in separate experiments and both were added to the bathing solution 20 min prior to EFS and were present throughout the remainder of the experiment.

In the last series of experiments, increasing concentrations of each PDE5 inhibitor (0.001–0.1 $\mu\text{mol/L}$) were assayed in carbachol-precontracted tissues stimulated repeatedly at 2 Hz (at 90 s intervals) and the duration of the nitrenergic response calculated as the time from 50% relaxation to 50% recovery. Before a second curve to GTN or EFS was constructed, the carbachol concentration was raised to 30 $\mu\text{mol/L}$ in the presence of the PDE5 inhibitors to attain similar levels of tone elicited in the absence of these drugs.

Assay for PDE activity

Anococcygeus muscle strips were frozen in liquid nitrogen, pulverized in a liquid nitrogen-cooled stainless-steel mortar and resuspended in 100 μL homogenization buffer containing 50 mmol/L triethanolamine (pH 7.5), 0.5 mmol/L EDTA, 7 mmol/L glutathione, 0.2 mmol/L phenylmethylsulphonyl fluoride, 1 $\mu\text{mol/L}$ pepstatin A and 1 $\mu\text{mol/L}$ leupeptin. The homogenate was then centrifuged for 10 min at 4000 g. The crude supernatant fractions (10–12 mg protein/mL) were aliquoted, frozen in liquid nitrogen and kept at -80°C until further analysis. The effect of PDE5 inhibitors on cGMP breakdown in anococcygeus muscle extracts was determined at 37°C in a total volume of 100 μL containing 20 mmol/L Tris-HCl, 20 mmol/L imidazole/HCl (pH 7.5), 3 mmol/L MgCl_2 , 15 mmol/L magnesium acetate, 250 nmol/L calmodulin, 250 $\mu\text{mol/L}$ CaCl_2 , 10 μL anococcygeus protein extract and, as a substrate, 10 μmol cGMP. The PDE5 inhibitors were added to a final concentration of 0.001–0.1 $\mu\text{mol/L}$, whereas in the control experiments only the solvent (dimethylsulphoxide; DMSO) was included. Additional samples were assayed in the absence of substrate or protein extract. Reactions were started by the addition of protein extract (30 μg) and stopped after 0, 5, 10 and 30 min by heating to 95°C . Phosphodiesterase activity was assayed using an ELISA with respect to a DMSO control (where the control without any inhibitor present indicated maximal GMP production under the assay conditions). Assays were performed in duplicate using different dilutions of samples. The pIC_{50} was defined as the negative logarithm of the molar concentration to produce 50% inhibition of GMP production compared with the DMSO control.

Western blot analysis

Rat anococcygeus muscle was removed as described above, snap-frozen in liquid nitrogen and kept at -80°C until use. Tissue segments were homogenized in a lysis buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, 1 mmol/L Na_3VO_4 and 1 mmol/L phenylmethylsulphonyl fluoride. The lysate was centrifuged at 10 000 g and the supernatant was collected. The protein concentration was determined using a BSA protein assay kit (Pierce Chemical, Rockford, IL, USA). An aliquot (40 μg) of protein from each sample was loaded per lane and resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were subsequently

transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked by treatment with 5% milk in Tris-buffered saline containing 0.05% Tween 20, probed with antibody against PDE5 (1:1000) and incubated with a horse-radish peroxidase-conjugated secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence autoradiography.

Drugs and chemicals

Bretylium tosylate, carbachol, forskolin, L-NAME and ODQ were purchased from Sigma Chemical (St Louis, MO, USA). Glyceryl trinitrate (Nitroglycerin; 5 mg/mL glass vials) was acquired from American Regent Laboratories (Shirley, NY, USA). Sildenafil citrate was obtained from Pfizer (New York, NY, USA). Vardenafil and tadalafil were obtained from commercial sources. Specifically, Levitra tablets (containing 10 mg vardenafil; Bayer) and Cialis tablets (containing 20 mg tadalafil; Eli Lilly) were used; in preparing the drugs for use in the present study, we took into account the molecular weight of the drugs and assumed purity of 8 and 5.44% for the Levitra and Cialis tablets, respectively. The anti-PDE V antibody and the human recombinant PDE type V were obtained from Chemicon (Temecula, CA, USA) and Calbiochem (San Diego, CA, USA), respectively. All other reagents were of analytical grade. Stock solutions of sildenafil, vardenafil and tadalafil were prepared in DMSO and dilutions were made up in deionized water immediately before use. The final concentration of the solvent did not exceed 0.1%. Preliminary experiments confirmed a lack of response to either vehicle in the concentrations assayed.

