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The phosphodiesterase inhibitory selectivity and the *in vitro* and *in vivo* potency of the new PDE5 inhibitor vardenafil

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We investigated the potency and the selectivity profile of vardenafil on phosphodiesterase (PDEs) enzymes, its ability to modify cGMP metabolism and cause relaxation of penile smooth muscle and its effect on erections *in vivo* under conditions of exogenous nitric oxide (NO) stimulation. PDE isozymes were extracted and purified from human platelets (PDE5) or bovine sources (PDEs 1, 2, 3, 4 and 6). The inhibition of these PDEs and of human recombinant PDEs by vardenafil was determined. The ability to potentiate NO-mediated relaxation and influence cGMP levels in human corpus cavernosum strips was measured *in vitro*, and erection-inducing activity was demonstrated in conscious rabbits after oral administration together with intravenous doses of sodium nitroprusside (SNP). The effects of vardenafil were compared with those of the well-recognized PDE5 inhibitor, sildenafil (values for sildenafil in brackets). Vardenafil specifically inhibited the hydrolysis of cGMP by PDE5 with an IC_{50} of 0.7 nM (6.6 nM). In contrast, the IC_{50} of vardenafil for PDE1 was 180 nM; for PDE6, 11 nM; for PDE2, PDE3 and PDE4, more than 1000 nM. Relative to PDE5, the ratios of the IC_{50} for PDE1 were 257 (60), for PDE6 16 (7.4). Vardenafil significantly enhanced the SNP-induced relaxation of human trabecular smooth muscle at 3 nM (10 nM). Vardenafil also significantly potentiated both ACh-induced and transmural electrical stimulation-induced relaxation of trabecular smooth muscle. The minimum concentration of vardenafil that significantly potentiated SNP-induced cGMP accumulation was 3 nM (30 nM). *In vivo* studies in rabbits showed that orally administered vardenafil dose-dependently potentiated erectile responses to intravenously administered SNP. The minimal effective dose that significantly potentiated erection was 0.1 mg/kg (1 mg/kg). The selectivity for PDE5, the potentiation of NO-induced relaxation and cGMP accumulation in human trabecular smooth muscle and the ability to enhance NO-induced erection *in vivo* indicate that vardenafil has the appropriate properties to be a potential compound for the treatment of erectile dysfunction. Vardenafil was more potent and selective than sildenafil on its inhibitory activity on PDE5. *International Journal of Impotence Research* (2001) **13,** 282–290.

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Introduction

Relaxation of arterial and trabecular smooth muscle is needed to achieve and maintain penile erection.[1] Indeed, vascular diseases in which impaired vasodilator responses have been recognized are commonly associated with the development of erectile dysfunction and impotence.[2–4]

Nitric oxide (NO) is a key mediator of penile smooth muscle relaxation[5–8] and is released by nonadrenergic, noncholinergic nerves within the trabecular and penile arterial tissues as well as the endothelia that line the lacunar spaces and the intima of penile arteries.[5] NO synthase-like immunoreactivity has been identified in nerves and endothelia in corpus cavernosum tissue, and the activity of the NO synthase has been characterized in corpus cavernosum tissue homogenates.[5] Inhibitors of NO synthase inhibit penile erection elicited by the stimulation of the pelvic nerves.[8,9]

NO exerts its relaxing action on corpus cavernosum and penile arteries by activating smooth muscle soluble guanylate cyclase and increasing the intracellular concentration of cGMP.[5,6,10] In second messenger systems which involve cyclic nucleo-

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tides, phosphodiesterases (PDEs) are key regulatory enzymes. Eleven different families of PDE isozymes have been reported,[11] with a specific distribution pattern in different organs and species.[12] The existence and activity of four distinct PDE isozymes (PDE2, PDE3, PDE4 and PDE5) in human corpus cavernosum tissue has been described,[13,14] although mRNAs from PDE1, PDE7, PDE8 and PDE9 have also been detected.[15] Four of these isozymes (PDE1, PDE2, PDE9 and PDE5) being capable of hydrolyzing cGMP, the latter is selective for this cyclic nucleotide at low K_m (Michaelis–Menten constant).[16] Therefore, the inhibition of cGMP-specific PDE could be expected to enhance the NO/cGMP signal specifically during sexual stimulation and thus facilitate penile smooth muscle relaxation.[17]

Furthermore, it has been shown that PDE5 inhibitors potentiate nonadrenergic, noncholinergic nerve-mediated relaxation of trabecular smooth muscle[6,18,19] and enhance penile erection in animal models[20–22] and patients.[23]

A high selectivity of the inhibitors for PDE5 is important since other PDE isozymes also play key regulatory roles in a number of systems and should be not affected. With this purpose, new pharmacological agents more potent and selective for PDE5, such as vardenafil, are being developed to treat erectile dysfunction.

