

## RESEARCH PAPER

NS11021, a novel opener of large-conductance  
Ca<sup>2+</sup>-activated K<sup>+</sup> channels, enhances erectile  
responses in ratsA Kun<sup>1</sup>, VV Matchkov<sup>2</sup>, E Stankevicius<sup>1</sup>, A Nardi<sup>3</sup>, AD Hughes<sup>4</sup>, HJ Kirkeby<sup>5</sup>, J Demnitz<sup>3</sup> and  
U Simonsen<sup>1</sup><sup>1</sup>Department of Pharmacology, University of Aarhus, Aarhus, Denmark, <sup>2</sup>Department of Physiology, University of Aarhus, Aarhus, Denmark, <sup>3</sup>NeuroSearch A/S, Ballerup, Denmark, <sup>4</sup>Department of Clinical Pharmacology, Imperial College, London, UK, and <sup>5</sup>Department of Urology, Aarhus University Hospital, Aarhus, Denmark

**Background and purpose:** Large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK<sub>Ca</sub>), located on the arterial and corporal smooth muscle, are potential targets for treatment of erectile dysfunction (ED). This study investigated whether NS11021 (1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-thiourea), a novel opener of BK<sub>Ca</sub> channels, relaxes erectile tissue *in vitro* and enhances erectile responses in intact rats. The effects were compared with sildenafil, an inhibitor of phosphodiesterase type 5.

**Experimental approach:** Patch clamp was used to record whole cell current in rat isolated corpus cavernosum smooth muscle cells (SMCs) and human umbilical vein endothelial cells (HUVECs). Isometric tension was measured in intracavernous arterial rings and corpus cavernosum strips isolated from rats and men, and simultaneous measurements of intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and tension were performed in intracavernous arteries. Erectile response was measured in anaesthetized rats.

**Key results:** In patch clamp recordings, NS11021 increased currents sensitive to the selective BK<sub>Ca</sub> channel blocker, iberiotoxin (IbTX) in SMCs, but did not modulate K<sup>+</sup> current in HUVECs. NS11021 reduced [Ca<sup>2+</sup>]<sub>i</sub> and tension in penile arteries. IbTX inhibited the vasorelaxation induced by NS11021 and sildenafil in human erectile tissue. NS11021 and sildenafil but not vehicle increased erectile responses in anaesthetized rats, an effect which was abolished after pretreatment with tetraethylammonium.

**Conclusions and implications:** NS11021 leads to relaxation of both intracavernous arteries and corpus cavernosum strips primarily through opening of BK<sub>Ca</sub> channels. It is also effective in facilitating erectile responses in anaesthetized rats. These results suggest a potential for use of BK<sub>Ca</sub> openers in the treatment of ED.

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**Keywords:** penile tissue; BK<sub>Ca</sub> channel; NS11021; sildenafil; vasodilation; erection

**Abbreviations:** [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; ADMA, asymmetric dimethylarginine; BK<sub>Ca</sub> channel, big-conductance calcium-activated potassium channel; DMSO, dimethylsulfoxide; HUVEC, human umbilical endothelial cells; IbTX, iberiotoxin; K<sub>ATP</sub>, ATP-sensitive potassium channel; MAP, mean arterial blood pressure; NS11021, 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-thiourea; PDE5, phosphodiesterase type 5; PICP, peak intracavernosal pressure; TEA, tetraethylammonium

## Introduction

Male erectile dysfunction (ED) is defined by the inability to attain or maintain penile erection sufficient for satisfactory sexual intercourse. The prevalence of ED is increased in

patients with ischaemic heart disease, diabetes and hypertension (Shiri *et al.*, 2003; Montorsi *et al.*, 2004; Vrentzos *et al.*, 2007). One of the underlying causes of ED of vascular origin is reduced nitric oxide (NO) bioavailability resulting in insufficient relaxation of smooth muscle in the penis (Azadzi *et al.*, 2005; Moore and Wang, 2006). Phosphodiesterase type 5 (PDE5) inhibitors increase cyclic GMP levels in penile smooth muscle followed by smooth muscle relaxation and erection. However, PDE5 inhibitors are less efficient in patients with diabetes and they are contraindicated in all men

Correspondence: Ulf Simonsen, Department of Pharmacology, Faculty of Health Sciences, University of Aarhus, The University Park, Wilhelm Meyers Allé 4, 8000 Aarhus C, Denmark. E-mail: us@farm.au.dk

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taking nitrate compounds (Jackson *et al.*, 2006). Therefore, novel pharmacological approaches are required for the treatment of ED in these patients.

Activation of K<sup>+</sup> channels followed by hyperpolarization and vasodilatation of penile arteries and relaxation of corporal smooth muscle cells (SMCs) is thought to be an important mechanism in erection (Prieto, 2008). Both ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub>) and big conductance calcium-activated (BK<sub>Ca</sub> or K<sub>Ca</sub>1.1) channels (nomenclature follows Alexander *et al.*, 2008) are expressed in corporal smooth muscle (Fan *et al.*, 1995; Lee and Kang, 2001; Spektor *et al.*, 2002). The BK<sub>Ca</sub> channels are directly involved in neurogenic NO-mediated relaxations in horse penile arteries (Simonsen *et al.*, 1995), inhibition of myogenic tone of rat penile arteries (Kun *et al.*, 2003) and relaxation to C-type natriuretic peptide in human penile arteries (Kun *et al.*, 2008). Moreover, mice lacking BK<sub>Ca</sub> channels have ED (Werner *et al.*, 2005), and ageing is associated with ED and causes cytoplasmic retention of BK<sub>Ca</sub> channels in rat corporal SMCs (Davies *et al.*, 2007). Intracavernosal injection of the *hSlo* gene, which encodes the BK<sub>Ca</sub> channel, enhances erectile responses in old and diabetic rats (Christ *et al.*, 2004), hence suggesting that activation of BK<sub>Ca</sub> channels may restore erectile function in patients with ED.

Both openers of BK<sub>Ca</sub> channels (Spektor *et al.*, 2002; Hewawasam *et al.*, 2003; 2004; Boy *et al.*, 2004) and of K<sub>ATP</sub> channels (Holmquist *et al.*, 1990; Venkateswarlu *et al.*, 2002) were found to relax corpus cavernosum strips. However, the K<sub>ATP</sub> channel openers, cromakalim and pinacidil, were mainly proposed for intracavernosal injection, since systemic administration was associated with hypotension and reflex tachycardia (Trigo-Rocha *et al.*, 1995; Moon *et al.*, 1999). Moreover, the selectivity of the benzimidazolone, NS1619 for BK<sub>Ca</sub> channels has been questioned, since at high concentrations, NS1619 directly inhibits L-type Ca<sup>2+</sup> channels in rat ventricular myocytes (Park *et al.*, 2007), calcium-activated chloride currents (Saleh *et al.*, 2007) and voltage-activated Ca<sup>2+</sup>, K<sup>+</sup> and Na<sup>+</sup> channels (Edwards *et al.*, 1994; Holland *et al.*, 1996). Recently, a novel BK<sub>Ca</sub> channel opener NS11021 (1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-thiourea) was found to be a more potent BK<sub>Ca</sub> channel activator than NS1619 and specific, as it did not affect L- and T-type calcium currents in native guinea pig ventricular cardiomyocytes (Bentzen *et al.*, 2007). Therefore, the present study proposed that NS11021 would relax erectile smooth muscle preparations and enhance erectile function. Thus, in the present study, we investigated the effect of NS11021 on: (i) K<sup>+</sup> current in smooth muscle and endothelial cells; (ii) intracavernous arteries and corpus cavernosum strips isolated from rat and man *in vitro*; (iii) smooth muscle intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and tension in intact penile arteries; and (iv) cardiovascular and erectile function in a rat model. The effect of NS11021 was compared to an inhibitor of PDE5, sildenafil. We found that NS11021 induces powerful relaxation *in vitro* and increases erectile function *in vivo*.