Statistical analysis

Experimental values were calculated relative to the maximal changes from the contraction produced by carbachol in each tissue, which was taken as 100%. Contractile responses were calculated as a percentage of KCl (80 mmol/L)-induced contraction. The pEC_{50} values for PDE5 inhibitors and GTN were determined as the negative logarithm of the molar concentration to produce 50% of the maximal relaxation in precontracted tissues. Data are shown as the percentage relaxation of n experiments, expressed as the mean $\pm$ SEM. Statistical comparisons were made using one-way ANOVA followed by Student–Newman–Keuls' multiple test or Student's t -test when appropriate. $P < 0.05$ was considered to indicate statistical significance. A program package was used for the statistical analysis of all data (GraphPad software, version 3.00; San Diego, CA, USA).

RESULTS

Relaxing activity of PDE5 inhibitors in the anococcygeus muscle

Carbachol (10 μ mol/L) caused a submaximal contraction in rat ano-coccygeus muscle preparations ($n = 72$) and generated active force of 54.3 ± 4.6 mN. The selective PDE5 inhibitors sildenafil, tadalafil and vardenafil (0.0001–10 μ mol/L) evoked concentration-dependent relaxations in precontracted muscle strips with pEC_{50} values of 7.72 ± 0.06 , 7.69 ± 0.05 and 8.11 ± 0.05 , respectively. Although no differences in maximal responses were obtained (85 ± 5 , 84 ± 6 and $95 \pm 6\%$ relaxation for sildenafil, tadalafil and vardenafil, respectively; $n = 19$), vardenafil was significantly more potent than the other two inhibitors ($P < 0.001$, one-way ANOVA followed by Student–Newman–Keuls' post hoc test), as attested by their calculated pEC_{50} values.

Effects of L-NAME and ODQ on relaxations induced by PDE5 inhibitors

Prior addition of L-NAME (100 μ mol/L; $n = 7$) did not significantly increase the tone of muscle strips precontracted with carbachol (56.7 ± 3.9 mN) and caused significant rightward shifts in the concentration–response curves (0.0001–10 μ mol/L) to sildenafil (threefold),

tadalafil (threefold) and vardenafil (fourfold) in the rat anococcygeus muscle. Similar to L-NAME, prior addition of the sGC inhibitor ODQ (10 $\mu\text{mol/L}$; $n = 7$) did not significantly increase the tone of carbachol-precontracted strips (57.4 ± 4.5 mN) and caused significant rightward displacement of the concentration–response curves constructed for sildenafil (threefold), tadalafil (fourfold) and vardenafil (sixfold; Table 1). Neither L-NAME nor ODQ had any significant effect on the maximal responses to the PDE5 inhibitors in the anococcygeus muscle (Table 1).

Effect of PDE5 inhibitors on GTN-induced relaxations

The NO donor GTN (0.01–10 $\mu\text{mol/L}$) evoked concentration-dependent relaxations of anococcygeus muscle strips that were fully blocked by ODQ (10 $\mu\text{mol/L}$; $n = 4$). The relaxant responses induced by GTN were shifted approximately 4.0-, 3.7- and 5.5-fold to the left by 0.1 $\mu\text{mol/L}$ sildenafil, 0.1 $\mu\text{mol/L}$ tadalafil and 0.1 $\mu\text{mol/L}$ vardenafil, respectively ($n = 6$; Fig. 1). The PDE5 inhibitors did not affect the maximal responses induced by GTN. In addition, none of the PDE5 inhibitors affected the relaxation induced by the adenylyl cyclase stimulator forskolin (0.0001–1 $\mu\text{mol/L}$; $n = 4$; data not shown).

Effect of PDE5 inhibitors on the magnitude and duration of nitrergic responses

Under the present experimental conditions, incubation of anococcygeus muscle strips with L-NAME (100 $\mu\text{mol/L}$; $n = 4$), ODQ (1 $\mu\text{mol/L}$; $n = 4$) or tetrodotoxin (1 $\mu\text{mol/L}$; $n = 5$) completely abolished the neurogenic relaxations evoked by EFS (1–32 Hz). At a concentration of 0.1 $\mu\text{mol/L}$, sildenafil ($n = 4$), tadalafil ($n = 4$) and vardenafil ($n = 5$) significantly enhanced the magnitude of EFS-evoked relaxations, particularly at lower frequencies of stimulation (1–4 Hz; Fig. 2). Most importantly, Fig. 3 shows that the duration of the nitrergic responses elicited by EFS was markedly increased in the presence of PDE5 inhibitors at any frequency investigated. Moreover, vardenafil was significantly more effective in increasing nitrergic duration than sildenafil and tadalafil (Table 2).

