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RESEARCH PAPER

Role of neuronal voltage-gated K^+ channels in the modulation of the nitrergic neurotransmission of the pig urinary bladder neck

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Background and purpose: As nitric oxide (NO) plays an essential role in the inhibitory neurotransmission of the bladder neck of several species, the current study investigates the mechanisms underlying the NO-induced relaxations in the pig urinary bladder neck.

Experimental approach: Urothelium-denuded bladder neck strips were dissected and mounted in isolated organ baths containing a physiological saline solution at 37 °C and continuously gassed with 5% CO_2 and 95% O_2, for isometric force recording. The relaxations to transmural nerve stimulation (EFS), or to exogenously applied acidified $NaNO_2$ solution were carried out on strips pre-contracted with phenylephrine, and treated with guanethidine and atropine, to block noradrenergic neurotransmission and muscarinic receptors, respectively.

Key results: EFS (0.2–1 Hz) and addition of acidified $NaNO_2$ solution (1 μM–1 mM) evoked frequency- and concentration-dependent relaxations, respectively. These responses were potently reduced by the blockade of guanylate cyclase and were not modified by the K^+ channel blockers iberiotoxin, charybdotoxin, apamin or glibenclamide. The voltage-gated K^+ (Kv) channels inhibitor 4-aminopyridine, greatly enhanced the nitrergic relaxations evoked by EFS, but did not affect the $NaNO_2$ solution-induced relaxations.

Conclusions and implications: NO, whose release is modulated by pre-junctional Kv channels, relaxes the pig urinary bladder neck through a mechanism dependent on the activation of guanylate cyclase, in which post-junctional K^+ channels do not seem to be involved. Modulation of Kv channels could be useful in the therapy of the urinary incontinence produced by intrinsic sphincteric deficiency.

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Keywords: NO; guanylate cyclase-dependent mechanism; K^+ channels; neuronal voltage-gated K^+ channels; pig urinary bladder neck

Abbreviations: 4-AP, 4-aminopyridine; ChTX, charybdotoxin; ω-CgTX, ω-conotoxin GVIA; EFS, electrical field stimulation; IbTX, iberiotoxin; K_{ATP} channels, ATP-dependent K^+ channels; K_{Ca} channels, Ca^{2+}-activated K^+ channels; K_V channels, voltage-gated K^+ channels; L-NOARG, N^G-nitro-L-arginine; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; NANC, non-adrenergic non-cholinergic; VOC, voltage-gated Ca^{2+} channels

Introduction

Nitric oxide (NO) plays an essential role in the relaxations of the lower urinary tract, by regulating the smooth muscle tone of the outflow region formed by the bladder neck and urethra (Andersson and Wein, 2004; Hedlund, 2005). Whereas the mechanisms underlying the NO-mediated relaxation in the mammalian urethra have extensively been investigated (Andersson and Wein, 2004), there is little information about such a relaxation in the urinary bladder neck (Hills *et al*., 1984; Thornbury *et al*., 1992). We have recently reported a modulation exerted by pre-junctional α_2-adrenoceptor stimulation on the nitrergic neurotransmission of the pig urinary bladder neck (Hernández *et al*., 2007).

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In addition to the NO-induced response, an NO-independent relaxation exerted by peptides such as pituitary adenylate cyclase-activating polypeptide 38 (PACAP 38), mainly released from capsaicin-sensitive primary afferents, has been described in the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission of the pig bladder neck (Hernández *et al*., 2006b). Both PACAP 38 and vasoactive intestinal peptide produce relaxation through muscle $VPAC_2$ receptors linked to the cAMP–PKA pathway and involving activation of voltage-gated K^+ (K_V) channels. Facilitatory PAC_1 receptors located at capsaicin-sensitive primary afferents and coupled to NO release, and inhibitory VPAC receptors at motor endings, are also involved in the relaxations to PACAP 38 and vasoactive intestinal peptide, respectively (Hernández *et al*., 2006a).

K^+ channels play a major role in the regulation of urinary bladder smooth muscle tension. Both Ca^{2+}-activated K^+ (K_{Ca}) (Heppner *et al*., 1997; Herrera *et al*., 2000) and K_V (Thorneloe and Nelson, 2003) channels are involved in the repolarization of the muscle action potential and in the maintenance of the resting membrane potential, inhibiting smooth muscle contraction. In addition, activation of ATP-dependent K^+ (K_{ATP}) channels relaxes bladder smooth muscle, to favour hyperpolarization and to exert an inhibitory action of membrane voltage-gated Ca^{2+} (VOC) channels (Foster *et al*., 1989; Bonev and Nelson, 1993). However, no data exist about the role of K^+ channels in the nitrergic neurotransmission of the urinary bladder neck. Therefore, the current study investigates the role of the guanylate cyclase pathway and K^+ channels in the relaxations of the pig urinary bladder neck to endogenously released or exogenously added NO.

