British Journal of Pharmacology (1997) 120, 609–616

Involvement of a glibenclamide-sensitive mechanism in the nitrergic neurotransmission of the pig intravesical ureter

*,[1]Medardo Hernández, *Dolores Prieto, *,†Luis M. Orensanz, *María Victoria Barahona, ‡Miguel Jiménez-Cidre, *Luis Rivera, *Albino García-Sacristán & #Ulf Simonsen

*Departamento de Fisiología, Facultad de Veterinaria UCM, 28040-Madrid; †Departamento de Investigación and ‡Servicio de Urología, Hospital Ramón y Cajal, 28034-Madrid, and #Institute of Pharmacology, Aarhus University, 8000-Aarhus C., Denmark

1 The present study was designed to investigate whether potassium (K^+) channels are involved in the relaxations to nitric oxide (NO) of pig intravesical ureteral preparations suspended in organ baths for isometric tension recordings. In ureteral strips treated with guanethidine (10^{-5} M) and atropine (10^{-7} M) to block adrenergic neurotransmission and muscarinic receptors, respectively, NO was either released from nitrergic nerves by electrical field stimulation (EFS, 0.5–10 Hz, 1 ms duration, 20 s trains), or exogenously-applied as an acidified solution of sodium nitrite ($NaNO_2$, 10^{-6}–10^{-3} M).

2 Incubation with an inhibitor of guanylate cyclase activation by NO, methylene blue (10^{-5} M) did not change the basal tension of intravesical ureteral strips but inhibited the relaxation induced by EFS or exogenous NO on ureteral preparations contracted with the thromboxane analogue U46619 (10^{-7} M).

3 Incubation with charybdotoxin (3×10^{-8} M) and apamin (5×10^{-7} M), which are inhibitors of large and small conductance calcium (Ca^{2+})-activated K^+ channels, respectively, did not modify basal tension or the relaxations induced by EFS and exogenous NO. Treatment with charybdotoxin or apamin plus methylene blue (10^{-5} M) significantly reduced the relaxations to EFS and exogenous NO. However, in both cases the reductions were similar to the inhibition evoked by methylene blue alone. The combined addition of charybdotoxin plus apamin did not change the relaxations to EFS or exogenously added NO of the porcine intravesical ureter.

4 Cromakalim (10^{-8}–3×10^{-6} M), an opener of ATP-sensitive K^+ channels, evoked a dose-dependent relaxation with a pD_2 of 7.3 ± 0.2 and maximum relaxant effect of a $71.8 \pm 4.2\%$ of the contraction induced by U46619 in the pig intravesical ureter. The blocker of ATP-sensitive K^+ channels, glibenclamide (10^{-6} M), inhibited markedly the relaxations to cromakalim.

5 Glibenclamide (10^{-6} M) had no effect on the basal tone of ureteral preparations but significantly reduced the relaxations induced by both EFS and exogenous NO. Combined treatment with methylene blue (10^{-5} M) and glibenclamide (10^{-6} M) did not exert an effect greater than that of methylene blue alone on either EFS- or NO-evoked relaxations of the pig ureter.

6 The present results suggest that NO acts as an inhibitory neurotransmitter in the pig intravesical ureter and relaxes smooth muscle through a guanylate cyclase-dependent mechanism which seems to favour the opening of glibenclamide-sensitive K^+ channels.

Keywords: Pig intravesical ureter; electrical field stimulation; nitric oxide; methylene blue; charybdotoxin; apamin; glibenclamide; cromakalim; K_{ATP} channels

Introduction

Ureteral peristalsis is a myogenic phenomenon in which the autonomic nervous system plays a modulatory role by regulating the transport of urine bolus throughout the ureter and its discharge into the urinary bladder (Weiss, 1992). The autonomic nervous system has a significant role in the distal ureter and ureterovesical junction as indicated by dense adrenergic and cholinergic innervation (Schulman, 1985; Prieto *et al*., 1993; 1994), and the observation that noradrenaline and acetylcholine induce contractions of the intravesical ureter and ureterovesical junction through activation of subtype specific receptors (Rivera *et al*., 1992a,b; Hernández *et al*., 1992; 1993; 1995a).

Nitric oxide (NO) has been proposed as a putative non-adrenergic non-cholinergic inhibitory neurotransmitter in both central and peripheral nerves (Bredt *et al*., 1990; Bredt & Snyder, 1992). Nitrergic fibres have been demonstrated in the human (Smet *et al*., 1994) and porcine (Hernández *et al*., 1995b) ureter. Thus, the NO donor, 3 morpholino-sydnonimine (SIN-1) produced relaxations in the human ureter (Stief *et al*., 1993), and both endogenously-released and exogenous NO relaxes the pig intravesical ureter (Hernández *et al*., 1995b).

NO causes relaxation by activating soluble guanylate cyclase resulting in accumulation of intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Ignarro *et al*., 1990). Cyclic GMP seems to cause relaxation of smooth muscle by lowering the intracellular calcium concentration by stimulating sarcoplasmic Ca^{2+}-ATPase activity, or through opening of K^+ channels leading to hyperpolarization (Lincoln & Cornwell, 1991; Robertson *et al*., 1993). In addition, NO can directly stimulate Ca^{2+}-activated K^+ channels in both vascular and colonic smooth muscle (Bolotina *et al*., 1994; Koh *et al*., 1995). Therefore, the purpose of the present study was to investigate whether K^+ channels are involved in the relaxation to NO of the pig intravesical ureter.

Methods

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders with attached ureters 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 preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection, as previously described

[1] Author for correspondence.

(Hernández *et al*., 1992). The ureteral strips were suspended horizontally and placed parallel between two platinum electrodes, with one end connected to an isometric transducer (Grass FT 03C) and the other to a displacement unit in 5 ml organ baths. The signal was continuously recorded on a polygraph (Graphtec Multicorder MC 6621). Passive tension of 2 g was applied to the ureteral preparations and they were allowed to equilibrate for 60 min.

Experimental procedures

The contractile ability of the preparations was determined by exposing the ureteral strips to 124 mM potassium-rich physiological saline solution (KPSS). Adrenergic neurotransmission and muscarinic receptors were blocked by incubation with guanethidine (10^{-5} M) and atropine (10^{-7} M), respectively, during a period of 1 h, washing every 20 min and these drugs were present throughout both electrical field stimulation (EFS) experiments and dose-response curves to exogenous NO. The preparations were contracted with the thromboxane analogue U46619 (10^{-7} M). EFS was performed with rectangular pulses (1 ms duration, 0.5–10 Hz, 20 s trains), at 3 min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain) with constant current output adjusted to 75 mA. A first frequency-response curve in U46619-contracted preparations was obtained. The porcine ureteral strips were repeatedly washed and allowed to equilibrate for at least 1 h before they were incubated with either methylene blue (10^{-5} M), charybdotoxin (3×10^{-8} M), apamin (5×10^{-7} M), glibenclamide (10^{-6} M) or the combination of either of the K^+ channel blockers with methylene blue. All tissues were incubated with modifying agents for a period of 30 min before a second frequency-response curve on U46619 contracted strips was performed. Relaxation curves to exogenous NO (added as acidified $NaNO_2$ solution) and cromakalim were obtained on U46619-precontracted ureteral preparations, which were then washed several times during 1.5 h, the strips were incubated with blocking agents for 30 min before a second curve was performed. Control curves were run in parallel. At the end of each experiment, papaverine (Pap, 10^{-4} M) was added to the organ bath with the aim of obtaining the maximal relaxation of the ureteral preparations.

