BJP British Journal of Pharmacology

DOI:10.1111/bph.12143
www.brjpharmacol.org

RESEARCH PAPER

# $Ca^{2+}$-activated $K^+$ channel ($K_{Ca}$) stimulation improves relaxant capacity of PDE5 inhibitors in human penile arteries and recovers the reduced efficacy of PDE5 inhibition in diabetic erectile dysfunction

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**Keywords**
calcium-activated potassium channels; PDE5; erectile dysfunction; diabetes; human penile resistance arteries; human corpus cavernosum; NS-8; NS1619; sildenafil

**Received**
15 November 2012
**Revised**
31 January 2013
**Accepted**
17 February 2013

## BACKGROUND AND PURPOSE

We have evaluated the influence of calcium-activated potassium channels ($K_{Ca}$) activation on cGMP-mediated relaxation in human penile tissues from non-diabetic and diabetic patients, and on the effects of PDE5 inhibitors on erectile responses in control and diabetic rats.

## EXPERIMENTAL APPROACH

Cavernosal tissues were collected from organ donors and from patients with erectile dysfunction (ED). Relaxations of corpus cavernosum strips (HCC) and penile resistance arteries (HPRA) obtained from these specimens were evaluated. Intracavernosal pressure (ICP) increases to cavernosal nerve electrical stimulation were determined in anaesthetized diabetic and non-diabetic rats.

## KEY RESULTS

Concentration-dependent vasodilation to the PDE5 inhibitor, sildenafil, in HPRA was sensitive to endothelium removal, NO/cGMP pathway inhibition and $K_{Ca}$ blockade. Accordingly, activation of $K_{Ca}$ with NS-8 (10 μM) significantly potentiated sildenafil-induced relaxations in HPRA ($EC_{50}$ 0.49 ± 0.22 vs. 5.21 ± 0.63 μM). In HCC, sildenafil-induced relaxation was unaffected by $K_{Ca}$ blockade or activation. Potentiating effects in HPRA were reproduced with an alternative PDE5 inhibitor (tadalafil) and $K_{Ca}$ activator (NS1619) and prevented by removing the endothelium. Large-conductance $K_{Ca}$ (BK) and intermediate-conductance $K_{Ca}$ (IK) contribute to NS-8-induced effects and were immunodetected in human and rat penile arteries. NS-8 potentiated sildenafil-induced enhancement of erectile responses in rats. Activation of $K_{Ca}$ recovered the impaired relaxation to sildenafil in diabetic HPRA while sildenafil completely reversed diabetes-induced ED in rats only when combined with $K_{Ca}$ activation.

## CONCLUSIONS AND IMPLICATIONS

Activation of $K_{Ca}$ improves vasodilatory capacity of PDE5 inhibitors in diabetic and non-diabetic HPRA, resulting in the recovery of erectile function in diabetic rats. These results suggest a therapeutic potential for $K_{Ca}$ activation in diabetic ED.

### Abbreviations

APA, apamin; BK, large-conductance $K_{Ca}$; ChTx, charybdotoxin; DMSO, dimethylsulfoxide; ED, erectile dysfunction; HCC, human corpus cavernosum; HPRA, human penile resistance arteries; HPβCD, hydroxy-propyl-β-cyclodextrin; IbTx, iberiotoxin; ICP, intracavernosal pressure; IK, intermediate-conductance $K_{Ca}$; $K_{ATP}$, ATP-sensitive $K^+$ channels; KPSS, equimolar substitution of NaCl for KCl in physiological salt solution; SK, small-conductance $K_{Ca}$

## Introduction

Vasodilation of penile arteries and relaxation of trabecular smooth muscle are required to achieve and maintain penile erection. NO is a key mediator of penile smooth muscle relaxation. Released from endothelia and nitrergic nerves, NO stimulates soluble guanylyl cyclase in smooth muscle, promoting cGMP generation. In turn, cGMP activates cGMP-dependent protein kinase (cGK) which induces smooth muscle relaxation acting on different intracellular mediators (Gratzke *et al.*, 2010). In addition, cGMP can directly act on specific ion channels in the cell membrane also facilitating relaxation of smooth muscle (Morgado *et al.*, 2012). PDE5 is the main cGMP-hydrolysing enzyme in human penile tissue. Inhibitors of PDE5 are the first-line therapy for the treatment of erectile dysfunction (ED). Despite the fact that these drugs display a high efficacy to overcome ED in general population (Goldstein *et al.*, 1998; Padma-Nathan *et al.*, 2001; Porst *et al.*, 2001), some specific groups of patients, such as diabetic men, are not responsive to this treatment in a significant percentage (Rendell *et al.*, 1999; Sáenz de Tejada *et al.*, 2002; Goldstein *et al.*, 2003). Thus, alternative pharmacological strategies for improving the efficacy of available treatments for diabetic ED would be required.

Hyperpolarization of smooth muscle cell also elicits relaxation by closing calcium channels and blocking calcium entry. Opening of potassium channels is a way to cause hyperpolarization of smooth muscle. There are a large number of different potassium channels that are grouped into several families with distinct characteristics. These include inwardly rectifying ($K_{IR}$), voltage-dependent ($K_v$), ATP-sensitive ($K_{ATP}$) and $Ca^{2+}$-activated potassium channels ($K_{Ca}$). This latter family comprises three subtypes with different conductance capacity: large-conductance $K_{Ca}$ (BK; MaxiK), small-conductance $K_{Ca}$ (SK) and intermediate-conductance $K_{Ca}$ (IK). $K_{ATP}$ and $K_{Ca}$ have been demonstrated at the functional level in human corpus cavernosum (HCC) smooth muscle cells (Christ *et al.*, 1993). Openers of $K_{ATP}$ have been shown to be potent relaxant agents in vascular and trabecular penile tissues (Venkateswarlu *et al.*, 2002; Ruiz-Rubio *et al.*, 2004) but activation of $K_{ATP}$ is known to cause hypotension as well (Mannhold, 2004). Activators of $K_{Ca}$ also have the capacity to relax human and animal penile smooth muscles (Spektor *et al.*, 2002; Hewawasam *et al.*, 2004). In addition, this type of channels has a key role in endothelium-derived hyperpolarizing factor-mediated relaxation of human penile arteries (Angulo *et al.*, 2003). Furthermore, the delivery of the gene for human BK (hSlo) into the penis has been shown to increase erectile responses in aged rats (Melman *et al.*, 2003) and to recover erectile function in diabetic rats (Christ *et al.*, 2004). The relevance of these channels in the physiology of erectile function is highlighted by the fact that mice lacking BK have ED (Werner *et al.*, 2005).

The aims of the present study were to analyse the effects of $K_{Ca}$ activation on relaxation induced by PDE5 inhibition in human penile arterial and trabecular tissues from non-diabetic and diabetic patients, as well as to evaluate the influence of $K_{Ca}$ stimulation on the efficacy of PDE5 inhibition to potentiate *in vivo* erectile responses in non-diabetic and diabetic rats.

## Methods

### *Human penile tissues*

Human penile tissue biopsies were obtained from 54 patients in Hospitals from Spain and Portugal. Tissues were obtained from men with ED who gave informed consent at the time of penile prosthesis insertion. Patients with infectious diseases or undergoing prosthesis re-implantation were excluded. Mean age was 56.7 ± 1.2 years (range from 38 to 76 years). Two or more cardiovascular risk factors (CVRFs) were present in 32.6% of the patients while 48.8% had one CVRF and 18.6% did not present any CVRF (ED of neurogenic aetiology). Nineteen patients had type 2 diabetes (35.2%). Hypertension was present in 29 patients (53.7%), hyperlipidaemia in 19 patients (35.2%) and 18 patients were smokers (33.3%). For the control of glycaemia, 11 diabetic patients were treated with insulin while 4 used hypoglycemiants and 2 were controlled by diet. Medication for the treatment of hypertension included angiotensin II type 1 receptor antagonists, ACE inhibitors, calcium antagonists, beta-blockers and diuretics. Almost half of hyperlipidaemic patients did not take any hypolipidemiant drug while the remaining patients were treated with statins. The study was approved by the local ethics committees of the hospitals where the tissues were collected (NC-009–2010). 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; $K_2HPO_4{\cdot}3H_2O$, 0.97 g; KCl, 0.112 g; $NaHCO_3$, 0.084 g) until used within 24 h from extraction (Angulo *et al.*, 2002).

### *Vascular reactivity of human penile resistance arteries*

Penile small arteries, helicine arteries (lumen diameter 150–400 μm), which are the terminal branches of deep penile

arteries, were dissected from human penile tissue specimens by carefully removing the adhering trabecular tissue, and arterial ring segments (2 mm long) were subsequently mounted on two 40 μm wires on microvascular Halpern-Mulvany myographs (J.P. Trading, Aarhus, Denmark) for isometric tension recordings. The vessels were allowed to equilibrate for 30 min in physiological salt solution of the following composition (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 bubbled with 95% $O_2$/5% $CO_2$ mixture to maintain a pH of 7.4. Passive tension and internal circumference of vascular segments when relaxed *in situ* under a transmural pressure of 100 mmHg ($L_{100}$), were determined. The arteries were then set to an internal circumference equivalent to 90% of $L_{100}$, at which the force development was close to maximal (Mulvany and Halpern, 1977). The preparations were then exposed to 120 mM $K^+$ (KPSS, equimolar substitution of NaCl for KCl in physiological salt solution) and the contractile response was measured. The arteries were contracted with 1 μM norepinephrine (80% of KPSS induced contraction, approximately) and relaxation responses were evaluated by cumulative additions of compounds to the chambers. Experiments were run in parallel. Concentration-response curves to the agents in arterial segments from the same patient receiving only vehicle (0.001% DMSO) were considered as controls for the evaluation of the effects of the different treatments.

Where stated, the endothelia from arterial segments were mechanically removed by repeatedly introducing a human hair by the lumen of the artery. The absence of a functional endothelium was confirmed by the lack of relaxant response to 10 μM ACh.