The objectives of this series of experiments were to determine the potency and inhibitory selectivity profile of vardenafil among PDE-isozymes, to evaluate its ability to raise cGMP levels and to relax the smooth muscle in human corpus cavernosum tissue, and to determine its efficacy in causing erections in an animal model.

Methods

Preparation of PDE isozymes

Bovine aorta and bovine heart were obtained freshly from the local slaughter house. All subsequent steps were performed at 2–6°C. Bovine heart was dissected from fat and connective tissue and minced with a commercial meat grinder, suspended 1 : 5 in buffer containing 20 mM K_2HPO_4 pH 6.8; 2 mM EDTA; 2 mM benzamidine; 3 mM 2-mercaptoethanol; 5 μg/ml leupeptin; 5 μg/l antipain; 5 μg/l chymostatin; 2 μg/l trassylol; 0.085 mg/l PMSF. The tissue was homogenized with a Polytron PT 1200 homogenizer (Kinematica AG, Littau, Switzerland). The homogenate was centrifuged for 30 min at 40 000 *g*. The supernatant was recovered and filtered. A similar procedure was used for the preparation of soluble fractions from bovine aorta.

The supernatant of 400 g bovine heart extract was stirred for 2–3 h with 400 ml of DEAE-Sephacel CI (Sigma). The DEAE-Sephacel was allowed to settle, the supernatant was decanted and the DEAE-Sephacel resuspended and stirred for 2 h in 1.5 l of separation buffer (20 mM Tris/HCl, pH 7.2; 2 mM EDTA; 2 mM benzamidine; 3 mM 2-mercaptoethanol). The procedure was repeated with 600 ml of separation buffer. Finally, the DEAE-Sephacel was poured into a column 5 × 30 cm (Pharmacia Ltd, Sweden). The PDE2, PDE3 and PDE4 were eluted in a NaCl gradient (0–0.8 M) in separation buffer at a flow rate of 5 ml/min. Fractions of 4 min were collected and tested for their PDE activity as described below. The fractions comprising the highest level of PDE3 and PDE4 were frozen without further purification and stored at −80°C. Fractions exerting PDE2 activity were pooled, dialyzed with separation buffer and rechromatographed on a 1.5 × 5 cm MONO Q-Sepharose column (Pharmacia Ltd, Sweden). PDE2 was eluted in NaCl gradient (0–0.4 M) in separation buffer at a flow rate of 3 ml/min. Fractions comprising the highest PDE2 activity were frozen and stored at −80°C.

For the isolation of PDE1, the soluble fraction of 200 g bovine aorta was adsorbed to 60 g DEAE-Sephacel and washed twice with 600 ml separation buffer PDE1/5 (20 mM KH_2PO_4, pH 6.8; 2 mM EDTA; 2 mM benzamidine; 5 mM DDT) and poured into a 5 × 30 cm column as described above. PDE1 was eluted in a NaCl gradient (0–0.3 M) in separation buffer at a flow rate of 5 ml/min. Fractions of 2.5 min were collected and tested for their PDE activity as described below. The PDE1-containing fractions were dialyzed against the PDE1 separation buffer and rechromatographed on a 1.5 × 5 cm Q-Sepharose column (Pharmacia Ltd, Sweden). PDE1 was eluted in a linear NaCl gradient at a flow rate of 3 ml/min. The fractions showing the highest PDE1 activity were frozen at −80°C.

PDE5 was isolated from human platelets. Human citrate blood (160 ml) was centrifuged at 126 *g* for 20 min; 56 ml of the supernatant was mixed with 14 ml of 44.8 mM sodium citrate, 20.9 mM citric acid, 74.1 mM glucose. The platelets were spun down at 370 *g* for 10 min and resuspended in 16 ml of 20 mM HEPES, pH 7.2, 0.25 M saccharose, 1 mM EDTA, 1 mM PMSF, sonicated for 60 s (cooled with ice) and centrifuged for 60 min at 75 000 g; 5 ml of the supernatant was applied to a 0.5 × 5 cm MONO Q Cl-column (Pharmacia Ltd), equilibrated with separation buffer A: 20 mM HEPES, pH 7.2 + 1 mM PMSF + 2 mM $MgCl_2$. The column was washed for 20 min with 1 ml/min of separation buffer. PDE5 was eluted in a gradient of NaCl (0–0.5 M) in 75-min fractions of 1 ml. Fractions 45–52 containing PDE5 were frozen in 100-μl aliquots and stored at −80°C.

PDE6 was purified from rod outer segments (ROS) of bovine retinae. Dark adapted ROS were mechanically broken at dim red light buffer A (10 mM Tris/HCl, pH 7.4, 2 mM $MgCl_2$ 120 mM KCl, 2 mM

DTT) and centrifuged at 90 000 *g* (Sorvall SW 28). The membrane pellet containing PDE6 was washed three times with buffer A. PDE6 was eluted from the membrane by incubation in buffer B (10 mM Tris/HCl, pH 7.4, 0.2 mM $MgCl_2$, 2 mM DTT) in daylight. Activation of PDE6 was achieved by mild trypsination for 25 min on ice (PDE6 : Trypsin 10 : 1 w/w). The reaction was stopped by addition of soybean trypsin inhibitor (Sigma). Trypsin-activated PDE6 was purified by anion exchange chromatography on a 0.5 × 5 cm Mono Q column (Pharmacia Ltd) in a linear gradient (0–0.5 M NaCl in buffer B). Fractions containing trypsin-activated PDE6 were frozen in liquid nitrogen and stored at −80°C.