Drugs and solutions

The following drugs were used: Apamin, atropine sulphate, charybdotoxin, cromakalim, glibenclamide, guanethidine, methylene blue, papaverine hydrochloride, 9, 11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). All drugs were dissolved in double distilled water, except glibenclamide and cromakalim which were dissolved in dimethyl sulphoxide (DMSO) and U46619 which was dissolved in 96% ethanol. These solvents in the amount applied had no effect on pig intravesical ureteral activity. 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, EDTA (ethylenediamine tetraacetic acid) 0.027. The solution was gassed at 37°C with 95% O_2 and 5% CO_2 to maintain pH at 7.4 KPSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in double-distilled water.

The 1 mM solution of $NaNO_2$ was prepared daily in distilled water with HCl giving a final pH of 2. This solution was placed on ice and protected from air. Further dilutions were made in this acidified solution (pH = 2). The acid solvent as well as the $NaNO_2$ solution at pH = 7.4 had no effect on pig intravesical ureteral smooth muscle.

Calculations and statistics

For each frequency- or concentration-response curve, the pulse frequency or drug concentration in the absence or presence of

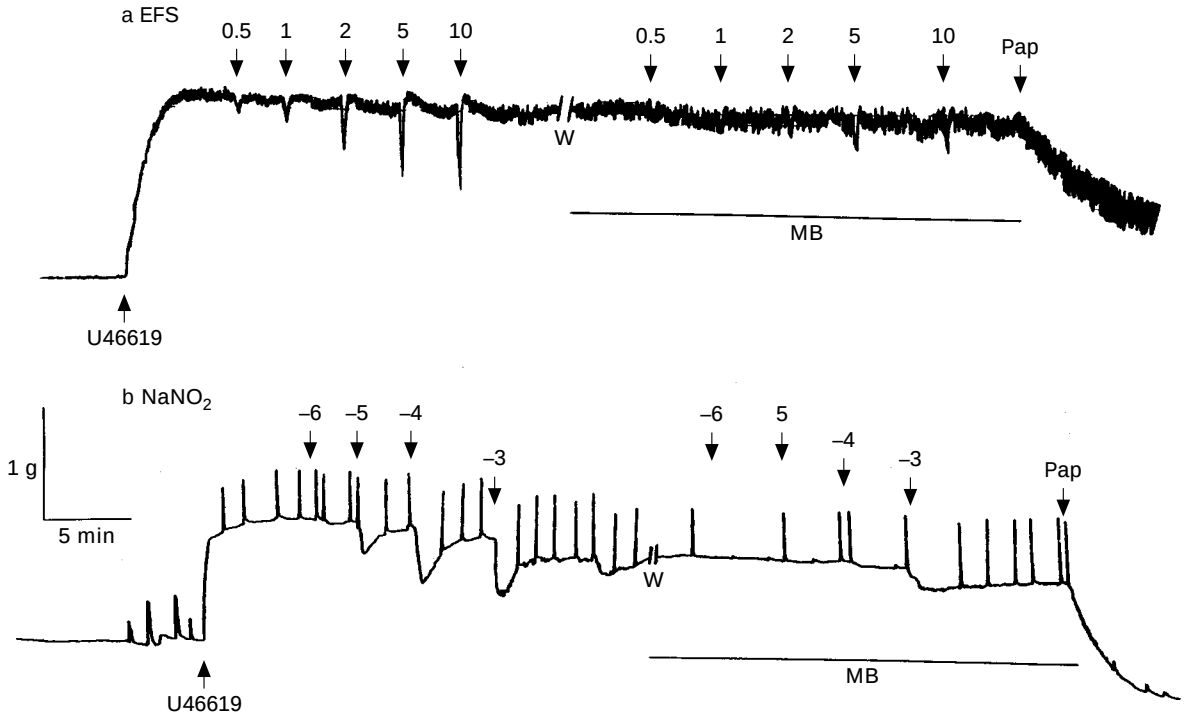

Figure 1 Isometric force recordings showing the effect of methylene blue (MB, 10^{-5} M) on the responses of pig intravesical ureteral strips to (a) electrical field stimulation (EFS, 0.5–10 Hz) and (b) exogenous addition of nitric oxide (NO, 10^{-6}–10^{-3} M, added as an acidified solution of $NaNO_2$). Guanethidine (10^{-5} M) and atropine (10^{-7} M) were present throughout the experiment, to block adrenergic neurotransmission and muscarinic receptors, respectively. The ureteral strips were contracted with the thromboxane analogue U46619 (10^{-7} M) and when a sustained tone was obtained, frequency- and/or concentration-response curves to EFS and NO were performed in the absence and presence of methylene blue (10^{-5} M). At the end of the experiment papaverine (Pap, 10^{-4} M) was added to obtain the maximal relaxation of the pig intravesical ureteral preparations. Numbers indicate frequency (Hz) or molar concentration in the bath W: wash out.

blocking agent required to give half-maximal relaxation (EF_{50} or EC_{50}, respectively) was determined by a computer programme (Graph Pad Inplot 4.1, San Diego, California, U.S.A.), fitting the data to the Hill equation: $E/E_{max} = A\ (M)^{n_H}/(A(M) + EC_{50}(M)^{n_H})$, where E/E_{max} is the relative response to the effective concentration of drug, A(M), and EC_{50}(M) are given in molar concentrations; n_H is a curve fitting parameter or Hill coefficient. The sensitivity and maximal relaxant responses of the exogenous NO and cromakalim were expressed in terms of pD_2 and E_{max}, respectively. pD_2 being defined as the negative logarithm of EC_{50} ($-\log EC_{50}$). Each parameter was determined from ureters of at least 4–5 different animals. Statistical differences were calculated by Student's *t* test, for paired observations for individual concentrations or frequencies and variance analysis (ANOVA) for multiple comparisons followed by an *a posteriori* Bonferroni test (Wallestein *et al*., 1980). Differences were considered significant with a probability level of $P<0.05$.