Materials and methods

Dissection and mounting

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders were removed immediately after the animals were killed, and kept in chilled physiological saline solution (PSS) at 4 °C. The adjacent connective and fatty tissues were removed with care and longitudinal strips were dissected out from the bladder neck, which is located below the trigone (8–9 mm from the urethral orifices) and 4–5 mm above the proximal urethra. Strips (4–6 mm long and 2–3 mm wide) were suspended horizontally and placed parallel between two platinum electrodes, with one end connected to an isometric force transducer (Grass FT 03C) and the other one to a micrometer screw, in 5 ml organ baths containing PSS at 37 °C gassed with carbogen (95% O_2 and 5% CO_2) to obtain a final pH of 7.4. The signal was continuously recorded on a polygraph (Graphtec Multicorder MC 6621). Passive tension of 2 g was applied to the strips and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile ability of the strips was determined by exposing them to potassium-rich (124 mM) PSS. In electrical field stimulation (EFS) experiments, noradrenergic neurotransmission and muscarinic receptors were blocked by pre-incubation with guanethidine (10 μM) and atropine (0.1 μM) for 1 h, replacing the solution every 20 min, and these drugs were present throughout the experiment. In strips pre-contracted with 1 μM phenylephrine, EFS was performed by delivering rectangular pulses (1 ms duration, 0.2–1 Hz, 20 s trains, with constant current output adjusted to 75 mA), at 4-min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain), parameters previously used to promote NO release from intramural nerves in the intravesical ureter (Hernández *et al*., 1995). EFS-induced relaxations were previously demonstrated to be abolished by the neuronal voltage-activated Na^+ channel blocker tetrodotoxin, thus indicating their neurogenic nature (Hernández *et al*., 2007). A first control–response curve was obtained for EFS and a second curve was obtained after incubation for 30 min with specific blockers (of neuronal VOC channels, guanylate cyclase, NO synthase (NOS) or K^+ channels). In experiments with $NaNO_2$-acidified solution, a first cumulative concentration–response curve (1 μM–1 mM) was obtained, the bath solution was then changed every 15 min for a period of 90 min, the preparations were incubated for 30 min with the specific treatments, and then a second relaxation curve was constructed.

Calculations and statistics

Sensitivity to acidified $NaNO_2$ solution is expressed in terms of pD_2, where $pD_2 = -\log EC_{50}$ and EC_{50} is the agonist concentration needed to produce half-maximal response. pD_2 was estimated by computerized nonlinear regression analysis (GraphPad Prism, San Diego, CA, USA). Differences were analysed by Student's *t*-test for paired and unpaired observations and by ANOVA and *a posteriori* Bonferroni method for multiple comparisons. Differences were considered significant with a probability level of $P<0.05$. *P*-values are shown in the figure legends.

Drugs and solutions

The following drugs were used: 4-aminopyridine (4-AP), apamin, atropine, charybdotoxin (ChTX), ω-conotoxin GVIA (ω-CgTX), guanethidine, iberiotoxin (IbTX), N^G-nitro-L-arginine (L-NOARG) and phenylephrine, all from Sigma (St Louis, MO, USA). Glibenclamide and 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were provided by Tocris (Bristol, UK). The composition of PSS was (mM): NaCl 119, KCl 4.6, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, $CaCl_2$ 1.5, KH_2PO_4 1.2 and EDTA 0.027. The solution was maintained at 37 °C and continuously gassed with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. The $NaNO_2$ solution (1 M) was prepared daily in double-distilled water with HCl (37%), obtaining a final pH of 2. This solution was placed over ice and protected from air.

Results

Urothelium-denuded strips of pig urinary bladder neck were allowed to equilibrate to a passive tension of 1.9 ± 0.2 g ($n = 65$). Phenylephrine (1 μM) induced a sustained contraction

above basal tension of 2.5 ± 0.4 g ($n = 65$). Under NANC conditions obtained by pre-incubation with guanethidine (10 μM) and atropine (0.1 μM), blockers of adrenergic neurotransmission and muscarinic receptors, respectively, low frequencies of EFS (0.2–1 Hz) evoked frequency-dependent relaxations (maximal relaxation of 68 ± 11% of the phenylephrine-induced contraction, $n = 65$, at 1 Hz).

Effects of blockade of guanylate cyclase, K_{Ca} and K_{ATP} channels on relaxations to EFS and exogenous NO

1H-[1,2,4]-Oxadiazolo[4,3-a]quinoxalin-1-one (5 μM), a guanylate cyclase inhibitor, almost abolished the relaxations induced by EFS (Figures 1a and b) and reduced those to addition of acidified $NaNO_2$ solution (Figures 1a and c). IbTX (100 nM), ChTX (100 nM) and apamin (0.5 μM), inhibitors of large-, intermediate- and small-conductance K_{Ca} channels, respectively, and glibenclamide, a K_{ATP} channel inhibitor, failed to modify the relaxations to EFS (Figures 2a, c, e and 3a) or acidified $NaNO_2$ solution (Figures 2b, d, f and 3b). Combined treatment of K_{Ca} and K_{ATP} channel blockers plus ODQ did not induce a greater inhibition than that evoked by ODQ alone (Figures 2a–f, 3a and b).