The fractions of the PDE isozymes were characterized based on their substrate specificity, effects of calcium calmodulin, the effect of cGMP on their hydrolytic activity or their inhibitory potency of selective inhibitors. Enzyme fractions exerting stimulation by 1 μM cGMP of at least factor four were classified as PDE2. Fractions inhibited to more than 90% by cGMP and inhibited by milrinone with an IC_{50} of about 0.5 μM were classified as PDE3. PDE4 was selective for cAMP and was inhibited by rolipram with an IC_{50} of 0.3 μM. Fractions hydrolyzing cGMP stimulated by calmodulin for at least 500% were classified as PDE1. Fractions which were inhibited to more than 90% by sildenafil and did not hydrolyze cAMP were classified as PDE5.

Enzyme inhibition

The commercially available ^{3}H-cAMP and ^{3}H-cGMP Scintillation Proximity Assay® (SPA) system from Amersham was used for enzyme inhibition studies. All tests were done in duplicate and were repeated at least three times. For PDE1 assays 3 mM $CaCl_2$ and 0.1 μM calmodulin were added. PDE2 assays were performed with ^{3}H-cAMP as substrate and 1 μM cGMP for the activation of the enzyme. PDE3 and PDE4 assays were done with ^{3}H-cAMP, PDE5 assays with ^{3}H-cGMP as substrate. These compounds were added in DMSO (final concentration of DMSO 0.1%). Incubation period was 15 min at 30°C. This method was used for additional enzyme inhibition assays using human recombinant PDE1C, PDE2A, PDE3B, PDE4B, PDE5A, PDE7B and PDE10, cloned by G Gallo, (BAYER, West Haven, Germany). PDE8 and PDE9 were of murine origin; the cDNAs were kindly provided by JA Beavo. The enzymes were expressed in a baculovirus SF9 system by H Apeler, U Brüggemeier (BAYER, Wuppertal, Germany).

Activity of highly purified trypsin-activated bovine PDE6 was measured using the commercially available Scintillation Proximity Assay system from Amersham, adding unlabelled cGMP to a final concentration of 10 μM. The incubation period was 10 min at 30°C, and vardenafil and sildenafil were added 5 min prior to the addition of the enzyme.

The IC_{50} values for the PDE inhibition were determined from sigmoidal curves, fitted to plots of enzyme activity *vs* log compound concentration using a GraphPad (San Diego, CA, USA) curve fitting program.

Human corpus cavernosum tissues

Human corpus cavernosum strips were obtained from impotent men at the time of penile prosthesis insertion. Tissues were maintained at 4–6°C in M-400 solution (composition per 100 ml: mannitol, 4.19 g; KH_2PO_4, 0.205 g; $K_2HPO_4{\cdot}3H_3O$, 0.97 g; KCl, 0.112 g; $NaHCO_3$, 0.084 g) until used (2–16 h after extraction).[24]

Organ chamber studies

Strips of corpus cavernosum tissue (3 × 3 × 7 mm) were immersed in 8-ml organ chambers containing physiological salt solution (PSS) in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, KH_2PO_4 1.2, EDTA 0.027 at 37°C, continuously gased with 95% O_2/5% CO_2 mixture to maintain a pH of 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 μM phenylephrine.[5,7] Preparations were then exposed to 125 mM K^+ (KPSS, equimolar substitution of NaCl for KCl in PSS). Contraction was elicited by 1 μM phenylephrine, and relaxation responses were evaluated by cumulative additions of compounds to the chambers. Transmural electrical stimulation (TES) was applied by means of two platinum electrodes on each side of the tissue, connected to a stimulator and a current amplifier (Cibertec, Madrid, Spain). Parameters of TES were square pulses of 0.5 ms, during 15 s with the voltage adjusted to obtain a current of 75 mA. Responses to TES were evaluated at increasing frequencies (0.5, 1, 2, 6 and 12 Hz). In all experiments, strips were incubated with atropine (0.1 μM) and guanethidine (10 μM) 45 min before contraction with phenylephrine. Treatments were added 30 min before evaluation of TES-induced responses. Relaxation responses are expressed as percentage of total relaxation (loss in tone) induced by the addition of 0.1 mM papaverine HCl to the chambers at the end of the experiment. All data are expressed as mean ± standard error of the mean (s.e.m.).