## Methods

### Animals

Animal care and experimental procedures were according to Danish Animal Law and regulations (permission 2007/561-

1265). Adult male Wistar rats (12–16 weeks old) were kept and cared for in standard cages under clean conditions in separate quarters in a 12–12 h light–dark cycle with free access to water and food pellets.

### Patch clamp on freshly isolated SMCs from rat corpus cavernosum

Strips of corpus cavernosum (about 1–2 mm long; see below for preparation) were stored overnight at 4°C in the solution for enzymatic digestion of the following composition (in mM): NaCl 110, KCl 5, MgCl<sub>2</sub> 2, KH<sub>2</sub>PO<sub>4</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.5, NaHCO<sub>3</sub> 10, CaCl<sub>2</sub> 0.16, EDTA 0.49, Na-HEPES 10, glucose 10, taurine 10, as well as 1.5 mg·mL<sup>-1</sup> papain, 1.6 mg·mL<sup>-1</sup> albumin, and 0.4 mg·mL<sup>-1</sup> dl-dithiothreitol; pH was adjusted to 7.0. The next day, the tissue was incubated in the enzymatic solution for 5–10 min at 37°C, and the single SMCs were released by intense pipetting and after attachment to a dish, they were patched at room temperature (22–24°C). Patch pipettes (resistance 2–8 MΩ) were prepared from borosilicate glass (PG15OT- 7.5; Harvard Apparatus) pulled on a P-97 puller (Sutter Instrument Co.). Membrane currents were recorded in the whole-cell configuration with an Axopatch 200B amplifier (Axon Instruments, Inc.) and the filtered (1 kHz) data were sampled in Microcal Origin software v. 5.0 for Windows (Microcal Software, Inc.). Series resistance and capacitive current were compensated before each experiment. The current amplitudes (recorded in pA) were normalized to the cell capacitance (recorded in pF) and expressed as current density (A·F<sup>-1</sup>). The cells were held at a holding potential of -50 mV. Current–voltage characteristics were obtained from a ramp protocol (Figure 1A). Voltage ramps were performed by stepping initially from -50 mV to -100 mV for 100 ms and, then, depolarizing the whole cell from -100 mV to +80 mV with a duration of 700 ms.

Calcium-activated potassium current was stimulated by dialysis of the cell with a pipette solution containing calcium buffered at ~500 nM, a concentration corresponding to submaximal activation of BK<sub>Ca</sub> channels (Sadoshima *et al.*, 1988). Free [Ca<sup>2+</sup>]<sub>i</sub> in the pipette solution was estimated using WEBMAXC v. 2.22 (Chris Patton, Stanford University, CA, USA). The patch pipette solution contained (in mM): NaCl 10, KCl 122, K-HEPES 10, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.072, BAPTA 0.1 at pH 7.35. The bath solution contained (in mM): NaCl 135, KCl 6, Na-HEPES 10, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1 at pH 7.4.

### Patch clamp on human umbilical vein endothelial cells (HUVECs)

This part of the investigation conformed to the principles outlined in the Declaration of Helsinki and was approved by the local Ethics Committee of Region Midt (permission 20040154). Umbilical cords were obtained immediately after delivery. HUVECs were isolated as previously described (Ostergaard *et al.*, 2007). In all experiments, cells were used at passages 2 or 3, where they did not express BK<sub>Ca</sub> channels in qPCR studies (Krøigaard and Simonsen, unpublished observation). The endothelial cell phenotype was confirmed using phase-contrast microscopy (i.e. cuboidal, cobblestone-appearance monolayer of cells) and positive immuno-

fluorescence staining with antibodies against von Willebrand factor (Ostergaard *et al.*, 2007).

In HUVECs, membrane currents were measured using conventional whole-cell patch clamp techniques. HUVECs were dispersed using a non-enzymatic technique and plated on coverslips in a recording chamber containing physiological extracellular solution (PES) containing (in mM): NaCl 105, NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.17, Na-HEPES 10, EGTA 0.026, glucose 5.5, pH 7.4 (with NaOH) to which 1.6 mM CaCl<sub>2</sub> was added for Ca<sup>2+</sup>-containing PES. Recording electrodes (resistance 2–5 MΩ) were fabricated from borosilicate glass and were filled with physiological intracellular solution containing (in mM): potassium-L-aspartate 100, KCl 30, EGTA 10, MgCl<sub>2</sub> 1, Na<sub>2</sub>ATP 3, HEPES-potassium salt 5, pH 7.2 (with KOH). Membrane currents were recorded at room temperature using an EPC-10 amplifier (HEKA) filtered at 1 kHz, digitized at 10 kHz and stored on a PC for subsequent analysis. Current–voltage (IV) relationships were generated in voltage-clamped cells led at a membrane potential of –60 mV and stepped from –60 mV to +180 mV in 40 mV increments.

#### Functional in vitro experiments

**Human erectile tissue.** All patients provided informed consent and this study was in accordance with Ethical Committee, Country of Region Midtjylland, Denmark. Human corpus cavernosum tissues were obtained from 50- to 70-year-old patients with ED of various origins (diabetes, prostatic cancer or neurogenic origin). Cavernous tissue strips were retrieved at the time of penile prosthesis insertion. Immediately after removal, tissues were placed in physiological saline solution (PSS; see composition below) and kept at 4°C from the time of surgery until they were used in experiments, within 24 h.

**Rat erectile tissue.** The animals were killed humanely by cervical dislocation and exsanguinated after decapitation. After cutting the crura corpora cavernosa at the point of adhesion to the lower pubic bone, the penis was removed and submerged in ice-cold (4°C) PSS, and the corpus cavernosum was microsurgically dissected free.

**Preparation of intracavernous artery.** Ring segments (ca. 2 mm long) of intracavernous artery were dissected and mounted on two 40 μm wires of isometric double myograph (410A, Danish Myotechnology, Aarhus, Denmark) as previously described (Kun *et al.*, 2003). The preparations were allowed to equilibrate in PSS solution, at 37°C, bubbled with a mixture of 5% CO<sub>2</sub> in air O<sub>2</sub> to maintain pH at 7.4. The relation between resting wall tension and internal circumference of the vessels was determined, and the internal circumference, L<sub>100</sub>, corresponding to a transmural pressure of 100 mm Hg in a relaxed vessel was calculated. Subsequently, the internal circumference of the vessels were set to L<sub>1</sub>, where L<sub>1</sub> = 0.9 × L<sub>100</sub>. The effective internal lumen diameter was determined as l<sub>1</sub> = L<sub>1</sub>/π.

