Presence of cyclic nucleotide-gated channels in the rat urethra and their involvement in nerve-mediated nitric relaxation

Domingo Triguero, María Sancho, Marta García-Flores, and Ángeles García-Pascual

Department of Physiology, Veterinary School, Complutense University, Madrid, Spain

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Triguero D, Sancho M, García-Flores M, García-Pascual A. Presence of cyclic nucleotide-gated channels in the rat urethra and their involvement in nerve-mediated nitric relaxation. Am J Physiol Renal Physiol 297: F1353–F1360, 2009. First published August 26, 2009; doi:10.1152/ajprenal.00403.2009.—We have addressed the distribution of cGMP-gated channels (CNG) in the rat urethra for the first time, as well as their putative role in mediating of the relaxation elicited by electrical field stimulation of nitric nerves. Functional studies have shown that specifically blocking CNG with L-cis-diltiazem leads to the rapid inhibition of urethral relaxation induced either by nitric oxide (NO) released by the nerves or by soluble guanylate cyclase activated with YC-1. By contrast, nerve-mediated noradrenergic contractions were only slowly and mildly reduced by L-cis-diltiazem. This effect was mimicked by lower concentrations of the n-diltiazem isomer, probably due to the non-specific inhibition of voltage-dependent calcium channels. However, n-diltiazem did not affect relaxation responses. The expression of heteromeric retinal-like CNGA1 channels was demonstrated by conventional PCR on mRNA from the rat urethra. These channels were located in a subpopulation of intramuscular interstitial cells of Cajal (ICC) as well as in smooth muscle cells, although they were less abundant in the latter. CNG channels could not be visualized in any nervous structure within the urethral wall, in agreement with the emerging view that a subset of ICC serves as a target for NO. These channels could provide a suitable ionic mechanism to associate the changes in cytosolic calcium with the activation of the nitric NO-cGMP pathway and relaxation although the precise mechanisms involved remain to be elucidated.

Urethral ICC are far from electrically quiescent since they generate spontaneous Ca\(^{2+}\) oscillations, supporting their role as pacemakers. In a tonic organ like the urethra, pacemaker cells have been suggested to support the asynchronous recruitment of muscle units to maintain tone, very much like in skeletal muscle (22). The ionic mechanism thought to underlie this activity is initiated by inositol 1,4,5-triphosphate (IP\(_3\))-mediated Ca\(^{2+}\) release from intracellular stores and the subsequent opening of Ca\(^{2+}\)-activated Cl\(^{-}\) channels in the rabbit urethra (17). In addition, extracellular Ca\(^{2+}\) entry through non-voltage-dependent Ca\(^{2+}\) channels is also needed for the spontaneous Ca\(^{2+}\) oscillations (24), and a Na\(^{+}/Ca\(^{2+}\) exchanger (NCX) working in a reverse mode has been suggested (4). The physiological relevance of the spontaneous ICC depolarization is reinforced by the fact that they can be modified by endogenous neurotransmitter release, suggesting that ICC act as mediators of neurotransmission as in the gut. Therefore, to define the involvement of ICC in the regulation of urinary motor function and their relationship with autonomic nervous control will require an analysis of the mechanisms of communication between all the cells involved in this pathway.

The hyperpolarization-activated (HCN) and the cyclic nucleotide-gated (CNG) channels are ion channels directly gated by the binding of intracellular cAMP and/or cGMP to a cytoplasmic cyclic nucleotide-binding domain. The functional roles of these ion channels are thought to be complex, and they are poorly understood (see updated reviews in Refs. 5 and 15). In clear contrast to the structurally related subfamily of HCN channels, CNG channels are weakly activated by changes in membrane potential, and their opening and gating are directly defined by the intracellular binding of cyclic nucleotides. These channels mainly carry an inward Na\(^{+}\) and Ca\(^{2+}\) current which provokes membrane depolarization or local changes in cytosolic Ca\(^{2+}\) concentrations. Although initially described in bovine rod photoreceptors (19), their involvement in the control of photoreceptor membrane depolarization is already found as early as in ciliate protozoa (29). Indeed, it is now generally accepted that they may be found in sensory receptors, epithelia, and blood vessels and even in spermatozoa (see Ref. 5), participating in a plethora of physiological processes from sensory transduction to the control of fluid reabsorption at the alveolar epithelia (16). However, in most of these cases the specific role of CNG channels is unknown. Although CNG channels can conduct currents carried by mono- and divalent cations, there is growing interest in this family as they provide an alternative pathway for Ca\(^{2+}\) entry that is virtually independent of membrane voltage and that couples the activity of Ca\(^{2+}\)-regulated proteins to cAMP/ cGMP signaling without involving protein kinases.