Determination of the amount of cyclic GMP in human corpus cavernosum tissue

Corpus cavernosum strips were immersed in 8-ml organ chambers containing physiological salt solution, maintained at 37°C and aerated with 5% CO_2/95% O_2, pH 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 μM phenylephrine. Then each tissue was allowed to incubate for 30 min. After this tissues were treated with drugs or vehicles for 5 min, then immediately frozen in liquid nitrogen and stored at −80°C until extraction for cyclic nucleotide assay. Tissues were extracted by homogenization in 6% trichloroacetic acid, followed by ether (H_2O-saturated) extraction and lyophilization. The amount of cGMP was determined by ELISA using a kit from Cayman Chemical Co. (Ann Arbor, MI, USA).

Protein determinations

Proteins were determined using the Bio-Rad Protein Assay Kit microtiter plate assay procedure (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard.

Measurement of penile erections in a conscious rabbit model

The method has been described elsewhere.[22] In brief, adult male chinchilla rabbits with an average weight of 3.5–4.5 kg were used. The rabbits were housed individually, and food and drink were available *ad libitum* for 2 h in the morning and 2 h in the afternoon. The rabbit penis is not visible when it is not erect.[25] However, when an erection occurs, it is possible to examine the pudendal area and measure the length of uncovered penile mucosa with sliding calipers. For this study, measurements were made at 0, 5, 10, 15, 30, 45, 60, 65, 70, 75, 90, 105 and 120 min after administration of the test compounds, respectively. Mean values of the length of exposed penile mucosa were calculated along with the s.e.m. The mean length was plotted against time, and the area under the curve (AUC) was calculated by an integration program (GraphPad Software, San Diego, CA, USA).

Each dose group consisted of six animals, which were used in a cross-over design for several studies. The time interval between studies was at least eight days. For oral administration, the respective compound was dissolved in a mixture of glycerol : water : polyethylene glycol 6 : 10 : 9.69 and given in a volume of 1 ml/kg by gavage. Controls were performed with corresponding solvents. Sodium nitroprusside (SNP) was dissolved in saline and administered into the animals' ear veins (0.5 ml/kg).

Drugs and materials

Acetylcholine chloride, phenylephrine, guanethidine, and SNP were obtained from Sigma Chemical Co. (St Louis, MO, USA). Atropine sulphate was obtained from Braun (Germany). Sildenafil and vardenafil were provided by BAYER (AG Pharma Chemical Research, Wuppertal, Germany). All drugs were dissolved in deionized water, except sildenafil and vardenafil, which were dissolved at 10 mM concentration in DMSO. The subsequent dilutions were made in deionized water.

Results

Potency and selectivity profile of vardenafil against different PDE isozymes

The IC_{50} values of vardenafil for the different isozymes are shown in Table 1. Vardenafil inhibited PDE5 from human platelets at subnanomolar concentrations, a potency approximately 10-fold higher than that of sildenafil (see Table 1). Vardenafil was 16-fold less potent against PDE6 and showed moderate activity against PDE1 from bovine aorta and very low potency against the PDE2, PDE3 and PDE4 from bovine heart.

The potency of vardenafil in inhibiting PDE5 from bovine tissues and the selectivity profile against other PDEs was confirmed by the data obtained with human recombinant PDEs (Table 2).

Table 1 Selectivity data of vardenafil and sildenafil for PDE5 inhibition

	PDE5	*PDE1*	*PDE2*	*PDE3*	*PDE4*	*PDE6 low [cGMP]*
Vardenafil						
IC_{50} (nM)	0.7	180	> 10000	2500	4000	11
Ratio X/5	1	257	> 10000	3600	5700	16
(*n*)	(12)	(12)	(4)	(4)	(4)	(3)
Sildenafil						
IC_{50} (nM)	6.6	396	> 10000	17000	12000	49
Ratio X/5	1	60	not cal.	2600	1800	7.4
(*n*)	(12)	(12)	(4)	(4)	(4)	(3)

IC_{50} is defined as the concentration of the inhibitor (in nM) required to reduce the cyclic nucleotide hydrolyzing activity of tested PDEs by 50%. The substrate concentrations for the assays were 10 μM cGMP in assays with low [cGMP]. The relative selectivity for PDE5 was determined by calculating the ratio of the IC_{50} for each PDE tested between the IC_{50} for PDE5 (Ratio X/5). *n* Indicates the number of determinations performed.