## Experiments with human corpus cavernosum tissue

Strips of corpus cavernosum tissue (3 × 3 × 7 mm) obtained from human penile tissue specimens 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. The preparations were then exposed to KPSS and the contractile response was measured. After an equilibration period, tissues were contracted with 0.5–3 μM phenylephrine (80% of KPSS induced contraction) and relaxation responses were evaluated by cumulative additions of compounds to the chambers. Experiments were done in parallel as described for human penile arteries.

## Experimental animals

Studies were performed in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by National Institutes of Health and were approved by the local Ethics Committee for Animal Welfare of the Hospital Universitario Ramón y Cajal (Exp. 11/2010). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total number of 60 male Sprague-Dawley rats under 12 h light/dark cycles with free access to food and water were used. Insulin-dependent diabetes was induced in 12-week-old male rats by a single administration of streptozotocin (60 mg·kg$^{-1}$; i.p.) dissolved in citric acid-trisodium citrate (0.1 M) buffer, pH 4.5. After one week, tail blood samples were obtained and glucose concentration was measured using an Accutrend glucometer (Boehringer Mannheim, Germany). Diabetes induction was considered successful when glycaemia was higher than 11 mM. Control non-diabetic animals were injected with citrate buffer solution and kept under identical conditions as diabetic animals. The experiments were performed 8 weeks after diabetes induction. *In vivo* alterations of vascular and erectile function have been observed after this period of diabetes (Angulo *et al.*, 1998; 2005). After evaluation of erectile responses rats were killed by an anaesthetic overdose.

## Erectile responses to cavernosal nerve stimulation in anaesthetized rats

Rats were anaesthetized with ketamine (60 mg·kg$^{-1}$) and diazepam (4 mg·kg$^{-1}$). The surgical procedure consisted of dissection and isolation of the right cavernous nerve through an abdominal midline incision and exposure of penile crura through a transverse perineal incision. Intracavernosal pressure (ICP) measurements were accomplished by insertion into the right crus of a 25-gauge needle connected to a disposable pressure transducer (Abbott, Sligo, Ireland) and a data acquisition system (ADInstruments, Castle Hill, Australia). Left carotid artery and right external jugular vein were catheterized for constant BP measurement and saline or drug infusion respectively. Electrical stimulation was applied by a delicate platinum bipolar hook electrode connected to a stimulator and current amplifier (Cibertec CS-9, Madrid, Spain). Parameters of electrical stimulation consisted of pulses with a duration of 1 ms and 1.5 mA of current intensity for 1 min. Frequency-response curves were performed by applying stimulation at 1, 3 and 10 Hz at 3 min intervals.

For evaluation of the acute effects of the treatments on erectile responses, a control frequency-response curve was performed and, after a stabilization period, sildenafil (0.1 mg·kg$^{-1}$ or 0.3 mg·kg$^{-1}$) or the vehicle [15% hydroxypropyl-β-cyclodextrin (HPβCD)] were intravenously administered. Thirty minutes later, NS-8 (0.3 mg·kg$^{-1}$), NS1619 (0.3 mg·kg$^{-1}$) or the vehicle (5% DMSO + 15% HPβCD) was intravenously administered, and the erectile responses were evaluated 15 min afterwards. Injection volume was 0.2 mL.

## Immunostaining

Human and rat corpus cavernosum specimens were fixed in 4% paraformaldehyde and included in paraffin blocks. Deparaffinized tissue sections were incubated in blocking buffer (0.1 M PBS containing 5% normal goat serum, 0.1% Triton X-100) for 60 min, and thereafter incubated overnight with rabbit polyclonal antibodies against human and rat pore forming α-subunit of $K_{Ca}$: anti-BK (peptide (C)STANRPNRPK-SRESRDK, corresponding to amino acid residues 1184–1200 in intracellular, C-terminal part), anti-IK (M20, N-terminal peptide GGDLVLGLGALRRRKC), anti-SK1 (L155, C-terminal peptide CSSPYRWTPVAPSDYG for human SK1 and rb200, peptide CSPQSHWLPTTASDYG for rat SK1),

anti-SK2 (M1, N-terminal peptide CRRSSSTAPPTSSESS) and anti-SK3 (M75, C-terminal peptide DTSGHFHDSGVGDLDC). Anti-BK and anti-IK antibodies were used at 1:50 dilution while anti-SK antibodies were used at 1:20 dilution. Anti-BK antibody was obtained from Alomone Labs (Jerusalem, Israel). The other antibodies were described previously (Chen *et al.*, 2004). The endogenous peroxidase was inactivated with hydrogen peroxide (0.03% in methanol). For immunodetection, tissues were incubated with biotin-goat anti-rabbit IgG (Zymed, San Francisco, CA, USA), 3-3-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) was used as chromagen. Controls without primary antibodies demonstrated lack of unspecific reactivity (data not shown).

## *Drugs and materials*

Phenylephrine, norepinephrine (arterenol), ACh, $N^G$-nitro-L-arginine (L-NNA), 8-bromo cyclic guanosin monophosphate, charybdotoxin (ChTx), apamin (APA), 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS1619), streptozotocin and HPβCD were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1*H*-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Iberiotoxin (IbTx) was obtained from Alomone Labs. 5[(2-chlorophenyl)(diphenyl)methyl]-1*H*-pyrazole (TRAM-34) was provided by GlaxoSmithKline (Stevenage, UK). 2-amino-5-(2-fluorophenyl)-4-methyl-1*H*-pyrrole-3-carbonitrile (NS-8) was provided by Dr. Esteve Laboratories (Barcelona, Spain). Sildenafil was a gift from Nitromed (Bedford, MA, USA) while tadalafil was provided by ICOS Corporation (Seattle, WA, USA). For *in vitro* experiments, all drugs were dissolved in deionized water, except for NS-8, NS1619 (Malysz *et al.*, 2004), sildenafil (Angulo *et al.*, 2010a) and tadalafil (Angulo *et al.*, 2012), which were dissolved at 10 mM concentration in dimethylsulfoxide (DMSO). The subsequent dilutions were made in deionized water. Final DMSO concentrations were 0.1% or lower.

## *Data analysis*

Relaxation responses are expressed as the percentage of total relaxation (loss in tone) induced by the addition of 0.1 mM papaverine to the chambers at the end of the experiment. $EC_{50}$ is defined as the concentration of sildenafil required to obtain 50% of maximal relaxation. It was graphically calculated for each individual curve. All data are expressed as mean ± standard error. Erectile responses were determined by measuring the AUC of the ICP increases (Total ΔICP) to rat cavernosal nerve stimulation normalized by mean arterial pressure values at the time of each stimulation. Complete concentration-response or frequency-response curves were obtained and compared by a two-factor ANOVA statistical test using StatView software for Apple computers (SAS, Cary, NC, USA).

# Results

## *Activation of $K_{Ca}$ causes relaxation of human penile resistance arteries and human corpus cavernosum*

Cumulative addition of $K_{Ca}$ activators NS-8 and NS1619 (1 nM to 10 μM) resulted in moderate vasodilation of human penile resistance arteries (HPRA) contracted with norepinephrine. Vasodilatory capacity of $K_{Ca}$ activators was significantly reduced when endothelium was mechanically removed in HPRA preparations (Figure 1A and B). Moderate relaxation was also induced in HCC strips contracted with phenylephrine by exposure to NS-8 and NS1619 (1 nM to 30 μM; Figure 1C and B). This relaxation was similar in magnitude to that observed in HPRA.

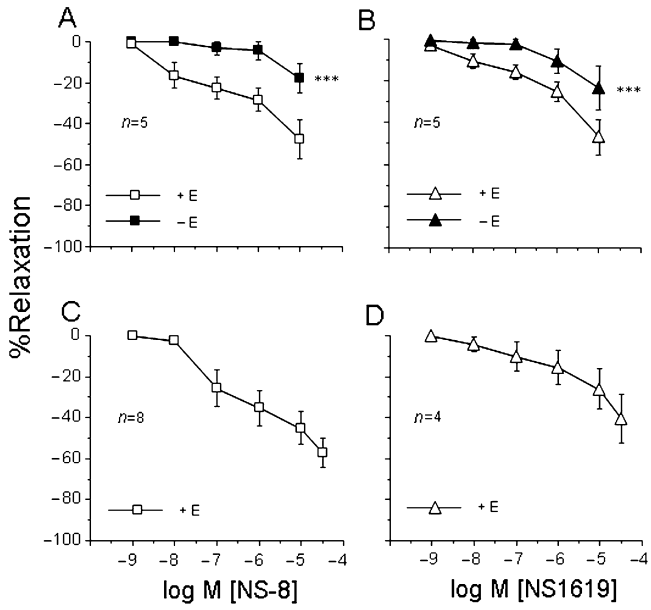
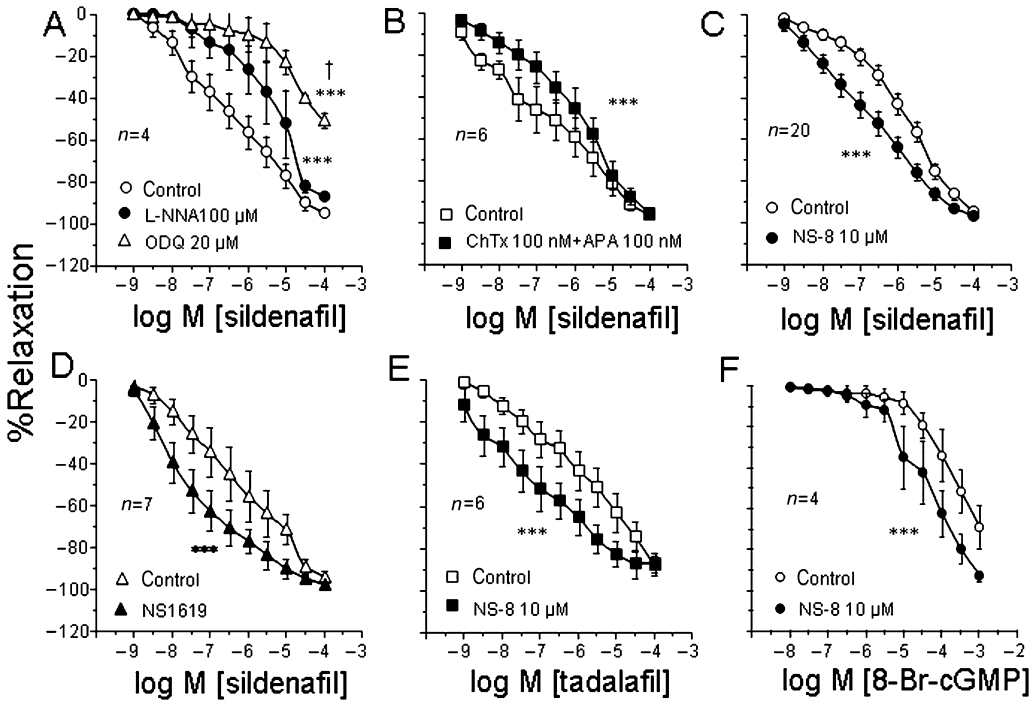



