Hydrogen Sulfide Plays a Key Role in the Inhibitory Neurotransmission to the Pig Intravesical Ureter

Vítor S. Fernandes¹, Ana S. F. Ribeiro¹, Pilar Martínez², María Elvira López-Oliva¹, María Victoria Barahona³, Luis M. Orensanz⁴, Ana Martínez-Sáenz¹, Paz Recio¹, Sara Benedito¹, Salvador Bustamante⁵, Albino García-Sacristán¹, Dolores Prieto¹, Medardo Hernández¹*

1. Departamento de Fisiología, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain, 2. Departamento de Anatomía y Anatomía Patológica Comparadas, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain, 3. Departamento de Toxicología y Farmacología, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain, 4. Departamento de Investigación, Hospital Universitario Ramón y Cajal, Madrid, Spain, 5. Departamento de Urología, Hospital Universitario Puerta de Hierro-Majadahonda, Madrid, Spain

*medardo@ucm.es

Abstract

According to previous observations nitric oxide (NO), as well as an unknown nature mediator are involved in the inhibitory neurotransmission to the intravesical ureter. This study investigates the hydrogen sulfide (H₂S) role in the neurogenic relaxation of the pig intravesical ureter. We have performed western blot and immunohistochemistry to study the expression of the H₂S synthesis enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), measurement of enzymatic production of H₂S and myographic studies for isometric force recording. Immunohistochemical assays showed a high CSE expression in the intravesical ureter muscular layer, as well as a strong CSE-immunoreactivity within nerve fibres distributed along smooth muscle bundles. CBS expression, however, was not consistently observed. On ureteral strips precontracted with thromboxane A₂ analogue U46619, electrical field stimulation (EFS) and the H₂S donor P-(4-methoxyphenyl)-P-4-morpholinoylphosphinodithioic acid (GYY4137) evoked frequency- and concentration-dependent relaxations. CSE inhibition with DL-propargylglycine (PPG) reduced EFS-elicited responses and a combined blockade of both CSE and NO synthesis enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), measurement of enzymatic production of H₂S and myographic studies for isometric force recording. Immunohistochemical assays showed a high CSE expression in the intravesical ureter muscular layer, as well as a strong CSE-immunoreactivity within nerve fibres distributed along smooth muscle bundles. CBS expression, however, was not consistently observed. On ureteral strips precontracted with thromboxane A₂ analogue U46619, electrical field stimulation (EFS) and the H₂S donor P-(4-methoxyphenyl)-P-4-morpholinoylphosphinodithioic acid (GYY4137) evoked frequency- and concentration-dependent relaxations. CSE inhibition with DL-propargylglycine (PPG) reduced EFS-elicited responses and a combined blockade of both CSE and NO synthase (NOS) with, respectively, PPG and N⁰-nitro-L-arginine (L-NOARG), greatly reduced such relaxations. Endogenous H₂S production rate was reduced by PPG, rescued by addition of GYY4137 and was not changed by L-NOARG. EFS and GYY4137 relaxations were also reduced by capsaicin-sensitive primary afferents (CSPA) desensitization with capsaicin and blockade of ATP-dependent K⁺ (K_ATP) channels, transient receptor potential A1

Citation: Fernandes VS, Ribeiro ASF, Martínez P, López-Oliva ME, Barahona MV, et al. (2014) Hydrogen Sulfide Plays a Key Role in the Inhibitory Neurotransmission to the Pig Intravesical Ureter. PLoS ONE 9(11): e113580. doi:10.1371/journal.pone.0113580

Editor: Agustín Guerrero-Hernandez, Cinvestav-IPN, Mexico

Received: June 30, 2014
Accepted: October 25, 2014
Published: November 21, 2014

Copyright: © 2014 Fernandes et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This work was supported by grants no. PSI09/00044 from the Ministerio de Ciencia e Innovación and PR6/13-18858 from the Universidad Complutense de Madrid (Santander-UCM) and by the Fundación para la Investigación en Urología from the Asociación Española de Urología, Spain. Vítor S. Fernandes is a research fellow (SFRH/BD/68460/2010) of Fundação para a Ciência e Tecnologia, Ministério da Educação e Ciência, Portugal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
(TRPA₁), transient receptor potential vanilloid 1 (TRPV₁), vasoactive intestinal peptide/pituitary adenyl cyclase-activating polypeptide (VIP/PACAP) and calcitonin gene-related peptide (CGRP) receptors with glibenclamide, HC030031, AMG9810, PACAP₆–₃₈ and CGRP₈–₃₇, respectively. These results suggest that H₂S, synthesized by CSE, is involved in the inhibitory neurotransmission to the pig intravesical ureter, through an NO-independent pathway, producing smooth muscle relaxation via K<sub>ATP</sub> channel activation. H₂S also promotes the release of inhibitory neuropeptides, as PACAP 38 and/or CGRP from CSPA through TRPA₁, TRPV₁ and related ion channel activation.

Introduction

Hydrogen sulfide (H₂S) is considered as the third endogenous gaseous transmitter besides nitric oxide (NO) and carbon monoxide (CO) [1, 2]. H₂S is synthesized from L-cysteine by the action of two pyridoxal-5′-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS) [1–5]. CBS activity is predominant in H₂S synthesis in the central nervous system whereas CSE is the major H₂S synthesis enzyme in the cardiovascular system [6, 7]. H₂S has been proposed as an antioxidant due to its ability to protect against oxidative stress and to react with oxidized thiols forming hydrodisulfide [8]. In spite of its therapeutic potential, the underlying mechanisms for its beneficial effects remain unclear due essentially to the lack of reliable methods for the detection of the sulfur-containing species [8].

In the lower urinary tract, H₂S donors produce a dual effect (contraction and/or relaxation) of smooth muscle. Thus, in rat bladder detrusor, the H₂S donor NaHS induces contraction via stimulation of capsaicin-sensitive primary afferents (CSPA), leading to release of tachykinins, such as substance P or neurokinin A [9, 10] whereas in bladder outflow region H₂S produces smooth muscle relaxation. In fact, in the pig bladder neck, H₂S, synthesized by CSE, acts as a signaling molecule in the inhibitory neurotransmission, producing smooth muscle relaxation via K<sub>ATP</sub> channel activation and favouring the release of the sensory neuropeptides [11, 12].