Effects of blockade of K_V channels, NOS and neuronal voltage-activated Ca^{2+} channels on relaxations to EFS and exogenous NO

4-Aminopyridine (3 mM), a blocker of K_V channels, potentiated the relaxations at low EFS frequencies (Figures 4a and b), but did not modify the responses to addition of acidified $NaNO_2$ solution (Figures 4a and c). Pretreatment with the NOS blocker L-NOARG (100 μM) plus 4-AP (Figures 5a and b), as well as with ω-CgTX (1 μM), a neuronal VOC channel inhibitor (Figures 6a and b), abolished the relaxations to EFS and prevented the enhancing effect of 4-AP. These blockers failed to modify the relaxations to acidified $NaNO_2$ solution (Figures 5c and 6c).

Discussion and conclusions

The present study was designed to investigate the mechanisms involved in the relaxations evoked by NO, endogenously released and exogenously added, in the pig urinary bladder neck, with special regard to the role of K^+ channels.

NO plays an essential role in the relaxations of the lower urinary tract and is directly implicated in the regulatory mechanisms of the smooth muscle tone of the outflow region of the urinary tract, reducing the resistance of the bladder neck and urethra during the emptying phase of the bladder (Andersson and Wein, 2004; Hedlund, 2005). Morphological studies have revealed the presence at the outlet of a high density of NOS immunoreactivity and NADPH diaphorase activity, localized to nerve cell bodies and nerve fibres distributed in the muscular layer, around blood vessels and close to the urothelium, such innervation being richer in the bladder neck and urethra than in the detrusor (Persson *et al*., 1993; Alm *et al*., 1995; Smet *et al*., 1996; Dixon *et al*., 1997). NOS-immunoreactive nerves are more prominent in the outlet region than in the detrusor, which suggests that bladder neck smooth muscle could behave more like urethral than detrusor smooth muscle (Crowe and Burnstock, 1989; Persson *et al*., 1995). Some of the NOS-immunoreactive nerves also stain for AChE, which

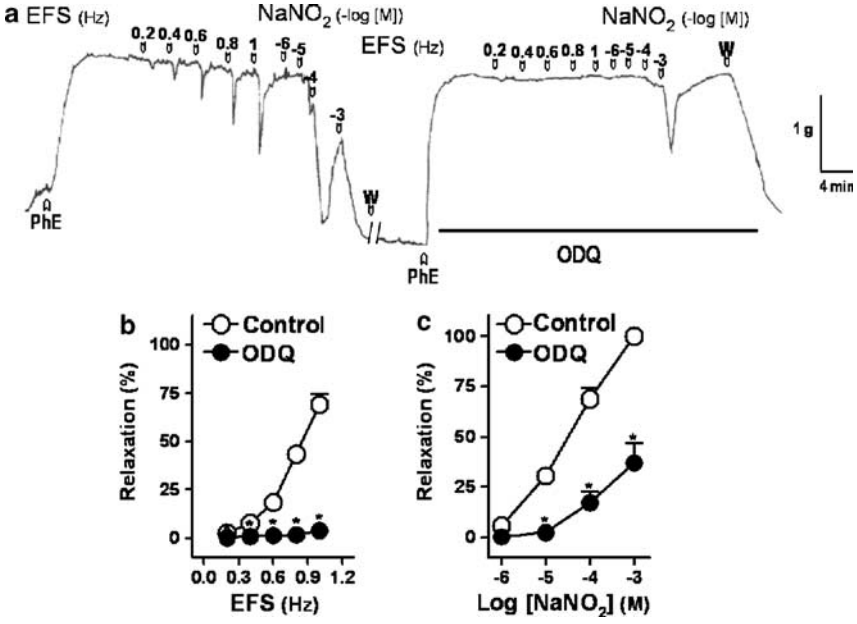

Figure 1 (**a**) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.2–1 Hz, 20 s trains) and addition of acidified $NaNO_2$ solution ($NaNO_2$, 1 μM–1 mM), in the absence or presence of 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM), on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine (PhE) and treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in grams and horizontal bar shows time in minutes. (**b**, **c**) Frequency–response and log concentration–response relaxation curves to EFS and addition of acidified $NaNO_2$ solution, respectively, in control conditions (open circles) and in the presence of ODQ (closed circles). Results are expressed as a percentage of the PhE-induced contraction and represent mean ± s.e.mean of nine preparations. *$P < 0.05$ versus control (paired *t*-test).