Results

Responses to EFS and exogenous NO

The pig intravesical ureteral strips were equilibrated to a passive tension of 1.8 ± 0.2 g ($n=65$). The thromboxane analogue U46619 (10^{-7} M) induced sustained contractions of 1.6 ± 0.2 g ($n=65$). EFS evoked frequency-response relaxations at 0.5–10 Hz which peaked at 10 s, with an $EF_{50}=0.8\pm0.1$ Hz and an E_{max} obtained at 10 Hz, which averaged $60.8\pm4.6\%$ ($n=34$) of the tone induced by U46619 (10^{-7} M) (Figures 1a and 2a). The relaxations to EFS were reproducible, giving EF_{50} values and E_{max} of 0.9 ± 0.1 Hz and $66.2\pm3.9\%$ and 0.88 ± 0.07 Hz and $62.1\pm5.4\%$, in a first and second frequency-response curve, respectively, performed in the same preparation ($n=11$).

Exogenous NO (10^{-6}–10^{-3} M), induced relaxations of pig intravesical ureter with pD_2 of 4.8 ± 0.2 and E_{max} of $58.3\pm5.7\%$ ($n=26$) of the U46619-induced contraction (Figures 1b and 2b). These relaxations were reproducible in a second curve with a pD_2 of 4.7 ± 0.2 and an E_{max} of $56.3\pm4.2\%$ ($n=7$).

Effect of methylene blue and potassium channel blockers

The inhibitor of soluble guanylate cyclase, methylene blue (10^{-5} M) had no effect on resting tension (1.8 ± 0.1 g, $n=14$) or contractions to U46619 in the pig intravesical ureter. However, it reduced significantly the relaxations to EFS at all frequencies applied (Figures 1a and 2a, Table 1). Similarly, incubation with methylene blue also inhibited the relaxations of the preparations to exogenous NO, although relaxations at the highest concentrations (10^{-4}–10^{-3} M) still persisted (Figures 1b and 2b, Table 1).

Charybdotoxin (3×10^{-8} M) and apamin (5×10^{-7} M), blockers of the large and small conductance Ca^{2+}-activated K^+ channels, respectively, had no effect on either resting tension or contractions to U46619. Thus, the resting tension was 1.7 ± 0.2 g ($n=7$) and 1.8 ± 0.1 g ($n=5$) in the presence of charybdotoxin or apamin, respectively, in the pig intravesical ureter. Moreover, both the sensitivity and maximal relaxations to EFS and exogenous NO added as acidified $NaNO_2$ were unchanged in the presence of the inhibitors of large or small Ca^{2+}-activated K^+ channels, charybdotoxin (Figures 3a and b, Table 1) and apamin (Figures 3c and d, Table 1), respectively. Incubation of the ureteral strips with both charybdotoxin plus methylene blue ($n=6$) or apamin plus methylene blue ($n=5$) inhibited the relaxations induced by both EFS and exogenous NO, but these inhibitions were not different to that observed with methylene blue alone (Figure 3a, b, c and d, Table 1). Combined treatment with charybdotoxin and apamin ($n=5$) did not modify the relaxation to endogenous or exogenous NO (Table 1).

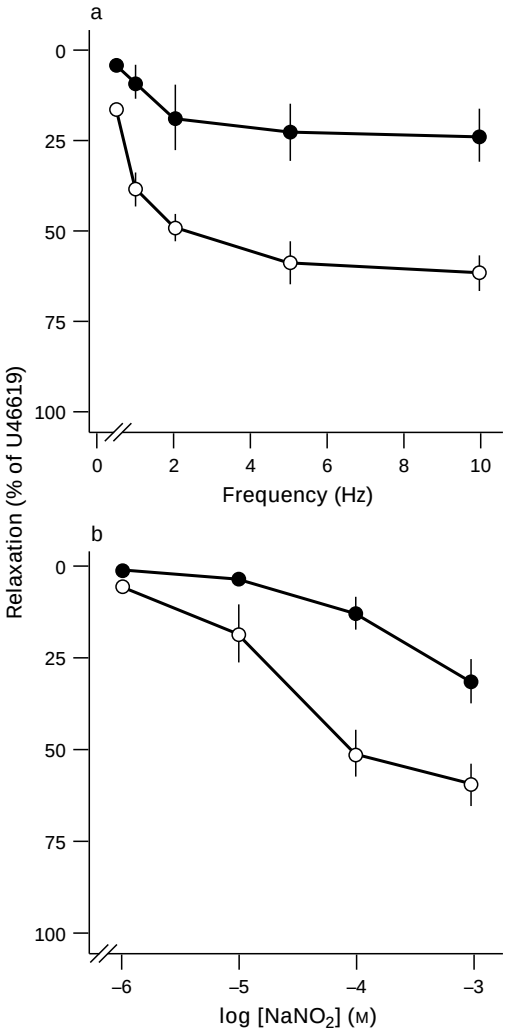

Figure 2 Frequency- (a) and concentration-response (b) relaxation curves to EFS- and $NaNO_2$ acidified solution, respectively, in U46619-precontracted pig intravesical ureteral preparations, in the absence (○) and presence (●) of methylene blue (10^{-5} M). Relaxations are expressed as a percentage of the tone induced by U46619 (10^{-7} M). Each point represents the mean and vertical lines show s.e.mean of 6–7 experiments.

In U46619-contracted pig intravesical ureteral strips, addition of an opener of ATP-sensitive K^+ channels, cromakalim (10^{-8}–3×10^{-6} M) ($n=7$) induced concentration-dependent relaxations with pD_2 values of 7.3 ± 0.2 and E_{max} of $71.8\pm4.2\%$ of the U46619 induced contraction. The inhibitor of ATP-sensitive K^+ channels, glibenclamide (10^{-6} M) caused significant rightwards shifts of the concentration-relaxation curves to cromakalim in pig intravesical ureter, the pD_2 values and E_{max} being 5.8 ± 0.2 and $20.3\pm6.4\%$, respectively ($P<0.05$, paired *t* test) ($n=7$) (Figures 4c and 5).

Glibenclamide (10^{-6} M) did not change the basal tension in the ureteral preparations (1.7 ± 0.2 g) ($n=11$) nor the contractions obtained to U46619. However, it reduced significantly the sensitivity to electrical field stimulation, while the maximal relaxations to EFS were unchanged in the presence of this K^+ channel inhibitor (Figures 4a and 6a, Table 1). In the presence of glibenclamide (10^{-6} M), exogenous NO added as acidified $NaNO_2$ also produced significantly smaller relaxations of U46619-contracted ureteral preparations at the lowest concentrations (10^{-6}–10^{-4} M), while the maximal relaxations obtained to 10^{-3} M acidified $NaNO_2$ were not changed (Figures 4b and 6b, Table 1). Incubating the preparations with

both glibenclamide and methylene blue caused a pronounced inhibition of the relaxations to both EFS and exogenous NO. However, these inhibitory effects were not significantly greater than those elicited by methylene blue alone (Figures 6a and b, Table 1).