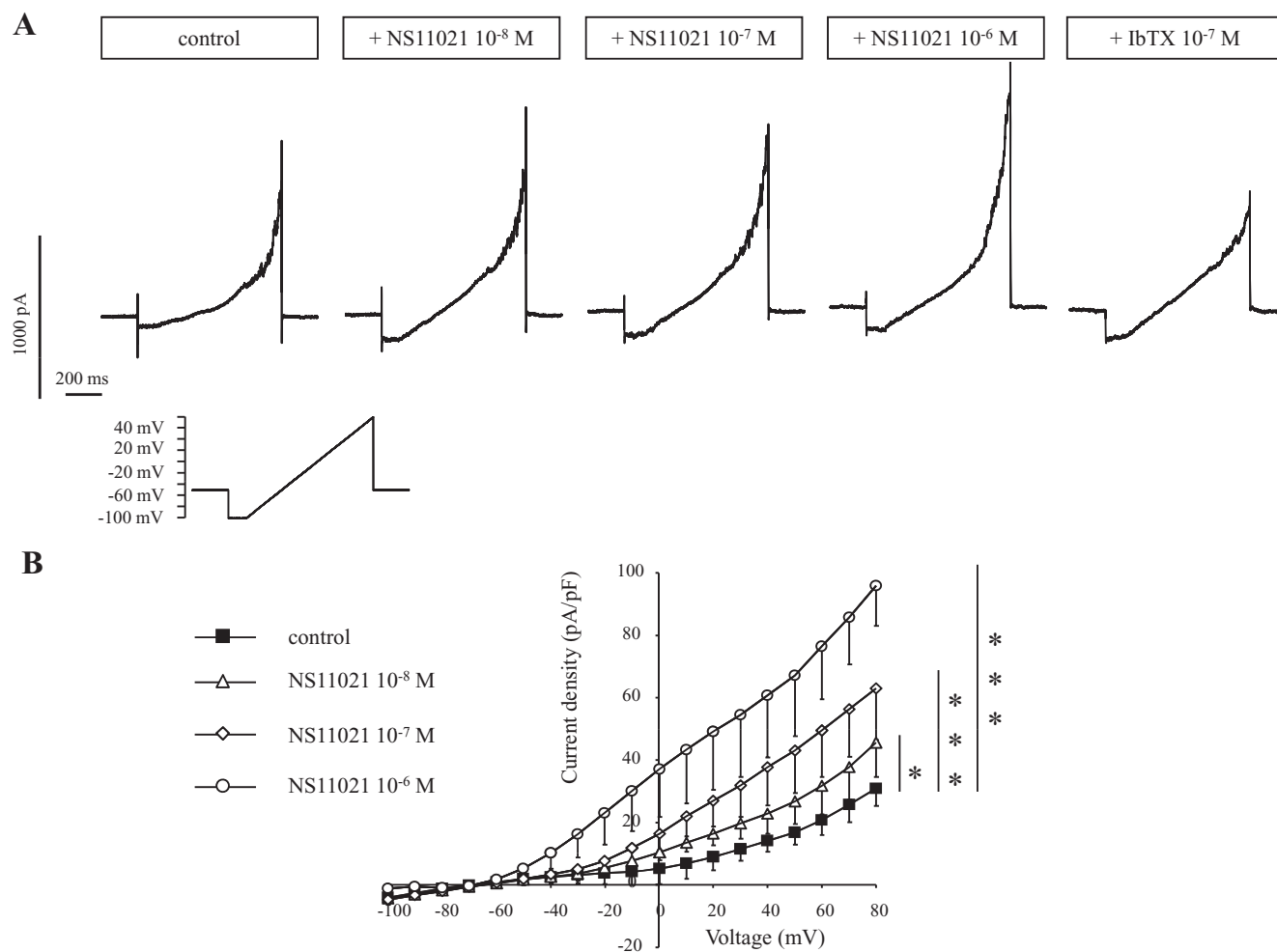
**Preparation of corpus cavernosum strips.** Change in isometric tension of human (1 × 1 × 4 mm) and rat (0.5 × 0.5 × 3 mm) corpus cavernosum strips were investigated in a tissue organ bath system (750TOBS, Danish Myotechnology, Aarhus, Denmark). Silk ligatures were applied at both ends of the strip

preparations, which were then suspended between two L-formed metal prongs in thermostatically controlled organ baths (5 mL, 37°C) containing PSS aerated with a mixture of 5% CO<sub>2</sub> in air (21% O<sub>2</sub>, pH 7.4). During an equilibration period of 60 min, tension was adjusted until a mean stable tension of, respectively, 1.4 mN and 1.2 mN was obtained for human and rat corpus cavernosum strips, as previously described (Hedlund *et al.*, 1999; Matsumoto *et al.*, 2005).

**Simultaneous measurements of [Ca<sup>2+</sup>]<sub>i</sub> and tension.** Simultaneous measurements of smooth muscle [Ca<sup>2+</sup>]<sub>i</sub> and tension were performed in intact arteries as previously described (Vilalba *et al.*, 2007). The arteries were mounted in a myograph as described above and loaded in the dark in PSS containing 8 μM fura 2-acetoxymethyl ester (fura 2-AM) and 0.1% cremophor EL for 3 h at 37°C. The bath solution was replaced with fresh PSS and FURA-2-AM after 90 min. Fluorescence measurements were performed using an inverted microscope equipped for dual-excitation wavelength fluorimetry (Deltascan, Photon Technology International, NJ, USA). The arteries were excited with alternating wavelengths of 340 and 380 nm, and the intensity of the emitted fluorescence was collected at a wavelength of 510 nm using a photomultiplier connected to a computer running software (Felix, Photon Technology International, USA). The 340/380 fluorescence ratio (F340/F380) was taken as a measure of [Ca<sup>2+</sup>]<sub>i</sub>. Ca<sup>2+</sup>-insensitive signals were determined after quenching with Mn<sup>2+</sup> (MnCl<sub>2</sub>) and subtracted.

**Experimental procedure.** To test contractility of the preparations, rat erectile tissues were exposed twice to 10 μM phenylephrine or 1 μM phenylephrine for human tissues. Arteries and corpus cavernosum strips were contracted with phenylephrine (1 μM), except human corpus cavernosum strips which were contracted with 0.5 μM phenylephrine, and cumulative concentration–response curves were constructed for NS11021 (0.1–100 μM), sildenafil (0.1 nM–30 μM) and vehicle for the drugs, or constructed in the presence of iberiotoxin (IbTX 0.1 μM), which was applied 30 min before obtaining the concentration–response curves and in a concentration causing full inhibition of BK<sub>Ca</sub> channels (Galvez *et al.*, 1990). Moreover, concentration–response curves for NS11021 were constructed in preparations contracted by 80 mM potassium-rich physiological salt solution, where NaCl was exchanged for KCl on an equivalent basis.

**Electrical field stimulation (EFS).** EFS was performed with platinum electrodes (J.P. Trading, Aarhus, Denmark), which were secured in plastic heads on both sides of the mounted segment, placed approximately 2 mm from the strips. The electrodes were connected to an electrical stimulator (CS200, Danish Myotechnology, Aarhus, Denmark) with submaximal constant current output. To examine the influence of drugs on neurogenic contractions, the preparations were incubated with asymmetric dimethylarginine (ADMA, 300 μM) and propranolol (1 μM) to inhibit concomitant NO release and β-adrenoceptors respectively (Kun *et al.*, 2003). The cumulative relaxant effect of drugs was investigated in strips contracted with 16 Hz EFS (supra-maximal current) applied with 4 min intervals.



**Figure 1** Patch clamp studies on smooth muscle cells from rat corpus cavernosum. Increasing concentrations of NS11021 ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M) stimulate membrane conductances which were then significantly inhibited by IbTX. (A) Typical experimental recordings from smooth muscle cell (membrane capacitance 17 pF) consequently stimulated with increasing concentrations of NS11021 and, then IbTX ( $0.1 \mu\text{M}$ ). Lower insert in (A) shows voltage ramp protocol used in these experiments. (B) shows average ( $n = 5$ ,  $C_m = 19 \pm 1$  pF) current-voltage recordings of IbTX-sensitive currents under control conditions and after cumulative increase of NS11021. The current-voltage curves achieved by subtraction of resting IbTX-insensitive current obtained at the end of each experiment. \*, \*\*\* $P < 0.05$ , 0.001 versus control IbTX-sensitive current by two-way ANOVA.