In order to better visualize the higher effects evoked by vardenafil in potentiating electrically induced responses in the anococcygeus muscle, repeated trains of EFS (2 Hz) were applied to preparations at 90 s intervals. The HTe PDE5 inhibitors were added after stabilization of EFS-induced relaxations, which was achieved when two repeated relaxant responses differed by less than 5% in amplitude. Nitrergic relaxations had a mean amplitude of $24 \pm 4\%$ (percentage of carbachol-induced contraction) and a mean duration (time from 50% relaxation to 50% recovery) of 18.7 ± 0.9 s. Under these conditions, the PDE5 inhibitors markedly potentiated the duration of the nitrergic responses in a concentration-dependent manner (Fig. 4; $n = 5$). At the highest concentration used (0.1 $\mu\text{mol/L}$), vardenafil was significantly more efficient in enhancing the duration of these responses (17.4 ± 1.1 and 41.3 ± 2.5 s under control and treated conditions, respectively; $P < 0.001$) compared with sildenafil (19.0 ± 0.6 and 34.1 ± 1.5 s under control and treated conditions, respectively; $P < 0.001$) and tadalafil (17.8 ± 1.0 and 29.2 ± 3.0 s under control and treated conditions, respectively; $P < 0.01$).

Effect of PDE5 inhibitors on electrically induced contractions

Contractions of anococcygeus muscle strips induced by EFS at supramaximal voltage were frequency dependent and consisted of rapidly developing and transient contractile responses. The mean amplitude of contractions evoked by 1, 2, 4, 8, 16 and 32 Hz were 3 ± 1 , 5 ± 1 , 33 ± 5 , 60 ± 5 , 81 ± 5 and $98 \pm 6\%$, respectively ($n = 20$). These contractile responses were fully blocked by either tetrodotoxin (1 $\mu\text{mol/L}$; $n = 4$) or phentolamine (1 $\mu\text{mol/L}$; $n = 4$). Addition of the PDE5 inhibitors (0.001–1 mol/L ; $n = 4$) sildenafil, tadalafil and vardenafil to the bathing medium had no significant effect on basal tension, but significantly reduced electrically induced contractions at all frequencies of stimulation in a concentration-

dependent manner. No significant differences were observed between the three PDE5 inhibitors. The percentage inhibition of these contractile responses is shown in Fig. 5.

In the presence of L-NAME (100 $\mu\text{mol/L}$), the magnitude of EFS-evoked contractions was slightly increased (but not significantly) at all frequencies (17, 19, 24, 13, 11 and 3% increase for 1, 2, 4, 8, 16 and 32 Hz, respectively). The inhibition of electrically induced contractions by the PDE5 inhibitors was not significantly affected by the presence of L-NAME ($n = 4$; data not shown).

Effect of PDE5 inhibitors on cGMP degradation

To confirm that the functional responses elicited by the PDE5 inhibitors correlated with enzyme inhibition, we examined the effects of sildenafil, vardenafil and tadalafil on cGMP levels in rat anococcygeus supernatant crude fractions, using 10 $\mu\text{mol/L}$ cGMP as a substrate to measure PDE activity. The cGMP was rapidly degraded when incubated in the protein extracts at 37°C, as measured at 5, 10 and 30 min following its addition to the reaction mixture, decreasing to approximately 35 ± 5 , 5 ± 3 and $2 \pm 1\%$ of the initial concentration, respectively. However, in the presence of PDE5 inhibitors, degradation of cGMP was significantly attenuated in a concentration-dependent manner. Vardenafil was approximately twofold more potent ($P < 0.05$) than sildenafil and tadalafil, with pIC_{50} values of 8.75 ± 0.06 , 8.33 ± 0.07 and 8.24 ± 0.08 , respectively (Fig. 6). In the concentration range 0.01–0.1 $\mu\text{mol/L}$, vardenafil was significantly more potent in inhibiting cGMP degradation compared with sildenafil and tadalafil. In the absence of the protein extract, no significant changes were observed in cGMP levels.

Expression of PDE5 in rat anococcygeus muscle

Figure 7 shows the protein expression of PDE5 in anococcygeus and corpus cavernosum muscle strips. The antibody against PDE5 recognized a 90/99 kDa doublet band, which was significantly stronger in the anococcygeus, when normalized against β -actin.

DISCUSSION

The advent of commercially available potent and specific PDE5 inhibitors to treat erectile dysfunction, such as sildenafil (Viagra; Pfizer), tadalafil (Cialis; Eli Lilly) and vardenafil (Levitra; Bayer), has provided new tools with which to investigate PDE5. The present study detailed a comparative analysis of the pharmacological properties of these three PDE5 inhibitors in the rat anococcygeus muscle. The findings give both functional and biochemical evidence for PDE5 expression and activity, regulating nitrergic signalling in this tissue.