Table 2 Selectivity data of vardenafil and sildenafil for human recombinant PDE5A inhibition

	PDE5A	*PDE1C*	*PDE2A*	*PDE3B*	*PDE4B*	*PDE7B*	*PDE8*	*PDE9A*	*PDE10*
Vardenafil									
IC_{50} (nM)	0.81	121	> 10000	2680	1910	4600	> 10000	3370	1000
Ratio X/5	1	149	> 10000	3308	2358	5290	> 10000	3870	1150
(*n*)	(6)	(6)	(3)	(3)	(3)	(4)	(3)	(5)	(3)
Sildenafil									
IC_{50} (nM)	12	350	> 10000	> 10000	2900	> 10000	> 10000	> 10000	3800
Ratio X/5	1	29	not cal.	not cal.	242	not cal.	not cal.	not cal.	317
(*n*)	(6)	(6)	(3)	(3)	(3)	(4)	(3)	(5)	(3)

IC_{50} is defined as the concentration of the inhibitor (in nM) required to reduce the cyclic nucleotide hydrolyzing activity of tested PDEs by 50%. The relative selectivity for PDE5 was determined by calculating the ratio of the IC_{50} for each PDE tested between the IC_{50} for PDE5A (Ratio X/5). *n* Indicates the number of determinations performed. All enzymes were human recombinant, except PDE8 and 9 which were of murine origin.

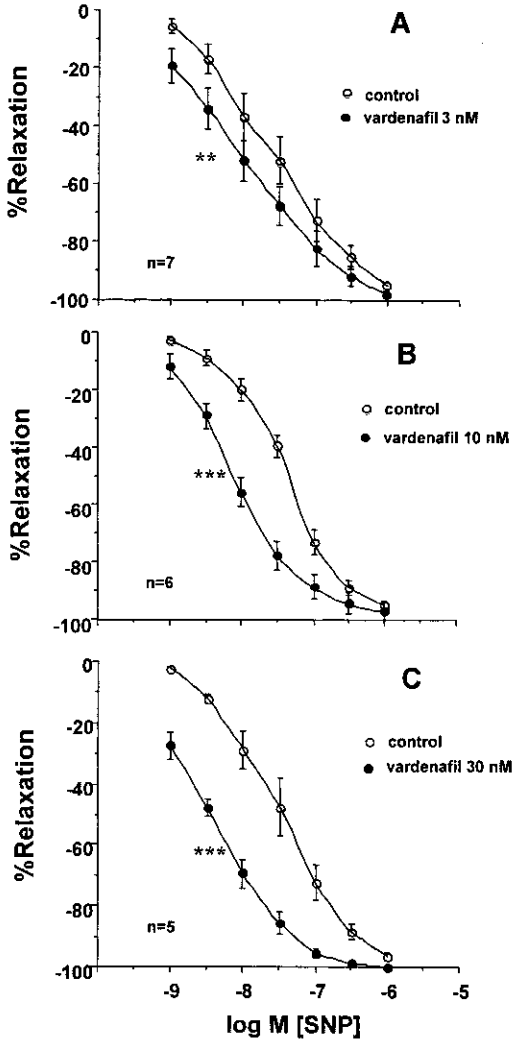

Figure 1 Effects of vardenafil at 3 nM (A), 10 nM (B) and 30 nM (C) concentrations on relaxations induced by sodium nitroprusside (SNP; 1 nM – 1 μM) in human trabecular smooth muscle strips contracted with phenylephrine (1 μM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. **Indicates $P < 0.01$ and ***$P < 0.005$ *vs* control curve by 2-factor ANOVA test.

Effects of vardenafil on SNP-induced relaxation in human trabecular smooth muscle

Vardenafil enhanced the concentration-dependent relaxation response curve to the nitrovasodilator SNP (1 nM to 1 μM) in human trabecular smooth muscle (Figure 1). A significant effect was observed at a concentration of 3 nM with a significant reduction of EC_{50} values for SNP. Sildenafil was effective at 10 nM (Table 3).

Effects of vardenafil on ACh-induced relaxation in human trabecular smooth muscle

Acetylcholine (ACh, 1 nM – 1 mM) caused concentration-dependent relaxations of trabecular smooth muscle contracted with phenylephrine (1 μM). Vehicle treatment did not modify ACh-induced responses (data not shown), whereas vardenafil (10 nM) significantly enhanced relaxant responses to ACh (Figure 2). Vardenafil increased the maximum relaxation induced by 3 μM ACh from 56.7 ± 8.1 to 80.4 ± 6.3%, $P < 0.01$, and reduced the EC_{50} values for ACh from 103.7 ± 26.3 to 32.8 ± 11.3 nM.

Table 3 Effects of vardenafil and sildenafil on EC_{50} values for SNP in human trabecular smooth muscle

	Vehicle	*Sildenafil*	*Vardenafil*	*(n)*
3 nM	1.33 ± 0.34	1.91 ± 0.39	2.90 ± 0.57*	(6)
10 nM	1.75 ± 0.38	3.36 ± 0.53*	6.16 ± 1.29**	(6)
30 nM	1.90 ± 0.33	10.66 ± 3.70*	12.80 ± 3.71*	(5)

Numbers express mean ± s.e.m. of the ratio EC_{50} for SNP obtained in the presence of the treatment by EC_{50} value for SNP obtained in the previous control curve. EC_{50} is defined as the concentration of SNP required to obtain 50% of the maximum relaxation. *n* Indicates the number of patients from whom the tissues were collected. *$P < 0.05$, **$P < 0.01$ *vs* vehicle effect by one-way ANOVA followed by Student-Newman-Keuls *post-hoc* test.