### In vivo experiments

**Measurements of intracavernous and mean arterial pressure (MAP).** The rats were anaesthetized with pentobarbital sodium (Sygehus Apoteket, Aarhus, Denmark;  $50 \text{ mg}\cdot\text{kg}^{-1}$ ; i.p.). During the experiment, the rats breathed spontaneously; the body temperature was monitored continuously and was maintained at  $37^\circ\text{C}$ . A heparinized ( $100 \text{ IE}\cdot\text{mL}^{-1}$ ) polyethylene catheter (PE 50) was introduced into the carotid artery to measure MAP. A 25 gauge needle attached to a heparinized polyethylene catheter was inserted into the crus corpus cavernosum to measure intracavernous pressure (ICP). Continuous direct measurements of MAP and ICP were performed with transducers (Disposable BP Transducer, ADInstruments, UK), and registered and analysed on a computerized data acquisition system (PowerLab, ADInstruments). A stabilizing period of 20–30 min was allowed before registration of basal ICP and MAP.

The cavernous nerve was isolated through a lower abdominal incision, and electrical stimulation was performed with a

slender bipolar platinum electrode, which was connected to a S48 stimulator (Grass Instrument Co., Boston, MA, USA). NS11021, sildenafil and vehicle were administered by injection in the jugular vein.

**Experimental procedure.** A first stimulation of the cavernous nerve at parameters eliciting the maximum amplitude of the erectile response (square wave pulses of 6 Volts, 10 Hz, 1 ms for 30 s) was performed, as described earlier (Giuliano *et al.*, 2003). Electrical parameters eliciting a submaximal erectile response were then sought between 0.6 and 1.55 Volts, other parameters remaining unchanged. Both NS11021 and sildenafil were injected at the doses of  $0.1$  and  $1 \text{ mg}\cdot\text{kg}^{-1}$  intravenously in volumes of maximum  $200 \mu\text{L}$ . One group was injected with vehicle alone. Submaximal electrical stimulation was applied 3, 13 and 23 min after the injection. At the end of each experiment, the maximal erectile response was repeated to ensure the cavernous nerve was intact.





EDTA 0.026 and glucose 5.5 (pH 7.4). The 80 mM K<sup>+</sup> solution had the same composition as PSS, but with NaCl replaced by KCl on an equimolar basis to attain the final 80 mM K<sup>+</sup> concentration.

The following drugs were used: NS11021 (NeuroSearch A/S, Ballerup, Denmark), sildenafil (gift from Pfizer Ltd.), ADMA, IbTX, phenylephrine hydrochloride, propranolol hydrochloride and tetraethylammonium chloride (Sigma, St. Louis, MO, USA). NS11021 was dissolved in DMSO, and then diluted in water. The maximal DMSO concentration (0.1%) applied *in vitro* did not modulate smooth muscle tone in control experiments. All other drugs were dissolved in distilled water. For *in vivo* experiments, NS11021 stock solution was diluted in sterile 0.9% NaCl; sildenafil was dissolved in sterile 0.9% NaCl.

## Results

### Patch clamp electrophysiology

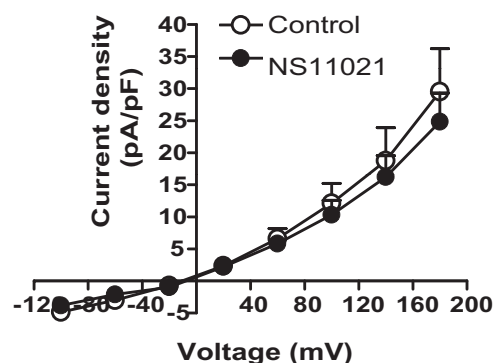
The effect of NS11021 on the membrane currents of freshly isolated SMCs from corpus cavernosum was tested using conventional whole-cell patch clamp technique (Figure 1). Initially, an outward rectifying membrane current with reversal potential at  $-21.5 \pm 4.5$  mV was observed. Cumulative addition of NS11021 increased whole-cell membrane current, primarily in the outward direction (Figure 1A). The current reversal potential in the presence of  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M NS11021 was shifted to the left ( $-28.0 \pm 5.8$  mV,  $-36.9 \pm 3.7$  mV and  $-48.0 \pm 4.6$  mV respectively). Addition of IbTX (0.1  $\mu$ M) inhibited membrane conductance in the outward direction. As the K<sup>+</sup> ion in the current solution composition (see Methods) was the only ion with negative equilibrium potential ( $-79$  mV), we suggested that NS11021 stimulated primarily BK<sub>Ca</sub> conductance which was inhibited by IbTX. When the resting IbTX-insensitive current was subtracted from the whole-cell conductance, a significant potentiation of IbTX-sensitive BK<sub>Ca</sub> current with NS11021 was observed (Figure 1B). The reversal potentials of IbTX-sensitive currents were  $-62 \pm 14$  mV,  $-63 \pm 8$  mV,  $-65 \pm 7$  mV and  $-71 \pm 6$  mV under control conditions and in the presence of  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M of NS11021 respectively.

To test the hypothesis that NS11021 specifically activates BK<sub>Ca</sub> current, SMCs were pretreated with IbTX (Figure 2) which significantly inhibited outward current (Figure 2A). The subsequent application of  $10^{-8}$  and  $10^{-7}$  M NS11021 had no effect on the membrane conductance but  $10^{-6}$  M NS11021 potentiated current in the outward direction (Figure 2B), an effect inhibited by tetraethylammonium (TEA, 1 mM).

To investigate the effect of NS11021 on membrane potential in HUVECs, the current–voltage relationships were determined in voltage-clamped endothelial cells. NS11021 (1  $\mu$ M) did not modulate the membrane current in HUVEC (Figure 3,  $n = 5$ ).

### Effect of NS11021 on [Ca<sup>2+</sup>]<sub>i</sub>

In phenylephrine (1  $\mu$ M)-contracted arteries, NS11021 evoked concentration-dependent relaxations and lowered [Ca<sup>2+</sup>]<sub>i</sub> (Figure 4A). In arteries contracted by 125 mM K<sup>+</sup>, higher con-



**Figure 3** Average effect of NS11021 ( $10^{-6}$  M) on the current–voltage relationships of voltage-clamped human umbilical vein endothelial cells (HUVECs,  $n = 5$ ).

centrations of NS11021 were required to lower calcium and relax the preparations (Figure 4B), and the concentration–response curves for NS11021 were markedly shifted to the right in preparations contracted by 125 mM K<sup>+</sup> compared with those contracted by phenylephrine (Figure 4C).