The level of PDE5 expression was found to be even higher in anococcygeus than in corpus cavernosum, supporting the notion that this can account, in part, for the relative targeting of PDE5 inhibitors for the erectile machinery. By examining the relaxant activity of the three inhibitors, we demonstrated a direct and concentration-dependent effect of the drugs in rat anococcygeus muscle. It is of note that vardenafil was the most potent PDE5 inhibitor assayed in the present study, which corroborates its higher potency to inhibit the enzyme, as described previously.³⁰ The relaxant potencies observed in our experiments with this preparation are higher than those reported for porcine and human urethra,³¹ and similar to those reported in rat aorta.^{32,33}

The therapeutic use of PDE5 inhibitors in erectile dysfunction is based on the selective inhibition of the enzyme, thus increasing the levels of intracellular cGMP in the corpus cavernosum in response to NO-induced sGC activation during sexual stimulation.³⁴ The present investigation provides several pieces of evidence supporting such a mechanism in

the anococcygeus muscle: (i) relaxations induced by the PDE5 inhibitors were decreased by either the NOS inhibitor L-NAME or the sGC inhibitor ODQ; (ii) both EFS- and GTN-induced relaxations were enhanced by the PDE5 inhibitors; and (iii) forskolin-evoked relaxations were not modified by any of the PDE5 inhibitors. The sensitivity of sildenafil, tadalafil or vardenafil to L-NAME and ODQ suggests a role for the endogenous NO–cGMP signalling pathway in the anococcygeus muscle. Similar results supporting the enhancement of the NO–cGMP signalling pathway by PDE5 inhibitors in vascular smooth muscle have been reported, including the rabbit pulmonary and basilar artery,^{33,35} rabbit corpus cavernosum,^{36,37} porcine coronary artery³⁸ and rat aorta.³² The partial inhibition of the relaxations induced by sildenafil, vardenafil and tadalafil suggests that additional NO-independent pathways of relaxation could be involved in these responses. Indeed, we have recently demonstrated that vardenafil partially relaxes the rat aorta and rabbit pulmonary artery through a mechanism involving inhibition of Ca^{2+} entry.^{32,33} These pharmacological effects of PDE5 inhibitors (i.e. direct blockade of ion channels at high concentrations) were not been addressed in the present study. It is unlikely that the cAMP pathway is involved because the PDE5 inhibitors did not affect the relaxations evoked by forskolin. These results are of interest because they suggest that there is little or no cross-talk between the cGMP and cAMP pathways, corroborating previous findings in the mouse anococcygeus.²⁷

Because sildenafil, tadalafil and vardenafil reduce cGMP breakdown, the potential exists for synergistic action between PDE5 inhibitors and NO. Our results showed that the PDE5 inhibitors not only relaxed the anococcygeus muscle due to accumulation of basally produced cGMP, but also potentiated NO-dependent relaxations to GTN and EFS of nitrergic nerves. These findings agree with previously reported enhancement of the relaxant effects of NO donors in isolated arteries^{38,39} and non-vascular smooth muscle,^{27,31,40} as well as NO-induced hypotension *in vivo*.⁴¹ Similar to the effects on the exogenous NO-mediated relaxations, sildenafil, tadalafil and vardenafil enhanced both the amplitude and duration of relaxations induced by EFS of the nitrergic nerves in the rat anococcygeus muscle. Their effects on amplitude were greater at lower frequencies of stimulation (1–4 Hz), whereas the increase in duration was much greater at higher frequencies (8–32 Hz). These results show that PDE5 inhibitors clearly potentiate relaxations of rat anococcygeus mediated by nitrergic EFS, with vardenafil being of particular interest because it was the most effective of the three inhibitors used in the present study. The prolonging effect of PDE5 inhibitors on the neurogenic relaxations can be ascribed to their delaying effect on the decline of cGMP to basal levels when nerve stimulation is halted. In terms of functional nerve responses, PDE5 inhibitors have been shown to increase both the magnitude and duration of nitrergic relaxation in rabbit corpus cavernosum,³⁴ rabbit clitoral smooth muscle,⁴² mouse anococcygeus,²⁷ bovine penile arteries³⁹ and porcine urethra.³¹ Furthermore, all three inhibitors significantly reduced adrenergic transmission in the rat anococcygeus, suggesting that PDE5 inhibitors are capable of modulating tone in favour of smooth muscle relaxation.