**Figure 1**

Vasodilation of norepinephrine-contracted HPRA (A and B) and relaxation of phenylephrine-contracted HCC (B and C) by addition of the $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) activators, NS-8 (A and C) and NS1619 (B and D). The effects of endothelium removal (-E) on vasodilations caused by NS-8 and NS1619 in HPRA are also shown in panels A and B respectively. Data are expressed as mean ± SEM 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. All data sets included two diabetic patients. *** indicates $P < 0.001$ versus control, $^{\dagger}P < 0.05$ versus L-NNA by a two-factor ANOVA test.

## *$K_{Ca}$ modulation influences relaxant capacity of PDE5 inhibitors in human penile resistance arteries but not in human corpus cavernosum*

The PDE5 inhibitor, sildenafil, caused concentration-dependent relaxation of HPRA contracted with norepinephrine. Sildenafil-induced vasodilations were inhibited by treating the arterial segments with the NO synthase inhibitor, $N^G$-nitro-L-arginine (L-NNA; 100 μM) or by adding an inhibitor of soluble guanylyl cyclase, ODQ (20 μM; Figure 2A), as well as by blocking calcium-activated potassium channels ($K_{Ca}$) with a combination of ChTx (100 nM) and APA (100 nM; Figure 2B). Conversely, activation of $K_{Ca}$ with NS-8 (10 μM) resulted in potentiation of the vasodilations caused by the PDE5 inhibitor (Figure 2C). A similar potentiation of




## Figure 2

Effects of inhibition of NO synthase with $N^G$-nitro-L-arginine (L-NNA; 100 µM) or inhibition of soluble guanylyl cyclase with ODQ (20 µM) (A), blockade of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) with ChTx (100 nM) plus APA (100 nM) (B) and activation of $K_{Ca}$ with NS-8 (10 µM) (C) or NS1619 (10 µM) (D) on relaxation induced by the PDE5 inhibitor, sildenafil (1 nM to 100 µM), in norepinephrine-contracted HPRA. The effects of activation of $K_{Ca}$ with NS-8 (10 µM) on relaxation induced by the PDE5 inhibitor, tadalafil (1 nM to 100 µM) (E) and by the stable cGMP analogue, 8-bromo-cGMP (8-Br-cGMP; 10 nM to 1 mM) (F) in norepinephrine-contracted HPRA are also shown. Data are expressed as mean ± SEM 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. Data sets included 1 (A and E), 2 (B, C and F) or 7 (C) diabetic patients. *** indicates $P < 0.001$ versus control, $^{\dagger}P < 0.05$ versus L-NNA by a two-factor ANOVA test.

sildenafil-induced relaxations was achieved by treating HPRA with a different $K_{Ca}$ activator, NS1619 (10 µM; Figure 2D). These effects were not related to alterations of the contractile capacity of norepinephrine since the treatment with NS-8 or NS1619 did not significantly influence contraction induced by norepinephrine in HPRA (11.4 ± 1.7 mN vs. 11.8 ± 1.4 mN for vehicle and NS-8, respectively, $n = 28$; and 15.1 ± 4.5 vs. 14.0 ± 5.2 mN for vehicle and NS1619 respectively, $n = 7$). Furthermore, the potentiating effects of NS-8 (10 µM) were maintained when the PDE5 inhibitor used was tadalafil instead of sildenafil (Figure 2E). The stable analogue of cGMP, 8-bromo-cGMP (8-Br-cGMP) also dilated HPRA, and these responses were again potentiated by the treatment with NS-8 (10 µM; Figure 2F).

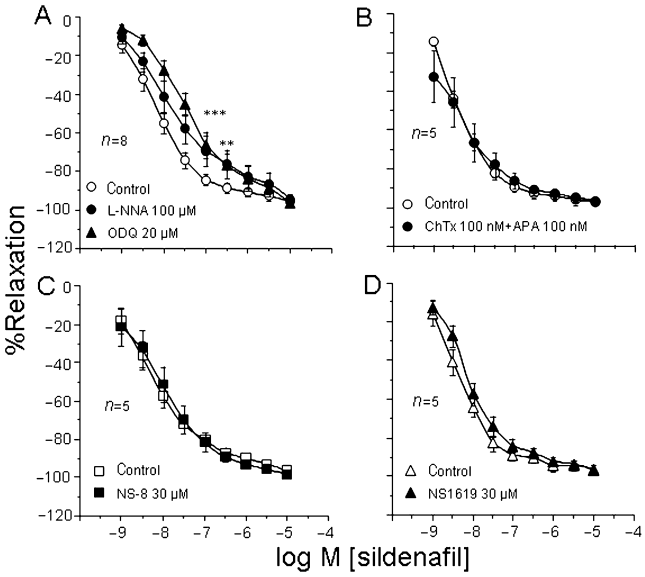
Sildenafil also relaxed HCC strips contracted with phenylephrine. This relaxation was significantly inhibited by L-NNA (100 µM) and ODQ (20 µM; Figure 3A), but in contrast to that observed in HPRA, it was unaffected by the blockade of $K_{Ca}$ with ChTx (100 nM) plus APA (100 nM; Figure 3B). In addition, even used at higher concentrations, both $K_{Ca}$ activators, NS-8 (30 µM) and NS1619 (30 µM), failed to modify sildenafil-induced relaxations in HCC (Figures 3C and D).

### *Involvement of endothelium in NS-8-induced potentiating effects on cGMP-mediated vasodilation of HPRA*

In HPRA, the mechanical removal of the endothelium caused a significant inhibition of sildenafil-induced vasodilations (Figure 4A) but did not affect those exerted by 8-Br-cGMP (Figure 4B). Endothelial removal also led to the disappearance of sensitivity of PDE5 inhibitor-induced relaxations to blockade of $K_{Ca}$ channels with ChTx (100 nM) plus APA (100 nM) (Figure 4C), and the potentiation effects of not only sildenafil- (Figure 4D) but also 8-Br-cGMP-induced relaxations in HPRA to NS-8 (Figure 4E).

### *Analysis of $K_{Ca}$ subtypes involved in NS-8-induced effects*

The potentiating effects of NS-8 on PDE5 inhibitor-induced relaxations were due to its activity on $K_{Ca}$ channels, since co-administration of ChTx (100 nM) and APA (100 nM) prevented the capacity of NS-8 to potentiate sildenafil-induced relaxations in HPRA (Figure 5A). With respect to the specific $K_{Ca}$ subtype involved, the blockade of BK channels with IbTx (100 nM) prevented the potentiating effects of NS-8 on PDE5




## Figure 3

Effects of inhibition of NO synthase with $N^G$-nitro-L-arginine (L-NNA; 100 µM) or inhibition of soluble guanylyl cyclase with ODQ (20 µM) (A), blockade of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) with ChTx (100 nM) plus APA (100 nM) (B), and activation of $K_{Ca}$ with NS-8 (30 µM) (C) or NS1619 (30 µM) (D) on relaxation induced by the PDE5 inhibitor, sildenafil (1 nM to 100 µM), in phenylephrine-contracted HCC strips. Data are expressed as mean ± SEM 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. Data sets included 1 (B) or 2 (A, C and D) diabetic patients. ** indicates $P < 0.01$, *** $P < 0.001$ versus control by a two-factor ANOVA test.

inhibitor-induced relaxations in HPRA (Figure 5B). Similarly, the blockade of IK channels with TRAM-34 (1 µM) resulted in the disappearance of NS-8-induced effects (Figure 5C). In contrast, sildenafil-induced relaxation of HPRA was potentiated by NS-8 despite the presence of the SK channel inhibitor, APA (100 nM) (Figure 5D).

### *Detection of $K_{Ca}$ channels in endothelium and smooth muscle of human and rat penile arteries and corpus cavernosum*

Endothelium of HPRA showed an intense expression of BK, IK and $SK_3$ subtypes as detected by immunostaining. These three subtypes were also present in arterial smooth muscle although the presence of BK was more intense (Figures 6A, B and E). SK1 and SK2 subtypes were not detected in HPRA (Figures 6C and 6D). BK was strongly manifested in the endothelium of rat penile arteries while IK and $SK_3$ were also detected but showing weaker intensity than BK (Figures 6F, G and H). Despite its lack of functional influence on PDE5 inhibitor-induced relaxations, BK, IK and $SK_3$ subtypes were intensely expressed at the protein level in endothelium and smooth muscle of HCC (Figures 6I, J and K). In rat corpus cavernosum, BK was present in endothelium and smooth muscle (Figure 6L) but IK and $SK_3$ were not detected (not shown).