Discussion

The present study reveals that EFS-released and exogenously-added NO induces relaxations of the ureteral smooth muscle through a guanylate cyclase-dependent mechanism which

Table 1 The effect of methylene blue (MB, 10^{-5} M), charybdotoxin (ChTX, 3×10^{-8} M), charybdotoxin plus methylene blue, apamin (Apa, 5×10^{-7} M), apamin plus methylene blue, charybdotoxin plus apamin, glibenclamide (Glib, 10^{-6} M) and glibenclamide plus methylene blue on frequencies (EF_{50}) and concentrations (pD_2) causing half-maximal, and maximal (E_{max}) relaxations to electrical field stimulation (EFS) and exogenous nitric oxide (NO) in pig intravesical ureter contracted to U46619 (10^{-7} M)

	EFS		*NO*	
	EF_{50} (Hz)	E_{max} (%)	pD_2	E_{max} (%)
Control	0.8±0.1 (6)	53.3±4.0 (6)	4.8±0.2 (6)	57.2±5.0 (6)
MB	1.2±0.1 (6)*	23.7±3.5 (6)*	3.7±0.1 (6)*	31.1±4.2 (6)*
Control	1.0±0.3 (7)	61.0±3.6 (7)	5.0±0.1 (7)	61.3±4.9 (7)
ChTX	1.0±0.3 (7)	57.6±4.8 (7)	5.0±0.2 (7)	59.6±4.2 (7)
ChTX + MB	1.5±0.2 (6)#	24.1±2.6 (6)#	4.2±0.1 (6)#	24.8±8.7 (6)#
Control	0.7±0.2 (5)	54.4±2.9 (5)	4.7±0.1 (5)	56.1±5.1 (5)
Apa	0.7±0.2 (5)	52.4±5.3 (5)	4.6±0.1 (5)	58.5±4.7 (5)
Apa + MB	1.4±0.1 (5)#	32.6±4.8 (5)#	3.9±0.0 (5)#	29.0±7.9 (5)#
Control	0.9±0.0 (5)	51.4±5.1 (5)	4.7±0.1 (5)	52.5±6.2 (5)
ChTX + Apa	0.9±0.1 (5)	45.7±6.9 (5)	4.6±0.1 (5)	50.2±5.9 (5)
Control	1.0±0.2 (8)	54.2±3.8 (8)	4.9±0.1 (6)	59.2±5.3 (6)
Glib	2.5±0.2 (8)#	49.6±6.5 (8)	4.1±0.1 (6)#	53.2±7.4 (6)
Glib + MB	1.5±0.3 (7)#	22.8±6.3 (7)#	4.1±0.1 (6)#	26.1±7.2 (6)#

Results are mean±s.e.mean of (n) experiments. *Parameter significantly ($P<0.05$) different compared to control value by paired t test. #Parameter significantly ($P<0.05$) different compared to control value by analysis of variance (ANOVA), followed by an *a posteriori* Bonferroni test. $pD_2 = -\log EC_{50}$ value. NO was added as acidified sodium nitrite ($NaNO_2$).

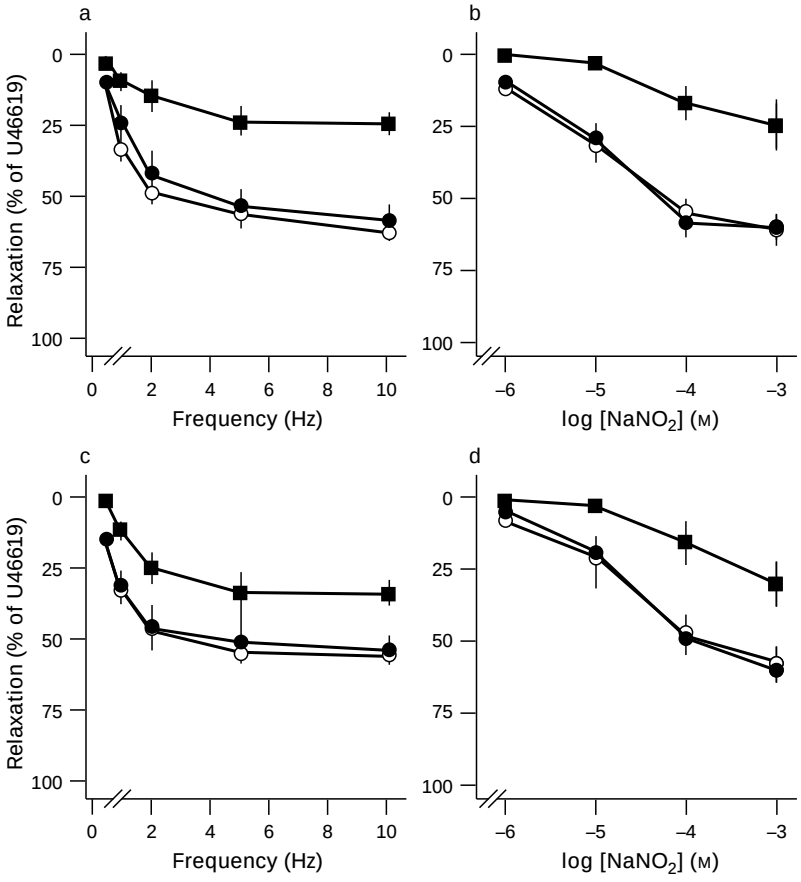

Figure 3 Frequency- (a, c) and concentration-response (b, d) relaxation curves to EFS and $NaNO_2$ acidified solution, respectively, in U46619-precontracted pig intravesical ureteral preparations in (a and b) the absence (○) and presence of either charybdotoxin (3×10^{-8} M) alone (●) or charybdotoxin (3×10^{-8} M) plus methylene blue (10^{-5} M) (■), and in (c and d) the absence (○) and presence of either apamin (5×10^{-7} M) alone (●) or apamin (5×10^{-7} M) plus methylene blue (10^{-5} M) (■) (c, d). Relaxations are expressed as a percentage of the tone induced by U46619 (10^{-7} M). Each point represents the mean and vertical lines s.e.mean of 5–7 experiments.

seems to favour the opening of glibenclamide-sensitive K^+ channels. Nitrergic nerves forming rich networks in the ureterovesical junction of man (Smet *et al*., 1994) and porcine ureter (Hernández *et al*., 1995b) were previously described, and exogenously NO added either as acidified $NaNO_2$ or sodium nitroprusside induces relaxation of porcine isolated ureteral strips (Hernández *et al*., 1995b). Moreover, the neurogenic relaxations evoked by EFS in porcine isolated ureteral strips were abolished in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine, suggesting a role for NO as inhibitory neurotransmitter in the porcine intravesical ureter (Hernández *et al*., 1995b).