One of the most widely used specific inhibitors of CNG-gated CNG channels is L-cis-diltiazem (15), while the n-isomer blocks voltage-gated Ca\(^{2+}\) channels. When analyzing the ef-
fect of synaptic vesicle depletion induced by the scorpion venom a-toxins in the sheep urethra, we observed that their depolarizing effect lead to pronounced NO synthase (NOS)-
and GC-mediated relaxation that could be inhibited by l-cis-
diltiazem (28). Similarly, l-cis-diltiazem was shown to inhibit relaxation elicited by electrical field stimulation (EFS) of intrinsic nitrergic nerves (28). These data strongly suggest that CNG channels are involved in the NO-cGMP signaling pathway active in the urethra.

In the present work, we further examine the possibility that retinal-like CNG channels (CNGA1), a functional subtype selectively gated by cGMP, are present in the rat urethra. We studied the cellular distribution of CNGA1 channels by immunofluorescence as well as the mRNA expression of the different subunits that form the functional channel. We show that CNGA1 channels are mainly present in a subpopulation of urethral ICC, and they are only weakly expressed in smooth muscle cells. In addition, the characterization of the functional effects of l-cis-diltiazem on both relaxant and contractile nerve-mediated responses induced by EFS provides new insights into the relevance of CNG channels in the regulation of urethral motility. We hypothesize that CNG could be a suitable link between the activation of the NO-cGMP pathway and the modulation of the nitrergic control of smooth muscle activity by the ICC.

**EXPERIMENTAL PROCEDURES**

**Drugs and Solutions**

Arginine vasopressin (AVP), atropine sulfate, guanethidine monosulfate, d-tubocurarine hydrochloride, 3-(5′-hydroxyethyl-2-furyl)-1-benzyl indazole (YC-1), and diltiazem were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). l-cis-Diltiazem was obtained from Biomol International. The drugs were all dissolved in distilled water and stored at −20°C, and the working concentrations were reached by dilution in 0.9% NaCl.

**Tissue Preparation**

The lower urinary tract was obtained from 60 female Wistar rats (6–8 wk old and weighing 200–300 g) killed by cervical dislocation followed by exsanguination. After opening of the abdomen, the whole lower urinary tract was removed and maintained at 4°C in Krebs solution (in mM): 119 NaCl, 4.6 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 15 NaHCO₃, 1.2 KH₂PO₄, 0.01 EDTA, and 11 glucose. All procedures were approved by the Complutense University Ethical Committee, and they were performed in accordance with European guidelines. Longitudinal strips (~3 mm wide and 5 mm long), or rings (3 mm wide) were obtained from the proximal urethra and used to study the relaxant and contractile responses, respectively.

**Recording of Isometric Tension**

Urethral preparations (strips or rings) were mounted between two stainless steel hooks in 5-ml organ baths containing Krebs solution at 37°C, and they were bubbled with a mixture of 95% O₂-5% CO₂ (pH 7.4). The isometric tension was recorded with Grass FT03C transducers (Grass Instruments, Quincy, MA) and displayed on a MacIntosh computer with a MacLab analog-to-digital converter, v 5.5 (AD Instruments, Hastings, East Sussex, UK). Preparations were equilibrated at a resting tension of 5 mN for 60 min, and their viability was tested by the contractile response elicited by exposure to high external K⁺ (120 mM).

