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Involvement of cyclic nucleotide-gated channels in spontaneous activity generated in isolated interstitial cells of Cajal from the rabbit urethra

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ABSTRACT

Cyclic nucleotide-gated (CNG) channels are non-selective cation channels that mediate influx of extracellular Na^+ and Ca^{2+} in various cell types. L-cis-Diltiazem, a CNG channel blocker, inhibits contraction of urethral smooth muscle (USM), however the mechanisms underlying this effect are still unclear. We investigated the possibility that CNG channels contribute to spontaneous pacemaker activity in freshly isolated interstitial cells of Cajal (ICC) isolated from the rabbit urethra (RUICC). Using immunocytochemistry, we found intense CNG1-immunoreactivity in vimentin-immunoreactive RUICC, mainly within patches of the cellular body and processes. In contrast, α-actin immunoreactive smooth muscle cells (SMC) did not show significant reactivity to a specific CNGA1 antibody. Freshly isolated RUICC, voltage clamped at −60 mV, developed spontaneous transient inward currents (STICs) that were inhibited by L-cis-Diltiazem (50 μM). Similarly, L-cis-Diltiazem (50 μM) also inhibited Ca^{2+} waves in isolated RUICC, recorded using a Nipkow spinning disk confocal microscope. L-cis-Diltiazem (50 μM) did not affect caffeine (10 mM)-induced Ca^{2+} transients, but significantly reduced phenylephrine–evoked Ca^{2+} oscillations and inward currents in in RUICC. L-type Ca^{2+} current amplitude in isolated SMC was reduced by ~18% in the presence of L-cis-Diltiazem (50 μM), however D-cis-Diltiazem, a recognised L-type Ca^{2+} channel blocker, abolished L-type Ca^{2+} current but did not affect Ca^{2+} waves or STICs in RUICC. These results indicate that the effects of L-cis-diltiazem on rabbit USM could be mediated by inhibition of CNG1 channels that are present in urethral ICC and therefore CNG channels contribute to spontaneous activity in these cells.

1. Introduction

Interstitial cells of Cajal (ICC) are pacemaker cells in the gastrointestinal tract that generate and conduct electrical slow waves which underlies rhythmicity in these tissues (Sanders et al., 2014). Similar cells have been reported in the smooth muscle layer of the urethra, where they are believed to act as specialised pacemaker cells that underlie spontaneous tone (Sergeant et al., 2000). Pacemaker activity in urethral ICC is characterised by the occurrence of spontaneous transient inward currents (STICs) under voltage clamp and spontaneous transient depolarisations (STDs), under current clamp (Sergeant et al., 2000, 2001). This activity results from underlying Ca^{2+} waves that are initiated by Ca^{2+} release from ryanodine receptors on the endoplasmic reticulum and are converted to propagating Ca^{2+} waves by activation of neighbouring inositol 1,4,5-triphosphate receptors (IP_3Rs, Sergeant et al., 2001, Johnson et al., 2005 & Drumm et al., 2015). These Ca^{2+} waves are also reliant on influx of Ca^{2+} across the plasma membrane via reverse mode NCX (Bradley et al., 2006; Drumm et al., 2015).

Cyclic nucleotide-gated (CNG) channels are directly gated by the binding of intracellular cAMP and/or cGMP to a cytoplasmic cyclic nucleotide-binding domain, but they are only weakly activated by changes in membrane potential (Craven and Zagotta, 2006). They are permeable to Na^+ and Ca^{2+} and CNG channel activation can induce membrane depolarization or local changes in cytosolic Ca^{2+} levels, providing an alternative pathway for Ca^{2+} entry that couples the activity of Ca^{2+}-regulated proteins to cAMP/cGMP signalling without involving protein kinases. All members of the CNG channel subfamily are hetero-tetramers of A and B subunits, which have different subtypes, and the varying stoichiometry of CNGA and CNGB subunits produces a wide variety of functional channels.

CNGA1 and CNGB1 subunits, which form functional CNGA1, or rod retinal-like CNG channels, are strongly expressed in a subpopulation of

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vimentin-positive ICC of the rat urethra, as demonstrated by intense CNG1A-ir (Triguero et al., 2009). In contrast, only weak and diffuse CNG1A-ir was evident in rat urethral smooth muscle cells (SMC, Triguero et al., 2009). In addition, L-cis-Diltiazem, a selective inhibitor of CNG channels, relaxed pre-contracted strips of rat urethra smooth muscle and inhibited EFS-induced contractions, however the mechanisms underlying these effects have yet to be established. One possibility, given that activation of CNG channels would facilitate Ca^{2+} and Na^{+} entry, is that CNG channels are involved in the generation of spontaneous activity in urethral ICC. We tested this possibility in the present study using the patch clamp technique and confocal Ca^{2+} imaging to examine the effect of L-cis-Diltiazem on spontaneous transient inward currents (STICs) and spontaneous Ca^{2+} waves in freshly isolated rabbit urethral ICC. Immunofluorescence experiments were also performed to compare the expression of CNG1 channels in freshly isolated SMC and ICC from the proximal rabbit urethra.

2. Materials and methods

Experiments in this study were approved by the Dundalk Institute of Technology Animal Use and Care Committee or the Ethical Committee at the Complutense University and performed in accordance with European guidelines (EU Directive 2010/63/EU).