All known PDE5 inhibitors were developed to compete with cGMP at the catalytic site of the enzyme. Although sildenafil, tadalafil and vardenafil are classified as PDE5 inhibitors, they differ slightly in their selectivity, pharmacokinetics and side-effect profile. In the enzyme assays using rat anococcygeus supernatant crude fractions, we demonstrated that vardenafil is approximately twofold more potent than sildenafil and tadalafil in inhibiting PDE5. Indeed, vardenafil is a potent and selective inhibitor of PDE5,⁴³ being approximately 25- and 48-fold more effective an inhibitor of human corpus cavernosum PDE5 than sildenafil and tadalafil, respectively.³⁰ Although vardenafil and sildenafil have a nitrogen-containing ring system in common that share similarities with the purine ring of cGMP, these compounds differ markedly in the strength of their interactions with PDE5.^{43,44} Molecular contacts of the three inhibitors within the catalytic site of the PDE5 have been

revealed recently by X-ray crystallography.⁴⁵ It is believed that the different core ring of vardenafil allows stronger contact with one or more amino acids of PDE5 that are thought to make contact with its core ring. Moreover, the different position of the nitrogen atom in the vardenafil structure could engender contact with an amino acid that is not contacted by sildenafil or it may have an indirect effect, such as changing the electron distribution in the core ring.⁴⁵ A recent study using four separate head-to-head assays also demonstrated that vardenafil is the most potent inhibitor because of its slow dissociation from PDE5.⁴⁶ Nevertheless, greater potency does not necessarily translate to greater therapeutic efficacy. Tadalafil, for example, has a much longer half-life compared with sildenafil and vardenafil.^{47,48} Other pharmacokinetic characteristics, such as bioavailability and metabolism, probably contribute to the net pharmacodynamic effects of these PDE5 inhibitors.

In conclusion, the present study has shown that PDE5 is highly expressed in rat anococcygeus muscle. The PDE5 inhibitors sildenafil, tadalafil and vardenafil potently relax this muscle and enhance exogenous and endogenous NO-mediated relaxations. These mechanisms may contribute to the reduction in electrically induced contractions. Furthermore, our findings identified vardenafil as the most potent PDE5 inhibitor based on both functional (direct relaxant effect and potentiation of NO-mediated responses) and biochemical (PDE activity experiments) assays.

Acknowledgments

This study was supported by grants from the National Institutes of Health (HL-71138, HL-74167). HAT is a fellow of the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). This paper is dedicated to the memory of Cleber E Teixeira, a wonderful friend and an excellent scientist.

References

- Andersson KE, Persson K. Nitric oxide synthase and nitric oxide-mediated effects in lower urinary tract smooth muscles. *World J Urol* 1994;12:274–80. [PubMed: 7532516]
- Gibson A. Phosphodiesterase 5 inhibitors and nitrergic transmission: From zaprinast to sildenafil. *Eur J Pharmacol* 2001;411:1–10. [PubMed: 11137852]
- Toda N, Okamura T. The pharmacology of nitric oxide in the peripheral nervous system of blood vessels. *Pharmacol Rev* 2003;55:271–324. [PubMed: 12773630]
- Andersson KE, Wagner G. Physiology of penile erection. *Physiol Rev* 1995;75:191–236. [PubMed: 7831397]
- Andersson KE. Pharmacology of penile erection. *Pharmacol Rev* 2001;53:417–50. [PubMed: 11546836]
- Leone AM, Palmer RM, Knowles RG, Francis PL, Ashton DS, Moncada S. Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J Biol Chem* 1991;266:23790–5. [PubMed: 1721054]
- Maurice DH, Palmer D, Tilley DG, et al. Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* 2003;64:533–46. [PubMed: 12920188]
- Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA. Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* 2003;93:280–91. [PubMed: 12933699]
- Francis SH, Turko IV, Corbin JD. Cyclic nucleotide phosphodiesterases: Relating structure and function. *Prog Nucleic Acid Res Mol Biol* 2001;65:1–52. [PubMed: 11008484]
- Juif DM, Soderling S, Burns F, Beavo JA. Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs). *Rev Physiol Biochem Pharmacol* 1999;135:67–104. [PubMed: 9932481]
- Boolell M, Allen MJ, Ballard SA, et al. Sildenafil: An orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996;8:47–52. [PubMed: 8858389]