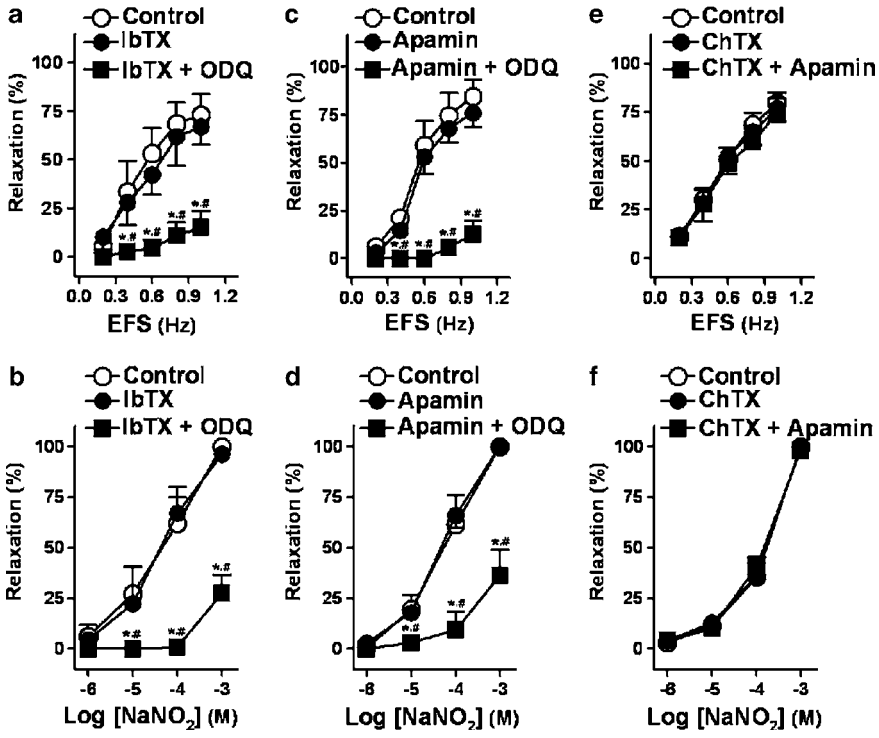

Figure 2 (**a**, **c**, **e**) Frequency–response and (**b**, **d**, **f**) log concentration–response relaxation curves to electrical field stimulation (EFS) and addition of acidified $NaNO_2$ solution, respectively, on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine and treated with guanethidine (10 μM) and atropine (0.1 μM), in control conditions and in the presence of (**a**, **b**) iberiotoxin (IbTX, 100 nM) and IbTX plus 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM), (**c**, **d**) apamin (0.5 μM) and apamin plus ODQ and (**e**, **f**) charybdotoxin (ChTX, 100 nM) and ChTX plus apamin. Results are expressed as a percentage of the phenylephrine-induced contraction and represent mean ± s.e.mean of 6–7 preparations. $^{*,\#}P<0.05$ versus control and IbTX, apamin and ChTX, depending on the group (ANOVA followed by Bonferroni test).

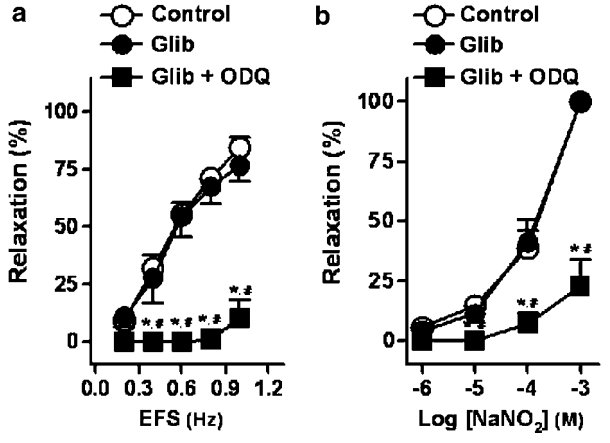

Figure 3 (**a**) Frequency–response and (**b**) log concentration–response relaxation curves to EFS and addition of acidified $NaNO_2$ solution, respectively, in control conditions and in the presence of glibenclamide (Glib; 1 μM) and glibenclamide plus 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM), on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine and treated with guanethidine (10 μM) and atropine (0.1 μM). Results are expressed as a percentage of the phenylephrine-induced contraction and represent mean ± s.e.mean of seven preparations. $^{*,\#}P<0.05$ versus control and glibenclamide, depending on the group (ANOVA followed by Bonferroni test).

suggests that NO may have a role both as a direct messenger and by interacting with a cholinergic transmitter (Persson *et al*., 1995). Functional studies have demonstrated potent relaxations exerted by NO, endogenously released, in the outlet flow region, with a clear differentiation between the bladder neck and the urethra. Thus, whereas the involvement of the NO/cGMP pathway has been demonstrated in the NANC inhibitory neurotransmission of the urethra of several species (Andersson and Wein, 2004), the involvement of NO in the NANC nerve relaxations of the urinary bladder neck has consistently been demonstrated only in sheep (Thornbury *et al*., 1992) and pig (Hernández *et al*., 2007) and just occasionally in humans (Ehrén *et al*., 1994). In sheep, a post-stimulus ('rebound') contraction has been observed, which is dependent more on the release of intracellular Ca^{2+} than on Ca^{2+} influx through L-type channels (Thornbury *et al*., 1995). This contractile component has not been demonstrated in pig (Hernández *et al*., 2007).