2.1. Cell isolation

Male and female New Zealand white rabbits (16–20 weeks old) were killed by lethal injection of pentobarbitone (i.v.). The most proximal urethra (3 cm) was removed and placed in Krebs' solution. This was then opened up longitudinally and the urothelium removed by sharp dissection. Strips of tissue, 0.5 cm in width were cut into 1 mm^3 pieces and stored in Ca^{2+}-free Hanks' solution for 30 min at 4 °C prior to cell dispersal. Dispersal medium consisted of Ca^{2+}-free Hanks' solution (see Solutions) containing (per 5 ml): 15 mg collagenase (type 1 A), 1 mg protease (type XXIV), 10 mg bovine serum albumin and 10 mg trypsin inhibitor (all from Sigma-Aldrich, Steinheim, Germany & Dublin, Ireland). Tissue pieces were incubated for 10–15 min at 37 °C, then transferred to Ca^{2+}-free Hanks' solution and stirred for 15–30 min to release single SMC and ICC. Dispersed cells were plated on glass coverslips for immunocytochemistry or in Petri dishes containing 100 μM Ca^{2+} Hanks' solution for Ca^{2+} imaging experiments. These were stored at 4 °C for use within 8 h.

2.2. Immunofluorescence of single SMC and ICC

Cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20 min, washed in PBS (3 × 5 min) at 4 °C and then incubated in normal donkey antiserum (10% in PBS + 0.05% Triton X-100) for 1 h at room temperature. They were incubated overnight at 4 °C in primary antibody: anti-CNG1 (affinity-purified rabbit antiserum; Chemicon International, Temecula, CA, USA), anti-vimentin (mouse monoclonal, clone V9; Chemicon International, Temecula, CA, USA) or anti-α-smooth muscle actin (mouse monoclonal antibody; Sigma-Aldrich, Steinheim, Germany). The antibodies were diluted 1:100 (anti-CNG and anti-vimentin) or 1:800 (anti-α-actin) in PBS containing 0.1% Triton X-100% and 5% donkey serum. Cells were then incubated with the secondary antibodies for 1 h at room temperature. Secondary antibodies used were 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 in PBS; Molecular Probes, Eugene OR). Controls were prepared by omitting the primary antibody from the first incubation solution. Specimens were examined under an Axioplan 2-fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany) equipped with the appropriate filter sets. They were photographed with a Spot-2 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA), and the images were stored digitally as 12-bit images using MetaMorph 6.1 software (MDS Analytical Technologies, Toronto, ON). Some cells were examined by confocal laser-scanning microscopy using a spectral confocal microscope (TCS-SP2, Leica Microsystems, Barcelona, Spain), and three-dimensional shadow projections were constructed from the resulting stacks using the Leica Confocal software (LCS, Leica Microsystems, Barcelona, Spain). Digital images were subsequently transferred to Adobe Photoshop 8.0.1 (San Jose, CA, USA).

2.3. Whole cell patch clamp recordings

Currents were recorded from freshly isolated ICC and SMC using the perforated patch configuration of the whole cell patch clamp technique (Rae et al., 1991). This circumvented the problem of current rundown encountered using the conventional whole cell configuration. The cell membrane was perforated using the antibiotic amphotericin B (600 μg/ml, Sigma). Patch pipettes were initially front filled by dipping into pipette solution and then back filled with the amphotericin B containing solution. Pipettes were pulled from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.17 mm inner diameter; Clark Medical Instruments) to a tip of diameter approximately 1–1.5 μm and resistance of 2–4 MΩ. Voltage clamp commands were delivered via an Axopatch 1D patch clamp amplifier (Axon Instruments) connected to a Digidata 1440 A AD/DA converter (Axon Instruments) interfaced to a computer running pClamp software (Axon Instruments). During experiments, the cell under study was continuously superfused with Hanks' solution by means of a close delivery system consisting of a pipette (tip diameter 200 μm) placed approximately 300 μm away. This could be switched, with a dead-space time of around five seconds, to a solution containing a drug. All experiments were carried out at 35–37 °C.

2.4. Calcium imaging of single ICC

Cells were allowed to settle in glass-bottomed Petri dishes until they had stuck down. They were then incubated in 0.5 μM fluo-4A.M. (Molecular Probes, Eugene OR) in Hanks' solution for 6 min at room temperature. The dish was constantly perfused with Hanks' solution to maintain a temperature between 35–37 °C. Additionally, the cell under study was continuously superfused with Hanks' solution by means of a close delivery system consisting of a pipette (tip diameter 200 μm) placed approximately 300 μm away. This could be switched, with a dead space of time of around 5 s, to a solution containing a drug. Cells were imaged using an iXon 887 EMCCD camera Andor Technology, Belfast, UK; 512 × 512 pixels, pixel size 16 × 16 μm (Coates et al., 2003) coupled to a Nipkow spinning disk confocal head (CSU22, Yokogawa, Japan). A krypton-argon laser (Melles Griot, UK) at 488 nm was used to excite the fluo-4, and the emitted light was detected at wavelengths >510 nm. Experiments were performed using a ×60 objective (Olympus) resulting in images of pixel size 0.266 × 0.266 μm and images were usually acquired at 5 frames/sec. Analysis of recordings was performed using iQ software (Andor, Belfast, UK). Movie files recorded using iQ were converted into a stack of TIFF (tagged image file format) images and imported into Image J (version 1.40, Bethesda, Maryland, USA) for *post hoc* analysis. Prior to analysis, background fluorescence was subtracted from the stack. A single pixel line was drawn along the mid-axis of the cell and a pseudo-line-scan image was produced using the "reslice" function in Image J, with distance along the cell (μm) on the vertical axis and time (sec) on the horizontal axis. Basal fluorescence was obtained from areas of the cell displaying the most uniform and least intense fluorescence (F_0). To analyze Ca^{2+} waves, a plot profile was generated by drawing a rectangle over an entire line scan and plotting the fluorescence intensity profile in Image J. The amplitude of waves was obtained by calculating the difference between basal and peak fluorescence, expressed as $\Delta F/F_0$. Waves were defined as events which were >25% of the maximum amplitude event