### *Effects of sildenafil and $K_{Ca}$ openers on systemic blood pressure in rats*

Intravenous administration of sildenafil caused a significant hypotensive effect in non-diabetic rats [–14.2 ± 2.7 and –21.1 ± 2.7% of change in mean arterial pressure (MAP) for 0.1 mg·kg$^{-1}$ and 0.3 mg·kg$^{-1}$ respectively]. In diabetic rats, sildenafil (0.3 mg·kg$^{-1}$) caused a similar BP drop (–19.0 ± 3.3%). Basal levels of MAP were recovered 15–25 min after sildenafil administration. The $K_{Ca}$ openers NS-8 and NS1619 did not significantly alter systemic arterial pressure at 0.3 mg·kg$^{-1}$ dose. In combination studies, NS-8 and NS1619 were administered 30 min after sildenafil administration, when the MAP levels were recovered after the hypotension induced by sildenafil. The pre-administration of this PDE5 inhibitor did not modify the effects of $K_{Ca}$ openers on systemic BP.

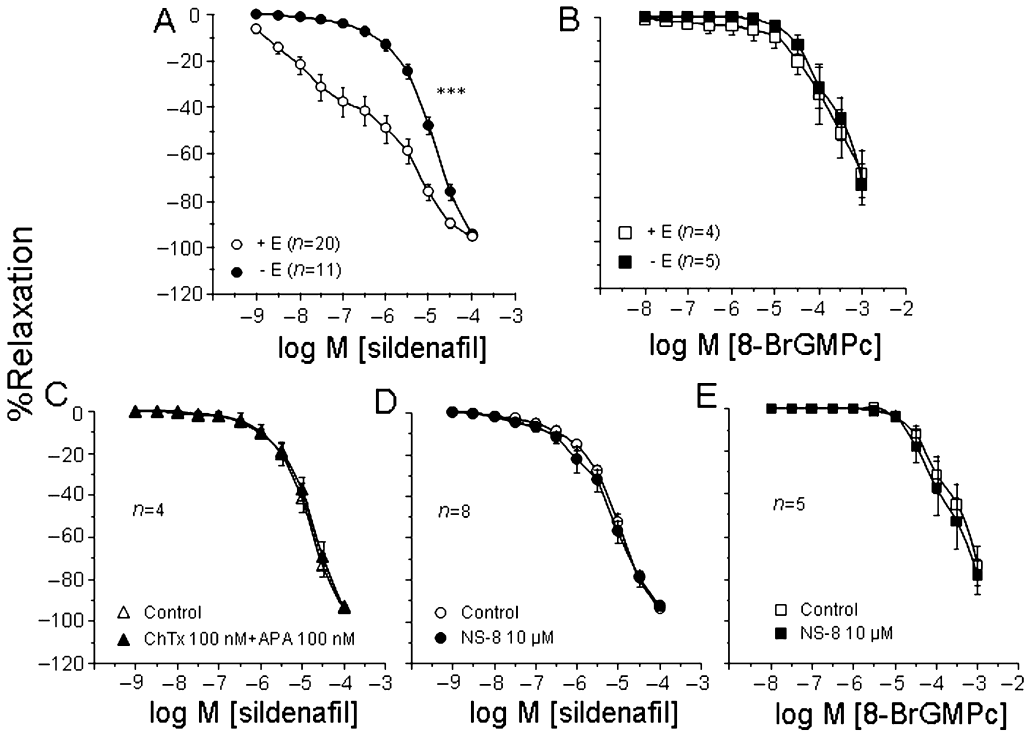
### *$K_{Ca}$ activation enhances the potentiation of erectile responses in rats induced by PDE5 inhibition*

Electrical stimulation of the cavernosal nerve in anaesthetized rats produced frequency-dependent increases of ICP. Erectile responses were not modified by i.v. administration of the vehicle (15% HPβCD) (data not shown). Intravenous administration of a subtherapeutic dose of sildenafil (0.1 mg·kg$^{-1}$) also failed to significantly alter erectile responses (Figure 7A), and a higher dose of this PDE5 inhibitor (0.3 mg·kg$^{-1}$) was required to produce a significant improvement of erectile responses in control (non-diabetic) rats (Figure 7B). Interestingly, although the $K_{Ca}$ activator, NS-8 (0.3 mg·kg$^{-1}$; i.v.) had no significant effect on erectile responses when individually administered (Figure 7C), the combined treatment with NS-8 (0.3 mg·kg$^{-1}$; i.v.) and the PDE5 inhibitor, sildenafil, at the previously inactive 0.1 mg·kg$^{-1}$ dose, resulted in a significant potentiation of erectile responses in control rats (Figure 7D).

### *$K_{Ca}$ activation reverses the impairment caused by diabetes on sildenafil-induced vasodilation of HPRA and augments the efficacy of PDE5 inhibition to recover erectile function in diabetic rats*

A significant impairment of sildenafil-induced vasodilations was observed in HPRA obtained from diabetic patients. Treatment with the $K_{Ca}$ activator, NS-8 (10 µM), produced a significant potentiation of sildenafil-induced relaxation of HPRA from both diabetic and non-diabetic patients (Figure 8). Furthermore, after treatment with NS-8, sildenafil-induced relaxations in HPRA from diabetic patients were not different from those obtained in HPRA from non-diabetic patients (Figure 8). Thus, NS-8 completely reversed the impairment of sildenafil-induced vasodilations caused by diabetes in HPRA.

After 8 weeks of diabetes, blood glucose concentrations were markedly elevated in diabetic rats (30.5 vs. 4.6 mM in diabetic and non-diabetic rats, respectively, $P < 0.001$). Diabetes caused a significant impairment of erectile responses to CNES in rats. Intravenous administration of NS-1619 or NS-8 (0.3 mg·kg$^{-1}$) did not significantly modify these responses in diabetic rats (data not shown). Erectile responses in diabetic




## Figure 4

Effects of the removal of the vascular endothelium on relaxation induced by the PDE5 inhibitor, sildenafil (1 nM to 100 µM) (A) and by the stable cGMP analogue, 8-bromo-cGMP (8-Br-cGMP; 10 nM to 1 mM) (B) in norepinephrine-contracted HPRA. Effects of blockade of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) with ChTx (100 nM) plus APA (100 nM) (C), and activation of $K_{Ca}$ with NS-8 (10 µM) (D) on sildenafil-induced relaxations as well as the effects of NS-8 (10 µM) on 8-Br-cGMP-induced relaxations (E) in de-endothelialized (-E) HPRA. Data are expressed as mean ± SEM 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. Data sets included 1 (C), 2 (B and E), 3 (D) or 7 (A) diabetic patients. *** indicates $P < 0.001$ versus HPRA with endothelium (+E).

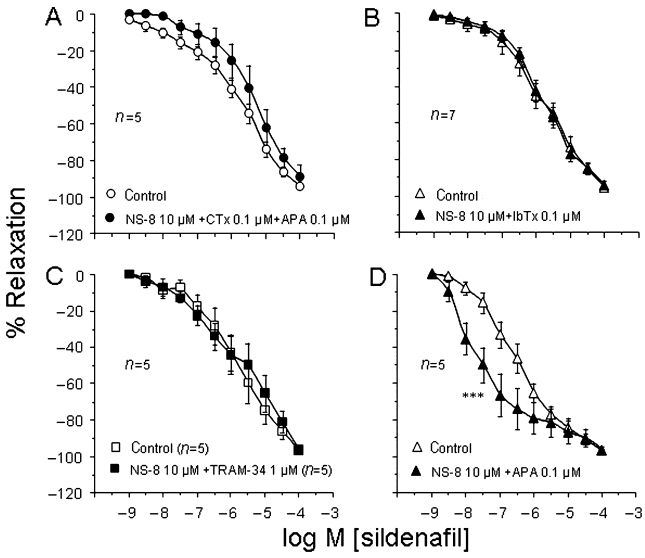
rats were slightly, but significantly, improved by administration of sildenafil (0.3 mg·kg$^{-1}$; i.v.). However, after sildenafil treatment, erectile responses in diabetic rats remained significantly reduced when compared to those in non-diabetic rats (Figure 9). In contrast, the combination of sildenafil with either the $K_{Ca}$ activator, NS-8 (0.3 mg·kg$^{-1}$) or NS1619 (0.3 mg·kg$^{-1}$) was able to completely reverse the impairment of erectile responses in diabetic rats (Figure 9).

## Discussion

The contribution of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) to cGMP-mediated relaxation in HPRA, which are essential players in penile erection, makes possible the potentiation of the vasodilatory capacity of PDE5 inhibitors in these arteries by stimulating $K_{Ca}$. BK and IK seem to be the responsible subtypes for such enhancement, being its protein expression demonstrated in human and rat penile arteries. Our data show that $K_{Ca}$ stimulation results in the potentiation of the relaxant capacity of PDE5 inhibitors, hence the recovery of its efficacy to vasodilate HPRA from diabetic patients. Similarly $K_{Ca}$ activation increases the capacity of PDE5 inhibition to enhance erectile responses in rats and allows the recovery of erectile function by PDE5 inhibition in diabetic rats.

Relaxation of HCC induced by exposure to $K_{Ca}$ activators has been demonstrated for NS1619 (Spektor *et al.*, 2002) and another novel activator, NS11021 (Kun *et al.*, 2009). In agreement with these findings, the $K_{Ca}$ activators, NS-8 and NS1619, caused relaxation of HCC. In addition, we provide evidence of the ability of these compounds to vasodilate human penile arteries, an effect mediated to a great extent by the endothelium. However, the magnitude of the relaxations produced by NS-8 and NS1619 is moderate in both HCC and penile arteries, which does not show great promise of these compounds for erectile function by themselves.