The density of the autonomic nerve fibers increases progressively from the upper ureter towards the bladder [13, 14]. In the proximal ureter, electric active pacemaker cells generate pyloureteric rhythmicity driving adjacent smooth muscle cells thus emphasizing the role of the interstitial cells of Cajal-like cells localized at this level [15]. These cells are involved in conducting and amplifying pacemaker activity in the upper urinary tract, producing electrical slow-wave potentials favouring the propagation of ureteral peristaltic activity [15]. In the distal ureter and ureterovesical junction, in contrast, there is a rich network of autonomic nerve fibers and numerous ganglion cells that play an important role in the coordination of the ureter and bladder activity at the ureterovesical
junction [13, 14, 16]. Thus, spontaneous peristaltic contractions of the upper ureter are initiated by a pacemaker activity at the renal pelvis and sustained essentially via myogenic mechanisms, whereas distal ureter activity is mainly regulated by autonomic nervous system. In fact, an NO dependent, as well as a neurogenic component of unknown nature has also been reported in the non-adrenergic, non-cholinergic (NANC) inhibitory transmission to the intravesical ureter [16, 17]. Knowledge of the mechanisms involved in the distal ureter smooth muscle relaxation is essential to provide useful therapeutic agents in the treatment of obstructive ureteral pathology produced by embedded calculi at the ureterovesical junction and in the vesico-ureteral reflux [16, 18].

H₂S has recently been identified as a powerful inhibitory neurotransmitter in the bladder base [11]. There are no available data, however, about the H₂S role in the distal ureter neurogenic relaxation. Therefore, the current study investigated the involvement of H₂S in the inhibitory neurotransmission to the pig intravesical ureter.

Materials and Methods

Adult pigs of either sex with no lesions in their urinary tract were selected from the Matadero Madrid Norte slaughterhouse. Urinary bladders with attached ureters were removed immediately after the animals were killed, and kept in chilled (4 °C) physiological saline solution (PSS). The protocol was carried out in the following 24 h. The adjacent connective and fatty tissues were carefully removed, and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were dissected from the bladder [19].

Western Blot

Intravesical ureter muscle was homogenized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate and 0.01% protease inhibitor cocktail (all from Sigma-Aldrich, St Louis, MO, USA). 50 µg protein were separated in a 15% polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). All membranes were blocked by 5% non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4 °C with rabbit anti-CSE or anti-CBS (1:1000 dilution, from Aviva Systems Biology, San Diego, USA) and mouse anti-β-actin (1:20000 dilution, from Santa Cruz Biotechnology Heidelberg, Germany) antibodies. Membranes were then washed in 0.05% Tween-20, incubated with HRP-conjugated secondary antibodies (Alexa Fluor 594 goat-antirabbit, 1:200 dilution, from Invitrogen, Paisley, UK) to detect CSE and CBS, for 1h at room temperature, and then washed and visualized by chemiluminescence (ECL advance-kit, GE Healthcare). Bands for CSE and CBS were normalized to those of β-actin. CSE and CBS expression in urinary bladder neck membranes were included as positive controls.
Immunohistochemistry

Intravesical ureter segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), for 2 to 4 h at 4°C, and subsequently placed in 30% sucrose in PB for cryoprotection. The tissue was embedded and frozen in OCT compound (Sakura Finetek, Europe BV), and stored at −80°C. Transversal sections 5 μm thick were obtained by means of a cryostat and preincubated in 10% normal goat serum in PB containing 0.3% Triton-X-100, for 2–3 h. Then, sections were incubated with either rabbit anti-CSE or anti-CBS antibodies at 4–8 μg/ml final concentration plus a mouse anti-protein gene product 9.5 (anti-PGP 9.5 from Abcam, Cambridge, UK), as neuronal marker, diluted 1:50, during 48 h at 4°C, washed and reacted with the secondary antibodies Alexa Fluor 594 goat-antirabbit (1:200 dilution) to detect CSE and CBS, and Alexa Fluor 488 goat-antimouse (1:200 dilution from Invitrogen, Paisley, UK), to detect PGP 9.5, for 2 h at room temperature. The slides were covered with a specific mounting medium with DAPI (Invitrogen), which stains all cell nuclei. Observations were made with a fluorescence microscope (Olympus IX51). No immunoreactivity could be detected in sections incubated in the absence of the primary antiserum [12].

Endogenous H₂S measurement

H₂S endogenous production was measured in intravesical ureter strips following the method previously described in the rat colon [20]. Briefly, the tissue was placed in a sealed polypropylene vial containing a Krebs incubation solution with 10 mM L-cysteine, 2 mM pyridoxal 5′-phosphate, 100 mM potassium phosphate buffer (pH 7.4), in the absence or in the presence of L-NOARG (100 μM), PPG (1 mM) and GYY4137 (10 μM), NO synthase (NOS) and CSE inhibitors and H₂S donor, respectively, which was connected to a 2 ml second vial containing 0.5 ml of 1% (w/v) zinc acetate. A gas mixture of 95% O₂ and 5% CO₂ was bubbled from the bottom of the first vial through the incubation solution. The reaction was started by transferring the vials from ice to a water bath at 37°C. The H₂S produced in the incubation chamber was then bubbled through the zinc acetate solution and trapped as zinc sulphide. The reaction was stopped at 30 min by injecting 0.5 ml of 50% (w/v) trichloroacetic acid into the incubation solution. Air flow was allowed to continue by an additional 30 min period, to ensure complete trapping of H₂S in the zinc acetate solution. The content of the second vial was transferred to test tubes containing 3.5 ml of de-ionized water, 0.4 ml of N,N-dimethyl-p-phenylenediamine sulphate (20 mM) in HCl (7.2 M) and 0.4 ml of FeCl₃ (30 mM) in HCl (1.2 M), for performing the methylene blue assay. The absorbance at 670 nm of the resulting solution was measured 20 min later by spectrophotometry (ELx800 microplate reader, Izasa). H₂S concentration was calculated against a calibration curve of the standard NaHS solutions.
Myographs for isometric force recordings

The intravesical ureter strips were suspended horizontally with one end connected to an isometric transducer and the other one to a micrometer screw, which regulates the tension applied to the preparations, in a myograph (DMT 820MS) containing PSS gassed with 5% CO₂ in O₂, giving a final pH of 7.4. Stretching of 2 g was applied to the preparations and they were allowed to equilibrate for 60 min.