The knowledge of the nature of the transmitters and/or modulators and of the mechanisms involved in the control of the bladder neck smooth muscle tone is essential for the therapeutic management of urinary incontinence. The existence of an open bladder neck strongly correlates with the presence of urinary stress incontinence due to intrinsic sphincteric deficiency (English *et al*., 1999). Recently, De Groat (2006) reported the bladder neck as an integrated part of a functional unit (outlet), the activity of which is regulated by a control system in the brain and spinal cord. In addition to this central nerve regulation, different candidates have been proposed to be involved in the NANC autonomic control of the urinary bladder neck tension (Hills

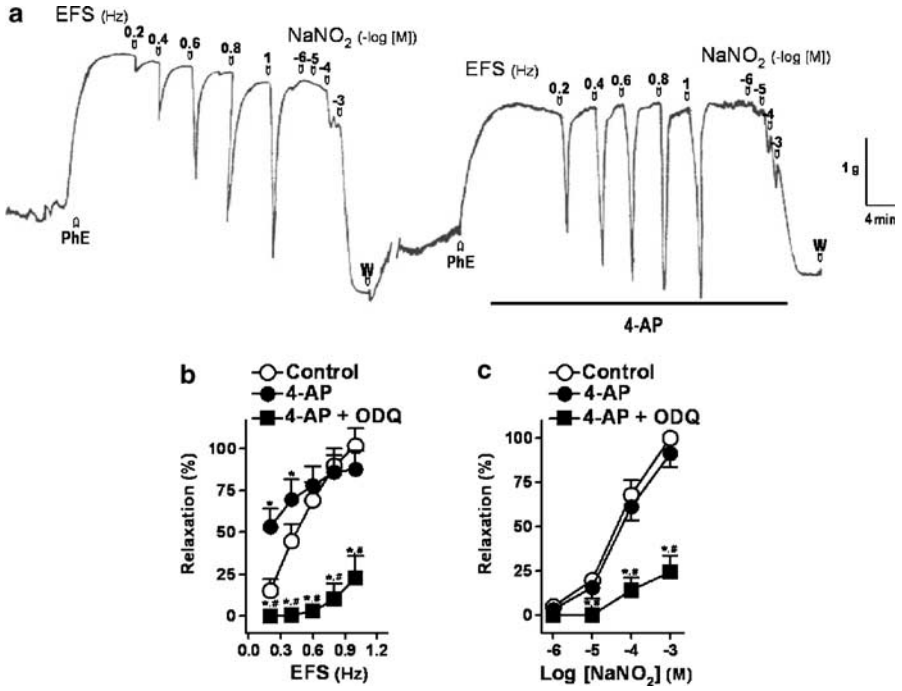

Figure 4 (**a**) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.2–1 Hz, 20 s trains) and addition of acidified $NaNO_2$ solution ($NaNO_2$, 1 μM–1 mM) in the absence or presence of 4-aminopyridine (4-AP, 3 mM), on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine (PhE) and treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in grams and horizontal bar shows time in minutes. (**b**) Frequency–response and (**c**) log concentration–response relaxation curves to EFS and addition of acidified $NaNO_2$ solution, respectively, in control conditions and in the presence of 4-AP and 4-AP plus 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM). Results are expressed as a percentage of the PhE-induced contraction and represent mean ± s.e.mean of nine preparations. $^{*,\#}P<0.05$ versus control and 4-AP, depending on the group (ANOVA followed by Bonferroni test).