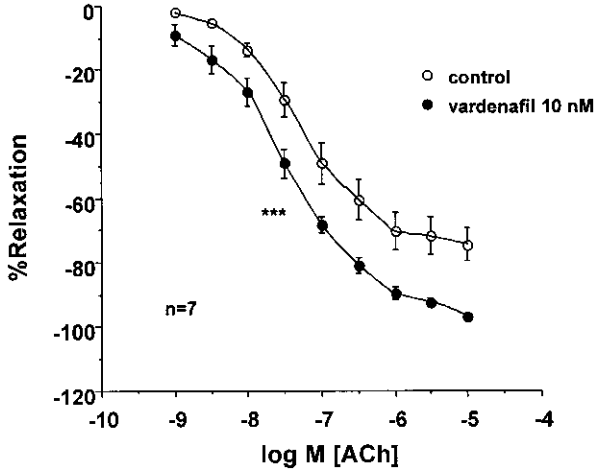

Figure 2 Effects of vardenafil (10 nM) on relaxations induced by acetylcholine (ACh; 1 nM – 3 μM) in human trabecular smooth muscle strips contracted with phenylephrine (1 μM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. ***Indicates $P < 0.005$ *vs* control curve by 2-factor ANOVA test.

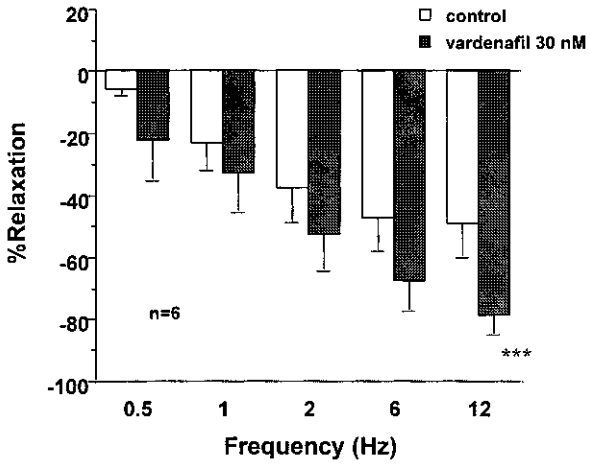

Figure 3 Effects of vardenafil (30 nM) on relaxations induced by transmural electrical stimulation (0.5 – 12 Hz) in human trabecular smooth muscle strips contracted with phenylephrine (1 μM) in the presence of guanethidine (10 μM) and atropine (0.1 μM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. ***Indicates $P < 0.005$ *vs* control curve by 2-factor ANOVA test.

Vardenafil enhances neurogenic responses induced by transmural electrical stimulation

Application of TES on human cavernosal strips contracted with phenylephrine produced frequency-dependent relaxations which were abolished by the treatment with tetrodotoxin (1 μM) and were not affected by the treatment with vehicle (data not shown). Vardenafil (30 nM) significantly potentiated the relaxation of human trabecular smooth muscle to TES (Figure 3).

Increase of cGMP content in human corpus cavernosum tissue

Vardenafil alone significantly augmented the amount of cGMP in corpus cavernosum at a concentration of 30 nM (Figure 4A). SNP alone at 1 μM increased the amount of cGMP about 2-fold. Vardenafil was found to significantly enhance this accumulation at the lowest concentration tested, 3 nM (Figure 4B). Sildenafil at 30 nM also produced a statistically significant potentiation of cGMP accumulation induced by 1 μM SNP, but not at 3 or 10 nM (Figure 4B).

Efficacy of vardenafil in inducing penile erections in the conscious rabbit

Vehicle administered to rabbits (control) did not produce erection. When SNP was given intravenously there was a brief and small response with a mean increment in penile length of 5 mm (Figure 5). When vardenafil was administered orally, there was no response for the first hour for the 0.3 mg/kg dose, but a small reponse was observed at the 1 mg/kg dose. After 60 min, when SNP was given intravenously, there was an immediate potentiation of the SNP effect, producing an erection with a maximum length of 20 mm for the 1 mg/kg dose (Figure 5A). The time to onset of action was similar to that of SNP alone. When erectile responses were expressed as AUC (mm × min), the effects induced by SNP were significantly enhanced by vardenafil at all tested doses. Sildenafil also potentiated the erectile response to SNP at the oral dose of 1 mg/kg, but not at lower doses (Figure 6).