The ability of PDE5 inhibitors to relax isolated horse and human penile arteries and, rabbit and HCC, has been previously observed (Taher *et al.*, 1997; Kalsi *et al.*, 2004; Ruiz-Rubio *et al.*, 2004; Angulo *et al.*, 2010a). Relaxation of contracted HPRA and HCC caused by PDE5 inhibition is sensitive to blockade of NO synthesis or inhibition of soluble guanylyl cyclase, confirming the involvement of the NO/cGMP pathway in these responses. Although this is applicable to both HPRA and HCC, these penile structures differ with respect to the contribution of $K_{Ca}$ to the relaxation induced by inhibition of PDE5. Blockade of $K_{Ca}$ significantly inhibits sildenafil-induced relaxations in HPRA but not in HCC, suggesting that $K_{Ca}$ contribute to the relaxant activity of PDE5 inhibition in HPRA but not in HCC. Substantial differ-




## Figure 5

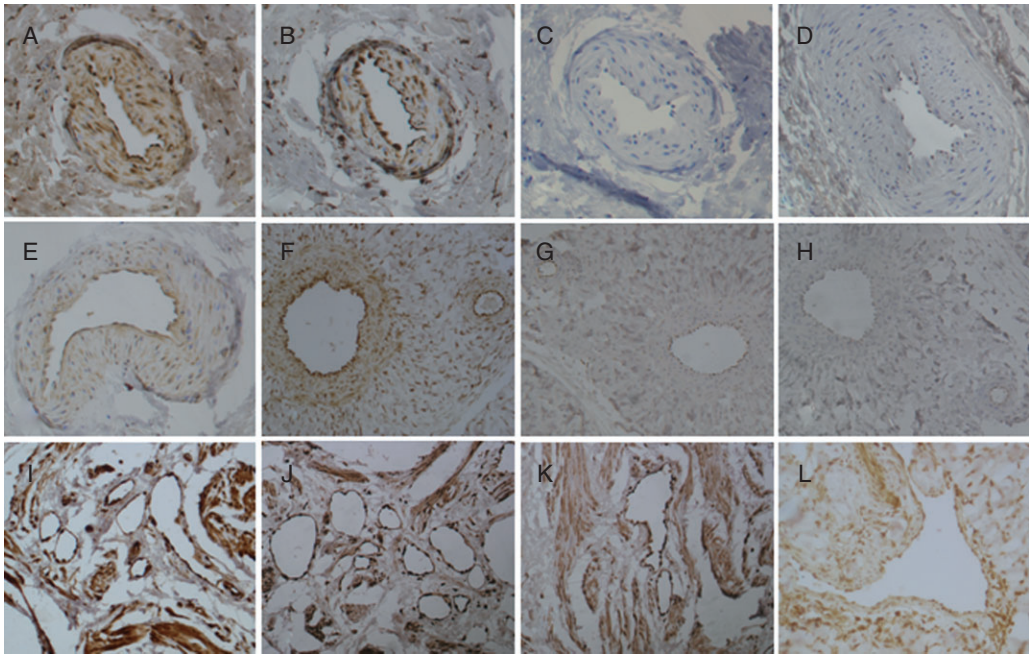
Effects of blockade of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) with ChTx (100 nM) plus APA (100 nM) (A), BK with IbTx (100 nM) (B), IK with TRAM-34 (1 μM) (C) and SK with APA (100 nM) (D) on the potentiating effects of NS-8 (10 μM) on relaxation induced by the PDE5 inhibitor, sildenafil (1 nM to 100 μM), in norepinephrine-contracted HPRA. Data are expressed as mean ± SEM 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. Data sets included 1 (A) or 2 (B, C and D) diabetic patients. *** indicates $P < 0.001$ versus control by a two-factor ANOVA test.

ences have been observed between HPRA and HCC with respect to the regulation of contractile tone. Functional differences between HPRA and HCC in response to vasoactive drugs have been previously described (Hedlund and Andersson, 1985; Angulo *et al.*, 2002). In addition, endothelium-dependent relaxation attributed to endothelium-derived hyperpolarizing factor plays a significant role in human penile arteries but not in corpus cavernosum (Angulo *et al.*, 2003). This endothelial process involving $K_{Ca}$, which is functional in penile arteries but not in trabecular tissue, is a functional divergence that could be related to the present findings.

Using specific antagonists, the involvement of $K_{Ca}$ in nitrergic relaxation of horse penile arteries (Simonsen *et al.*, 1995) and in the relaxation induced by PDE5 inhibitors in horse and rat penile arteries has been suggested (Prieto *et al.*, 2006; Sánchez *et al.*, 2008). These reports are consistent with the present findings of the contribution of $K_{Ca}$ to PDE5 inhibitor-induced vasodilatation of HPRA. The lack of influence of $K_{Ca}$ blockade on relaxations driven by PDE5 inhibition in HCC is, however, in contradiction to the previously reported sensitivity of sildenafil-induced relaxation to IbTx in HCC (Kun *et al.*, 2009), and the suggested participation of $K_{Ca}$ in PDE5 inhibitor-induced relaxation in rabbit clitoris (Gragasin *et al.*, 2004) and mouse corpus cavernosum (Werner *et al.*, 2008). Our results nevertheless were strengthened by the absence of significant effects of NS-8 or NS1619 on sildenafil-induced relaxation of HCC. In contrast, $K_{Ca}$ activation potentiated the relaxant responses to PDE5 inhibition in HPRA, demonstrating tissue specificity. This potentiating effect is not limited to a pharmacological interaction of two specific molecules, since two different well-known activators of $K_{Ca}$ channels, NS1619 and NS-8 (Malysz *et al.*, 2004) both potentiate sildenafil-induced relaxation. Similarly, vasodilatory responses to two different PDE5 inhibitors, sildenafil and tadalafil, are both enhanced by NS-8. This potentiating effect does not seem to be related to increased NO/cGMP production or further reduced hydrolysis since the activation of $K_{Ca}$ by NS-8 also potentiated relaxation to the stable analogue of cGMP, 8-Br-cGMP, in HPRA.

Previous studies carried out in different tissues, including erectile tissues, have described intracellular activation of $K_{Ca}$ by cGMP generated in smooth muscle, contributing to NO/cGMP-mediated relaxation (Sausbier *et al.*, 2000; Lee and Kang, 2001; Gragasin *et al.*, 2004; Tanaka *et al.*, 2004). It has been proposed that the cGMP-induced activation of BK channels is mediated by cGMP-dependent kinase (Schubert and Nelson, 2001; Zhou *et al.*, 2001). The interaction between NO/cGMP and $K_{Ca}$ reported here could perhaps involve a different process. This hypothesis is based mainly on the findings that the endothelial function is required for the potentiation of sildenafil-induced relaxation in penile arteries by $K_{Ca}$ activation as well as for the inhibition of these responses by $K_{Ca}$ blockade. The lack of effects of NS-8 also on 8-Br-cGMP-induced responses in arterial segments without endothelium makes it hard to believe that the disappearance of the effect of NS-8 on sildenafil-induced responses in arterial preparations without endothelium is merely due to the lack of NO/cGMP production under these conditions. Thus, a direct effect of activators on $K_{Ca}$ on smooth muscle cells is unlikely to be responsible for the enhancement of the effects of PDE5 inhibition in HPRA. Participation of endothelial $K_{Ca}$ would be consistent with a previous study describing the modulation of NO-mediated responses and neurogenic contractions by endothelial $K_{Ca}$ in rat penile arteries (Kun *et al.*, 2003).

With respect to the specific subtype of $K_{Ca}$ channel responsible for mediating NS-8-induced potentiation of the vasodilatory capacity of PDE5 inhibition in HPRA, both BK and IK channels seem to be involved. This is inferred from the fact that the individual treatment with IbTx, a specific blocker of BK, as well as with TRAM-34, a specific blocker of IK, results in the abolition of the effects induced by NS-8. In contrast, relaxant responses to sildenafil in HPRA are potentiated despite the presence of the blocker of SK, APA. In ion efflux *in vitro* assays, $EC_{50}$ for BK has reported to be in 1–4 μM range for NS1619 and NS-8 (Parihar *et al.*, 2003b). Most data reporting the specificity of these molecules have been generated in functional studies where a dependency on BK activation in the NS-8- or NS1619-induced effects is abolished by BK-selective toxins (Holland *et al.*, 1996; Malysz *et al.*, 2004; Soder and Petkov, 2011). Although NS-8 is a known activator of BK (Parihar *et al.*, 2003b; Malysz *et al.*, 2004), NS1619 is considered to be the only selective BK opener by some authors (Ghatta *et al.*, 2006) without any effect on $K_{ATP}$, voltage-gated $K^+$, $Na^+$ or $Ca^{2+}$ channels (Olesen *et al.*, 1994). According to previous results (Parihar *et al.*, 2003b; Malysz *et al.*, 2004), there are no qualitative differences between the effects induced by NS1619 and NS-8 in our experimental

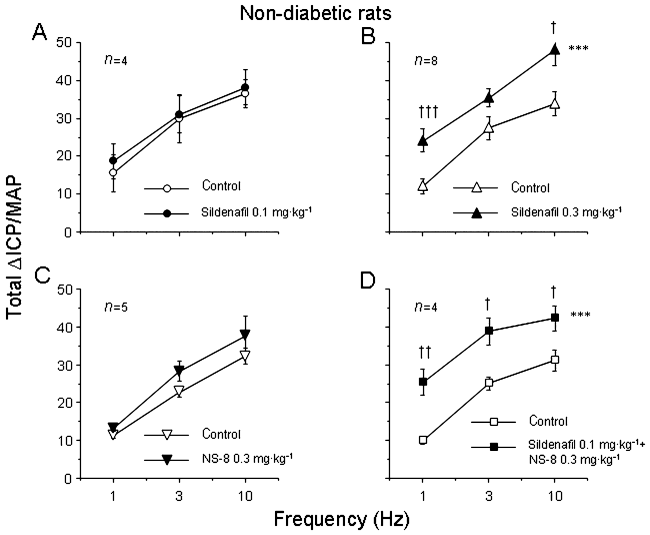



## Figure 6

Immunohistochemical localization of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$) subtypes in human and rat erectile tissues. BK (A) and IK (B) are detected in endothelium and smooth muscle of HPRA but expression of IK is more intense in endothelium. SK1 (C) and SK2 (D) were not detected while SK3 (E) is expressed in HPRA, preferentially in endothelium. As in HPRA, BK were detected in endothelium and smooth muscle of rat penile arteries (F) while IK (G) and SK3 (H) were only detected in the endothelium of these vessels. BK (I), IK (J) and SK3 (K) are present in smooth muscle fibres and the endothelia of lacunar spaces in HCC while only BK were detected in endothelium and smooth muscle in rat corpus cavernosum (L). Positive cells are stained in brown. Microphotographs are representative from at least three different patients or animals. Original magnification ×200.