Cyclic GMP and cyclic adenosine 3′:5′-monophosphate (cyclic AMP) are important intracellular messengers in smooth muscle cells, and NO causes relaxation by activating soluble guanylate cyclase resulting in accumulation of intracellular cyclic GMP (Ignarro *et al*., 1990). The inhibitor of the NO-elicited soluble guanylate cyclase activation and cyclic GMP accumulation, methylene blue (Martin *et al*., 1985), induced a significant reduction of the relaxations evoked by both EFS and exogenous NO in the pig intravesical ureter. Similar observations have been obtained previously for the pig trigone (Persson & Andersson, 1992) and urethra of several species (see Andersson, 1993). Moreover, Iselin *et al*. (1996) observed that the inhibition exerted by exogenous NO on 5-hydroxytryptamine-induced contraction of the pig ureter is associated with an increase in the cyclic GMP levels. Taken together, these data suggest that NO released from nitrergic nerves relaxes U46619-contracted ureteral smooth muscle tone by activation of guanylate cyclase, with the subsequent accumulation of cyclic GMP.

NO may relax some blood vessels via activation of potassium channels, either through cyclic GMP-dependent protein kinase (Robertson *et al*., 1993) or by direct opening of

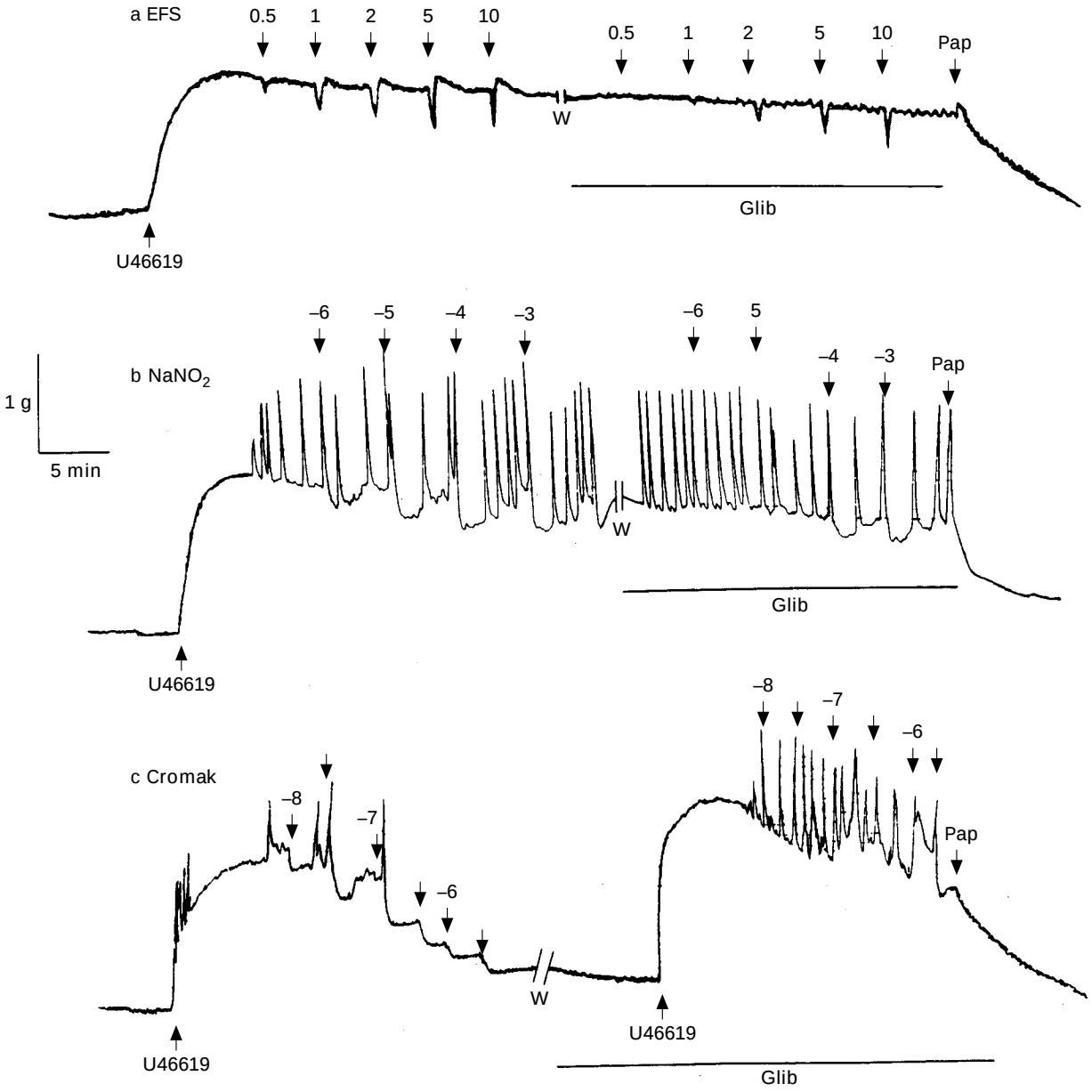

Figure 4 Isometric force recordings showing the effect of glibenclamide (Glib, 10^{-6} M) on the response of pig intravesical ureteral strips to (a) EFS (0.5–10 Hz), (b) exogenous addition of NO ($NaNO_2$, 10^{-6}–10^{-3} M) and (c) cromakalim (Cromak, 10^{-8}–3×10^{-6} M). Guanethidine (10^{-5} M) and atropine (10^{-7} M) were present throughout the experiment to block adrenergic neurotransmission and muscarinic receptors, respectively. The ureteral strips were contracted to the thromboxane analogue, U46619 (10^{-7} M) and when a sustained tone was obtained, frequency- and/or concentration-response curves to EFS, NO and cromakalim were performed in the absence and the presence of glibenclamide (Glib, 10^{-6} M). At the end of the experiment papaverine (Pap, 10^{-4} M) was added to obtain the maximal relaxation of the pig intravesical ureteral preparations. Numbers indicate frequency (Hz) or molar concentration in the bath. W: wash out.

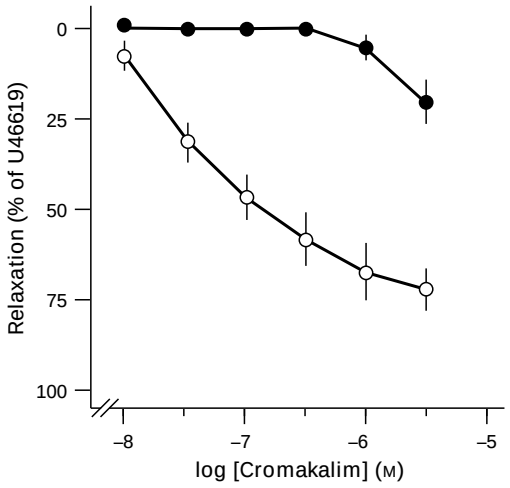

Figure 5 Average concentration-response curves for the relaxant effect of cromakalim in U46619-precontracted pig intravesical ureteral preparations, in the absence (○) and presence (●) of glibenclamide (10^{-6} M). Relaxations are expressed as a percentage of the tone induced by U46619 (10^{-7} M). Each point represents the mean and vertical lines s.e.mean of 7 experiments.