Urethral relaxation of nitrergic origin was achieved through EFS of strip preparations precontracted with AVP (0.1 μM), and in the continued presence of atropine (1 μM) and guanethidine (50 μM) to avoid cholinergic and adrenergic excitatory influences. d-Tubocurarine (10 μM) was also added to block the activation of urethral striated muscle. EFS was achieved with a Grass S-48 stimulator (Grass Instruments) connected to platinum electrodes placed parallel to the preparation and coupled to a Med-Lab stimulus splitter (Med-Lab Instruments, Loveland, CO). To construct relaxant frequency-response curves, square-wave pulses of 0.8 ms at supramaximal voltage (current strength, 200 mA) were delivered at 2-min intervals in trains of 5 s at a frequency ranging from 0.5 to 12 Hz. Subsequently, long-train duration (60 s) single relaxations at a frequency of 2 and 35 Hz were performed at 1-min intervals. After washing, the preparations were pretreated for 30 min with either l-cis-diltiazem (50 μM) or d-diltiazem (1 μM) before the contraction (AVP)-relaxation (EFS) protocol was again followed in the continued presence of these inhibitors. In a separate set of experiments, AVP-precontracted preparations were subjected to repetitive EFS (5 s, 10 Hz, at 1-min intervals) and l-cis-diltiazem (50 μM) was added at the peak of contraction while the repetitive EFS was maintained.

Urethral contractile responses were assessed on ring preparations by either EFS or exposure to a 120 mM K⁺ solution. EFS delivered on basal tone consisted of 10–12 stimuli of 0.8-ms pulses in 5-s trains at a frequency of 35 Hz and at 1-min intervals. This stimulation paradigm was repeated at 10-min intervals, during which the preparations were incubated with increasing concentrations of l-cis-diltiazem (0.01 to 50 μM). Finally, the effect of l-cis-diltiazem (50 μM) on repetitive EFS was studied in a similar way to that described previously, except that the EFS was delivered at a frequency of 35 Hz on basal tone.

Depolarization induced by high external K⁺ (120 mM) was used to assess the possible effect of both diltiazem isomers on voltage-dependent calcium channel activation. After challenging twice with K⁺ (120 mM), preparations were incubated for 30 min with atropine (1 μM) and guanethidine (50 μM), which remained present for the rest of the experiment to avoid the release of excitatory neurotransmitters by the depolarizing stimuli. The preparations were then challenged again with high K⁺, pretreated for 30 min with either l-cis- or d-diltiazem isomers (50 and 1 μM, respectively), and contracted again with 120 mM K⁺. After extensive washing, the reversibility of the diltiazem inhibition was tested by a final exposure to high K⁺. The effect of YC-1 (50 μM), a specific activator of soluble GC, was studied when added at the peak of contraction induced by AVP (0.1 μM) in the absence of or after a 15-min incubation with l-cis-diltiazem (50 μM).

Control preparations that were not exposed to drugs were run in parallel and subjected to the same protocols.

**Immunofluorescence**

Urethral preparations were processed for immunofluorescence studies as described previously (11). In brief, the tissue was fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.0), cryoprotected at 4°C with increasing concentrations of sucrose in PB (10 to 30%), snap-frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for up to 15 days. Cryostat sections (10 μm: CM1850 UV, Leica Microsystems, Barcelona, Spain) of the urethra embedded in Tissue-Tek OCT compound were thawed onto poly-L-lysine-coated slides. From each urethra, consecutive sections were collected on separate slides to obtain 10–15 serial sections from the same animal. The slides were air-dried at room temperature for 12–24 h and then processed directly or stored at −80°C for no more than 30 days.