The contractile ability of the strips was determined by exposing them to a 124 mM potassium 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 precontracted with 0.1 μM U46619, a thromboxane A₂ receptor agonist, EFS was performed by delivering rectangular pulses (1 ms duration, 0.5–16 Hz, 20 s trains, with constant current output adjusted to 75 mA), at 4 min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain). These EFS parameters have previously been used to elicit neurogenic relaxations in the intravesical ureter [16]. A first control response curve to EFS or to the H₂S donor P-(4-methoxyphenyl)-P-4-morpholinyphosphinodithioic acid (GYY4137, 0.1 nM- 30 μM) addition was obtained. The bath solution was then changed every 15 min for a period of 90 min, the preparations were incubated with the specific treatments for 30 min, and then a second relaxation curve was constructed. The concentration of the agents used was chosen on the basis of previous studies [11,12]. Control curves were run in parallel.

To desensitize capsaicin-sensitive primary afferents (CSPA), strips were pre-incubated in 10 μM capsaicin for 1 h, replacing the solution every 20 min, and then experiments were conducted in the continuous presence of capsaicin [21].

Drugs and solutions

The following drugs were used: (2E)-N-(2, 3-dihydro-1, 4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide (AMG9810), atropine, and DL-propargylglycine (PPG), guanethidine, indomethacin, NG-nitro-L-arginine (L-NOARG) and O-(carboxymethyl)hydroxylamine (AOAA), NaHS, L-cysteine, pyridoxal 5'-phosphate, zinc acetate trichloroacetatic acid, N,N-dimethyl-p-phénylénediamine sulphate, HCl, FeCl₃ all from Sigma (St Louis, MO, USA). Calcitonin gene-related peptide 8-37 (CGRP₈₋₃₇), capsaicin, glibenclamide, P-(4-methoxyphenyl)-P-4-morpholinyphosphinodithioic acid (GYY4137), 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropyl phenyl)acacetamide (HC030031), (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-] benzodiazocine -10-carboxylic acid (KT5720), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), pituitary adenyl cyclase-activating polypeptide 6-38 (PACAP₆₋₃₈) and 9,11-dideoxy-9α,11a-methanoepoxy prostaglandin F₂α (U46619) from Tocris (Bristol, UK). AMG9810, AOAA, CGRP₈₋₃₇, PPG, glibenclamide, GYY4137, HC030031, KT5720, ODQ and PACAP₆₋₃₈ were dissolved in dimethylsulphoxide. Indomethacin and U46619 were dissolved in
ethanol. The other drugs were dissolved in distilled water. The solvents used had no effect on the contractility of the bladder neck preparations.

The composition of PSS was (mM): NaCl 119, KCl 4.6, MgCl₂ 1.2, NaHCO₃ 24.9, glucose 11, CaCl₂ 1.5, KH₂PO₄ 1.2, ethylenediamine tetraacetic acid (EDTA) 0.027. The solution was maintained at 37°C and continuously gassed with 95% O₂ and 5% CO₂ to maintain pH at 7.4. K⁺-enriched PSS was PSS in which NaCl was exchanged for KCl on an equimolar basis.

**Calculations and Statistics**

Sensitivity to GYY4137 is expressed in terms of pD₂, where pD₂ = -log EC₅₀ and EC₅₀ is the agonist concentration needed to produce half-maximal response. pD₂ was estimated by computerized non-linear regression analysis (GraphPad Prism, USA). Results are expressed as a percentage reversal of U46619- or KPSS-induced contraction, and represent the mean ± s.e.m. of n (number of preparations, 1-2 strips per animal). Differences were analyzed by Student’s t-test for paired observations and by analysis of variance and a posteriori Bonferroni method for multiple comparisons. The differences were considered significant with a probability level of P<0.05. P values are shown in the Figure legends.

**Results**

**Expression of CSE**

By western blot, a CSE antibody recognized a band of approximately 45 kDa, which corresponded to the expected molecular weight, suggesting CSE protein expression in intravesical ureter smooth muscle (Fig. 1A) (n=4 from 4 pigs). CSE and CBS expression in the intravesical ureter was also investigated by using CSE and CBS selective antibodies combined with the neuronal marker PGP 9.5. CSE immunoreactivity was observed colocalized with the neuronal marker PGP 9.5 within nerve fibers widely distributed in the smooth muscle layer running parallel to the smooth muscle bundles (Fig. 1B–E) (n=5 from 5 pigs), and around the small arteries supplying the intravesical ureter (data not shown). CBS expression was not consistently detected in intravesical ureter membranes (Fig. 1F–J).

**Functional studies**

Urothelium-denuded strips of pig intravesical ureter were allowed to equilibrate to a passive tension of 1.5±0.1 g (n=75 preparations from 47 pigs). U46619 (0.1 μM) induced a sustained contraction above basal tension of 1.7±0.1 g (n=75).

**Relaxations to EFS and GYY4137**

Under NANC conditions, EFS (0.5–16 Hz) evoked reproducible frequency-dependent relaxations (maximal relaxation at 16 Hz of 75±7% of the U44619-induced contraction, n=12 from 9 pigs). The H₂S donor GYY4137 (0.1 nM–30 μM) induced potent concentration-dependent relaxations (pD₂ and Emax
values of $7.7 \pm 0.1$ and $81 \pm 7\%$, $n=12$ from 9 pigs), which were not changed as a consequence of urothelium mechanical removal.

