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SPECIAL REPORT

Inhibition of neuroeffector transmission in human vas deferens by sildenafil

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Sildenafil (0.1–30 μM), a cyclic GMP phosphodiesterase 5 (PDE 5) inhibitor, induced inhibition of electrically evoked contractions of ring segments of human vas deferens from 34 vasectomies. Zaprinast (0.1–100 μM), another PDE 5 inhibitor, and the nitric oxide (NO) donor sodium nitroprusside (SNP) (0.1–100 μM) had no effect on neurogenic contractions. The inhibition induced by sildenafil was not modified by the inhibitor of guanylate cyclase 1H-[1,2,4]oxadiazolo[4,3-a] quinoxaline-1-one (ODQ) (1–30 μM) but it was abolished by the K^+ channel blockers tetraethylammonium (TEA, 1 mM), iberiotoxin (0.1 μM) and charybdotoxin (0.1 μM). Sildenafil, zaprinast and SNP did not affect the contractions induced by noradrenaline. SNP (10 μM) caused elevation of cyclic GMP levels that was potentiated by sildenafil (10 μM) and zaprinast (100 μM). ODQ (10 μM) inhibited the increase in cyclic GMP. Sildenafil inhibits adrenergic neurotransmission in human vas deferens. The inhibition is not related to accumulation of cyclic GMP but is probably due to activation of prejunctional large-conductance Ca^{2+}-activated K^+ channels.
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Abbreviations: cyclic GMP, guanosine 3′5′ cyclic monophosphate; NO, nitric oxide; ODQ, 1-H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one; PDE, phosphodiesterase(s); SIN-1, linsidomine chlorhydrate; SNP, sodium nitroprusside; TEA, tetraethylammonium

Introduction Sildenafil, a selective inhibitor of type 5 cyclic GMP phosphodiesterase (PDE) (Boolell *et al*., 1996; Ballard *et al*., 1998) has proved to be effective in the treatment of erectile dysfunction after oral administration in man (Goldstein *et al*., 1998). The enhancement of penile erection by sildenafil involves potentiation of the NO-stimulated cyclic GMP signal mediating relaxation of both cavernosal (Ballard *et al*., 1998) and penile vessels (Medina *et al*., 2000a) smooth muscle. We have recently reported that sildenafil potentiates SNP-induced relaxation in human coronary, internal mammary and radial arteries and in forearm vein (cephalic) obtained from multiorgan donors (Medina *et al*., 2000b). In addition to enhance the NO-stimulated cyclic GMP signal, sildenafil may cause relaxation of human vessels through mechanisms independent of NO formation, which include inhibition of sympathetic contraction and smooth muscle relaxation. Immunohistochemical studies have revealed the presence of nitric oxide (NO) synthase, the enzyme responsible for the formation of NO, within nerve terminals supplying the human vas deferens (Jen *et al*., 1999). Thus the possibility exists that sildenafil could enhance the NO-cyclic GMP signal and influence vas deferens motility by attenuating sympathetic contraction. However, a role for NO in modulation of excitatory neurotransmission in the human vas deferens has not yet been investigated. The present work was designed to investigate the effects of sildenafil given alone or in combination with the NO donor SNP on nerve-and noradrenaline-induced contractions in human vas deferens. The PDE 5 inhibitor zaprinast (Beavo & Reifsnyder, 1990) was included in the study for comparison.

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Methods Segments (15–20 mm long) of the epididymal part of the vas deferens were taken from 34 men (aged 31–48 years) who were sterilized by elective vasectomy. The study was approved by the Ethics Committee of our institution and informed consent was obtained from each subject before the study. The specimens were placed in chilled isotonic NaCl, and were divided into ring preparations 3–4 mm long.

Organ-bath experiments Ring preparations were suspended between two L-shaped stainless steel pins. Changes in isometric force were recorded on a Macintosh computer by use of Chart *vs* 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, Hastings, East Sussex, U.K.). Each preparation was set up in a 4-ml bath containing modified Krebs-Henseleit solution of the following composition (mM): NaCl 115, KCl 4.6, $MgCl_2$ $6H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1 and disodium EDTA 0.01. The solution was equilibrated with 95% O_2 and 5% CO_2. The preparations were allowed to equilibrate for 2 h and during this time tension was adjusted to a final tension of 20 mN.

Tissues were stimulated electrically *via* two platinum electrodes positioned on each side and parallel to the axis of the ring. Single square wave pulses (20 V, 0.25 ms pulse duration, 20 Hz) were used. The train duration was 5 s and the stimulation interval 180 s. The stimulation parameters used elicit contractile responses that are abolished by tetrodotoxin (1 μM) or prazosin (1 μM) (Medina *et al*., 1998).