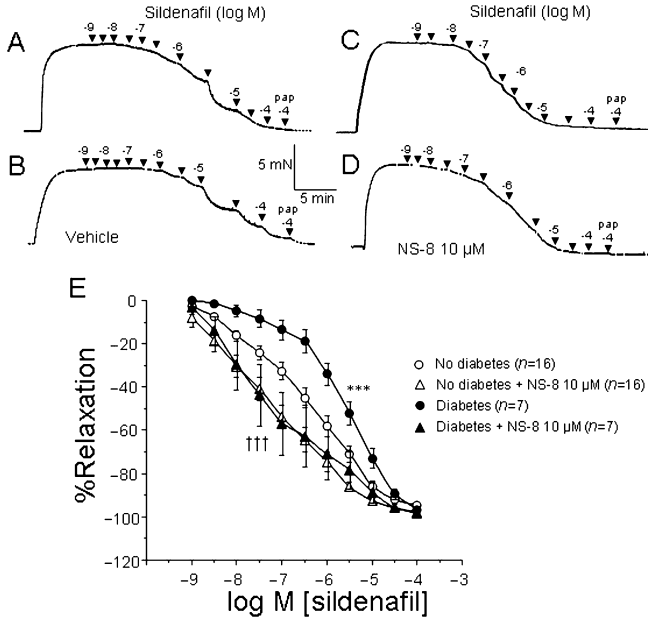
settings, suggesting that differences in selectivity between these molecules do not exist or are not relevant for the present findings. On the other hand, some authors suggest that, in general, BK activators have only moderate selectivity (Shieh *et al.*, 2000, Nardi and Olesen, 2008), which leaves the possibility of simultaneous IK activation. However, in functional *in vitro* assays, the lack of effect of NS-8 on IK activity has been demonstrated (Parihar *et al.*, 2003a). Similarly, it has been shown that NS1619 did not activate IK in bovine aortic endothelial cells, and even inhibited this channel at high concentrations in inside-out experiments (Cai *et al.*, 1998). In the present report, NS-8-induced effects are prevented by either the BK or IK inhibitors separately. This is unlikely due to the lack of selectivity of TRAM-34, as it is above 1000-fold selective for IK over BK (Wulff *et al.*, 2000). In fact, TRAM-34 $K_d$ for BK is above 25 μM (Wulff *et al.*, 2000), which would anticipate an $IC_{50}$ even higher, making a definite effect of 1 μM TRAM-34 on BK unlikely. Although the activation of IK by NS-8 due to a lack of specificity of this activator cannot be completely discarded, a possible explanation for this complex mechanism that also requires the contribution of the endothelium could rely on a sequential activation of IK after the activation of BK by NS-8 in the same or different cell type that would be consistent with the fact that the individual blockade of BK or IK results in the complete loss of NS-8-induced effects. Results obtained in vascular preparations (Climent *et al.*, 2012) would point to the involvement of smooth muscle BK and endothelial IK in the effects induced by $K_{Ca}$ activation.

Immunostaining revealed an intense expression of BK and IK in endothelium of HPRA that supports the possible role of these channel subtypes in vasodilation of HPRA. IK channels have been reported to be expressed in endothelial cells from different human vessels (Kohler *et al.*, 2000; Brakemeier *et al.*, 2003; Grgic *et al.*, 2005). The data regarding the presence of BK channels in the vascular endothelium are controversial. It has been reported not to be expressed in bovine coronary endothelial cells (Gauthier *et al.*, 2002) and only in human mesenteric artery endothelium in pathological conditions (Kohler *et al.*, 2000). On the other hand, BK channel expression has been observed in bovine aortic endothelial cells (Wang *et al.*, 2005) and human dermal microvascular endothelial cells (Grgic *et al.*, 2005). Our results are consistent with the expression of BK in vascular endothelium. Interestingly, endothelium in lacunar spaces and surrounding smooth muscle in trabecular tissue also express BK, IK and SK channels. In fact, an intense expression of IK channels is observed in our HCC specimens despite using the same antibody as Chen *et al.*, who did not find IK channel expression in HCC (Chen *et al.*, 2004). Thus, it seems that the absence of the effects of $K_{Ca}$ activators on NO/cGMP pathway in HCC is due to a functional difference rather to the absence of any of the channels involved. It is important to note that all these antibodies are targeted to pore-forming




## Figure 7

Effects of i.v. administration of the PDE5 inhibitor, sildenafil at 0.1 mg kg $^{-1}$ (A) or 0.3 mg kg $^{-1}$ dose (B), the $K_{Ca}$ channel opener, NS-8 (0.3 mg kg $^{-1}$) (C), or the combination of sildenafil (0.1 mg kg $^{-1}$) plus NS-8 (0.3 mg kg $^{-1}$) (D) on erectile responses induced by cavernosal nerve electrical stimulation (1 to 10 Hz) in anaesthetized rats. Data are expressed as mean ± SEM of the AUC of the ICP increase (Total ΔICP) normalized by mean arterial pressure (MAP) value at each stimulation. *n* indicates the number of animals used for the experiments. ***$P < 0.001$ versus control responses by a two-factor ANOVA test.




## Figure 8

Representative tracings showing vasodilations induced by sildenafil (1 to 100 μM) in norepinephrine-contracted HPRA from one non-diabetic (A) and one diabetic (B) patient. C and D panels show the tracings of sildenafil-induced relaxations after treatment with NS-8 (10 μM) in parallel arterial segments from the same patients than in A and B respectively. Panel E shows the effects of diabetes and the treatment with the activator of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$), NS-8 (10 μM) on vasodilation induced by the PDE5 inhibitor, sildenafil in HPRA. Data are expressed as mean ± SEM 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. Two of non-diabetic patients did not present any cardiovascular risk factor (CVRF), eight had one single CVRF and six had two or more CVRF. Four diabetic patients had diabetes as the only CVRF while three had one or more additional CVRF. Average age was 57.1 ± 3.1 years and 51.9 ± 2.6 years for non-diabetic and diabetic patients respectively ($P > 0.25$). *** indicates $P < 0.001$ versus No diabetes, $^{†††}P < 0.001$ versus Diabetes (without NS-8) by a two-factor ANOVA test.

subunits, and differential expression of regulatory subunits of the channels could be possible.

$K_{Ca}$ expression in rat corpus cavernosum is different from that in HCC since only BK is detected. In contrast, the expression of BK, IK and SK3 was demonstrated in the endothelium of rat penile arteries, similarly to that observed in HPRA, which provides the structural basis for considering the rat an acceptable model for the evaluation of the possible actions of $K_{Ca}$ channel activators on erectile responses. In fact, the $K_{Ca}$ activator-induced potentiation of vasodilation evoked by PDE5 inhibition in HPRA was translated *in vivo* to an enhancement of the pro-erectile capacity of sildenafil, as demonstrated by the reduction of the dose of sildenafil required to significantly enhance erectile responses in rats. Importantly, this potentiating effect was achieved at a dose of $K_{Ca}$ activator that did not significantly impact arterial pressure, a characteristic that represents an advantage over the $K_{ATP}$ activation. Although NS-8 did not significantly augment erectile responses by itself at the dose used in the present study, a positive modulation of erectile responses in rats by BK stimulation with the novel activator, NS11021 has been reported (Kun *et al.*, 2009).

The increased vasodilatory and pro-erectile activities of PDE5 obtained after pharmacological stimulation of $K_{Ca}$ would have special relevance in conditions where the efficacy of PDE5 inhibitors to treat ED is compromised. One of these conditions is diabetic ED. The efficacy of PDE5 inhibitors for treating ED is lower in diabetic patients (Vickers and Satyanarayana, 2002; El-Sakka, 2004). It has been recently demonstrated that capacity of sildenafil to relax HPRA is reduced in diabetic patients with ED (Angulo *et al.*, 2010a). In addition to confirm the impaired vasodilatory capacity of sildenafil in HPRA from diabetic patients, the present results show that the activation of $K_{Ca}$ with NS-8 potentiates the relaxant responses to PDE5 inhibition on HPRA from both diabetic and non-diabetic men. Furthermore, this effect leads to comparable sildenafil-induced vasodilations in diabetic and non-diabetic HPRA after treatment with NS-8, recovering the efficacy of PDE5 inhibition in HPRA from diabetic patients. This improvement of efficacy demonstrated *in vitro* is again corroborated *in vivo*. While, accordingly with previous observations (Bivalacqua *et al.*, 2004; Angulo *et al.*, 2005; Zhang *et al.*, 2006; Angulo *et al.*, 2010b), the impairment of erectile responses caused by diabetes is not reversed after PDE5 inhibition, ED in diabetic rats is completely reversed when PDE5 inhibition is combined with stimulation of $K_{Ca}$.