**Effect of CSE and CBS blockade in the absence or presence of NOS inhibitor on EFS and GYY4137 relaxations**

To assess whether H$_2$S plays a role in the inhibitory neurotransmission of the intravesical ureter, ureteral preparations were treated with PPG and AOAA, inhibitors of, respectively, CSE and CBS. PPG (1 mM) reduced EFS-induced relaxations (Fig. 2A and B), whereas AOAA (1 mM) failed to modify these responses (Table 1). Pretreatment with L-NOARG (100 $\mu$M) reduced the EFS relaxations (Fig. 3B). Incubation of ureteral strips with PPG along with L-
NOARG greatly reduced the EFS responses (13% of control value at 16 Hz frequency) (Fig. 3A and B). Treatment with PPG (Fig. 2C), L-NOARG (Fig. 3C), PPG plus L-NOARG (Fig. 3C), or AOAA (Table 2) failed to modify GYY4137 relaxations. All these results suggest that H₂S produced by CSE acting in concert with NO is responsible for the EFS induced relaxation of the intravesical ureter under NANC conditions.

Effect of NOS and CSE inhibition and of the H₂S donor GYY4137 on endogenous H₂S production

CSE protein expression (n=4 from 4 pigs) (Fig. 3D) and endogenous H₂S production (n=8 from 7 pigs) (Fig. 3E) in intravesical ureter smooth muscle was not changed by pretreatment with the NOS inhibitor, L-NOARG (100 μM) (4.8±0.5 nM.min⁻¹.g⁻¹ and 4.4±0.5 nM.min⁻¹.g⁻¹, in the absence or presence of L-NOARG (P>0.05, versus control value, analysis of variance followed by Bonferroni method). The generated H₂S level, however, was reduced by CSE blockade with PPG (1 mM) (2.9±0.3 nM.min⁻¹.g⁻¹, n=8) and restored by addition of the H₂S donor, GYY4137 (10 μM) (3.8±0.5 nM.min⁻¹.g⁻¹#, n=8) (#P<0.05, versus control and PPG value, respectively, analysis of variance followed by Bonferroni method) (Fig. 3E).

Effect of soluble guanylyl cyclase, COX, PKA and K⁺ATP channel blockade on EFS and GYY4137 relaxations

The soluble guanylyl cyclase inhibitor ODQ (5 μM) reduced the EFS relaxations (Table 1) and failed to modify the GYY4137 responses (Table 2). Moreover, indomethacin (3 μM) and KT5720 (3 μM), blockers of, respectively, COX and PKA, did not change EFS (Table 1) or GYY4137 (Table 2) relaxations.

Raising extracellular K⁺ to 80 mM induced a sustained tone of 1.7±0.1 g (n=6). GYY4137 induced concentration-dependent relaxations on 80 mM K⁺ PSS-precontracted strips which were lower than those obtained on 0.1 μM U46619-contracted preparations (pD₂ and Emax values of 7.8±0.1 and 83±8% and 7.7±0.1 and 63±7%*, in 0.1 μM U46619- or 80 mM K⁺ PSS-precontracted strips, respectively, *P<0.05 versus control, paired t-test, n=7 from 4 pigs).

Glibenclamide (1 μM), a K⁺ATP channel inhibitor, reduced both EFS and GYY4137 relaxations (Fig. 4), thus indicating a K⁺ATP channel involvement in the H₂S relaxant responses.

Effect of capsaicin-sensitive primary afferent desensitization and of TRPA₁ and TRPV₁, receptor blockade on EFS and GYY4137 relaxations

Capsaicin (10 μM) (Fig. 5A and D), a CSPA neurotoxin, as well as HC030031 (60 μM) (Fig. 5B and E) and AMG9810 (10 μM) (Fig. 5C and F), antagonists of transient receptor potential A1 (TRPA₁) and transient receptor potential vanilloid 1 (TRPV₁), respectively, receptors, reduced both EFS and GYY4137 responses. These data indicate that H₂S relaxations are partly produced through TRPA₁, TRPV₁ and/or related ion channel activation-mediated release of inhibitory neuropeptides from CSPA.
Figure 2. Involvement of H2S, synthesized by CSE, in the inhibitory neurotransmission to the intravesical ureter. (A) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.5–16 Hz, 20 s trains) and GYY4137 (0.1 nM–30 μM), in the absence or presence of DL-propargylglycine (PPG, 1 mM), cystathionine γ-lyase inhibitor, on 0.1 μM U46619-precontracted pig intravesical ureter strips treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in g and horizontal bar time in min. W: wash. (B, C) Frequency- and concentration-response relaxation curves to EFS (B) and GYY4137 (C) in the absence (control, open circles) or in the presence (closed circles) of PPG. Results are expressed as a percentage reversal of the U46619-induced contraction and represent mean ± s.e.m. of 8 preparations from 4 pigs.*P<0.05, versus control (paired t-test).

Table 1. Effects of inhibitors of CBS, guanylyl cyclease, COX and PKA on relaxations induced by electrical field stimulation (EFS, 0.5–16 Hz) in the pig intravesical ureter.

|              | n  | 0.5  | 1    | 2    | 4    | 8    | 16   |
|--------------|----|------|------|------|------|------|------|
| Control      | 9  | 20±4 | 48±4 | 65±3 | 77±4 | 84±4 | 85±4 |
| AOAA (1 mM)  | 9  | 24±4 | 53±4 | 69±3 | 78±2 | 85±3 | 85±3 |
| Control      | 7  | 25±4 | 43±2 | 53±4 | 64±4 | 73±5 | 76±5 |
| ODQ (5 μM)   | 7  | 4±2  | 18±4 | 27±6 | 39±6*| 52±5*| 55±4*|
| Control      | 6  | 29±2 | 50±3 | 70±2 | 78±2 | 82±3 | 86±4 |
| Indomethacin (3 μM) | 6  | 32±6 | 51±5 | 68±2 | 76±3 | 81±3 | 84±3 |
| Control      | 6  | 19±3 | 42±4 | 65±3 | 76±3 | 81±3 | 82±2 |
| KT5720 (3 μM) | 6  | 18±4 | 39±5 | 63±3 | 75±3 | 79±3 | 79±3 |