Discussion

Inhibition of PDE5 activity in human penile smooth muscle is able to treat many men with erectile dysfunction.[26] The benefit of PDE5 inhibition is from the enhancement of the increase of cGMP concentration caused by the action of NO, which is released from nonadrenergic, noncholinergic nerves during sexual arousal and also from the endothelium of penile arteries and lacunar spaces,[5] further facilitating smooth muscle relaxation[6,7] and penile erection.[8,19] Impaired relaxation of penile smooth muscle results in erectile dysfunction.[3] Indeed, pathophysiological studies have shown an impairment of neurogenic and endothelium-dependent

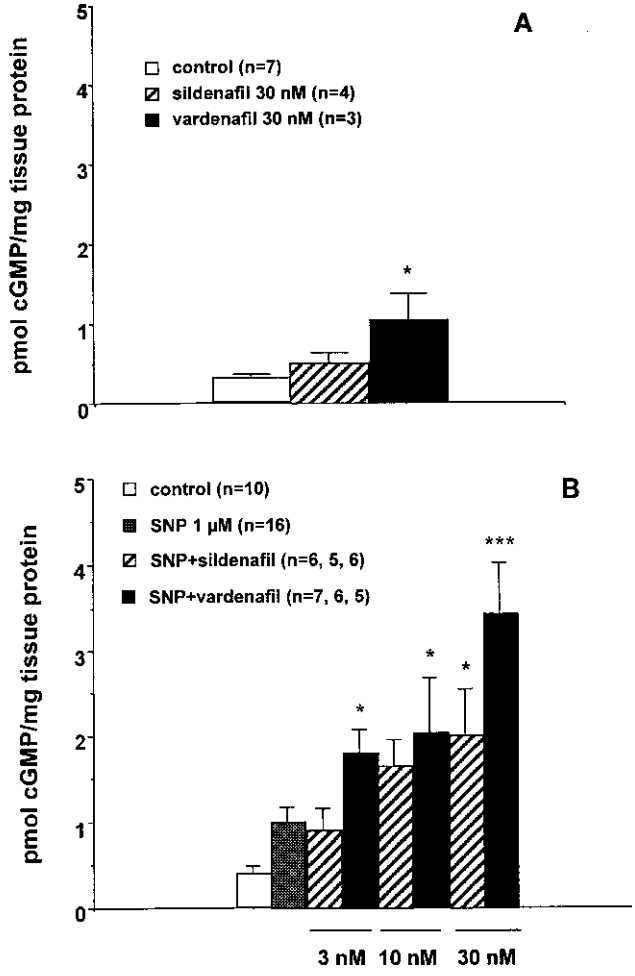

Figure 4 Effects of vardenafil (30 nM) and sildenafil (30 nM) on the cGMP tissue content of human corpus cavernosum tissue (A), and effects of vardenafil (3, 10 and 30 nM) and sildenafil (3, 10 and 30 nM) on the increase of cGMP levels in this tissue induced by sodium nitroprusside (SNP; 1 μM) (B). Data are expressed as mean ± s.e.m. of pmol cGMP per mg of tissue protein content. *n* Indicates the number of patients from whom the tissues were collected for the experiments. In A, *Indicates $P < 0.05$ *vs* control, and in B, *Indicates $P < 0.05$, **$P < 0.01$ and ***$P < 0.005$ *vs* SNP 1 μM by one-way ANOVA followed by student-Newman-Keuls *post-hoc* test.

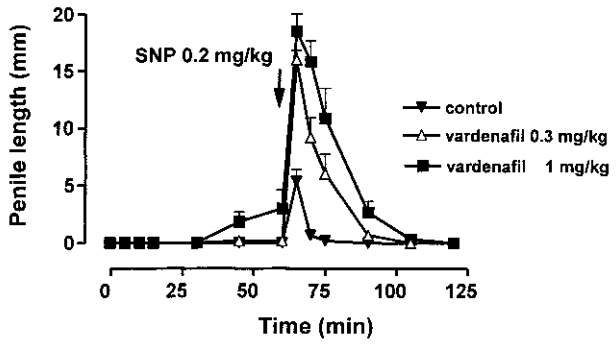

Figure 5 Effects of orally administered vardenafil (0.3 and 1 mg/kg) on the time course of erectile responses in conscious rabbits. Sixty minutes later, sodium nitroprusside (SNP; 0.2 mg/kg) was injected intravenously. For comparison, the response to SNP alone is shown. The length of uncovered penile mucosa was measured at the indicated times. Values are mean ± s.e.m., $n = 6$.