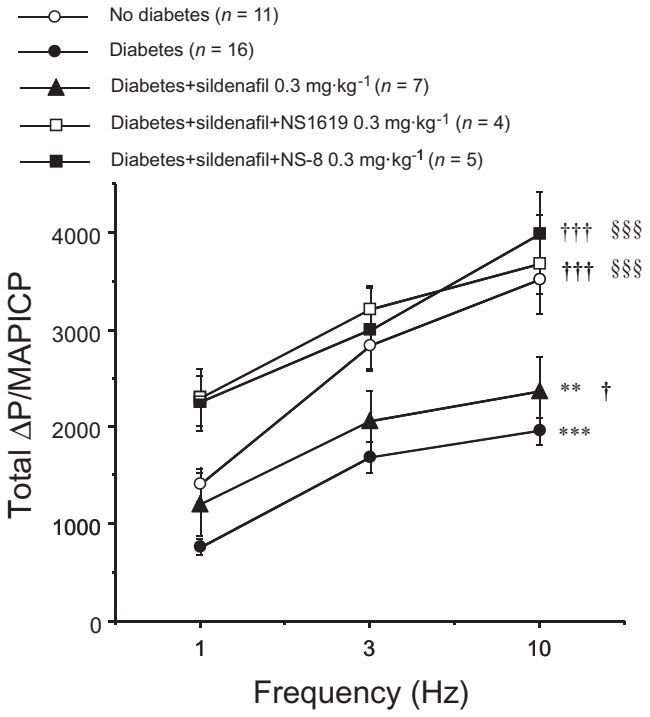



**Figure 9**

Effects of i.v. administration of the PDE5 inhibitor, sildenafil (0.3 mg kg $^{-1}$), alone or in combination with the $K_{Ca}$ channel openers, NS1619 (0.3 mg kg $^{-1}$) or NS-8 (0.3 mg kg $^{-1}$) on erectile responses induced by cavernosal nerve electrical stimulation (1 to 10 Hz) in anaesthetized streptozotocin-induced diabetic rats. Erectile responses in non-diabetic rats (No diabetes) are displayed for comparisons. Data are expressed as mean ± SEM of the AUC of the ICP increase (Total ΔICP) normalized by mean arterial pressure (MAP) value at each stimulation. *n* indicates the number of animals used for the experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus No diabetes, $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ versus Diabetes, and $^{\S\S\S}P < 0.001$ versus sildenafil alone by a two-factor ANOVA test.

Elevated prevalence of ED has been reported in type 1 and type 2 diabetic patients although ED was slightly more prevalent in type 1 (Fedele *et al.*, 2000). In fact, reduced response rates to therapy with PDE5 inhibitors have been observed in populations of either type 1 (Boulton *et al.*, 2001; Ziegler *et al.*, 2006) or type 2 (Stuckey *et al.*, 2003) diabetic men. Positive effects here obtained with $K_{Ca}$ activators in tissues from type 2 diabetic patients as well as *in vivo* in an animal model of type 1 diabetes suggest that results could be generalizable to both types of diabetes. Although the lack of accurate information on the glycaemic control of diabetic patients (ideally $HbA_{1c}$ levels) should be considered as a limitation of the study, the fact that NS-8 is effective in diabetic and non-diabetic tissues and animals suggest that the effects of $K_{Ca}$ activation on vasodilatory capacity of PDE5 inhibitors are not dependent on glycaemic status.

In conclusion, activation of $K_{Ca}$ produces an endothelium-mediated potentiation of the NO/cGMP-mediated relaxation in HPRA that results in improved vasodilatory capacity of PDE5 inhibitors in these human vessels. This potentiating effect that involves BK and IK subtypes results in increased efficacy of PDE5 inhibition to enhance erectile responses *in vivo*. Furthermore, $K_{Ca}$ stimulation recovers the impaired vasodilatory efficacy in diabetic HPRA and makes it possible to restore PDE5 inhibition for ED in diabetic rats. These results together with the low impact of $K_{Ca}$ stimulators on arterial pressure provide a rationale for considering the therapeutic potential of $K_{Ca}$ activators to improve the efficacy of PDE5 inhibitors in the management of diabetic ED.

## Acknowledgements

We thank Drs. Derek Trezise, David Davis, Graham Robertson and Chris Plumpton for assistance in obtaining important supplies for this work. The work was partially supported by an unrestricted grant from Esteve Laboratories, Spain.

## Conflict of interests

None of the authors have any conflict of interests.

## References

Angulo J, Rodriguez-Mañas L, Peiró C, Neira M, Marín J, Sánchez-Ferrer CF (1998). Impairment of nitric oxide mediated relaxations in anaesthetized autoperfused streptozotocin-induced diabetic rats. Naunyn Schmiedebergs Arch Pharmacol 358: 529–537.

Angulo J, Cuevas P, La Fuente JM, Pomerol JM, Ruiz-Castañé E, Puigvert A *et al.* (2002). Regulation of human penile smooth muscle tone by prostanoid receptors. Br J Pharmacol 136: 23–30.

Angulo J, Cuevas P, Fernandez A, Gabancho S, Videla S, Sáenz de Tejada I (2003). Calcium dobesilate potentiates endothelium-derived hyperpolarizing factor-mediated relaxation of human penile resistance arteries. Br J Pharmacol 139: 854–862.

Angulo J, Cuevas P, Gabancho S, Gonzalez-Corrochano R, Videla S, Saenz de Tejada I (2005). Enhancement of both, endothelium-derived hyperpolarizing factor and nitric oxide/cyclic guanosin-monophosphate pathways reverses erectile dysfunction in diabetic rats. J Sex Med 2: 317–322.

Angulo J, González-Corrochano R, Cuevas P, Fernández A, La Fuente JM, Rolo F *et al.* (2010a). Diabetes exacerbates the functional deficiency of NO/cGMP pathway associated with erectile dysfunction in human corpus cavernosum and penile arteries. J Sex Med 7: 758–768.

Angulo J, Wright HM, Cuevas P, González-Corrochano R, Fernández A, Cuevas B *et al.* (2010b). Nebivolol dilates penile arteries and reverses erectile dysfunction in diabetic rats through enhancement of nitric oxide signaling. J Sex Med 7: 2681–2697.

Angulo J, Cuevas P, Fernández A, La Fuente JM, Allona A, Moncada I *et al.* (2012). Tadalafil enhances the inhibitory effects of tamsulosin on neurogenic contractions of human prostate and bladder neck. J Sex Med 9: 2293–2306.

Bivalacqua TJ, Usta MF, Champion HC, Leungwattanakij S, Dabisch PA, McNamara DB *et al.* (2004). Effect of combination

endothelial nitric oxide synthase gene therapy and sildenafil on erectile function in diabetic rats. Int J Impot Res 16: 21–29.

Boulton AJ, Selam JL, Sweeney M, Ziegler D (2001). Sildenafil citrate for the treatment of erectile dysfunction in men with type II diabetes mellitus. Diabetologia 44: 1296–1301.

Brakemeier S, Kersten A, Eichler I, Grgic I, Zakrzewicz A, Hopp H *et al*. (2003). Shear stress-induced up-regulation of the intermediate conductance $Ca^{2+}$-activated $K^+$ channel in human endothelium. Cardiovasc Res 60: 488–496.

Cai S, Garneau L, Sauvé R (1998). Single-channel characterization of the pharmacological properties of the K($Ca^{2+}$) channel of intermediate conductance in bovine aortic endothelial cells. J Membr Biol 163: 147–158.

Chen MX, Gorman SA, Benson B, Singh K, Hieble JP, Michel MC *et al*. (2004). Small and intermediate conductance $Ca^{2+}$-activated $K^+$ channels confer distinctive patterns of distribution in human tissues and differential cellular localisation in the colon and corpus cavernosum. Naunyn Schmiedebergs Arch Pharmacol 369: 602–615.

Christ GJ, Spray DC, Brink PR (1993). Characterization of K currents in cultured human corporal smooth muscle cells. J Androl 14: 319–328.

Christ GJ, Day N, Santizo C, Sato Y, Zhao W, Sclafani T *et al*. (2004). Intracorporal injection of hSlo cDNA restores erectile capacity in STZ-diabetic F-344 rats in vivo. Am J Physiol Heart Circ Physiol 287: H1544–H1553.

Climent B, Schubert R, Stankevicius E, García-Sacristán A, Simonsen U, Rivera L (2012). Large conductance Ca2+-activated K+ channels modulate endothelial cell outward currents and nitric oxide release in the intact rat superior mesenteric artery. Biochem Biophys Res Commun 417: 1007–1013.

El-Sakka AI (2004). Efficacy of sildenafil citrate in treatment of erectile dysfunction: effect of type 2 diabetes. Eur Urol 46: 503–509.

Fedele D, Bortolotti A, Coscelli C, Santeusanio F, Chatenoud L, Colli E *et al*. (2000). Erectile dysfunction in type 1 and type 2 diabetics in Italy. On behalf of Gruppo Italiano Studio Deficit Erettile nei Diabetici. Int J Epidemiol 29: 524–531.

Gauthier KM, Liu C, Popovic A, Albarwani S, Rusch NJ (2002). Freshly isolated bovine coronary endothelial cells do not express the $BK_{Ca}$ channel gene. J Physiol 545: 829–836.

Ghatta S, Nimmagadda D, Xu X, O'Rourke ST (2006). Large-conductance, calcium-activated potassium channels: structural and functional implications. Pharmacol Ther 110: 103–116.

Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA (1998). Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. N Engl J Med 338: 1397–1404.

Goldstein I, Young JM, Fischer J, Bangerter K, Segerson T, Taylor T (2003). Vardenafil, a new phosphodiesterase type 5 inhibitor, in the treatment of erectile dysfunction in men with diabetes: a multicenter double-blind placebo-controlled fixed-dose study. Diabetes Care 26: 777–783.

Gragasin FS, Michelakis ED, Hogan A, Moudgil R, Hashimoto K, Wu X *et al*. (2004). The neurovascular mechanism of clitoral erection: nitric oxide and cGMP-stimulated activation of $BK_{Ca}$ channels. FASEB J 18: 1382–1391.

Gratzke C, Angulo J, Chitaley K, Dai Y-T, Kim NN, Paick J-S *et al*. (2010). Anatomy, physiology, and pathophysiology of erectile dysfunction. J Sex Med 7: 445–475.

Grgic I, Eichler I, Heinau P, Si H, Brakemeier S, Hoyer J *et al*. (2005). Selective blockade of the intermediate-conductance $Ca^{2+}$-activated $K^+$ channel suppresses proliferation of microvascular and macrovascular endothelial cells and angiogenesis in vivo. Arterioscler Thromb Vasc Biol 25: 704–709.

Hedlund H, Andersson KE (1985). Comparison of the responses to drugs acting on adrenoreceptors and muscarinic receptors in human isolated corpus cavernosum and cavernous artery. J Auton Pharmacol 5: 81–88.