Results are expressed as a percentage reversal of the 0.1 μM U46619-induced contraction and represent the mean ± s.e.m. of n preparations from 4-5 pigs. *P<0.05 versus control (paired t-test).
Figure 3. H2S and NO are involved in the NANC neurogenic relaxations to the intravesical ureter. (A) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.5–16 Hz, 20 s trains) and GYY4137 (0.1 nM–30 μM), in the absence or presence of DL-propargylglycine (PPG, 1 mM) plus Nω-nitro-L-arginine (L-NOARG, 100 μM), inhibitors of, respectively, cystathionine γ-lyase (CSE) and nitric oxide synthase, on 0.1 μM U46619-precontracted pig intravesical ureter strips treated with guanethidine (10 μM) and atropine (0.1 μM). Vertical bar shows tension in g and horizontal bar time in min. W: wash. (B, C) Frequency- and concentration-response relaxation curves to EFS (B) and GYY4137 (C) in the absence (control, open circles) or in the presence of L-NOARG (closed circles) and PPG plus L-NOARG (closed triangles). Results are expressed as a percentage reversal of the U46619-induced contraction and represent mean ± s.e.m. of 7 preparations from 4 pigs. *P<0.05, versus control and L-NOARG value, respectively (analysis of variance followed by Bonferroni method). (D) Western blot intravesical ureter membranes from smooth muscle incubated with a CSE antibody in the absence and the presence of L-NOARG (100 μM). Protein levels were normalized to β-actin. Bars represent mean ± s.e.m. of 4 preparations from 4 pigs (E) Level of H2S generated in the absence or presence of L-NOARG (100 μM), PPG (1 mM) and PPG plus GYY4137 (10 μM). Results represent mean ± s.e.m. of 8 preparations from 8 pigs. *P<0.05, versus control and PPG value, respectively (analysis of variance followed by Bonferroni method).

doi:10.1371/journal.pone.0113580.g003

Table 2. Effects of inhibitors of CBS, guanylyl cyclase, COX and PKA on relaxations evoked by the H2S donor GYY4137 (0.1 nM–30 μM).

|                        | Control | 9     | 8.7 ± 0.1 | 91 ± 5 |
|------------------------|---------|-------|-----------|--------|
| GYY4137 n              | 9       |       | 8.6 ± 0.1 | 88 ± 5 |
| AOAA (1 mM)            | 9       |       | 8.8 ± 0.2 | 94 ± 2 |
| Control                | 7       | 8.8 ± 0.1 | 92 ± 3 |
| ODQ (5 μM)             | 7       | 8.3 ± 0.1 | 99 ± 1 |
| Control                | 6       | 8.2 ± 0.1 | 98 ± 1 |
| Indomethacin (3 μM)    | 6       | 8.4 ± 0.2 | 98 ± 1 |
| Control                | 6       | 8.4 ± 0.1 | 98 ± 1 |

Results represent the mean ± s.e.m. of n preparations from 4–5 pigs. Emax is the maximal relaxation, expressed as a percentage reversal of the 0.1 μM U46619-induced contraction, obtained for each drug. pD2 = -log EC50, where EC50 is the concentration of agonist producing 50% of the Emax.

doi:10.1371/journal.pone.0113580.t002
Effect of VIP/PACAP and CGRP receptor blockade on EFS and GYY4137 relaxations

PACAP 6–38 (3 μM) (Fig. 6A and C) and CGRP 8–37 (3 μM) (Fig. 6B and D), antagonists of VIP/PACAP and CGRP, respectively, receptors, reduced both EFS and GYY4137 responses, thus suggesting that a part of H₂S relaxation might be due to PACAP 38 and CGRP.

Discussion

Our results provide morphological and functional evidence that neuronal H₂S, synthesized by CSE, is involved in the NO-independent NANC inhibitory transmission to the pig intravesical ureter. H₂S induces smooth muscle relaxation via K₅₇₆ channel activation and also promotes the release of inhibitory
neuropeptides PACAP 38 and CGRP from CSPA, through sensory nerve TRPA1, TRPV1 and/or related ion channel activation. This conclusion is supported by the following observations: (1) The presence of CSE within nerve fibers widely distributed in the smooth muscle layer of the intravesical ureter. (2) The neurogenic relaxation elicited by EFS was inhibited by PPG. (3) EFS and GYY4137 responses were reduced by blockade of K_ATP channels, desensitization of CSPA and inhibition of TRPA1 and TRPV1 channels and of PACAP and CGRP receptors.

In the pig intravesical ureter, only CSE expression was consistently observed. Western blot assays showed a band compatible with that expected for CSE in the muscular layer, and immunostaining of ureteral samples revealed a labeling for CSE protein within nerve fibers widely distributed among smooth muscle.
bundles. The high density and the distribution of CSE immunoreactivity observed in the intravesical ureter agree with that found in pig bladder neck [12]. In the current study, the existence of CSE-immunoreactive elements around small arteries also suggests a role for H2S in the regulation of intravesical ureter blood flow, thus supporting an important role for H2S in vascular tone modulation [22].