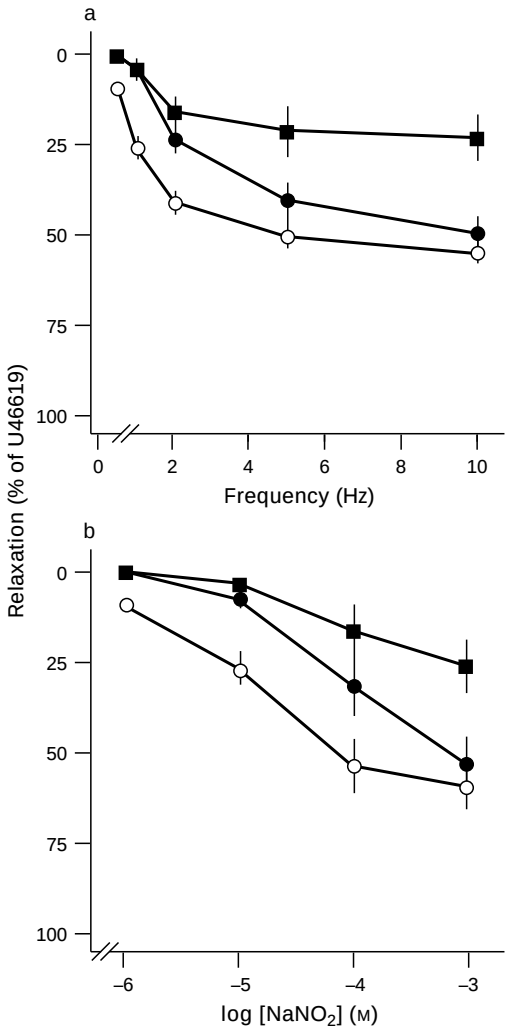

Figure 6 Frequency- (a) and concentration-response (b) relaxation curves to EFS- and $NaNO_2$ acidified solution, respectively, in U46619-precontracted pig intravesical ureteral preparations in control conditions (○) and in the presence of either glibenclamide alone (10^{-6} M) (●) or glibenclamide (10^{-6} M) plus methylene blue (10^{-5} M) (■). Relaxations are expressed as a percentage of the tone induced by U46619 (10^{-7} M). Each point represents the mean and vertical lines s.e.mean of 6–8 preparations.

Ca^{2+}-activated K^+ channels which does not require cyclic GMP (Bolotina *et al*., 1994). Cyclic GMP has been suggested to cause relaxation of smooth muscle by lowering the intracellular calcium concentration by either stimulating Ca^{2+}-ATPase activity or through opening of K^+ channels leading to hyperpolarization and subsequent reduction of Ca^{2+} influx through voltage-operated Ca^{2+} channels (Lincoln & Cornwell, 1991; Robertson *et al*., 1993). Thus, large conductance Ca^{2+}-activated K^+ channels mediate the relaxations to NO in rabbit aorta (Bolotina *et al*., 1994), and to both endogenous and exogenous NO in guinea-pig ileum (Osthaus & Galligan, 1992). Small conductance Ca^{2+}-activated K^+ channels were shown to mediate the hyperpolarization and relaxations to EFS and S-nitrosocysteine in the rat gastric fundus (Kitamura *et al*., 1993), and to EFS-induced NO-release and NO donors in guinea-pig trachea (Ellis & Conanan, 1994). In contrast, in the present study the inhibitors of the large (Giménez-Gallego *et al*., 1988) and small (Weir & Weston, 1986) conductance Ca^{2+}-activated K^+ channels, charybdotoxin and apamin, respectively, did not change the relaxations induced by either EFS or exogenous NO. Moreover, in the presence of methylene blue neither charybdotoxin nor apamin produced an additional inhibitory effect on the relaxation to either endogenous or exogenous NO. Recently, it has been found that the combined treatment of charybdotoxin plus apamin produces an inhibitory effect on endothelium-dependent vasodilatation in the rat hepatic artery (Zygmunt & Högestätt, 1996). This contrasts with results in the present study, where the incubation with the combination of inhibitors of the Ca^{2+}-sensitive K^+ channels failed to modify the relaxations evoked by EFS or exogenous NO. Such data suggest that Ca^{2+}-sensitive K^+ channels are not involved in the response to inhibitory neurotransmission evoked by NO in the pig intravesical ureter.

ATP-sensitive K^+ channels (Cook & Quast, 1990) play an important role in the regulation of the smooth muscle of the urinary tract (see Andersson, 1993). Thus, an opener of ATP-sensitive K^+ channels, cromakalim, reduces bladder mechanical activity in guinea-pig and rat (Foster *et al*., 1989a; Malmgren *et al*., 1989), as well as contractions in normal and unstable human and pig detrusor (Foster *et al*., 1989b). Cromakalim induces smooth muscle hyperpolarization and reduces the open probability of voltage-sensitive calcium channels (Foster *et al*., 1989b; Cook & Quast, 1990; Maggi *et al*., 1994a,b). In isolated preparations of guinea-pig urinary bladder (Fujii *et al*., 1990), renal pelvis and ureter (Maggi *et al*., 1994a), cromakalin reduced both spontaneous and electrically-evoked contractions. The inhibitor of ATP-sensitive K^+ channels, glibenclamide (Ashcroft & Ashcroft, 1990; Edwards *et al*., 1991), reversed the effect of cromakalim in the guinea-pig urinary bladder, ureter and renal pelvis (Fujii *et al*., 1990, Bonev & Nelson, 1993; Trivedi *et al*., 1994; Maggi *et al*., 1994a). Cromakalim also induced concentration-dependent relaxations of U46619-contracted pig intravesical ureteral strips, and these relaxations were inhibited by glibenclamide, suggesting the presence of ATP-sensitive K^+ channels in the porcine intravesical ureter.