Urethral sections were washed with PB (3x, 5 min) and incubated for 60 min with 3% normal donkey antiserum (Chemicon International, Temecula, CA) containing 0.3% Triton X-100. The sections were incubated with primary antibodies diluted in 2% normal donkey serum and 0.3% Triton X-100 for 24 h at 4°C in a humidified chamber. The primary antibodies used were raised against CNGA1 (affinity-purified rabbit antiserum, 1:100) and vimentin (a mouse...
monoclonal, clone V9; 1:100), both from Chemicon International, and α-smooth muscle actin (a mouse monoclonal antibody, 1:1,000) from Sigma-Aldrich Chemie. The sections were then incubated with the secondary antibodies for 2 h in the dark in a humidified chamber at room temperature. The secondary antibodies were used appropriately matched to the species in which the primary antibody was raised: donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 594 (both diluted at 1:200; Molecular Probes, Eugene, OR). After washing (3 times, 10 min each) with PB, the sections were washed again and mounted with Prolong Gold antifade reagent (Molecular Probes). In all cases, a number of controls were performed in which the specificity of the immunoreactions was established by omitting the primary antibodies.

The labeled sections were examined under an Axioplan 2 fluorescence microscope (Carl Zeiss Microimaging, Götttingen, Germany) equipped with the appropriate filter sets. They were photographed with a Spot-2 digital camera (Diagnostic Instruments, Sterling Heights, MI), and the images were stored digitally as 12-bit images with MetaMorph 6.1 software (MDS Analytical Technologies, Toronto, ON). Digital images were subsequently transferred to Adobe Photoshop 8.0 (San Jose, CA).

**RT-PCR**

After dissection, samples of female rat urethra and retina were immediately frozen in liquid N₂ and total RNA was extracted with a Qiagen Rneasy Fibrous Tissue minikit. Conventional RT-PCR to amplify transcripts of CNGA1 and CNGB1 subunits was performed on a PerkinElmer thermocycler (Gene Amp PCR System 2400) in a 25-μl reaction volume using the Access RT-PCR system (Promega). The RT-PCR protocol involved: an incubation at 45°C for 45 min, and then at 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 45 s, and 68°C for 45 s; an elongation step was at 68°C for 7 min. Specific primers for CNGA1 amplification were designed based on the published sequence (accession number: NM_053497): forward primer 5'-GTGGTGGATTCCGAGTATGT-3' (positions 1081–1100) and reverse primer 5'-GCTTGAGTTTCTGCTGCATC-3' (1923–1942). An aliquot of 0.5 μl from the first reaction was used as a template for a second round of amplification of 15 cycles, using two previously described nested primers (18); the forward primer was 5'-GCCACCATTGTGGTTCTGGGTAT-3' (positions 1141–1163), and the reverse primer was 5'-TCATACCTCACAGGAGTGGCGC-3' (1895–1918). To amplify CNGB1, we used the primers designed by Bönigk et al. (2): forward primer 5'-TGACGTCACCTCAGGAGG-3' (positions 612–631) and reverse primer 5'-TGAGGCTTTTGGAGGATG-3' (1187–1206). An additional round of amplification was performed over a further 15 cycles with the same primers using 2 μl of the first PCR product. Similar procedures were performed on the retina, except for the second round of amplification, which was omitted. The sizes of the amplified fragments were verified in a 2% agarose gel stained with SYBR gold and visualized in a Bio-Rad Fluor-S Multilmager (Hercules CA).

**Data Analysis**

Urethral relaxation was expressed as the percentage of the tension elicited by AVP immediately before each stimulation, while contractions were expressed as a percentage of that elicited by control 120 mM K⁺. The results are given as means ± SE from n experiments (from n different animals), and one-way ANOVA was used for multiple comparisons followed by the unpaired Student’s t-test. Data were compared using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

**RESULTS**

**Effects of L-cis-Diltiazem and D-Diltiazem on Nitrergic Relaxation**

The exposure of AVP (0.1 μM) precontracted rat urethral preparations to L-cis-diltiazem (50 μM) significantly inhibited the relaxation induced by nitrergic stimulation at all frequencies tested, following both short and long trains of EFS (Fig. 1, A and B). It should be noted that these rat urethral preparations were more sensitive to the effect of L-cis-diltiazem than sheep preparations, which showed a similar inhibition at 300 μM L-cis-diltiazem (28).