NO-dependent and independent NANC neurogenic relaxations in the intravesical ureter have previously been reported [16,17]. In the current investigation, isometric force recording experiments showed that the CSE inhibitor PPG reduced the EFS-elicited neurogenic relaxations, whereas that the CBS inhibitor AOAA failed to modify these responses, reinforcing the validity of observations made about the lack of CBS immunoreactivity in the intravesical

Figure 6. PACAP and CGRP might be involved in the H2S relaxations. Frequency- and concentration-response relaxation curves to electrical field stimulation (EFS, 1 ms duration, 0.5–16 Hz, 20 s trains) (A, B) and GYY4137 (0.1 nM–30 μM) (C, D) in the absence (control, open circles) or in the presence (closed circles) of PACAP6–38 (3 μM) (A, C) and CGRP8–37 (3 μM) (B, D). VIP/PACAP and CGRP receptor antagonists, respectively, on 0.1 μM U46619-precontracted pig intravesical ureter strips treated with guanethidine (10 μM) and atropine (0.1 μM). Results are expressed as a percentage reversal of the U46619-induced contraction and represent mean ± s.e.m. of 7–8 preparations from 4 pigs. *P<0.05, versus control (paired t-test).

doi:10.1371/journal.pone.0113580.g006
ureteral wall. These results, together with the reduction of endogenous H$_2$S production elicited by PPG and its recovery in response to GYY4137, clearly indicate that neuronally-released endogenous H$_2$S synthesized by CSE is responsible for a considerable part of the NANC inhibitory transmission to the intravesical ureter. The fact that incubation with the NO synthase inhibitor L-NOARG plus PPG abolished the EFS relaxations indicates that, in addition with NO, H$_2$S plays a key role in ureteral inhibitory neurotransmission, and might therefore directly be involved in the regulatory mechanisms of the smooth muscle tone, thus reducing the ureteral resistance during bladder filling. In the intravesical ureter, in addition to the predominant longitudinal smooth muscle fibers, circular and helical fibers have also been described [13, 14], so that other mechanisms might be involved in the regulation of the ureteral smooth muscle contractility. In the current study, an effect of GYY4137 on the amplitude and frequency of the U46619 contractions was not consistently observed. Further in vivo studies would be necessary to assess the changes induced by H$_2$S in the distal ureter urodynamic parameters.

NO has been proposed as an inducer or as a molecular switch for endogenous H$_2$S production for regulating of vascular smooth muscle tension [2]. In the current study, the fact that endogenous H$_2$S production rate was not modified under conditions of NOS blockade suggests the involvement of a NO-independent pathway in the intravesical ureter endogenous H$_2$S generation. These results agree with those obtained in bladder neck, where both H$_2$S [12] and NO [23] neuronal pathways promote smooth muscle relaxation. Current results showing the mediation of H$_2$S, together with NO, in the intravesical ureter neurogenic relaxation reinforces the role of the autonomic nervous system in the regulation to the distal ureter tension in contrast with the myogenic electrical activity characteristic of the pyeloureteral segments [13, 14, 16, 17].

In our study, GYY4137, a donor which in the cardiovascular system slowly releases H$_2$S, both in vivo and in vitro [24], produced a potent relaxation (pD$_2$ value of 7.7), slow in onset and sustained, which was similar to that previously obtained in bladder neck [12] indicating an essential role for H$_2$S in the ureteral smooth muscle relaxation. The fact that PPG failed to modify the GYY4137 relaxations may be explained on the basis that PPG is an inhibitor of the endogenous H$_2$S synthesis enzyme CSE, and therefore does not seem probable that it can reduce the responses to the exogenously-added H$_2$S donors. Current results agree with those obtained in bladder neck, where CSE selective blockade did not change the GYY4137 responses [12]. Urothelium mechanical removal, as well as pretreatment with the NO enzyme synthesis inhibitor L-NOARG did not change the GYY4137 relaxations, thus suggesting that H$_2$S produces smooth muscle relaxation via urothelium- or NO-independent mechanisms.

Like neuronal- and endothelial-NOS, CSE activity is Ca$^{2+}$-calmodulin dependent [25] and H$_2$S generated from L-cysteine by CSE exerts its biological action by sulfhydrating target proteins, process that may augment guanylyl cyclase activity, thus increasing [cGMP], and relaxing smooth muscle [26]. In the present study, relaxations to EFS were reduced by ODQ, a soluble guanylyl cyclase
inhibitor. This is consistent with previous findings in the intravesical ureter showing that NO-mediated NANC neurogenic relaxation is produced, in part, via activation of guanylyl cyclase [17]. ODQ, however, failed to modify the GYY4137 responses, thus initially ruling out an involvement of the cGMP/NO-dependent mechanisms in the H₂S relaxations. These results agree with those previously described in vascular smooth muscle, where unlike the intracellular signaling responsible for the vasodilator action induced by NO and CO, H₂S relaxations were produced in a guanylyl cyclase activation-independent way [27].

H₂S has previously been reported to inhibit superoxide anions formation via adenylyl cyclase-PKA pathway in pig pulmonary arterial endothelial cells [28]. In the current study, however, the lack of effect shown by the PKA inhibitor KT5720 on EFS or GYY4137 responses seems to rule out the involvement of the PKA pathway in H₂S relaxations.

Kₐᵥ₅₃ channel activation mediates the H₂S-induced relaxation in both vascular and visceral smooth muscle. Thus, Kₐᵥ₅₃ channel opening-mediated H₂S responses have been described in rat aorta and mesenteric arteries [3, 5, 22] or pig bladder neck [11]. In the intravesical ureter, GYY4137 relaxations were reduced in 80 mM K⁺ PSS-precontracted strips. Extracellular [K⁺] elevation inhibits K⁺ efflux through membrane K⁺ channels, and since glibenclamide, a Kₐᵥ₅₃ channel inhibitor, reduced the EFS or GYY4137 responses, it seems likely that ionic conductance modifications via Kₐᵥ₅₃ channels are involved in H₂S relaxations. Interestingly, this signaling pathway is also involved in the neuronal NO-mediated relaxation of the pig intravesical ureter [17].

The COX pathway is involved in bladder physiology and pathology, and several studies have demonstrated a role for COX-derived prostanoids in the neural control of bladder smooth muscle tone [11, 29, 30, 31]. In the current study, indomethacin, a COX inhibitor, failed to modify the EFS or GYY4137 relaxations, thus indicating that COX-derived prostanoids are not likely to be involved in the H₂S responses.