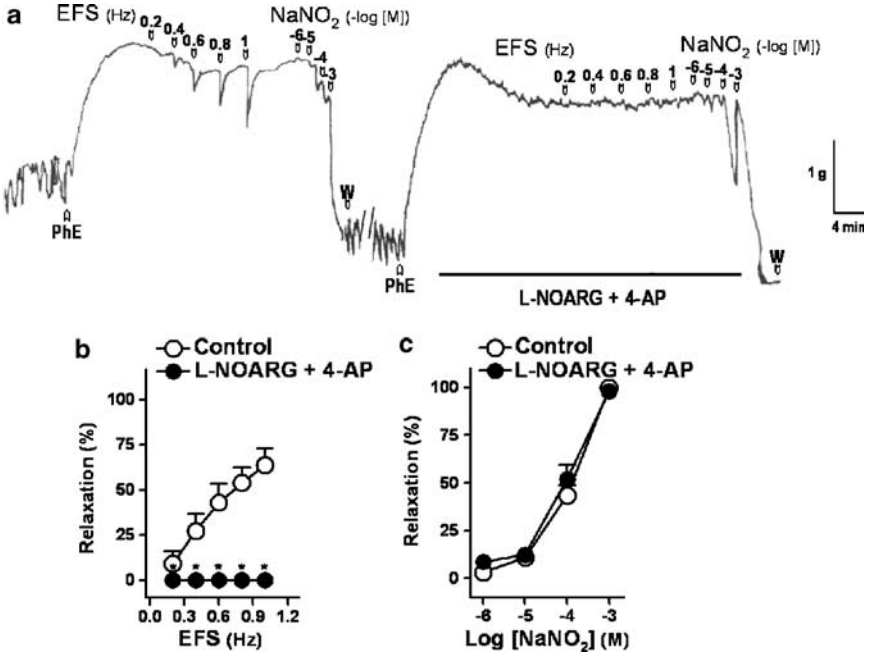

Figure 5 (**a**) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.2–1 Hz, 20 s trains) and addition of acidified $NaNO_2$ solution ($NaNO_2$, 1 μM–1 mM) in the absence or presence of N^G-nitro-L-arginine (L-NOARG, 100 μM) plus 4-aminopyridine (4-AP, 3 mM), on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine (PhE) and treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in grams and horizontal bar shows time in minutes. (**b**) Frequency–response and (**c**) log concentration–response relaxation curves to EFS and addition of acidified $NaNO_2$ solution, respectively, in control conditions and in the presence of L-NOARG (100 μM) plus 4-AP (3 mM). Results are expressed as a percentage of the PhE-induced contraction and represent mean ± s.e.mean of seven preparations. $^{*}P<0.05$ versus control (paired *t*-test).

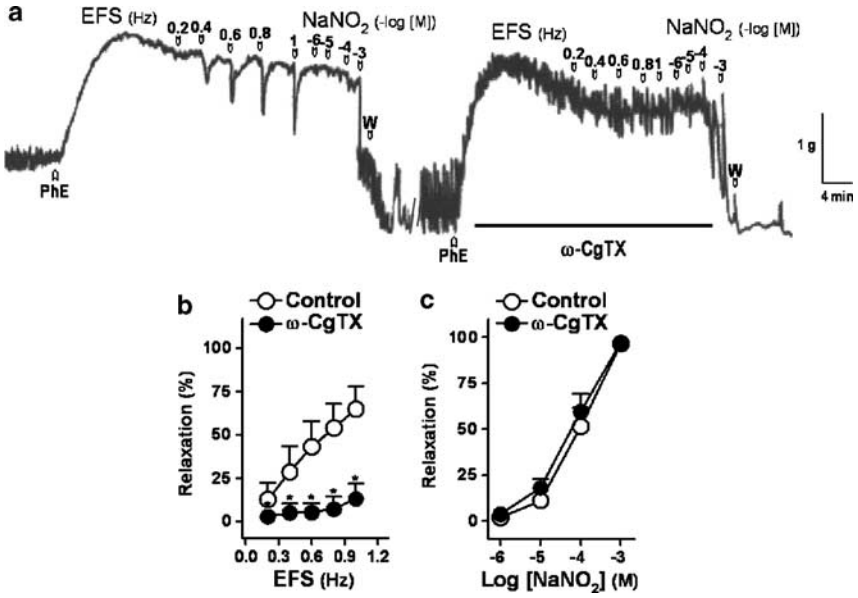

Figure 6 (**a**) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.2–1 Hz, 20 s trains) and addition of acidified $NaNO_2$ solution ($NaNO_2$, 1 μM–1 mM) in the absence or presence of ω-conotoxin GVIA (ω-CgTX, 1 μM), on pig urinary bladder neck strips, pre-contracted with 1 μM phenylephrine (PhE) and treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in grams and horizontal bar shows time in minutes. (**b**) Frequency–response and (**c**) log concentration–response relaxation curves to EFS and addition of acidified $NaNO_2$ solution, respectively, in control conditions and in the presence of ω-CgTX (1 μM). Results are expressed as a percentage of the PhE-induced contraction and represent mean ± s.e.mean of seven preparations. $^*P<0.05$ versus control (paired *t*-test).

et al., 1984). For this reason, the current study was designed to investigate the mechanisms involved in the relaxations elicited by endogenously released and exogenously added NO in the pig urinary bladder neck.

In addition to the work under established NANC experimental conditions, a critical point of the current investigation has been to stimulate the preparations at low (0.2 and 1 Hz) frequencies, so as to induce a selective release of NO from nerves, as frequencies higher than 2 Hz evoke the release of other non-nitrergic mediators (Hernández *et al*., 2006b). In fact at 10–16 Hz frequencies, the main release corresponds to peptides such as PACAP 38, released from capsaicin-sensitive primary afferents, producing relaxation of the pig bladder neck through mediation of neuronal PAC_1 and smooth muscle $VPAC_2$ receptors, the later coupled to the cAMP–PKA pathway and involving activation of K_V channels (Hernández *et al*., 2006a, b). In addition to the release of PACAP 38, other non-nitrergic mediators of unknown nature are also involved in the potent nerve relaxations evoked by high frequencies of stimulation (Hernández *et al*., 2006b).