Hewawasam P, Fan W, Cook DA, Newberry KS, Boissard CG, Gribkoff VK *et al*. (2004). 4-Aryl-3-(mercapto)quinolin-2-ones: novel maxi-K channel opening relaxants of corporal smooth muscle. Bioorg Med Chem Lett 14: 4479–4482.

Holland M, Langton PD, Standen NB, Boyle JP (1996). Effects of the $BK_{Ca}$ channel activator, NS1619, on rat cerebral artery smooth muscle. Br J Pharmacol 117: 119–129.

Kalsi JS, Kell PD, Cellek S, Ralph DJ (2004). NCX-911, a novel nitric oxide-releasing PDE5 inhibitor relaxes rabbit corpus cavernosum in the absence of endogenous nitric oxide. Int J Impot Res 16: 195–200.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Kohler R, Degenhardt C, Kuhn M, Runkel N, Paul M, Hoyer J (2000). Expression and function of endothelial $Ca^{2+}$-activated $K^+$ channels in human mesenteric artery. A single-cell reverse transcriptase-polymerase chain reaction and electrophysiological study in situ. Circ Res 87: 496–503.

Kun A, Martínez AC, Tanko LB, Pataricza J, Papp JG, Simonsen U (2003). $Ca^{2+}$-activated $K^+$ channels in the endothelial cell layer involved in modulation of neurogenic contractions in rat penile arteries. Eur J Pharmacol 474: 103–115.

Kun A, Matchkov VV, Stankevicius E, Nardi A, Hughes AD, Kirkeby HJ *et al*. (2009). NS11021, a novel opener of large-conductance $Ca^{2+}$-activated $K^+$ channels, enhances erectile responses in rats. Br J Pharmacol 158: 1465–1476.

Lee SW, Kang TM (2001). Effects of nitric oxide on the $Ca^{2+}$-activated potassium channels in smooth muscle cells of the human corpus cavernosum. Urol Res 29: 359–365.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Malysz J, Buckner SA, Daza AV, Milicic I, Perez-Medrano A, Gopalakrishnan M (2004). Functional characterization of large conductance calcium-activated $K^+$ channel openers in bladder and vascular smooth muscle. Naunyn Schmiedebergs Arch Pharmacol 369: 481–489.

Mannhold R (2004). $K_{ATP}$ channel openers: structure-activity relationships and therapeutic potential. Med Res Rev 24: 213–266.

Melman A, Zhao W, Davies KP, Bakal R, Christ GJ (2003). The succesful long-term treatment of age related erectile dysfunction with hSlo cDNA in rats in vivo. J Urol 170: 285–290.

Morgado M, Cairrao E, Santos-Silva AJ, Verde I (2012). Cyclc nucleotide-dependent relaxation pathways in vascular smooth muscle. Cell Mol Life Sci 69: 247–266.

Mulvany MJ, Halpern W (1977). Contractile properties of small resistance arteries in spontaneously hypertensive and normotensive rats. Circ Res 41: 19–26.

Nardi A, Olesen SP (2008). BK channel modulators: a comprehensive overview. Curr Med Chem 15: 1126–1146.

Olesen SP, Munch E, Moldt P, Drejer J (1994). Selective activation of $Ca^{2+}$-dependent $K^+$ channels by novel benzimidazolone. Eur J Pharmacol 251: 53–59.

Padma-Nathan H, McMurray JG, Pullman WE, Whitaker JS, Saoud JB, Ferguson KM *et al.* (2001). On-demand IC351 (Cialis) enhances erectile function in patients with erectile dysfunction. Int J Impot Res 13: 2–9.

Parihar AS, Coghlan MJ, Gopalakrishnan M, Shieh CC (2003a). Effects of intermediate-conductance $Ca^{2+}$-activated $K^+$ channel modulators on human prostate cancer cell proliferation. Eur J Pharmacol 471: 157–164.

Parihar AS, Groebe DR, Scott VE, Feng J, Zhang XF, Warrior U *et al.* (2003b). Functional analysis of large conductance $Ca^{2+}$-activated $K^+$ channels: ion flux studies by atomic absorption spectrometry. Assay Drug Dev Technol 1: 647–654.

Porst H, Rosen R, Padma-Nathan H, Goldstein I, Giuliano F, Ulbrich E *et al.* (2001). The efficacy and tolerability of vardenafil, a new, oral, selective phosphodiesterase type 5 inhibitor, in patients with erectile dysfunction: the first at-home clinical trial. Int J Impot Res 13: 192–199.

Prieto D, Rivera L, Benedito S, Recio P, Villalba N, Hernandez M *et al.* (2006). $Ca^{2+}$-activated $K^+$ (KCa) channels are involved in the relaxation elicited by sildenafil in penile resistance arteries. Eur J Pharmacol 531: 232–237.

Rendell MS, Rajfer J, Wicker PA, Smith MD, for the Sildenafil Diabetes Study Group (1999). Sildenafil for treatment of erectile dysfunction in men with diabetes. A randomized controlled trial. JAMA 281: 421–426.

Ruiz-Rubio JL, Hernández M, Rivera de los Arcos L, Benedito S, Recio P, García-Sacristán A *et al.* (2004). Role of ATP-sensitive $K^+$ channels in relaxation of penile resistance arteries. Urology 63: 800–805.

Sáenz de Tejada I, Anglin G, Knight JR, Emmick JT (2002). Effects of tadalafil on erectile dysfunction in men with diabetes. Diabetes Care 25: 2159–2164.

Sánchez A, Villalba N, Martínez AC, García-Sacristán A, Hernández M, Prieto D (2008). Mechanisms of the relaxant effect of vardenafil in rat penile arteries. Eur J Pharmacol 586: 283–287.

Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M *et al.* (2000). Mechanisms of NO/cGMP-dependent vasorelaxation. Circ Res 87: 825–830.

Schubert R, Nelson MT (2001). Protein kinases: tuners of the BKCa channel in smooth muscle. Trends Pharmacol Sci 22: 505–512.

Shieh CC, Coghlan M, Sullivan JP, Gopalakrishnan M (2000). Potassium channels: molecular defects, diseases, and therapeutic opportunities. Pharmacol Rev 52: 557–594.

Simonsen U, Prieto D, Sáenz de Tejada I, García-Sacristán A (1995). Involvement of nitric oxide in the non-adrenergic non-cholinergic neurotransmission of horse deep penile arteries: role of charybdotoxin-sensitive $K^+$-channels. Br J Pharmacol 116: 2582–2590.

Soder RP, Petkov GV (2011). Large conductance $Ca^{2+}$-activated $K^+$ channel activation with NS1619 decreases myogenic and neurogenic contraction of rat detrusor smooth muscle. Eur J Pharmacol 670: 252–259.

Spektor M, Rodríguez R, Rosenbaum RS, Wang H-Z, Melman A, Christ GJ (2002). Potassium channels and human corporeal smooth muscle cell tone: further evidence of the physiological relevance of the maxi-K channel subtype to the regulation of human corporeal smooth muscle tone in vitro. J Urol 167: 2628–2635.

Stuckey BG, Jadzinsky MN, Murphy LJ, Montorsi F, Kadioglu A, Fraige F *et al.* (2003). Sildenafil citrate for treatment of erectile dysfunction in men with type 1 diabetes: results of a randomized controlled trial. Diabetes Care 26: 279–284.

Taher A, Meyer M, Stief CG, Jonas U, Forssmann WG (1997). Cyclic nucleotide phosphodiesterase in human cavernous smooth muscle. World J Urol 15: 32–35.

Tanaka Y, Koike K, Toro L (2004). MaxiK channel roles in blood vessel relaxations induced by endothelium-derived relaxing factors and their molecular mechanisms. J Smooth Muscle Res 40: 125–153.

Venkateswarlu K, Giraldi A, Zhao W, Wang H-Z, Melman A, Spektor M *et al.* (2002). Potassium channels and human corporeal smooth muscle cell tone: diabetes and relaxation of human corpus cavernosum smooth muscle by adenosine triphosphate sensitive potassium channel openers. J Urol 168: 355–361.

Vickers MA, Satyanarayana R (2002). Phosphodiesterase type 5 inhibitors for the treatment of erectile dysfunction in patients with diabetes mellitus. Int J Impot Res 14: 466–471.

Wang XL, Ye D, Peterson TE, Cao S, Shah VH, Katusic ZS *et al.* (2005). Caveolae targeting and regulation of large conductance $Ca^{2+}$-activated $K^+$ channels in vascular endothelial cells. J Biol Chem 280: 11656–11664.

Werner ME, Zvara P, Meredith AL, Aldrich RW, Nelson MT (2005). Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. J Physiol 567: 545–566.

Werner ME, Meredith AL, Aldrich RW, Nelson MT (2008). Hypercontractility and impaired sildenafil relaxations in the BKCa channel deletion model of erectile dysfunction. Am J Physiol Regul Integr Comp Physiol 295: R181–R188.

Wulff H, Miller MJ, Hänsel W, Grissmer S, Cahalan MD, Chandy KG (2000). Design of a potent and selective inhibitor of the intermediate-conductance $Ca^{2+}$-activated $K^+$ channel, *IKCa1*: a potential immunosuppressant. Proc Natl Acad Sci U S A 97: 8151–8156.

Zhang XH, Filippi S, Morelli A, Vignozzi L, Luconi M, Donati S *et al.* (2006). Testosterone restores diabetes-induced erectile dysfunction and sildenafil responsiveness in two distinct animal models of chemical diabetes. J Sex Med 3: 253–264.

Zhou X-B, Arntz C, Kamm S, Motejlek K, Sausbier U, Wang G-X *et al.* (2001). A molecular switch for specific stimulation of the $BK_{Ca}$ channel by cGMP and cAMP kinase. J Biol Chem 276: 43239–43245.

Ziegler D, Merfort F, van Ahlen H, Yassin A, Reblin T, Neureither M (2006). Efficacy and safety of flexible-dose vardenafil in men with type 1 diabetes and erectile dysfunction. J Sex Med 3: 883–891.