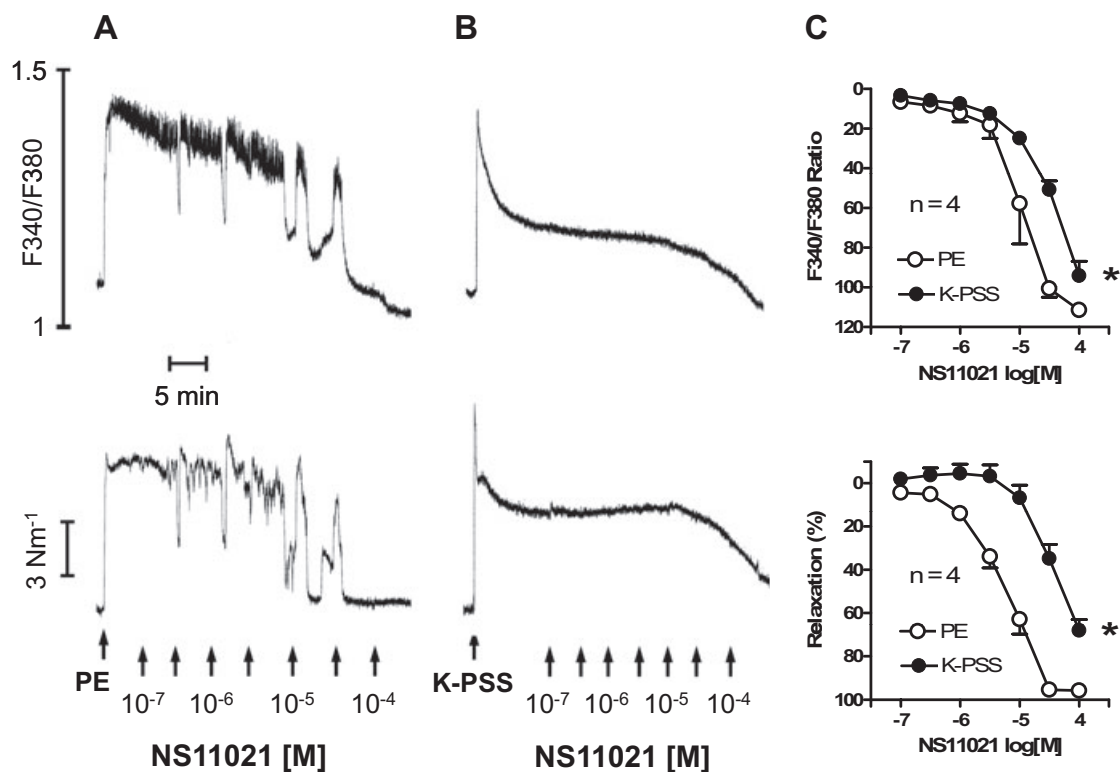
### In vitro investigations on rat tissues

In penile arteries with internal diameters of  $195 \pm 5$   $\mu$ M ( $n = 24$ ) and corpus cavernosum strips isolated from rat penile tissue, phenylephrine (10  $\mu$ M) induced contractions of, respectively,  $2.1 \pm 0.1$  N·m<sup>-1</sup> ( $n = 24$ ) and  $4.5 \pm 0.2$  mN ( $n = 87$ ).

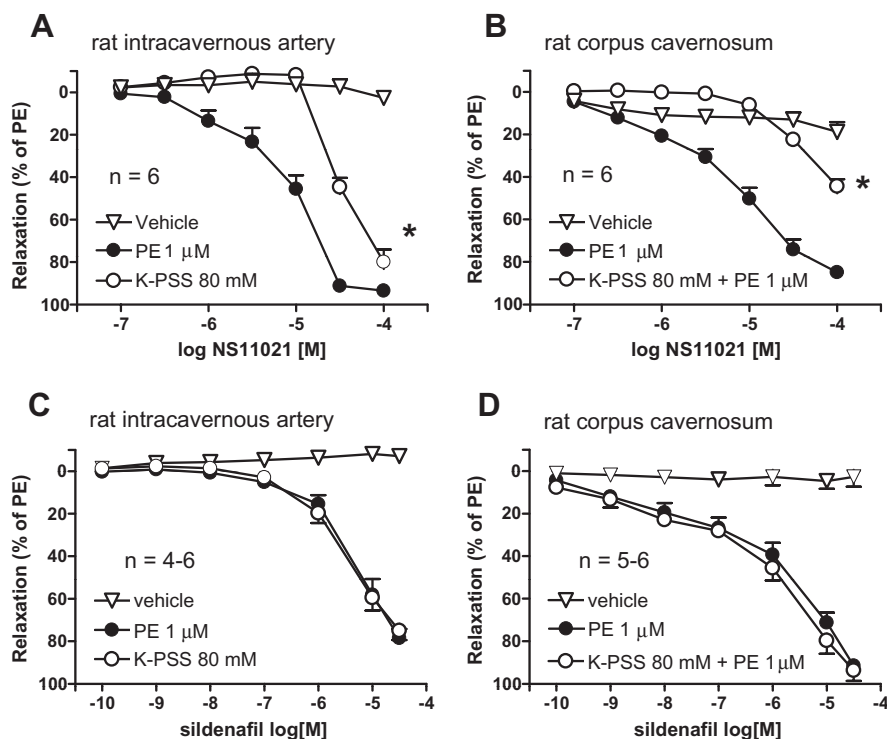
NS11021 (0.1–100  $\mu$ M) relaxed phenylephrine-contracted (1  $\mu$ M) penile arteries and corpus cavernosum strip preparations in a concentration-dependent manner (Figure 5A and B respectively) ( $n = 6$ ). The pD<sub>2</sub> values for NS11021 in phenylephrine-activated penile arteries and corpus cavernosum preparations were  $5.1 \pm 0.1$  and  $4.8 \pm 0.1$  respectively ( $n = 6$ ). In arteries and corpus cavernosum strips contracted with 80 mM K<sup>+</sup> or 80 mM K<sup>+</sup> plus 1  $\mu$ M phenylephrine, NS11021 relaxation was reduced, compared with preparations contracted with 1  $\mu$ M phenylephrine (Figure 5A and B) ( $n = 4$ ). The pD<sub>2</sub> values in intracavernous artery and corpus cavernosum preparations contracted with 80 mM K<sup>+</sup> were  $4.4 \pm 0.1$  and  $4.0 \pm 0.1$  respectively ( $n = 4$ ). Vehicle had no effect (Figure 5A and B) ( $n = 6$ ).

Sildenafil (0.1 nM–30  $\mu$ M), an inhibitor of PDE5, induced relaxations in both rat penile arteries and corpus cavernosum strips contracted with 1  $\mu$ M phenylephrine as well as in 80 mM K<sup>+</sup>-activated preparations (Figure 5C and D). The pD<sub>2</sub> values for sildenafil in penile arteries contracted with 1  $\mu$ M phenylephrine and 80 mM K<sup>+</sup> were, respectively,  $5.2 \pm 0.1$  and  $5.5 \pm 0.1$  ( $n = 4$ ) and in corpus cavernosum strips, respectively,  $5.7 \pm 0.1$  and  $6.0 \pm 0.1$  ( $n = 5$ ). The vehicle of sildenafil had no effect in these preparations (Figure 5C and D).

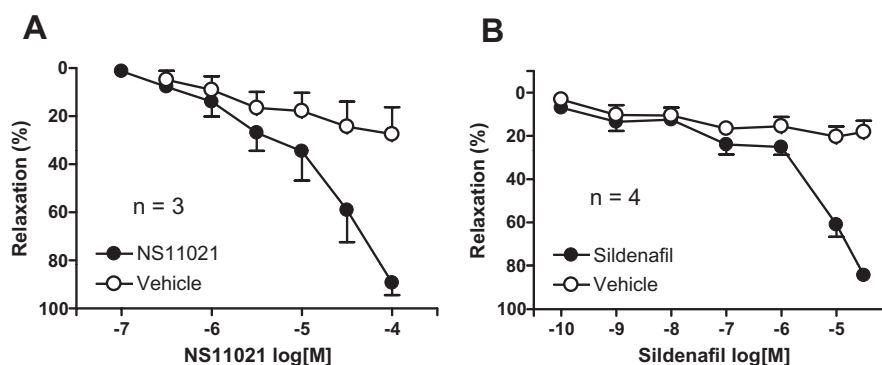
In corpus cavernosum preparations, incubated with ADMA (300  $\mu$ M) and propranolol (1  $\mu$ M), EFS (16 Hz) produced reproducible, frequency-dependent contractions producing an average force of  $3.4 \pm 0.2$  mN ( $n = 15$ ). Prazosin (1  $\mu$ M) inhibited the EFS-evoked contractions by  $99.9 \pm 0.1\%$  ( $n = 3$ , data not shown). NS11021 induced concentration-dependent (0.1–100  $\mu$ M) inhibition of EFS-induced contractions. The



**Figure 4** Effects of NS11021 on  $[Ca^{2+}]_i$  and tension in rat intracavernous artery. Effect of NS11021 in (A) phenylephrine (PE, 1  $\mu$ M)- and (B) 125 mM  $K^+$ -contracted arteries. (C) Average data showing the decrease in  $[Ca^{2+}]_i$  and tension in PE- and K-PSS-contracted preparations in response to cumulative administration of NS11021 ( $n = 4$ ). \* $P < 0.05$  by two-way ANOVA.