Calcitonin gene-related peptide (CGRP), which acts as an inhibitory neurotransmitter in the mammalian ureter (Maggi & Giuliani, 1991; Sann *et al*., 1993), increases cyclic AMP and causes hyperpolarization and relaxation through the opening of ATP-sensitive K^+ channels in both ureteral and arterial smooth muscle (Maggi *et al*., 1994b; 1995; Santicioli & Maggi, 1994; Quayle *et al*., 1994). These effects could be mimicked by an activator of adenylate cyclase, forskolin, suggesting that elevation of cyclic AMP may be involved in the opening of ATP-sensitive K^+ channels in both guinea-pig ureter (Maggi *et al*., 1995) and arterial smooth muscle (Quayle *et al*., 1994). On the other hand, NO, which activates guanylate cyclase and increases cyclic GMP, was shown to induce hyperpolarization of mesenteric arteries through ATP-sensitive K^+ channels (Murphy & Brayden, 1995). In the present study of the pig intravesical ureter, glibenclamide significantly reduced the re-

laxations elicited by EFS. This inhibitory effect appeared to be postjunctional, since glibenclamide also effectively inhibited the relaxations to exogenous NO. These results collectively suggest that in addition to activation of glibenclamide-sensitive K^+ channels by the cyclic AMP pathway in guinea-pig ureter (Maggi *et al*., 1994b; 1995), both endogenous NO released by EFS and exogenous NO produce relaxations of the pig intravesical ureter in part via opening of glibenclamide-sensitive K^+ channels.

In the present investigation, part of the relaxations induced by either EFS or exogenous NO were resistant to methylene blue suggesting the existence of a cyclic GMP-independent mechanism. As previously stated, NO can relax smooth muscle via activation of K^+ channels, either by activation of a cyclic GMP-dependent protein kinase (Robertson *et al*., 1993) or the direct opening of K^+ channels without the requirement of cyclic GMP (Bolotina *et al*., 1994). If the latter occurred in the ureter the inhibitory effects of methylene blue and glibenclamide would be expected to be additive. However, incubation of pig ureteral strips with both methylene blue and glibenclamide did not produce a significant additional inhibition of the relaxations induced by either EFS or exogenous addition of NO to that induced by methylene blue alone. Such findings thus suggest that both EFS-released NO and exogenous NO activate guanylate cyclase and that the subsequent increase in cyclic GMP favours the opening of glibenclamide-sensitive K^+ channels, hyperpolarization and relaxation of pig intravesical ureter.

It has been shown that the increase in cyclic GMP may cause apamin-resistant hyperpolarization but the apamin-sensitive hyperpolarization induced by EFS or S-nitrosocysteine is mediated by another mechanism in the rat gastric fundus (Kitamura *et al*., 1993). Recently, Takeuchi and colleagues (1996) observed that NO induced relaxations of rat proximal colon by an unknown mechanism which were not associated with changes in cyclic GMP content and membrane potential of the smooth muscle. In the present study, there was persistence of some NO-induced relaxation in the presence of methylene blue and glibenclamide. This also suggests that another mechanism, perhaps similar to that in the gastric fundus (Kitamura *et al*., 1993) or proximal colon (Takeuchi *et al*., 1996) of the rat, might also contribute to the relaxations obtained to NO in the pig intravesical ureter.

In summary, the present results suggest that the inhibitory neurotransmission in the pig intravesical ureter is in part mediated by NO, causing relaxation through a guanylate cyclase-dependent mechanism which seems to favour the opening of glibenclamide-sensitive K^+ channels.

The authors thank Mr Francisco Puente and Mr Manuel Perales for their technical assistance. They also wish to thank GIPISA slaughterhouse (Pozuelo de Alarcón, Madrid) for kindly donating the ureters.

References

ANDERSSON, K.-E. (1993). Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol. Rev*., **45,** 253 – 308.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP sensitive K channels. *Cell. Signalling*, **2,** 197 – 214.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850 – 853.

BONEV, A.D. & NELSON, M.T. (1993). ATP-sensitive potassium channels in smooth muscle cells from guinea-pig urinary bladder. *Am. J. Physiol*., **264,** C1190 – C1200.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347,** 768 – 806.

BREDT, D.S. & SNYDER, S.H. (1992). Nitric oxide: A novel neuronal messenger. *Neuron*, **8,** 8 – 11.

COOK, N.S. & QUAST, U. (1990). Potassium channel pharmacology. In *Potassium Channels*. ed. Cook, N.S. pp. 181 – 258. Chichester, England: Ellis Horwood Series in Pharmaceutical Technology.

EDWARDS, G., HENSHAW, M., MILLER, M. & WESTON, A.H. (1991). Comparison of the effects of several potassium-channel openers on rat bladder and rat portal vein *in vitro*. *Br. J. Pharmacol*., **102,** 679 – 686.

ELLIS, J.L. & CONANAN, N.D. (1994). Effect of potassium channel blockers on relaxations to a nitric oxide donor and to nonadrenergic nerve stimulation in guinea pig trachea. *J. Pharmacol. Exp. Ther*., **271,** 782 – 786.

FOSTER, C.D., FUJII, K., KINGDON, J. & BRADING, A.F. (1989a). The effect of cromakalim on the smooth muscle of the guinea-pig urinary bladder. *Br. J. Pharmacol*., **97,** 281 – 291.

FOSTER, C.D., SPEAKMAN, M.J., FUJII, K. & BRADING, A.F. (1989b). The effects of cromakalim on the detrusor muscle of human and pig urinary bladder. *Br. J. Urol*., **63,** 284 – 294.

FUJII, K., FOSTER, C.D., BRADING, A.F. & PAREKH, A.B. (1990). Potassium channel blockers and the effects of cromakalim on the smooth muscle of the guinea-pig bladder. *Br. J. Pharmacol*., **99,** 779 – 785.

GIMENEZ-GALLEGO, G., NAVIA, N.M., REUBEN, J.P., KATZ, G.M., KACZOROWSKI, G.J. & GARCIA, M.L. (1988). Purification, sequence and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U.S.A*., **85,** 3329 – 3333.

HERNÁNDEZ, M., GARCIA-SACRISTAN, A. & ORENSANZ, L.M. (1995a). Muscarinic binding sites of the pig intravesical ureter. *J. Auton. Pharmacol*., **15,** 351 – 359.

HERNÁNDEZ, M., PRIETO, D., ORENSANZ, L.M., BARAHONA, M.V., GARCIA-SACRISTAN, A. & SIMONSEN, U. (1995b). Nitric oxide is involved in the non-adrenergic, non-cholinergic inhibitory neurotransmission of the pig intravesical ureter. *Neurosci. Lett*., **186,** 33 – 36.

HERNÁNDEZ, M., PRIETO, D., SIMONSEN, U., RIVERA, L., BARAHONA, M.V. & GARCIA-SACRISTAN, A. (1992). Noradrenaline modulates smooth muscle activity of the isolated intravesical ureter of the pig through different types of adrenoceptors. *Br. J. Pharmacol*., **107,** 924 – 931.

HERNÁNDEZ, M., SIMONSEN, U., PRIETO, D., RIVERA, L., GARCIA, P., ORDAZ, E. & GARCIA-SACRISTAN, A. (1993). Different muscarinic receptor subtypes mediating the phasic activity and basal tone of pig isolated intravesical ureter. *Br. J. Pharmacol*., **110,** 1413 – 1420.