H₂S donors produce contraction of rat detrusor via release of tachykinins such as substance P or neurokinin A from CSPA, by activating non-selective cation channel TRPV₁, TRPA₁ and/or related ion channels in the sensory nerves [9, 10, 32]. Sensory neuropeptides, such as pituitary adenylyl cyclase-activating polypeptide 38 (PACAP 38) relax the intravesical ureter [33]. In the pig bladder neck, the H₂S relaxant responses are produced, in part, via PACAP 38 and calcitonin gene-related peptide (CGRP) release from CSPA [11]. For this reason, we investigated whether in the intravesical ureter, the release of sensory neuropeptides such as PACAP 38 and/or CGRP could be involved in the H₂S relaxations. The protocol of capsaicin desensitization carried out in our investigation produces an intravesical ureter CSPA functional blockade [21]. Thus, the reduction of the EFS or GYY4137 relaxations caused by capsaicin would indicate that these responses are produced, in part, by inhibitory peptides released from CSPA. TRPA₁ are recognized as the main target for H₂S in sensory neurons [34]. In the current study, the inhibition produced by HC030031, a TRPA₁ selective antagonist, on EFS or GYY4137 responses, suggests the involvement of
TRPA1 receptors in H2S relaxations. Moreover, the H2S response reduction produced by blockade of TRPV1 with AMG9810 indicates the mediation of these receptors. The fact that capsaicin inhibition of the EFS or GYY4137 relaxations was higher than that exerted by HC030031 and AMG9810 suggests that in addition to the TRPA1 and TRPV1, the possible role of related ion channels located on sensory neurons. The EFS or GYY4137 response inhibition produced by VIP/PACAP and CGRP receptor blockade, suggests that H2S may promote intravesical ureter smooth muscle relaxation via PACAP 38 and/or CGRP release from CSPA. These results agree with those found in the pig bladder neck, where part of H2S relaxations are indirectly produced via inhibitory neuropeptide release from sensory nerves [11].

H2S donors have been proposed as helpful therapeutic tools for unilateral ureteric obstruction-induced renal damage by attenuating fibrosis, oxidative stress and inflammation [35]. Neurogenic mechanisms play an essential role in distal ureteral motility. In fact, intravesical ureter efferent and afferent innervation, including cholinergic, adrenergic and NANC components, is much dense than that in the upper ureter in humans [36, 37]. Most urinary stones are frequently located distally [38], therefore a better understanding of the neurogenic mechanisms involved in distal ureteral smooth muscle relaxation could lead to the discovery of new drugs useful in relieving ureteral colic, facilitating spontaneous stone passage, relieving symptoms or preparing the ureter for ureteroscopy. Our
lab previously demonstrated the involvement of NO and an unknown nature mediator/s in the intravesical ureter neurogenic relaxation \[16, 17\]. Current results show that, beside NO, H\(_2\)S is responsible for the intravesical ureter NANC inhibitory neurotransmission, thus suggesting that H\(_2\)S-mediated neurotransmission might be useful as a therapeutic target in the obstructive ureteral pathology and in the vesico-ureteral reflux.

In conclusion, present results suggest that H\(_2\)S, synthesized by CSE, acts as a potent inhibitory neurotransmitter to the pig intravesical ureter through a NO-independent mechanism, producing smooth muscle relaxation via K\(_{\text{ATP}}\) channel activation. H\(_2\)S also promotes the release of PACAP 38 and CGRP from CSPA through activation of TRPA\(_1\), TRPV\(_1\) and/or related ion channels in the sensory nerves (Fig. 7). To our knowledge this is the first study showing the involvement of H\(_2\)S in the neurogenic relaxation of the intravesical ureter.

**Acknowledgments**

The authors wish to thank Dr. Marcel Jiménez from Department of Cell Biology, Universitat Autónoma de Barcelona, for his valuable scientific support in the CSE activity assay. Moreover, authors thank Ms. Macarena Martín, Mr. Francisco Puente and Mr. Manuel Perales for their technical assistance. They also thank Matadero Madrid Norte S.A. (San Agustín de Guadalix, Madrid) for kindly donating the urinary bladders.

**Author Contributions**

Conceived and designed the experiments: VSF AGS DP MH. Performed the experiments: VSF ASFR PM MELO MVB PR S. Benedito S. Bustamante MH. Analyzed the data: VSF ASFR PM MELO MVB LMO AMS PR S. Benedito S. Bustamante AGS DP MH. Contributed reagents/materials/analysis tools: VSF ASFR PM MELO MVB LMO AMS PR S. Benedito S. Bustamante AGS DP MH. Wrote the paper: VSF ASFR PM MELO MVB LMO AGS DP MH.

**References**

1. Moore PK, Bhatia M, Moolchala S (2003) Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol Sci 24: 609–611.
2. Wang R (2002) Two’s company, three’s a crowd: can H\(_2\)S be the third endogenous gaseous transmitter? FASEB J 16: 1792–1798.
3. Wang R (2003) The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal 5: 493–501.
4. Zhao W, Wang R (2002) H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 283: H474–H480.
5. Zhao W, Zhang J, Lu Y, Wang R (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 20: 6006–6016.
6. Bhatia M (2005) Hydrogen sulfide as a vasodilator. IUBMB Life 57: 603–606.
7. **Lowicka E, Beltowski J** (2007) Hydrogen sulfide (H2S) - the third gas of interest for pharmacologists. Pharmacol Rep 59: 4–24.

8. **Li Q, Lancaster JR Jr** (2013) Chemical foundations of hydrogen sulfide biology. Nitric Oxide 35: 21–34.

9. **Patacchini R, Santicoli P, Giuliani S, Maggi CA** (2004) Hydrogen sulfide (H2S) stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder. Br J Pharmacol 142: 31–34.

10. **Patacchini R, Santicoli P, Giuliani S, Maggi CA** (2005) Pharmacological investigation of hydrogen sulfide (H2S) contractile activity in rat detrusor muscle. Eur J Pharmacol 509: 171–177.

11. **Fernandes VS, Ribeiro AS, Barahona MV, Orensanz LM, Martinez-Sainz A, et al.** (2013) Hydrogen sulfide-mediated inhibitory neurotransmission to the pig bladder neck: Role of KATP channels, sensory nerves and calcium signaling. J Urol 190: 746–756.