**Figure 5** Average NS11021 (A, B) and sildenafil (C, D) induced relaxation in phenylephrine (PE)-contracted and high potassium (125 mM  $K^+$ )-contracted intracavernous artery (A, C,  $n = 4-6$ ) and corpus cavernosum strips (B, D,  $n = 5-6$ ) isolated from rat penis. \* $P < 0.05$  by two-way ANOVA.



**Figure 6** Average effect of (A) NS11021, (B) sildenafil, and vehicle on electrical field stimulation-evoked neurogenic contractions in rat corpus cavernosum strips ( $n = 3-4$ ).

maximal inhibitory effect of  $89 \pm 5\%$  was obtained at  $100 \mu\text{M}$  NS11021 ( $n = 3$ ) (Figure 6A). The  $\text{pD}_2$  value for NS11021 on contractions produced by EFS was  $4.7 \pm 0.3$  ( $n = 3$ ). Sildenafil ( $0.1 \text{ nM}-30 \mu\text{M}$ ) induced almost full inhibition, similar to NS11021, on EFS-induced contractions with a maximum of  $84 \pm 3\%$  ( $n = 4$ ) (Figure 6B). The  $\text{pD}_2$  value for sildenafil was  $5.1 \pm 0.1$  ( $n = 4$ ). Vehicle did not affect the EFS-evoked contractions.

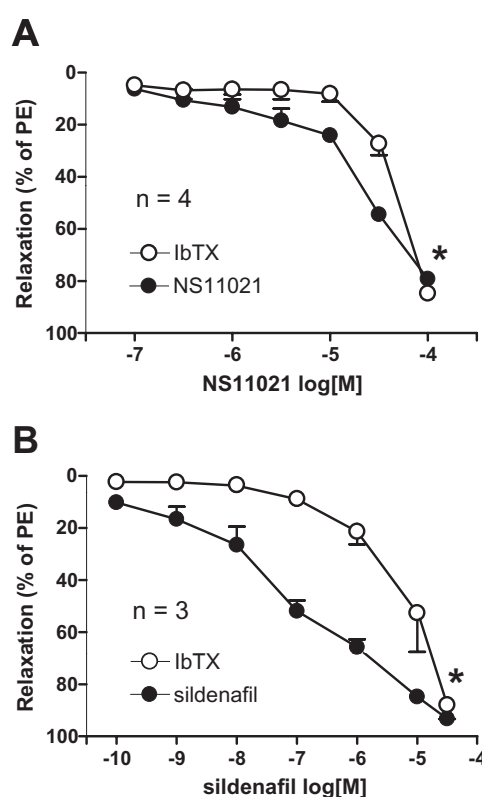
#### In vitro investigations on human tissues

In intracavernous arteries (with internal diameter of  $202 \pm 21 \mu\text{M}$ ,  $n = 2$ ), phenylephrine ( $10 \mu\text{M}$ ) increased active tension by  $4.1 \pm 1.0 \text{ Nm}^{-1}$  ( $n = 2$ ). In two representative experiments, NS11021 and sildenafil induced concentration-dependent relaxations in penile small intracavernous arteries isolated from human tissue, while vehicle failed to alter phenylephrine contraction (data not shown).

In corpus cavernosum strips, phenylephrine ( $1 \mu\text{M}$ ) increased force by  $20.5 \pm 1.8 \text{ mN}$  ( $n = 16$ ). NS11021 ( $0.1-100 \mu\text{M}$ ) induced IbTX-sensitive concentration-dependent relaxations in phenylephrine ( $0.5 \mu\text{M}$ )-contracted preparations (Figure 7A). The  $\text{pD}_2$  values for NS11021 in the absence and presence of IbTX were  $4.5 \pm 0.1$  and  $4.3 \pm 0.1$  respectively ( $n = 4$ ). Sildenafil-induced relaxations were also inhibited by IbTX (Figure 7B,  $n = 3$ ).  $\text{pD}_2$  values for sildenafil in the absence and presence of IbTX were  $7.0 \pm 0.1$  and  $5.2 \pm 0.2$ .

#### In vivo experiments

Original traces show the experimental procedure (Figure 8A) and the magnification of an erectile response to electrical stimulation (Figure 8B). Administration of vehicle did not influence the erectile response or blood pressure (Figure 9A,  $n = 5$ ), but sildenafil ( $0.1 \text{ mg}\cdot\text{kg}^{-1}$ ) slightly increased, while a higher dose of sildenafil ( $1 \text{ mg}\cdot\text{kg}^{-1}$ ) resulted in a significant increase of the erectile response compared with the control response (Figure 9B,  $n = 5$ ). Erectile responses in anaesthetized rats were significantly increased after injection of both  $0.1$  and  $1 \text{ mg}\cdot\text{kg}^{-1}$  NS11021 (Figure 9C,  $n = 5$ ). A non-selective blocker of  $\text{K}_{\text{Ca}}$  channels, TEA was applied as intravenous injection ( $6 \text{ mg}\cdot\text{kg}^{-1}$ ), and caused an initial decrease in erectile responses as well as in MAP, but at the third electrical stimulation, the erectile response and MAP had recovered to

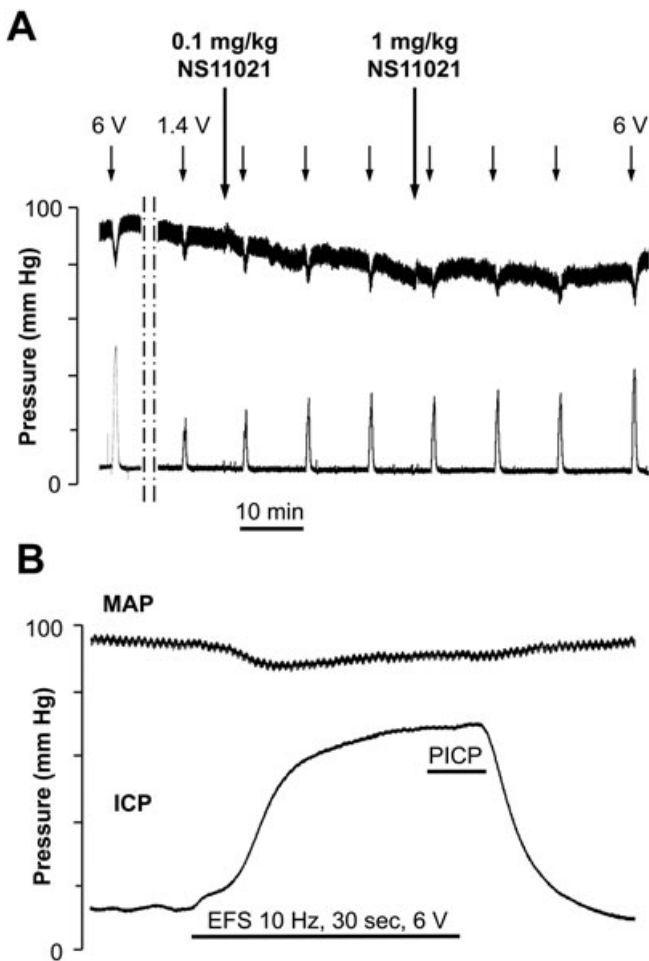


**Figure 7** Average NS11021 (A,  $n = 4$ ) and sildenafil (B,  $n = 3$ ) induced relaxations in phenylephrine (PE,  $0.5 \mu\text{M}$ )-contracted human corpus cavernosum strips in the presence and absence of iberiotoxin (IbTX,  $0.1 \mu\text{M}$ ).  $*P < 0.05$  by two-way ANOVA.

control values (Figure 9D,  $n = 4$ ). However, the facilitating effect of NS11021 ( $1 \text{ mg}\cdot\text{kg}^{-1}$ ) on erectile responses was abolished in the presence of TEA (Figure 9D).