In the current investigation, in urothelium-denuded pre-contracted strips from pig urinary bladder neck, EFS and exogenous NO evoked frequency- and concentration-dependent relaxations. The electrically induced relaxations were previously demonstrated to be abolished by the neuronal voltage-activated Na^+ channel blocker tetrodotoxin as well as by L-NOARG, thus indicating their neurogenic nitrergic nature (Hernández *et al*., 2007). As in our experimental protocol, to induce NO release from intramural nerves, adrenergic neurotransmission was blocked by pretreatment with guanethidine, the electrically induced relaxations could suggest that nerves liberating NO are likely to be parasympathetic and/or nitrergic in nature. These results are consistent with the high density and coexistence of cholinergic and nitrergic innervation in some of the nerves at this level (Crowe and Burnstock, 1989; Persson *et al*., 1995).

cGMP is considered to be the most important intracellular second messenger in promoting relaxant responses in various smooth muscle cells, including those of the urinary tract (Hedlund, 2005). NO causes smooth muscle relaxation by activating soluble guanylate cyclase, resulting in the accumulation of intracellular cGMP (Ignarro *et al*., 1990). In our study, ODQ, a blocker of the NO-elicited soluble guanylate cyclase activation and cGMP accumulation, promoted a powerful reduction of the relaxations evoked by both EFS and addition of acidified $NaNO_2$ solution. These results suggest that, under our experimental conditions, NO, released from intramural nerves, relaxes phenylephrine-pre-contracted pig urinary bladder neck smooth muscle tone by activation of guanylate cyclase, with the subsequent accumulation of cGMP. These results agree with those obtained in the pig trigone (Persson and Andersson, 1992) and intravesical ureter (Hernández *et al*., 1997) and in the urethra of several species (Andersson and Wein, 2004), where NO relaxes through cGMP-dependent mechanisms.

NO also relaxes smooth muscle via activation of K^+ channels, either through cGMP-dependent protein kinase (Robertson *et al*., 1993) or by direct opening of K_{Ca} channels that does not require cGMP (Bolotina *et al*., 1994). cGMP-dependent opening of K^+ channels leads to hyperpolarization and subsequent reduction of Ca^{2+} influx through VOC channels (Lincoln and Cornwell, 1991; Robertson *et al*., 1993). In urinary bladder, large- and small-conductance K_{Ca} channels play an essential role in the repolarization of the action potential and in the maintenance of the resting membrane potential, limiting the amplitude and duration of

smooth muscle contractile responses (Heppner *et al*., 1997; Herrera *et al*., 2000). Large-conductance K_{Ca} channels act as negative feedback regulators by decreasing voltage-dependent extracellular Ca^{2+} entry (Imai *et al*., 2001; Herrera and Nelson, 2002). Alterations in the expression of K_{Ca} channels may cause urinary dysfunctions, such as overactive bladder and urinary incontinence (Herrera *et al*., 2005). In our study, IbTX, ChTX and apamin, blockers of large, intermediate and small, respectively, K_{Ca} channels, and ChTX plus apamin, failed to modify the relaxations induced by EFS or addition of acidified $NaNO_2$ solution. Moreover, pretreatment with IbTX or apamin plus ODQ did not produce an additional inhibitory effect over that evoked by ODQ alone, on the relaxations to either EFS or acidified $NaNO_2$ solution. These data suggest that activation of K_{Ca} channels is not involved in the NO-mediated relaxations of the pig urinary bladder neck.

K_{ATP} channels play an essential role in the regulation of urinary tract smooth muscle (Brading, 1992). Thus, cromakalim, an activator of K_{ATP} channels, reduces bladder mechanical activity in guinea-pig and rat, as well as contractions in normal and unstable human and pig detrusor, to promote smooth muscle hyperpolarization, and reduces the open probability of voltage-sensitive Ca^{2+} channels (Foster *et al*., 1989; Bonev and Nelson, 1993). The efficacy of K_{ATP} channel openers to inhibit spontaneous bladder contractions favours the development of these drugs as therapeutic tools to treat overactive bladder symptoms (Buckner *et al*., 2002). K_{ATP} channel activation is also involved in the relaxations evoked by NO released from nitrergic nerves in the smooth muscle of the pig intravesical ureter (Hernández *et al*., 1997). In the present work, however, glibenclamide, an inhibitor of K_{ATP} channels, did not change the relaxations to either EFS or addition of acidified $NaNO_2$ solution. Moreover, the incubation of glibenclamide plus ODQ did not produce a blockade higher than that evoked by ODQ alone. These results seem to rule out the involvement of K_{ATP} channels in the nitrergic relaxations of the pig urinary bladder neck.