The inhibitory effect of L-cis-diltiazem (50 μM) on nitrergic relaxation had a rapid onset (Fig. 2). Repetitive short EFS (5 s, 10 Hz, at 1-min intervals) elicited a fairly constant and rapid urethral relaxation with a mild and progressive decay in the contractile tension (Fig. 2, A and B). The onset of the inhibitory effect of L-cis-diltiazem (50 μM) was rapid, inducing significant inhibition after the fourth EFS (P < 0.05), its effect being completed within the next six EFS (Fig. 2B).

When rat urethral preparations were exposed to D-diltiazem at a concentration routinely used to effectively block voltage-gated Ca²⁺ channels in urethral preparations (1 μM) (8), nitrergic relaxation remained unaffected at all the frequencies and durations of EFS tested (Fig. 3, A and B).

**Effects of L-cis-Diltiazem and D-Diltiazem on Nerve-Mediated Contractile Responses**

L-cis-diltiazem exerted a dose-dependent inhibition of EFS-induced contractions (10–12 stimuli, 5 s, 35 Hz, 1-min intervals, Fig. 4A). Contractile responses were almost abolished on exposure to 50 μM L-cis-diltiazem, while a similar effect was obtained with D-diltiazem at 1 μM (data not shown). The time-dependent effect of L-cis-diltiazem (50 μM) was then tested by its addition after the fourth EFS in a protocol of short repetitive EFS (5 s, 35 Hz, 1-min intervals) on basal tone (Fig. 4B). As can be seen, the effect of L-cis-diltiazem on EFS-induced contractions had a much longer latency than on EFS.

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For a comprehensive understanding, please refer to the full article for detailed methodology and results.
induced relaxations since it required an average of 15 additional EFS to significantly inhibit urethral contractions (P < 0.05, Fig. 4B).

**Effects of L-Cis-Diltiazem and D-Diltiazem on 120 mM K⁺-Induced Contractions**

The effect of both diltiazem isomers on contraction elicited by the depolarization induced by high extracellular K⁺ concentrations was subsequently tested. As can be seen, the contractile response induced by 120 mM K⁺ was mildly decreased by blocking excitatory influences with atropine and guanethidine (Fig. 5, A and B). The remaining contraction can therefore be considered as due to direct smooth muscle depolarization. Preincubation with either L-cis-diltiazem or D-diltiazem (50 and 1 μM, respectively) significantly inhibited this residual high-K⁺ contraction, an effect that was partially reversed by extensive washout of both isomers (Fig. 5, A and B).

Thus L-cis-diltiazem seems to also affect L-type voltage-gated Ca²⁺ channel properties, although it seems to be >50 times weaker than the D-isomer.

**Effects of L-Cis-Diltiazem on YC-1-Induced Relaxations**

Exposure of AVP-precontracted urethral preparations to YC-1, a NO-independent specific activator of soluble GC, elicits a rapid relaxation that is thought to be due to the rise in intracellular cGMP levels subsequent to GC activation (Fig. 6, A and B). Such relaxation was completely abolished by preincubation with L-cis-diltiazem (50 μM), suggesting that it is mediated by the binding of intracellular cGMP to the cyclic binding domain of the putative CNG channel.

**CNGA1 and CNGB1 mRNA Expression in the Rat Urethra**

No CNG channels have been formerly identified in the urethra, although the functional results obtained in this study...

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**Fig. 2. Fast inhibitory effect of L-cis-diltiazem on nitrergic relaxation.** A: representative traces showing the relaxation elicited by repetitive EFS (5 s, 10 Hz, at 1-min interval) in AVP (0.1 μM)-precontracted rat urethral preparations in control conditions (top) and when L-cis-diltiazem (50 μM) was added after the fourth EFS (bottom). B: temporal evolution of rat urethral relaxations induced by repetitive EFS (5 s, 10 Hz, 1-min interval; # indicates the stimulus number) in control preparations (open symbols) and following L-cis-diltiazem addition (50 μM, arrow, filled symbols). Values are means ± SE (n = 6–8 from different animals). *P < 0.05, **P < 0.01, ***P < 0.001: significantly different from controls (1-way ANOVA followed by Student’s t-test for unpaired observations).