When electrically induced phasic contractions were stable (after 10–20 min) the cyclic GMP-PDE 5 inhibitors sildenafil (0.1–30 μM) and zaprinast (0.1–100 μM), the inhibitor of guanylate cyclase ODQ (1–30 μM), or the NO donors SNP (0.01–10 μM) and SIN-1 (0.1–100 μM), were added cumulatively to the preparations and the effects of electrical field stimulation were recorded. In another group of experiments, the preparations were preincubated for 15 min with TEA (1 mM), charybdotoxin (0.1 μM), iberiotoxin (0.1 μM), apamin (1 μM) or glibenclamide (10 μM).

Concentration-response curves to noradrenaline were determined in a cumulative manner in the absence and in the presence of either sildenafil (10 μM), zaprinast (100 μM), SNP (10 μM) or ODQ (10 μM). Control and experimental responses were obtained from separate preparations.

Cyclic GMP measurement Vasa deferentia were placed in Krebs solution and continuously gassed with 95% O_2 plus 5% CO_2 at 37°C. One set of rings was removed at the end of the 60 min equilibration period and served as the untreated controls. The second set of rings was incubated for 20 min with either sildenafil, zaprinast, SNP or ODQ. The effects of sildenafil (1 μM), zaprinast (100 μM) or ODQ (10 μM), on SNP-induced cyclic GMP accumulation were tested by incubating the tissues in the presence of the drug for 20 min prior to the addition of SNP (10 μM). After the incubation period, the specimens were rapidly frozen in liquid nitrogen and stored at −80°C until being homogenized in 1 ml of 10% (w v^{-1}) trichloroacetic acid by mechanical homogenizer. The homogenate was centrifuged at 10,000 × *g* for 5 min and the supernatant was removed and extracted four times with 5 ml of water-saturated diethylether, and the cyclic GMP content was then assayed by using an enzymeimmunoassay kit (Biotrak, Amersham Pharmacia Biotech Ltd., Buckinghamshire, U.K.). The pellet resuspended in 1 ml of 0.5 M NaOH was incubated overnight for the estimation of protein concentration (Lowry *et al*., 1951). All results are expressed as fmol cyclic GMP mg^{-1} protein.

Drugs The following drugs were used: noradrenaline hydrochloride, prazosin hydrochloride, tetrodotoxin, sodium nitroprusside (SNP), zaprinast, tetraethylammonium bromide (TEA), charybdotoxin, iberiotoxin, apamin, glibenclamide (Sigma Chemical Co., St. Louis, MO, U.S.A.), sildenafil hydrochloride (Pfizer Ltd., Sandwich, Kent, U.K.), linsidomine chlorhydrate (SIN-1) (ICN Pharmaceuticals Inc., Costa Mesa, CA, U.S.A.) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Tocris Cookson Ltd. Avonmouth, Bristol, U.K.). All drugs were dissolved in Krebs solution except zaprinast and ODQ, which were dissolved in dimethyl sulphoxide.

Data analysis All values are expressed as means ± s.e.mean. Contractions are reported as absolute values (mN) or as percentages of control responses. pD_2 values (negative logarithm of the molar concentration at which half-maximum contraction occurs) were determined from individual concentration-response curves by non-linear regression analysis. The responses obtained in each subject were averaged to yield a single value. Differences between untreated and treated groups were assessed by two-way analysis of variance (ANOVA). Differences between means were identified by *t*-test. Statistical significance was accepted at $P<0.05$.

Results *Effects of PDE 5 inhibitors* Sildenafil (0.1–30 μM) induced concentration-dependent inhibition of electrically evoked contractions (Figure 1A). In contrast to a 90% inhibition induced by sildenafil, zaprinast (0.1–100 μM) did not modify the contractions (Figure 1A). The inhibition induced by sildenafil was not modified ($P>0.05$) in the presence of the inhibitor of guanylate cyclase ODQ (1–30 μM) (Figure 1B). However, in the presence of TEA (1 mM), iberiotoxin (0.1 μM) or charybdotoxin (0.1 μM) the inhibitory effects of sildenafil were significantly reduced (Figure 1B). Apamin (1 μM) or glibenclamide (10 μM) did not modify the inhibitory effect of sildenafil on neurogenic contractions ($n=5$, results not shown). The NO donors SNP (0.1–10 μM) and SIN-1 (0.1–100 μM), given alone or after pretreatment with 10 μM sildenafil, had no effect on neurogenic contractions ($n=6$ for each NO donor, results not shown).