12. Rosen RC, Kostis JB. Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am J Cardiol* 2003;92:M9–18.
13. Montorsi F, Briganti A, Salonia A, Montorsi P, Rigatti P. The use of phosphodiesterase type 5 inhibitors for erectile dysfunction. *Curr Opin Urol* 2004;14:357–9. [PubMed: 15626879]
14. Jackson G, Gillies H, Osterloh I. Past, present, and future: A 7-year update of Viagra (sildenafil citrate). *Int J Clin Pract* 2005;59:680–91. [PubMed: 15924597]
15. Carson CC, Hatzichristou DG, Carrier S, et al. Erectile response with vardenafil in sildenafil nonresponders: A multicentre, double-blind, 12-week, flexible-dose, placebo-controlled erectile dysfunction clinical trial. *BJU Int* 2004;94:1301–9. [PubMed: 15610110]
16. Giuliano F, Donatucci C, Montorsi F, et al. Vardenafil is effective and well-tolerated for treating erectile dysfunction in a broad population of men, irrespective of age. *BJU Int* 2005;95:110–16. [PubMed: 15638906]
17. Kulkarni SK, Sharma A. Rat anococcygeus: A dynamic smooth muscle preparation for experimental pharmacology. *Methods Find Exp Clin Pharmacol* 1994;16:379–85. [PubMed: 7837828]
18. Gibson A, McFadzean L. Biology of the anococcygeus muscle. *Int Rev Cytol* 2001;205:1–35. [PubMed: 11336390]
19. Dail WG, Carrillo Y, Walton G. Innervation of the anococcygeus muscle of the rat. *Cell Tissue Res* 1990;259:139–46. [PubMed: 2297780]
20. Gillespie JS, Liu XR, Martin W. The effects of L-arginine and N^G -monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br J Pharmacol* 1989;98:1080–2. [PubMed: 2611482]
21. Li CG, Rand MJ. Evidence for a role of nitric oxide in the neuro-transmitter system mediating relaxation of the rat anococcygeus muscle. *Clin Exp Pharmacol Physiol* 1989;16:933–8. [PubMed: 2558828]
22. Song ZM, Brookes SJ, Costa M. NADPH-diaphorase reactivity in nerves supplying the rat anococcygeus muscle. *Neurosci Lett* 1993;158:221–4. [PubMed: 7694208]
23. Mitchell JA, Sheng H, Forstermann U, Murad F. Characterization of nitric oxide synthases in non-adrenergic non-cholinergic nerve containing tissue from the rat anococcygeus muscle. *Br J Pharmacol* 1991;104:289–91. [PubMed: 1724623]
24. Cellek S, Kasakov L, Moncada S. Inhibition of nitrenergic relaxations by a selective inhibitor of the soluble guanylate cyclase. *Br J Pharmacol* 1996;118:137–40. [PubMed: 8733586]
25. Fonseca M, Uddin N, Gibson A. No evidence for a significant non-nitrenergic, hyperpolarising factor contribution to field stimulation-induced relaxation of the mouse anococcygeus. *Br J Pharmacol* 1998;124:524–8. [PubMed: 9647477]
26. Teixeira CE, Priviero FB, Claudino MA, et al. Stimulation of soluble guanylyl cyclase by BAY 41-2272 relaxes anococcygeus muscle: Interaction with nitric oxide. *Eur J Pharmacol* 2006a; 530:157–65. [PubMed: 16371226]
27. Frith D, Gibson A. Sildenafil citrate on nitrenergic transmission in anococcygeus muscles from the urogenital system of male and female mice. *Eur J Pharmacol* 2000;400:305–12. [PubMed: 10988348]
28. Gillespie JS. The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br J Pharmacol* 1972;45:404–16. [PubMed: 5072226]
29. Teixeira CE, Jin L, Ying Z, Palmer T, Webb RC. Ca^{2+} sensitization and the regulation of contractility in rat anococcygeus and retractor penis muscle. *Biochem Pharmacol* 2005;69:1483–92. [PubMed: 15857612]
30. Briganti A, Salonia A, Gallina A, et al. Drug insight: Oral phosphodiesterase type 5 inhibitors for erectile dysfunction. *Nat Clin Pract Urol* 2005;2:239–47. [PubMed: 16474835]
31. Werkstrom V, Svensson A, Andersson KE, Hedlund P. Phosphodiesterase 5 in the female pig and human urethra: Morphological and functional aspects. *BJU Int* 2006;98:414–23. [PubMed: 16626307]
32. Teixeira CE, Priviero FB, Webb RC. Differential effects of the phosphodiesterase type 5 inhibitors sildenafil, vardenafil, and tadalafil in rat aorta. *J Pharmacol Exp Ther* 2006;316:654–61. [PubMed: 16204472]