NS11021 ( $0.1 \text{ mg}\cdot\text{kg}^{-1}$ ) did not change MAP, but a higher dose ( $1 \text{ mg}\cdot\text{kg}^{-1}$ ) induced a transient drop in MAP (maximum fall of  $9.0 \pm 2.5 \text{ mmHg}$ ,  $n = 5$ ), which recovered to pre-injection values after  $41.8 \pm 15.2 \text{ s}$  (data not shown). TEA prevented the transient drop in blood pressure after administration of  $1 \text{ mg}\cdot\text{kg}^{-1}$  NS11021 (data not shown). Sildenafil ( $0.1 \text{ mg}\cdot\text{kg}^{-1}$ ) induced a sustained drop in MAP of  $24.9 \pm 1.4 \text{ mmHg}$ , while a higher dose ( $1 \text{ mg}\cdot\text{kg}^{-1}$ ) caused a





**Figure 8** (A) Original trace of mean arterial pressure and intracavernous pressure recordings obtained in male Wistar rat anaesthetized with pentobarbital. NS11021 (0.1 and 1 mg·kg<sup>-1</sup>) was administered (↓) after establishing a maximal (10 Hz, 1 ms, 6 V) and submaximal response (10 Hz, 1 ms, 0.6 to 1.55 V) to cavernous nerve stimulation for 30 seconds. Submaximal stimulation was repeated 3, 13 and 23 min after drug administration. Upper trace shows the mean arterial pressure (MAP), lower trace represents the intracavernous pressure (ICP). (B) Represent a magnification of an erectile response induced by electrical field stimulation (EFS) of the cavernous nerve. The plateau response considered for calculation of the peak intracavernous pressure (PICP) during the erectile response. Erectile response was calculated by PICP(mm Hg)/MAP(mm Hg) × 100.

transient decrease in blood pressure of  $27.3 \pm 4.7$  mm Hg ( $n = 5$ ).

## Discussion

The main findings of the present study are that NS11021, a novel selective opener of BK<sub>Ca</sub> channels, increased IbTX-sensitive potassium currents in vascular smooth muscle, induced potent relaxations of penile arteries and corpus cavernosum strips, and facilitated erectile responses in anaesthetized rats. The magnitude of the effect of NS11021 was comparable with that of sildenafil, a potent inhibitor of PDE5.

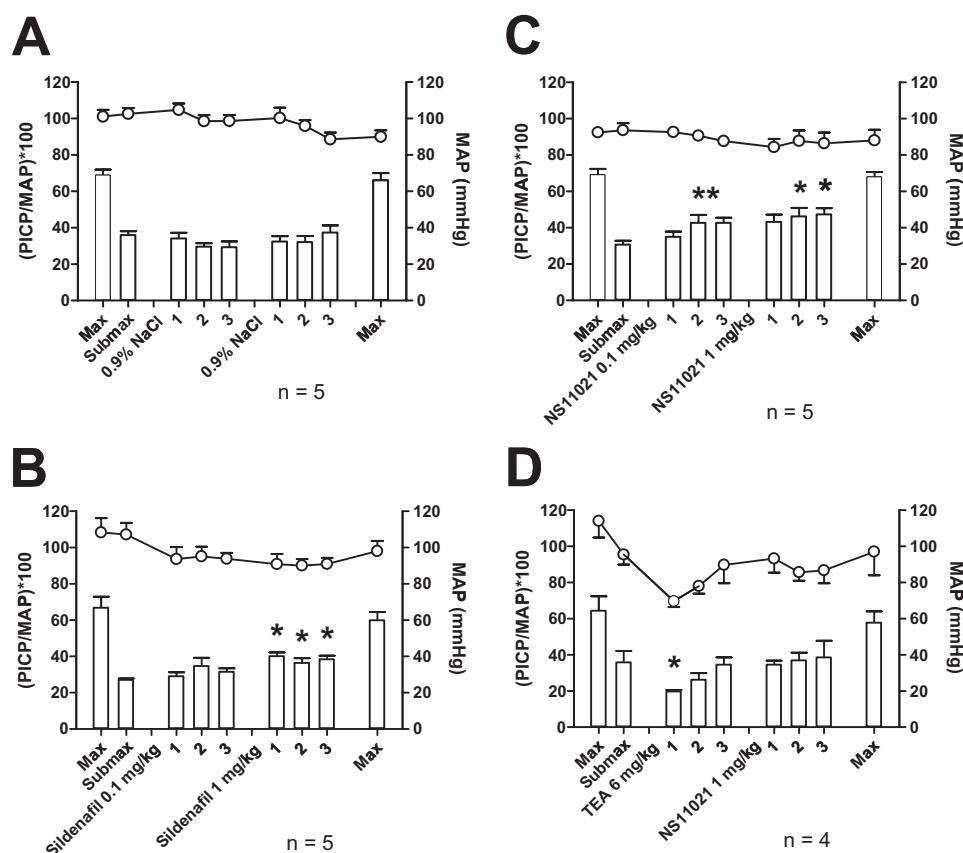
NS11021 was recently found to activate BK<sub>Ca</sub> channels in single-channel recordings and two-electrode voltage clamp

electrophysiological recordings of monoclonal HEK293 cells (Bentzen *et al.*, 2007). In our experiments, NS11021 increased the K<sup>+</sup> currents in a concentration-dependent manner (10 nM–1 μM) in rat freshly isolated corpus cavernosum SMCs, and IbTX inhibited the effect of low NS11021 concentrations ( $\leq 1$  μM), suggesting NS11021 increases BK<sub>Ca</sub> current. NS11021 up to 1 μM did not change membrane current in HUVECs, suggesting that the effect on BK<sub>Ca</sub> channels is specific. However, higher concentrations of NS11021 ( $\geq 1$  μM) activated an additional outward rectifying potassium current which was sensitive to TEA (1 mM). Low and intermediate TEA concentrations (0.1–3 mM) can also block voltage-gated K<sup>+</sup> channels of the K<sub>v</sub>7.2 and K<sub>v</sub>7.4 sub-type (Hadley *et al.*, 2000). Moreover, in oocyte studies, NS11021 (30 μM) was found to increase voltage-gated K<sub>v</sub>7.4 current, an effect which was blocked by TEA (Bentzen *et al.*, 2007). Therefore, the present patch clamp experiments suggest that at submicromolar concentrations, NS11021 increases BK<sub>Ca</sub> current while at higher concentrations, activation of voltage-gated K<sup>+</sup> current may also contribute to the effect of NS11021 in rat penile smooth muscle.

In the present study, NS11021-evoked maximal relaxation was inhibited but not abolished contraction induced by high extracellular potassium concentration in intracavernous arteries and corpus cavernosum strips. Moreover, the reduction in [Ca<sup>2+</sup>]<sub>i</sub> and tension was inhibited in the presence of high potassium solution only at low concentrations of NS11021 (<10 μM). Although in guinea pig ventricular cardiomyocytes, NS11021 had no effect on L- and T-type calcium currents (Bentzen *et al.*, 2007), we can not exclude the possibility of modulation of Ca<sup>2+</sup> currents in cavernosal SMCs. Therefore, in addition to the effect on BK<sub>Ca</sub> channels at submicromolar concentrations, on K<sub>v</sub> channels at supramicromolar concentrations, blocking of calcium entry appear to contribute to the relaxant effect of high concentrations (>10 μM) of NS11021.