Several functionally relevant subunits of K_V channels are expressed in urinary bladder (Thorneloe and Nelson, 2003). K_V channels contribute to the regulation of muscle myogenic contraction (Imai *et al*., 2001), and have been proposed to mediate repolarization of the smooth muscle action potential, and to regulate the resting membrane potential (Thorneloe and Nelson, 2003). K_V channels have been proposed to mediate the relaxations induced by PACAP 38 and vasoactive intestinal peptide in pig urinary bladder neck. These peptides relax the bladder neck through muscle $VPAC_2$ receptors coupled to the cAMP–PKA pathway and involving activation of K_V channels (Hernández *et al*., 2006a). In the current study, the potentiation produced by the K_V channel inhibitor 4-AP on the relaxations evoked by low-field stimulation frequencies, but not on the relaxations to acidified $NaNO_2$ solution, suggests a pre-junctional modulatory role of K_V channels. The abolition of the electrically induced relaxations by pretreatment with L-NOARG plus 4-AP, or with the neuronal VOC channel blocker, ω-CgTX, along with the lack of inhibitory effect of these blockers on exogenous NO relaxations seems to rule out a post-junctional role of K_V channels in such responses and indicates that the modulation is on nerve NO release, which is essentially dependent on Ca^{2+} influx to the nerve endings through neuronal VOC channels. NO is synthesized on demand and is neither stored in synaptic vesicles nor released by exocytosis, but simply diffuses from nerve terminals. The most important regulator of neural NOS (nNOS) seems to be free cytosolic Ca^{2+}, which stimulates nNOS through interaction with calmodulin (Esplugues, 2002). Arrival of action potentials activates VOC channels in the membrane and stimulates Ca^{2+} influx. The results obtained in the present study suggest that in addition to membrane VOC channels, there is an activation of neuronal K_V channels that would be involved in the inhibition of VOC channels and thus would modulate the NO release from nerves (Figure 7). We have recently demonstrated that pre-junctional α_2-adrenoceptors downregulate neural NO release in the pig bladder neck (Hernández *et al*., 2007). Further studies are needed to clarify whether the inhibitory effect of α_2-adrenoceptors is linked to inhibition of VOC channels and/or activation of K_V channels in the nitrergic nerve terminals. This modulatory role of K_V on neurotransmitter release would agree with observations in the CNS, specifically in rat hippocampal neurons where linopirdine, a cognition-enhancing drug, increased cholinergic neurotransmitter release, in part, through blockade of K_V currents (Schnee and Brown, 1998).

In conclusion, our results suggest that NO, the release of which is modulated by pre-junctional K_V channels, relaxes the pig urinary bladder neck through a guanylate cyclase activation-dependent mechanism in which post-junctional

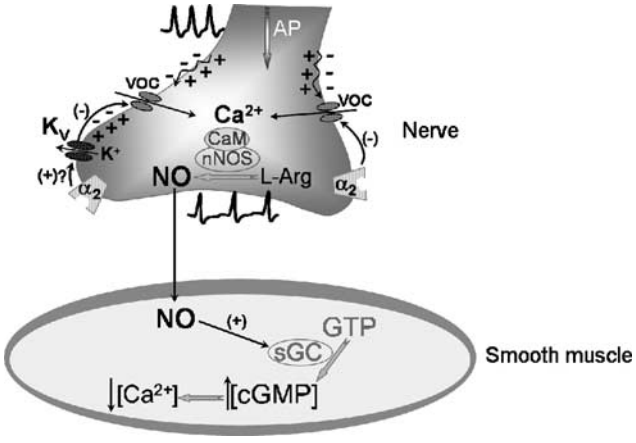

Figure 7 Proposed mechanism for NO release from nerve terminals and its action on smooth muscle, in pig urinary bladder neck. Arrival of action potentials (APs) at the nerve ending evokes membrane depolarization and activation of voltage-gated Ca^{2+} (VOC) channels with the subsequent Ca^{2+} influx. Increased cytosolic Ca^{2+} would stimulate neural NO synthase (nNOS) through interaction with calmodulin (CaM), and would favour NO synthesis from L-arginine (L-Arg) and release from nerves. In addition to the opening of VOC channels, an activation of neuronal K_V channels would downregulate the NO release, probably through inhibition of VOC channels, thus increasing the hyperpolarizing post-potential phase. Pre-junctional α_2-adrenoceptors inhibit NO release possibly through inhibition of VOC channels and/or activation of K_V channels. NO diffusion to smooth muscle produces activation of soluble guanylate cyclase (sGC) and increased cGMP, thus initiating muscle relaxation.

K^+ channels do not seem to be involved. Modulation of K_V channels could be useful in the pharmacological management of urinary incontinence produced by intrinsic sphincteric deficiency.

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Conflict of interest

The authors state no conflict of interest.

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