33. Toque HA, Teixeira CE, Priviero FB, Morganti RP, Antunes E, De Nucci G. Vardenafil, but not sildenafil or tadalafil, has calcium-channel blocking activity in rabbit isolated pulmonary artery and human washed platelets. *Br J Pharmacol* 2008;154:787–96. [PubMed: 18536732]
34. Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM. Effects of sildenafil on the relaxation of human corpus cavernosum tissue *in vitro* and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* 1998;159:2164–71. [PubMed: 9598563]
35. Salom JB, Burguete MC, Perez-Asensio FJ, Castello-Ruiz M, Torregrosa G, Alborch E. Relaxant effect of sildenafil in the rabbit basilar artery. *Vasc Pharmacol* 2006;44:10–16.
36. Giuliano F, Bernabe J, Alexandre L, Niewoehner U, Haning H, Bischoff E. Pro-erectile effect of vardenafil: *In vitro* experiments in rabbits and *in vivo* comparison with sildenafil in rats. *Eur Urol* 2003;44:731–6. [PubMed: 14644128]
37. Lau LC, Adaikan PG. Mechanisms of direct relaxant effect of sildenafil, tadalafil and vardenafil on corpus cavernosum. *Eur J Pharmacol* 2006;541:184–90. [PubMed: 16777087]
38. Sakuma I, Akaishi Y, Tomioka H, Sato A, Kitabatake A, Hattori Y. Interactions of sildenafil with various coronary vasodilators in isolated porcine coronary artery. *Eur J Pharmacol* 2002;437:155–63. [PubMed: 11890904]
39. Simonsen U, Contreras J, Garcia-Sacristán A, Martinez AC. Effect of sildenafil on non-adrenergic non-cholinergic neurotransmission in bovine penile small arteries. *Eur J Pharmacol* 2001;412:155–69. [PubMed: 11165227]
40. Thompson CS, Mumtaz FH, Khan MA, et al. The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. *Eur J Pharmacol* 2001;425:57–64. [PubMed: 11672575]
41. Yoo KY, Kim HS, Moon JD, Lee J. Sildenafil (Viagra) augments sodium nitroprusside-induced but not nitroglycerin-induced hypotension in dogs. *Anesth Analg* 2002;94:1505–9. [PubMed: 12032016]
42. Vemulapalli S, Kurowski S. Sildenafil relaxes rabbit clitoral corpus cavernosum. *Life Sci* 2000;67:23–9. [PubMed: 10896025]
43. Saenz de Tejada I, Angulo J, Cuevas P, et al. The phosphodiesterase inhibitory selectivity and the *in vitro* and *in vivo* potency of the new PDE5 inhibitor vardenafil. *Int J Impot Res* 2001;13:282–90. [PubMed: 11890515]
44. Corbin JD, Blount MA, Weeks JL, et al. [³H]Sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous, and stimulated by cGMP. *Mol Pharmacol* 2003;63:1364–72. [PubMed: 12761347]
45. Sung BJ, Hwang KY, Jeon YH, et al. Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. *Nature* 2003;425:98–102. [PubMed: 12955149]
46. Blount MA, Beasley A, Zoraghi R, et al. Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* 2004;66:144–52. [PubMed: 15213306]
47. Carrier S. Pharmacology of phosphodiesterase 5 inhibitors. *Can J Urol Suppl* 2003;1:12–16.
48. Carson CC, Lue TF. Phosphodiesterase type 5 inhibitors for erectile dysfunction. *BJU Int* 2005;96:257–80. [PubMed: 16042713]

Fig. 1.

Effects of the phosphodiesterase (PDE) 5 inhibitors (a) sildenafil, (b) tadalafil and (c) vardenafil on the relaxations induced by glyceryl trinitrate (GTN) in rat anococcygeus muscle preparations. (●), control; (■), in the presence of 0.1 μmol/L PDE5 inhibitor. Muscles were precontracted with carbachol (10 μmol/L) and experimental values were calculated relative to the maximal changes from the contraction produced by carbachol in each tissue, which was taken as 100%. Data are the mean $\pm$ SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$ compared with respective control values (Student's t -test).

Fig. 2.

Effects of the phosphodiesterase (PDE) 5 inhibitors (a) sildenafil, (b) tadalafil and (c) vardenafil on electrical field stimulation (EFS)-induced relaxations of rat anococcygeus muscle strips precontracted with carbachol (10 μ mol/L). (●), control; (■), in the presence of 0.1 μ mol/L PDE5 inhibitor. Experimental values were calculated relative to the maximal changes from the contraction produced by carbachol in each tissue, which was taken as 100%. Data are the mean $\pm$ SEM ($n = 4-5$). * $P < 0.05$, ** $P < 0.01$ compared with respective control values (Student's t -test).

Fig. 3. Representative tracings of frequency-dependent relaxations to electrical field stimulation (EFS) in the absence and in the presence of the phosphodiesterase (PDE) 5 inhibitors (a) sildenafil, (b) tadalafil and (c) vardenafil (0.1 $\mu\text{mol/L}$; $n = 4$ each). Enhancement of the magnitude and duration of EFS-induced relaxations were observed in the presence of the PDE5 inhibitors. Nitroergic duration was determined as the time elapsed from 50% relaxation to 50% recovery.

Fig. 4.

Effect of increasing concentrations of sildenafil (●), tadalafil (■) and vardenafil (○) on the duration of nitroergic relaxations evoked by electrical field stimulation (EFS; 2 Hz, 50 V, 10 s trains every 90 s) in rat anococcygeus muscle strips contracted with carbachol (10 μ mol/L). The duration of EFS-induced responses was determined as the time elapsed from 50% relaxation to 50% recovery. Data are the mean $\pm$ SEM ($n = 4$). * $P < 0.05$ compared with sildenafil and tadalafil; $^{\dagger}P < 0.05$ compared with tadalafil (one-way ANOVA followed by Student–Newman–Keuls' post hoc test).