**Fig. 4. Effects of L-cis-diltiazem on nerve-mediated contractile responses.** A: representative traces showing the rat urethral contractions elicited by 5 pulses train EFS (5 s, 35 Hz, 1-min interval) on basal tension every 10 min in control conditions (top) and following 10-min incubation with increasing doses of L-cis-diltiazem. B: temporal evolution of rat urethral contractions induced by repetitive single EFS (5 s, 35 Hz, 1-min interval; # indicates the stimulus number) in control preparations (open symbols) and following L-cis-diltiazem addition (50 μM, arrow, filled symbols). Values are means ± SE (n = 6 from different animals). **P < 0.01, ***P < 0.001: significantly different from controls (1-way ANOVA followed by Student’s t-test for unpaired observations).
suggest that the heteromeric retinal-like CNG channel is likely to exist in this tissue. We carried out RT-PCR using specific primers based on the known sequences of the mRNA encoding CNGA1 and CNGB1 channel subunits, and a band of the predicted size was amplified that showed 99% identity with the rodtype CNGA1 sequence when sequenced. Another band was also identified of a similar size to that of the CNGB1 fragment obtained in the retina used as a positive control (Fig. 7). No bands were detected when the reactions were carried out without reverse transcriptase (data not shown), eliminating the possibility of genomic DNA contaminating the preparation. Nested primers were used in a second round of CNGA1 amplification to eliminate nonspecific fragments, and two rounds of amplification with the same primers pair were sufficient to obtain a visible band corresponding to CNGB1.

**Immunofluorescence Detection of CNG1 Channels in the Urethral Wall**

Specific CNG1 channel immunoreactivity (ir) was evident in two cell types in the rat urethra (Fig. 8), and it was not detected in the absence of the primary antibody (data not shown). Weak and diffuse CNG1-ir was present in smooth muscle cells where it colocalized with α-actin (Fig. 8, A–C). These CNG1-ir cells were a subpopulation of the vimentin-ir cells present in the rat urethra (Fig. 8, D–F), confirming that they were ICC. These cells had long and thin cytoplasmic prolongations, usually running parallel to the corresponding smooth muscle fibers. CNG1-ir was not detected in any nervous structure such as nerve fibers, nerve trunks, or ganglia (data not shown).

**DISCUSSION**

In the present study, we further extend our understanding of the role of functional CNG channels in the urethra and in the relaxation response mediated by the NO-cGMP pathway. This activity was largely based on the effect of L-cis-diltiazem, a specific blocker of most cGMP-regulated CNG channels (15). Here, we demonstrate inhibition by L-cis-diltiazem in rat urethral preparations, which was fivefold more potent than that previously observed in sheep (28), being within the concentration range described in intact rod photoreceptors (27). Moreover, we observed a similar effect of L-cis-diltiazem in preliminary experiments on mouse preparations (Triguero D, unpublished observations). Hence this effect might be a general feature in different species.

By contrast, rat urethral noradrenergic contractions elicited by EFS (8) were only slowly and mildly inhibited by the same concentration of L-cis-diltiazem. This effect probably comes from a nonspecific action of L-cis-diltiazem rather than by inhibiting noradrenergic neurotransmission. In fact, L-cis-diltiazem inhibited contractile responses induced by either NE (28)

Fig. 5. Effects of L-cis-diltiazem and cis-diltiazem on 120 mM K+–induced contractions. A: representative traces showing the rat urethral contractions induced by 120 mM K+ addition (triangle, from left to right) in control conditions, in the presence of guanethidine (10 μM) and atropine (1 μM), after treatment with t-cis-diltiazem (50 μM, top tracings) or cis-diltiazem (1 μM, bottom tracings); and after washout of the diltiazem isomers. B: mean values of the 120 mM K+–induced contraction in rat urethral preparations under the different experimental conditions detailed in A. Values are means ± SE (n = 6 from different animals). **P < 0.01, ***P < 0.001: significantly different from controls. †††P < 0.001 compared with guanethidine + atropine. ﬀ ﬀ ﬀ P < 0.01 compared with the presence of diltiazem isomers (1-way ANOVA followed by Student’s t-test for unpaired observations).