IGNARRO, L.J., BRUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun*., **170,** 843 – 850.

ISELIN, C.E., ALM, P., SCHAAD, N.C., LARSSON, B., GRABER, P. & ANDERSSON, K.-E. (1996). Nitric oxide inhibits contraction of isolated pig ureteral smooth muscle. *J. Urol*., **155,** 763 – 767.

KITAMURA, K., LIAN, Q., CARL, A. & KURIYAMA, H. (1993). S-nitrosocysteine, but not sodium nitroprusside, produces apamin-sensitive hyperpolarization in rat gastric fundus. *Br. J. Pharmacol*., **109,** 415 – 423.

KOH, S.D., CAMPBELL, J.D., CARL, A. & SANDERS, K.M. (1995). Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. *J. Physiol*., **489,** 743 – 753.

LINCOLN, T.M. & CORNWELL, T.L. (1991). Towards and understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. *Blood Vessels*, **28,** 129 – 137.

MAGGI, C.A. & GIULIANI, S. (1991). The neurotransmitter role of CGRP in the rat and guinea-pig ureter: effect of a CGRP antagonist and species-related differences in the action of omega conotoxin on CGRP release from primary afferents. *Neuroscience*, **43,** 261 – 271.

MAGGI, C.A., GIULIANI, S. & SANTICIOLI, P. (1994a). Effect of cromakalim and glibenclamide on spontaneous and evoked motility of the guinea-pig isolated renal pelvis and ureter. *Br. J. Pharmacol.*, **111,** 687–694.

MAGGI, C.A., GIULIANI, S. & SANTICIOLI, P. (1994b). Multiple mechanisms in the smooth muscle relaxant action of calcitonin gene-related peptide (CGRP) in the guinea-pig ureter. *Naunyn-Schmiedeberg's Arch Pharmacol.*, **350,** 537–547.

MAGGI, C.A., SANTICIOLI, P. & GIULIANI, S. (1995). Role of cyclic AMP and protein kinase A in K^+-channel activation by calcitonin gene-related peptide (CGRP) in the guinea-pig ureter. *J. Auton. Pharmacol.*, **15,** 403–419.

MALMGREN, A., ANDERSSON, K.-E., SJOGREN, C. & ANDERSSON, P.O. (1989). Effects of pinacidil and cromakalim (BRL 34915) on bladder function in rats with detrusor instability. *J. Urol.*, **142,** 1124–1138.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232,** 708–716.

MURPHY, M.E. & BRAYDEN, J.E. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J. Physiol.*, **486,** 47–58.

OSTHAUS, L.E. & GALLIGAN, J.J. (1992). Antagonists of nitric oxide synthesis inhibit nerve-mediated relaxations of longitudinal muscle in guinea-pig ileum. *J. Pharmacol. Exp. Ther.*, **260,** 140–145.

PERSSON, K. & ANDERSSON, K.-E. (1992). Nitric oxide and relaxation of pig lower urinary tract. *Br. J. Pharmacol.*, **106,** 416–422.

PRIETO, D., HERNÁNDEZ, M., RIVERA, L., ORDAZ, E. & GARCIA-SACRISTAN, A. (1993). Catecholaminergic innervation of the equine ureter. *Res. Vet. Sci.*, **54,** 312–318.

PRIETO, D., SIMONSEN, U., MARTIN, J., HERNÁNDEZ, M., RIVERA, L., LEMA, L., GARCIA, P. & GARCIA-SACRISTAN, A. (1994). Histochemical and functional evidence for a cholinergic innervation of the equine ureter. *J. Auton. Nerv. Syst.*, **47,** 159–170.

QUAYLE, J.M., BONEV, A.D., BRAYDEN, J.E. & NELSON, M.T. (1994). Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase A. *J. Physiol.*, **475,** 9–13.

RIVERA, L., HERNÁNDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992a). Mediation of contraction and relaxation by alpha- and beta-adrenoceptors in ureterovesical junction of the sheep. *Res. Vet. Sci.*, **52,** 57–61.

RIVERA, L., HERNÁNDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992b). Mediation of contraction by cholinergic muscarinic receptors in the ureterovesical junction. *J. Auton. Pharmacol.*, **12,** 175–182
.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.*, **265,** C299–C303.

SANN, H., ROSSLER, W., HAMMER, K. & PIERAU, Fr, K. (1993). SP and CGRP in the ureter of chicken, and guinea-pig: distribution, binding sites and possible functions. *Neuroscience*, **49,** 699–713.

SANTICIOLI, P. & MAGGI, C.A. (1994). Inhibitory transmitter action of calcitonin gene-related peptide in guinea-pig ureter via activation of glibenclamide-sensitive K channels. *Br. J. Pharmacol.*, **113,** 588–592.

SCHULMAN, C.C. (1985). Innervation of the ureter: a histochemical and ultrastructural study. In *Urodynamics. Upper and Lower Urinary Tract II*. ed. Lutzeyer, W & Hannapel, I. pp. 292–361. Berlin: Springer-Verlag.

SMET, P.J., EDYVANE, K.A., JONAVICIUS, J. & MARSHALL, V.R. (1994). Colocalization of nitric oxide synthase with vasoactive intestinal peptide, neuropeptide Y, and tyrosine hydroxylase in nerves supplying the human ureter. *J. Urol.*, **152,** 1292–1296.

STIEF, C.G., TAHER, A., MEYER, M., KIRCHER, M., FORSSMANN, W., MAYER, B. & JONAS, U. (1993). A possible role of nitric oxide (NO) in the relaxation of renal pelvis and ureter. *J. Urol.*, **149,** 492A.

TAKEUCHI, T., KISHI, M., ISHII, T. NISHIO, H. & HATA, F. (1996). Nitric oxide mediated relaxation without concomitant changes in cyclic GMP content of rat proximal colon. *Br. J. Pharmacol.*, **117,** 1204–1208.

TRIVEDI, S., STETZ, S., LEVIN, R., LI, J. & KAU, S. (1994). Effect of cromakalim and pinacidil on ^{86}Rb efflux from guinea pig urinary bladder smooth muscle. *Pharmacology*, **49,** 159–166.

WALLESTEIN, S., ZUCKER, G.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47,** 1–9.

WEIR, S.W. & WESTON, A.H. (1986). Effect of apamin on response to BRL 34915, nicorandil and other relaxants in the guinea-pig taenia caeci. *Br. J. Pharmacol.*, **88,** 113–120.

WEISS, R.M. (1992). Physiology and pharmacology of renal pelvis and ureter. In *Campbell's Urology*. ed. Walsh, P.C., Retik, A.B. Stamey, T.A. & Vaughan, E.D. Vol. I, pp. 113–144. Philadelphia PA: W.B. Saunders Co.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117,** 1600–1606.

(Received June 13, 1996
Revised October 25, 1996
Accepted November 5, 1996)