Fig. 5. Effects of the phosphodiesterase (PDE) 5 inhibitors (a) sildenafil, (b) tadalafil and (c) vardenafil on the contractions induced by electrical field stimulation (EFS) in rat anococcygeus muscle preparations. ($\square$), 0.001 $\mu\text{mol/L}$; (▨), 0.01 $\mu\text{mol/L}$; ($\blacksquare$), 0.1 $\mu\text{mol/L}$; (▤), 1 $\mu\text{mol/L}$. Data were calculated relative to the contraction produced by each frequency of stimulation under control conditions (i.e. in the absence of inhibitor). Data are the mean $\pm\text{SEM}$.

Fig. 6.

Degradation of cGMP in the presence of rat anococcygeus cytosolic extracts in the absence or presence of (a) sildenafil, (b) tadalafil and (c) vardenafil. (●), control; (○), 0.001 $\mu\text{mol/L}$; (■), 0.01 $\mu\text{mol/L}$; (□), 0.1 $\mu\text{mol/L}$. Cyclic GMP (10 $\mu\text{mol/L}$) was incubated in the presence of cytosolic extract (30 μg protein) at 37°C (see Methods) and cyclic nucleotide content (expressed in $\mu\text{mol/L}$) was determined after 0, 5, 10 and 30 min of incubation. Data are the mean $\pm$ SEM ($n = 4$). The dashed line represents the initial concentration of cGMP (10 $\mu\text{mol/L}$) for reference. The pIC_{50} values for sildenafil, tadalafil and vardenafil were 8.33 ± 0.07 , 8.24 ± 0.08 and 8.75 ± 0.08 , respectively.

Fig. 7.

Western blot analysis of phosphodiesterase (PDE) 5 in rat anococcygeus (AcM) and corpus cavernosum (CC). Marks indicate the approximate molecular weight. Tissue extracts were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by western blotting with anti-phosphodiesterase V antibody, as described in the Methods. Blots were detected with an Advance Chemiluminescence Detection Kit ($n = 6$). Summarized data of densitometrical analysis are also represented. Each set of determinations was performed in triplicate. H, human recombinant PDE5. ** $P < 0.01$ compared with AcM (Student's t -test).

Table 1

Potency (pEC_{50}) and maximum response (E_{max}) derived from concentration–response curves to the phosphodiesterase 5 inhibitors sildenafil, tadalafil and vardenafil (0.0001–10 $\mu\text{mol/L}$) in rat anococcygeus muscle strips precontracted with carbachol (10 $\mu\text{mol/L}$)

	Sildenafil		Tadalafil		Vardenafil	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Control	7.72 ± 0.06	85 ± 5	7.69 ± 0.05	84 ± 6	8.11 ± 0.05	95 ± 6
L-NAME	$7.24 \pm 0.06^*$	73 ± 5	$7.21 \pm 0.12^*$	77 ± 4	$7.49 \pm 0.05^*$	97 ± 3
ODQ	$7.21 \pm 0.07^{**}$	74 ± 6	$7.13 \pm 0.08^{**}$	78 ± 5	$7.33 \pm 0.09^{**}$	88 ± 7

Curves were constructed in the absence and presence of the nitric oxide synthesis inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; 100 $\mu\text{mol/L}$; $n = 7$) or the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 $\mu\text{mol/L}$; $n = 7$).

Data are the mean $\pm$ SEM.

* $P < 0.05$,

** $P < 0.01$ compared with the respective control (one-way ANOVA followed by Student–Newman–Keuls’ post hoc test).

Table 2

Percentage increases in nitrenergic duration induced by the phosphodiesterase 5 inhibitors sildenafil, tadalafil and vardenafil (0.1 $\mu\text{mol/L}$)

Frequency (Hz)	Increase in nitrenergic duration (%)		
	Sildenafil	Tadalafil	Vardenafil
1	61 $\pm$ 10	50 $\pm$ 5	85 $\pm$ 10
2	69 $\pm$ 4	60 $\pm$ 9	93 $\pm$ 3 *
4	94 $\pm$ 15	67 $\pm$ 6	107 $\pm$ 10 **
8	123 $\pm$ 16	86 $\pm$ 19	153 $\pm$ 14 **
16	134 $\pm$ 12	97 $\pm$ 13	189 $\pm$ 14 *
32	139 $\pm$ 15	112 $\pm$ 24	216 $\pm$ 18 *

Nitrenergic responses were elicited by electrical field stimulation (EFS) at varying frequencies (1–32 Hz).

Data are the mean $\pm$ SEM ($n = 4$ –5).

* $P < 0.05$ compared with sildenafil and tadalafil (one-way ANOVA);

** $P < 0.05$ compared with tadalafil (Student's t -test).