Fig. 6. A: representative traces showing the relaxant effect of YC-1 (50 μM) added at the peak (dot) AVP (0.1 μM)-precontracted rat urethral preparations (top tracing) and its inhibition by treatment with L-cis-diltiazem (50 μM, bottom tracing). B: bars graphs showing the effect of L-cis-diltiazem (50 μM, filled bar) on YC-1-induced relaxation (open bar). Values are means ± SE (n = 6 from different animals). **P < 0.001 significantly different from control (Student’s t-test for unpaired observations).

Fig. 7. Amplification of CNGA1 and CNGB1 cyclic nucleotide-gated channel subunits visualized in 2% agarose gels stained with SYBR gold. The mRNA transcripts of appropriate sizes for both subunits were expressed in rat urethral tissues from different animals (U1 and U2; 778 bp for CNGA1 and 595 bp for CNGB1; middle lane, 100-bp ladder) compared with the transcripts in the rat retina (R) used as controls.

Fig. 8. Immunofluorescence micrographs of control sections. A: representative micrographs showing the distribution of CNG1-ir in the urethral wall. B: representative micrographs showing the distribution of CNG1-ir in the urethral wall. C: representative micrographs showing the distribution of CNG1-ir in the urethral wall.
or AVP (present study). In addition, direct smooth muscle depolarization induced by high K+ was similarly inhibited by l-cis-diltiazem, possibly due to its structural similarity with d-diltiazem, the most representative benzothiazepine calcium channel blocker. Indeed, while d-diltiazem is two orders of magnitude more potent than l-diltiazem in inhibiting the urethral contraction induced by both EFS and high K+, it is completely ineffective on EFS-induced relaxations. In addition, the temporal profile of the inhibition elicited by l-cis-diltiazem, being extremely rapid on relaxations, could reflect the action of l-cis-diltiazem on different targets: a rapid blockade of the cyclic nucleotide-binding site of a CNG channel and a slower inhibition of L-type Ca2+ channels. Significantly, inhibition of cGMP-gated currents by low-micromolar concentrations of l-cis-diltiazem applied extracellularly in patch-clamp studies was described to be limited by the time required to change the solution, while d-diltiazem had little effect when applied intracellularly even at millimolar concentrations (27).

Alternatively, l-cis-diltiazem could abrogate the relaxation elicited by YC-1 in rat urethral preparations. YC-1 has been used to increase cGMP by directly and strongly activating soluble GC by NO-independent mechanisms (7). Therefore, the almost complete inhibition of the YC-1-induced relaxation of the rat urethra by l-cis-diltiazem strongly favors our proposal that its main effect on relaxation is mediated by the specific blockade of CNG channels.

All the members of the CNG channel subfamily are heterotetramers of the A and B subunits, with different subtypes, and the varying stoichiometries produce a wide variety of functional channels (15). Our RT-PCR experiments confirmed the expression of mRNA for the CNGA1 and CNGB1 subunits in the rat urethra which have been described to form the functional CNGA1 channel, usually referred as retinal-like CNG. The CNGA1 channel is considered to be specifically activated by cGMP, as it has a preferential Ca2+ conductance and it is specifically blocked by micromolar l-cis-diltiazem when applied intracellularly. However, this inhibition requires the presence of both subunits with a stoichiometry of 3 CNGA1:1 CNGB1 (15). Although not quantified, the apparent expression of transcripts for both subunits mRNAs suggests that the CNGA1 subunit is more strongly expressed. Although this channel is highly expressed in retinal cells, few CNG transcripts were found in a variety of tissues as brain, testis, kidney, and heart (3) as seems to occur in the urethra since two rounds of amplification were needed to visualize both bands. Such weak expression was initially surprising given the prominent inhibitory effect of l-cis-diltiazem on EFS-induced relaxation, suggesting their association to the abundant urethral nitricergic innervation.