Cumulative addition of noradrenaline (1–300 μM) induced repetitive phasic, concentration-dependent contractions with a pD_2 value of 5.24 ± 0.06. Pretreatment with sildenafil (10 μM), zaprinast (100 μM), SNP or ODQ (10 μM) did not affect the contractions induced by noradrenaline (Figure 2).

Effects of PDE 5 inhibitors and ODQ on SNP-induced cyclic GMP accumulation SNP (10 μM) caused a significant elevation of cyclic GMP levels (Table 1). Sildenafil (10 μM) or zaprinast (100 μM), which alone did not affect the basal cyclic GMP level, increased cyclic GMP in the presence of SNP (10 μM).

ODQ (10 μM) completely abolished cyclic GMP accumulation in response to SNP, and inhibited the increased cyclic GMP levels induced by SNP together with either sildenafil or zaprinast (Table 1).

Discussion Concentration of cyclic GMP is controlled through the rate of synthesis of soluble guanylate cyclase and the rate of hydrolytic breakdown to guanosine 5′-monophosphate by cyclic nucleotide phosphodiesterases

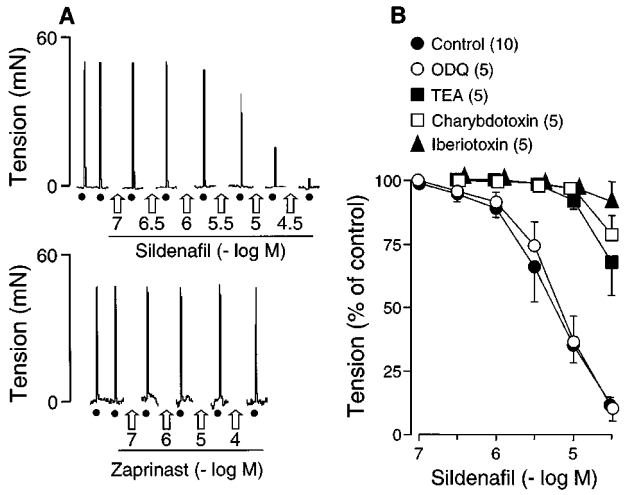

Figure 1 (A) Recordings illustrating the contractile responses to electrical field stimulation (●, 20 Hz) in the absence and in the presence of increasing concentrations of sildenafil or zaprinast. (B) Inhibition of electrical field stimulation-induced contraction by increasing concentrations of sildenafil in the absence (control) and in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM), tetraethylammonium (TEA; 1 mM), charybdotoxin (0.1 μM) and iberiotoxin (0.1 μM). Values in B are shown as the means ± s.e.mean. Numbers in parentheses indicate the number of subjects in each group.

(Beavo, 1995). SNP has been known to evoke relaxation of smooth muscle *via* the release of NO and subsequent elevation of cyclic GMP content (Katsuki *et al*., 1977). In the present experiments, SNP produced an increase in cyclic GMP levels but had no effect on contractions induced by electrical stimulation or noradrenaline. ODQ, a potent and selective inhibitor of guanylate cyclase (Garthwaite *et al*., 1995) did not modify the contractile responses induced by electrical field stimulation but totally inhibited SNP-stimulated cyclic GMP accumulation. Thus the lack of effects of SNP on contractile responses cannot be explained on the basis of failure to elevate cyclic GMP levels.

Both sildenafil and zaprinast, two inhibitors of PDE 5 (Beavo & Reifsnyder, 1990; Boolell *et al*., 1996; Ballard *et al*., 1998), increased SNP-stimulated cyclic GMP accumulation but only sildenafil inhibited neurogenic contractions. Noradrenaline-induced contractions were not modified by sildenafil thus suggesting that the inhibition of neurogenic contractions was due to a prejunctional effect. Moreover, ODQ inhibited the increase in cyclic GMP induced by SNP together with sildenafil, but did not change the inhibitory effects of sildenafil on neurogenic contractions. Therefore, the inhibitory effect of sildenafil on contractions induced by electrical field stimulations seems unlikely to be related to increased formation of cyclic GMP. These results do not support the proposal that the action of sildenafil is only dependent on pre-existing activation of the NO-cyclic GMP system (Ballard *et al*., 1998). On the contrary, we observe that the inhibition of neurogenic contraction induced by sildenafil was similar in the absence and in the presence of a NO donor.