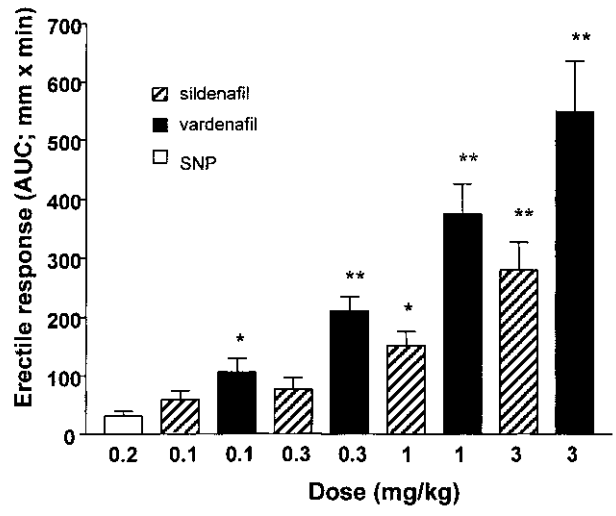

Figure 6 Erectile response after oral administration of sildenafil and vardenafil in the indicated doses, followed by an injection of 0.2 mg/kg of SNP, was expressed as the product of the time (min) and the length of the uncovered penile mucosa (mm), representing the area under the curve (AUC). The period of time 0–120 min was used for the calculation. Data are expressed as mean ± s.e.m., $n = 6$. *$P < 0.05$. **$P < 0.01$ *vs* SNP by one-way ANOVA followed by Student-Newman-Keuls *post-hoc* test.

relaxations of penile smooth muscle in disease with a high prevalence of erectile dysfunction such as diabetes, hypercholesterolemia and atherosclerosis, in animal models and in patients.[4,27,28]

Vardenafil is a new PDE5 inhibitor currently under clinical development. It has been shown to be a well tolerated[29,30] and effective oral treatment for male erectile dysfunction in preliminary studies.[30–33] In this study, we have characterized the activity of vardenafil *in vitro* and *in vivo* and compared it with that of sildenafil, which has been shown previously to potentiate NO-mediated relaxation of trabecular smooth muscle[19] and to enhance penile erections in animal models[21] and patients.[22]

According to these studies, vardenafil is a highly selective PDE5 inhibitor with a potency about 10-fold higher than that of sidenafil. The selective effect exerted by vardenafil on PDE5 activity is important to minimize possible side effects at the estimated therapeutic doses for the treatment of erectile dysfunction. The closest IC_{50} values were for PDE1 and PDE6. The other PDEs were all minimally affected by vardenafil, with IC_{50} values greater than 1000-fold more than for PDE5. Vardenafil has over 200-fold less effect on PDE1 compared with PDE5. Assays with PDE6 using a cGMP concentration of

10 μM were performed because of the difference in the IC_{50} values for sildenafil obtained under the different conditions in previously published studies.[19,34] Independent of the different substrate concentrations, vardenafil was 2-fold more selective than sildenafil. The potency and selectivity for PDE5 is similar in inhibition assays with tissue extracted enzymes and in those with human recombinant enzymes.

According to its mechanism of action as a PDE5 inhibitor, vardenafil was capable of amplifying relaxant responses elicited by agents that stimulate the NO/cGMP pathway. Vardenafil enhanced the relaxation of human trabecular smooth muscle produced by SNP, an exogenous source of NO, showing efficacy at concentrations as low as 3 nM. Also vardenafil enhanced neurogenic relaxation and endothelium-dependent ACh-induced relaxation of cavernosal strips.

The results in functional analysis of smooth muscle relaxation had further confirmation in the data from the determination of cyclic nucleotide content in cavernosal tissue, where 3 nM vardenafil was able to significantly enhance the increase in cGMP level. A 10-fold higher concentration of sildenafil was necessary to produce a similar effect.

Taken together, these results demonstrate that vardenafil is an efficient enhancer of the NO-mediated relaxation of human trabecular smooth muscle, probably by increasing cGMP accumulation in this tissue. Of note, the potentiation of NO-mediated relaxation in penile smooth muscle is produced at concentrations of vardenafil below the free plasma levels of this molecule in man after oral administration of 20 mg.[29,30] Potentiation of the NO/cGMP pathway by vardenafil has clinical relevance because it could allow a more effective penile smooth muscle relaxation when active release of NO is produced in response to sexual arousal.

The results observed in human trabecular smooth muscle have a correlation with results obtained *in vivo* using a conscious rabbit model to determine erectile responses. Oral vardenafil is efficacious in improving penile erections induced by SNP, and the effective doses are in the range of doses which are under clinical investigation. In this model, the higher potency vardenafil with respect to sildenafil in this animal model may be explained by its higher efficacy in increasing cGMP content and NO-mediated relaxation of trabecular smooth muscle.

In conclusion, our studies clearly demonstrate that vardenafil is a potent and selective inhibitor of PDE5. Vardenafil enhances penile smooth muscle relaxation induced by exogenous and endogenous NO. Furthermore, when orally administered, vardenafil enhances potently NO-mediated penile erection in an animal model. Vardenafil is consistently more potent and also more selective for PDE5 than sildenafil. These data suggest that vardenafil may have the biochemical and physiological characteristics necessary for treating erectile dysfunction. In Phase II trials the clinical efficacy of vardenafil in treatment of patients with erectile dysfunction has been demonstrated.[30–33]

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Note added in proof

After the acceptance of this manuscript, vardenafils selectivity against human recombinant PDE11A has been determined with the same methods which were used for other PDE isoenzymes. The selectivity ratio IC_{50} for PDE11A/IC_{50} for PDE5A is 346.

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