CNGA1-ir was localized in a subpopulation of spindle-shaped vimentin-ir cells, the intramuscular urethral ICC, and more weakly in smooth muscle cells. However, CNGA1-ir was absent from nervous structures. This distribution of CNGA1-ir is very similar to that found for cGMP-ir in rat urethral preparations challenged with EFS or NO donors (11). Thus the same cells that produce an increase in cGMP in response to NO also express CNGA1, strongly suggesting that these channels mediate the effect of cGMP. One of the main known effectors of cGMP is cGMP-dependent protein kinase I (PKG I). In the mouse urethra, PKG I expression is restricted to smooth muscle and some non-nitricergic nerves, and it has not been described in ICC-like cells (20). Preferential expression of CNGA1 channels in ICC, while PKG-mediated mechanisms were more relevant in smooth muscle, might explain our results. New tools acting selectively on CNG channels and PKG are needed to define the relative importance of these two functional pathways in the downstream effects of cGMP in the urethra.

We believe that the most relevant result presented here is that CNG channel activation has a key role in nitricergic relaxation and that this control is mainly exerted through ICC. The morphology and distribution of urethral CNGA1-ir ICC resemble that of vimentin/cGMP-ir ICC, which has a close relationship with neuronal NOS- or PGP 9.5-ir nerve structures (11), suggesting a regulatory role for ICC in nitricergic transmission through CNG channels. Indeed, ICC in the urethra have been suggested to mediate neurotransmission (14, 23, 24) and to act
as pacemakers of the spontaneous myogenic tone (22). However, whether both functions are related and mediated by common mechanisms is unclear. It has been shown that ICC electrical activity is increased by neurally released NE through the activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (23) while it is inhibited by NO donors and GC activators, probably by inhibiting the IP\(_3\)-mediated Ca\(^{2+}\) release (14, 24). Although the role of Ca\(^{2+}\) stores in the spontaneous transient depolarization of urethral ICC has been firmly established, some caveats remain (see 25). It has been suggested that capacitative calcium entry could contribute to the Ca\(^{2+}\)-propagating waves (17). However, the dependence of ICC on Ca\(^{2+}\) entry by alternative routes has also been highlighted. One of the proposed pathways involves Ca\(^{2+}\) influx through the NCX working in a reverse mode (4). However, they do not explain the physiological conditions that could change the ionic balance in the vicinity of the NCX to reverse its activity. The presence of CNG channels in ICC could provide another pathway for Ca\(^{2+}\) entry, participating in the generation and maintenance of Ca\(^{2+}\) waves.

It has to be stressed that our results indicate that CNG channel activation is involved in cGMP-mediated relaxation, which presupposes a decrease rather an increase in intracellular Ca\(^{2+}\) concentrations. Thus Ca\(^{2+}\) entry through CNG should not affect contractile filaments. In the gastrointestinal tract, it has been suggested that the ICC communicate with smooth muscle cells by electrical coupling through gap junctions (6). In addition, diffusible mediators released by ICC act on smooth muscle, and synapse-like junctions have been observed between ICC and smooth muscle cells (13). In previous studies, we suggested that the urethral nitrergic transmitter is not NO but a more stable nitrocompound, based on the fact that it was not sensitive to superoxide generators and to an extracellular NO scavenger (9, 10). An alternative explanation is that NO was released at tightly associated one-to-one communication sites between nerve terminals and the ICC, where it is not readily accessible to exogenous NO destructors, while another chemical mediator is released by the ICC to finally induce the smooth muscle relaxation. The cGMP-mediated entry of Ca\(^{2+}\) through CNG channels strategically located near to the release sites of ICC may be the stimulus needed to couple NO-cGMP responses to secretion. Indeed, control of exocytosis by CNG channels has been demonstrated previously at the cone synapses (21).

Conclusions

In conclusion, we have described the expression of CNG channels of the retinal-like type (CNGA1) in the rat urethra located in smooth muscle cells and, more remarkably, in a subpopulation of ICC. These channels seem to mediate the urethral relaxation elicited by functional activation of the NO-cGMP pathway, and they could be viewed as a suitable link between NOS activity in nitrergic nerves and the balance of ions such as Ca\(^{2+}\) in effector cells. The possible involvement of CNG channels in either the pacemaker function of ICC or in their function as mediators of neurotransmission should be considered.

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