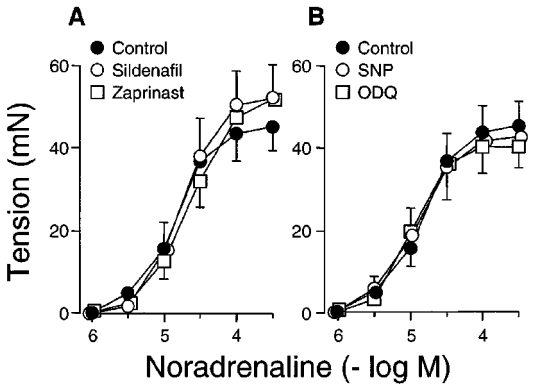

Figure 2 (A) Concentration-response curves to noradrenaline in the absence (control; $n=10$) and in the presence of 10 μM sildenafil ($n=6$) or 100 μM zaprinast ($n=6$). (B) Concentration-response curves to noradrenaline in the absence and in the presence of 10 μM sodium nitroprusside (SNP; $n=6$) or 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; $n=5$). Values are shown as the means$\pm$s.e. mean.

Table 1 Effects of various treatments on basal and sodium nitroprusside (SNP)-stimulated cyclic GMP levels

	cyclic GMP, fmol per mg of protein	
Treatment	*Basal*	*SNP* (1 μM)
None	41$\pm$11 (7)	152$\pm$20* (7)
Sildenafil (10 μM)	50$\pm$12 (7)	332$\pm$55* (6)
Zaprinast (100 μM)	49$\pm$9 (6)	335$\pm$40* (6)
ODQ (10 μM)	42$\pm$11 (6)	40$\pm$12 (6)
+ sildenafil (10 μM)	–	43$\pm$15 (6)
+ zaprinast (100 μM)	–	42$\pm$17 (6)

Results are absolute cyclic GMP levels from individual rings from (n) subjects expressed as means$\pm$s.e.mean. *$P<0.05$ versus rings without treatment on basal conditions.

It has been shown that K^+ channels are involved in the prejunctional inhibitory effects of atrial natriuretic factor in the rabbit isolated vas deferens (Kanwal & Trachte, 1993) and in the α_2-adrenoceptor-mediated inhibition in rat vas deferens (Docherty & Brady, 1995). We tested the hypothesis that sildenafil may exert its neuromodulatory effects by activation of K^+ channels in the human vas deferens. Our results show that glibenclamide, a blocker of ATP-sensitive K^+ channels (Sturgess *et al*., 1985), and apamin, a blocker of small-conductance Ca^{2+}-activated K^+ channels (Murphy & Brayden, 1995), failed to alter the inhibition caused by sildenafil on neurogenic contractions. On the other hand, TEA, a nonspecific K^+ channel blocker (Benham *et al*., 1985), iberiotoxin, an inhibitor of large conductance Ca^{2+}-activated K^+ channels (Galvez *et al*., 1989) and charybdotoxin, an inhibitor of both large and intermediate conductance Ca^{2+}-activated K^+ channels (Garcia *et al*., 1995) inhibited the effects of sildenafil on electrical field stimulation. These results are consistent with the proposal that sildenafil activates the opening of prejunctional K^+ channels to reduce adrenergic neurotransmission. The K^+ channel involved in the effect of sildenafil appears to be a large conductance Ca^{2+}-activated K^+ channel.

In summary, the present results indicate that sildenafil, but not zaprinast, inhibits adrenergic neurotransmission in the human vas deferens. The inhibition of neurogenic contractile responses induced by sildenafil are not related to accumulation of cyclic GMP but are probably due to activation of prejunctional Ca^{2+}-activated K^+ channels sensitive to iberiotoxin and charybdotoxin. Moreover, the modulation by sildenafil of NO-mediated relaxation on corpus cavernosum (Ballard *et al*., 1998) and penile blood vessels (Medina *et al*., 2000a) does not occur in the vas deferens, probably because NO donors do not affect neurotransmission in the human vas deferens. Whether the inhibition of sympathetic contractions by sildenafil represents a diminished vas deferens motility *in vivo* remains speculative. It is of interest to note that sildenafil inhibits K^+ channels at concentrations approximately 15 fold higher than the concentration needed to inhibit sympathetic neurotransmission in penile blood vessels (Medina *et al*., 2000a). A recent report shows that sildenafil can prolong cardiac repolarization by blocking the rapid component of the delayed rectifier potassium current (Geelen *et al*., 2000), an effect that may be seen after drug overdose or in the presence of impaired drug elimination. Thus the effects of sildenfil appear to have tissue selectivity when high concentrations are used.

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