12. **Fernandes VS, Ribeiro AS, Martinez MP, Orensanz LM, Barahona MV, et al.** (2013) Endogenous hydrogen sulfide has a powerful role in inhibitory neurotransmission to the pig bladder neck. J Urol 189: 1567–1573.

13. **Prieto D, Hernández M, Rivera L, Ordaz E, García-Sacristán A** (1993) Catecholaminergic innervation of the equine ureter. Res Vet Sci 54: 312–318.

14. **Prieto D, Simonsen U, Martín J, Hernández M, Rivera L, et al.** (1994) Histochemical and functional evidence for a cholinergic innervation of the equine ureter. J Auton Nerv Syst 47: 158–170.

15. **Di Benedetto A, Arena S, Nicotina PA, Mucciardi G, Gali A, et al.** (2013) Pacemakers in the upper urinary tract. Neurourol Urodyn 32: 349–353.

16. **Hernández M, Prieto D, Orensanz LM, Barahona MV, García-Sacristán A, et al.** (1995) Nitric oxide is involved in the non-adrenergic, non-cholinergic inhibitory neurotransmission of the pig intravesical ureter. Neurosci Lett 186: 33–36.

17. **Hernández M, Prieto D, Orensanz LM, Barahona MV, Jiménez-Cidre M, et al.** (1997) Involvement of a glibenclamide-sensitive mechanism in the nitrergic neurotransmission of the pig intravesical ureter. Br J Pharmacol 120: 609–616.

18. **Blok C, Van Venrooij GE, Mokhless I, Coolsaet BL** (1985) Dynamics of the ureterovesical junction: its fluid transport mechanism in the pig. J Urol 134: 175–178.

19. **Hernández M, Prieto D, Simonsen U, Rivera L, Barahona MV, et al.** (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.

20. **Gil V, Gallego D, Jiménez M** (2011) Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility. Br J Pharmacol 164: 485–498.

21. **Bustamante S, Orensanz LM, Barahona MV, Contreras J, García-Sacristán A, et al.** (2000) Tachykinergic excitatory neurotransmission in the pig intravesical ureter. J Urol 164: 1371–1375.

22. **Tang G, Wu L, Liang W, Wang R** (2005) Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. Mol Pharmacol 68: 1757–1764.

23. **Hernández M, Barahona MV, Recio P, Navarro-Dorado J, Bustamante S, et al.** (2008) Role of neuronal voltage-gated K(+) channels in the modulation of the nitrergic neurotransmission of the pig urinary bladder neck. Br J Pharmacol 153: 1251–1258.

24. **Li L, Whitteman M, Guan YY, Neo KL, Cheng Y, et al.** (2008) Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): New insights into the biology of hydrogen sulfide. Circulation 117: 2351–2360.

25. **Yang G, Wu L, Jiang B, Yang W, Qi J, et al.** (2008) H2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine γ-lyase. Science 322: 587–590.

26. **Mustafa AK, Gadalla MM, Snyder SH** (2009) Signaling by gasotransmitters. Sci Signal 2(68): re2.

27. **Kajimura M, Fukuda R, Bateman RM, Yamamoto T, Suematsu M** (2010) Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H2S gas biology. Antioxid Redox Signal 13: 157–192.

28. **Muzaffar S, Jeremy JY, Sparatore A, Del Soldato P, Angelini GD, et al.** (2008) H2S-donating sildenafil (ACS6) inhibits superoxide formation and gp91phox expression in arterial endothelial cells: role of protein kinases A and G. Br J Pharmacol 155: 984–994.
29. Andersson KE (2010) Detrusor myocyte activity and afferent signaling. Neurourol Urodyn 29: 97–106.

30. de Jongh R, van Koeveerige GA, van Kerrebrokeck PE, Markerink-van Ittersum M, de Vente J, et al. (2007) The effects of exogenous prostaglandins and the identification of constitutive cyclooxygenase I and II immunoreactivity in the normal guinea pig bladder. BJU Int 100: 419–429.

31. Martínez-Saez A, Barahona MV, Orensanz LM, Recio P, Bustamante S, et al. (2011) Mechanisms involved in the nitric oxide independent inhibitory neurotransmission to the pig urinary bladder neck. Neurourol Urodyn 30: 151–157.

32. Streng T, Axelsson HE, Hedlund P, Andersson DA, Jordt SE, et al. (2008) Distribution and function of the hydrogen sulfide-sensitive TRPA1 ion channel in rat urinary bladder. Eur Urol 53: 391–399.

33. Hernández M, Barahona MV, Recio P, Rivera L, Benedito S, et al. (2004) Heterogeneity of neuronal and smooth muscle receptors involved in the VIP- and PACAP-induced relaxations of the pig intravesical ureter. Br J Pharmacol 141: 123–131.

34. Ogawa H, Takahashi K, Miura S, Imagawa T, Saito S, et al. (2012) H(2)S functions as a nociceptive messenger through transient receptor potential ankyrin 1 (TRPA1) activation. Neuroscience 218: 335–343.

35. Jung KJ, Jang HS, Kim JI, Han SJ, Park JW, et al. (2013) Involvement of hydrogen sulfide and homocysteine transsulfuration pathway in the progression of kidney fibrosis after ureteral obstruction. Biochim Biophys Acta 1832: 1989–1997.

36. Edyvane KA, Trussell DC, Jonavicius J, Henwood A, Marshall VR (1992) Presence and regional variation in peptide-containing nerves in the human ureter. J Auton Nerv Syst 39: 127–137.

37. Santicioli P, Maggi CA (1998) Myogenic and neurogenic factors in the control of pyeloureteral motility and ureteral peristalsis. Pharmacol Rev 50: 683–722.

38. Porpiglia F, Destefanis P, Fiori C, Fontana D (2000) Effectiveness of nifedipine and deflazacort in the management of distal ureter stones. Urology 56: 579–582.