As mentioned in the Introduction, several lines of evidence support an important role for BK<sub>Ca</sub> channels in erection (Prieto, 2008) and putative openers of BK<sub>Ca</sub> channels, NS1619, 4-aryl-3-(hydroxyalkyl)quinolin-2-ones and 3-thioquinolinones applied in drug concentrations of 10 μM have previously been found to relax corpus cavernosum strips by 20–50% (Spektor *et al.*, 2002; Hewawasam *et al.*, 2003; Boy *et al.*, 2004). In the present study, NS11021 (1–10 μM) induced significant relaxations both in intracavernous arteries and corpus cavernosum strips. Moreover, IbTX induced a rightward shift in the concentration–response curves for NS11021 in human corpus cavernosum strips. These observations suggest that NS11021 is a potent and selective opener of BK<sub>Ca</sub> channels in erectile smooth muscle.

Sildenafil, by inhibition of PDE5, increases cyclic GMP leading to activation of cyclic GMP-dependent protein kinase which, through phosphorylation, leads to increased Ca<sup>2+</sup>-ATPase activity and sarcoplasmic calcium uptake, activation of myosin light chain phosphatase and opening of BK<sub>Ca</sub> channels (Simonsen *et al.*, 2002; Prieto, 2008). Thus, the relaxant effect of sildenafil in horse penile resistance arteries is due in part to cyclic GMP accumulation and to the increased effects of basally released endothelial NO (Prieto *et al.*, 2006b), and sildenafil relaxes rabbit corpus cavernosum smooth muscle by



**Figure 9** Average effect of vehicle (A), sildenafil (B) and NS11021 (C) on mean arterial pressure and erectile response obtained in male Wistar rats anaesthetized with pentobarbital. Drugs or vehicle were administered after establishing the maximal (10 Hz, 1 ms, 6 V) and submaximal responses (10 Hz, 1 ms, 0.6 to 1.55 V) to cavernous nerve stimulation for 30 s. Submaximal stimulation was repeated 3, 13 and 23 min after drug or vehicle administration. (D) Effect of intravenous tetraethylammonium (TEA, 6 mg·kg<sup>-1</sup>) pre-treatment on mean arterial pressure, erectile response and on effect of 1 mg·kg<sup>-1</sup> NS11021. Upper traces show the mean arterial pressure (MAP), bars represent the erectile response ((PICP/MAP)\*100), where PICP is the peak intracavernous pressure. \*  $P < 0.05$  compared with submaximal control response using one-way ANOVA followed by Dunnett's test.

amplifying the effects of the endogenous cyclic GMP-dependent relaxation mechanisms (Chuang *et al.*, 1998). In previous studies, high K<sup>+</sup> solutions and IbTX inhibited the relaxation evoked by sildenafil in horse penile small arteries and rabbit corpus cavernosum strips (Prieto *et al.*, 2006a; El-Metwally *et al.*, 2007), and sildenafil enhanced the NO-induced activation of BK<sub>Ca</sub> channels in SMCs of human corpus cavernosum (Lee and Kang, 2001). The present results also suggest that the relaxant responses elicited by sildenafil involve the activation of BK<sub>Ca</sub> channels in both intracavernous artery and human corpus cavernosum strips, as sildenafil relaxations were inhibited in the presence of IbTX. However, in the present study, high K<sup>+</sup> solutions inhibited NS11021 but not sildenafil-induced relaxation in rat penile arteries and corpus cavernosum strips, and IbTX did not modify sildenafil relaxation in rat erectile tissue (data not shown). These findings suggest that there are species differences with regard to involvement of BK<sub>Ca</sub> channels in sildenafil relaxation in erectile tissue.

In diabetes, smooth muscle relaxation mechanisms are often impaired and vasoconstriction in erectile tissue increased (Sáenz de Tejada *et al.*, 2005; Wingard *et al.*, 2007).  $\alpha$ -Adrenoceptor-mediated contraction maintains the penis in

the flaccid state (Simonsen *et al.*, 2002). In the present study, blockade of EPS-evoked contractions by prazosin also supported the proposal that the neurogenic contraction of corpus cavernosum was elicited by activation of post-junctional  $\alpha_1$ -adrenoceptors. Both sildenafil and NS11021 caused inhibition of neurogenic contractions in erectile tissue in the present study. Therefore, in addition to the augmenting effect of NS11021 in basally released NO-mediated activation of the BK<sub>Ca</sub> channels (Taniguchi *et al.*, 1993), inhibition of  $\alpha$ -adrenoceptor-mediated contraction due to direct activation of BK<sub>Ca</sub> channels may also play a role in the relaxant effect of NS11021.

In anaesthetized rats, electrical stimulation of the cavernous nerve induces erection and increases the intracavernous pressure. However, the PICP during the erectile response and MAP are correlated; therefore, increases in intracavernous pressure are expressed as the ratio of the PICP over the corresponding MAP during the response (Matsumoto *et al.*, 2005). This allows analysis of the effect of the drug treatment on the erectile response independently of their effect on blood pressure. In our experiments, NS11021, administered intravenously in 0.1 mg·kg<sup>-1</sup> and 1 mg·kg<sup>-1</sup> doses, significantly increased the erectile response in rats without changing MAP.

Moreover, TEA abolished the effect of NS11021 1 mg·kg<sup>-1</sup> on the erectile response. Although TEA can block several K<sub>Ca</sub> channels subtypes (Sheng and Braun, 2007), the role of BK<sub>Ca</sub> channels in the regulation of the circulation *in vivo*, investigated by TEA, is described in different animal (Eduardo da Silva-Santos and Assreuy, 1999), as well as in human studies (Bellien *et al.*, 2005). Taken together with the findings that IbTX blocks NS11021 *in vitro*, our results suggest that NS11021 enhances erectile function in intact animals primarily through activation of BK<sub>Ca</sub> channels.

In the present study, sildenafil was able to facilitate significantly the erectile response only at the 1 mg·kg<sup>-1</sup> dose. These findings agree with previous studies in rats where erectile responses were also unchanged after administration of 0.1 mg·kg<sup>-1</sup> dose of sildenafil (Giuliano *et al.*, 2003). The *in vitro* results suggest that NS11021 and sildenafil are equipotent, and taking the difference in molar weight of NS11021 (367 g·mol<sup>-1</sup>) and sildenafil (474.6 g·mol<sup>-1</sup>) into account, the *in vivo* effect on erection of the two compounds is also of comparable magnitude.

Augmented contractility and/or impaired relaxation of arterial and corporal smooth muscle are a primary cause of ED. The fact that NS11021, an activator of BK<sub>Ca</sub> channels, enhanced the erectile response in rats *in vivo* is consistent with previous findings showing increased erectile responses in old and diabetic rats after intracavernosal injection of the *hSlo* gene, which encodes the BK<sub>Ca</sub> channel (Christ *et al.*, 2004). Moreover, altered BK<sub>Ca</sub> channels function may contribute to the development of other vascular diseases, such as hypertension, diabetes and atherosclerosis (Sobey, 2001). Therefore, activators of this channel may have a therapeutic potential in treatment of ED and cardiovascular disease.

In summary, the present study provides evidence suggesting that NS11021, a novel opener of BK<sub>Ca</sub> channels, causes potent relaxations of erectile smooth muscle *in vitro* and facilitates erectile response *in vivo*, at doses devoid of cardiovascular side effects. These responses are comparable to those elicited by sildenafil, suggesting a potential for use of BK<sub>Ca</sub> openers in the treatment of ED.

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## Conflict of interest

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