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Contribution of reverse Na^+–Ca^{2+} exchange to spontaneous activity in interstitial cells of Cajal in the rabbit urethra

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Interstitial cells of Cajal (ICC) isolated from the rabbit urethra exhibit regular Ca^{2+} oscillations that are associated with spontaneous transient inward currents (STICs) recorded under voltage clamp. Their frequency is known to be very sensitive to external Ca^{2+} concentration but the mechanism of this has yet to be elucidated. In the present study experiments were performed to assess the role of Na^+–Ca^{2+} exchange (NCX) in this process. Membrane currents were recorded using the patch clamp technique and measurements of intracellular Ca^{2+} were made using fast confocal microscopy. When reverse mode NCX was enhanced by decreasing the external Na^+ concentration $[Na^+]_o$ from 130 to 13 mM, the frequency of global Ca^{2+} oscillations and STICs increased. Conversely, inhibition of reverse mode NCX by KB-R7943 and SEA0400 decreased the frequency of Ca^{2+} oscillations and STICs. Application of caffeine (10 mM) and noradrenaline (10 μM) induced transient Ca^{2+}-activated chloride currents (I_{ClCa}) at −60 mV due to release of Ca^{2+} from ryanodine- and inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores, respectively, but these responses were not blocked by KB-R7943 or SEA0400 suggesting that neither drug blocked Ca^{2+}-activated chloride channels or Ca^{2+} release from stores. Intact strips of rabbit urethra smooth muscle develop spontaneous myogenic tone. This tone was relaxed by application of SEA0400 in a concentration-dependent fashion. Finally, single cell RT-PCR experiments revealed that isolated ICC from the rabbit urethra only express the type 3 isoform of the Na^+–Ca^{2+} exchanger (NCX3). These results suggest that frequency of spontaneous activity in urethral ICC can be modulated by Ca^{2+} entry via reverse NCX.

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Spontaneous myogenic tone generated by the smooth muscle wall of the urethra is associated with the occurrence of spontaneous electrical slow waves (Hashitani *et al.* 1996; Hashitani & Edwards, 1999). These events are similar to those which have been recorded in the gastro-intestinal (GI) tract where they originate in specialized pacemaker cells known as interstitial cells of Cajal (ICC; Sanders, 1996). The electrical activity in the urethra is also now believed to result from the rhythmic firing of spontaneous transient depolarizations (STDs) from ICC, previously referred to as interstitial cells (IC) by Sergeant *et al.* (2000). STDs in urethra ICC arise due to periodic activation of I_{ClCa} by release of Ca^{2+} from intracellular stores (Sergeant *et al.* 2001). This process is thought to be sustained by Ca^{2+} influx, as Johnston *et al.* (2005) reported that Ca^{2+} oscillations cease immediately upon removal of Ca^{2+} from the bathing media. In addition, when the Ca^{2+} concentration was raised the frequency of the oscillations increased (Johnston *et al.* 2005). The identity of the Ca^{2+} entry pathways involved remain unknown, though the failure of nifedipine (10 μM) to affect STICs at −60 mV or Ca^{2+} oscillations (Sergeant *et al.* 2001; Johnston *et al.* 2005) suggests that L-type Ca^{2+} channels are not involved. Bradley *et al.* (2005) investigated the hypothesis that Ca^{2+} oscillations in urethra ICC were driven by capacitative Ca^{2+} entry (CCE). However, the results of this study showed that although these cells possess a CCE pathway, inhibitors which blocked it had no effect on pacemaker activity.

Putney (2001) noted that in cells which do not manifest CCE, replenishment of Ca^{2+} stores is accomplished by plasma membrane NCX. Although the Na^+–Ca^{2+}

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exchanger is typically thought of as a Ca^{2+} extrusion mechanism, it is in fact a bidirectional ion transport protein which can mediate Ca^{2+} entry depending on the net electrochemical driving force (Blaustein & Lederer, 1999). Ca^{2+} influx via reverse NCX is now known to be involved in numerous processes such as proliferation and contraction of smooth muscle cells (Zhang *et al.* 2005; Saleh *et al.* 2005), excitation–contraction coupling in the heart (Leblanc & Hume, 1990) and neurotransmitter release from presynaptic nerve terminals (Luther *et al.* 1992). The aim of the present study was to investigate if Ca^{2+} influx via reverse NCX contributes to pacemaker activity in urethral ICC.

Methods

Cell dispersal

All experiments conducted in this study were approved by the Dundalk Institute of Technology Animal Use and Care Committee. The urethra was removed from male New Zealand white rabbits immediately after they had been killed by lethal injection of pentobarbitone. The most proximal 1.5 cm of the urethra was removed and placed in Krebs solution. Strips of tissue, 0.5 cm in width were dissected, cut into 1 mm^3 pieces and stored in Ca^{2+}-free Hanks' solution for 30 min prior to cell dispersal. Tissue pieces were incubated in dispersal medium containing (per 5 ml) of Ca^{2+}-free Hanks' solution (see Solutions): 15 mg collagenase (Sigma type 1A), 1 mg protease (Sigma type XXIV), 10 mg bovine serum albumin (Sigma) and 10 mg trypsin inhibitor (Sigma) for 10–15 min at 37°C. Tissue was then transferred to Ca^{2+}-free Hanks' solution and stirred for a further 15–30 min to release single smooth muscle cells and ICC. These were plated in Petri dishes containing 100 μM Ca^{2+} Hanks' solution and stored at 4°C for use within 8 h. During experiments, the dish containing the cells was continuously perfused with Hanks' solution at 36 ± 1°C. Additionally the cell under study was continuously superfused by means of a custom built close delivery system with a pipette of tip diameter 200 μm placed approximately 300 μm from the cell. The Hanks' solution in the close delivery system could be switched to a drug-containing solution with a dead space time of less than 5 s.

Perforated patch recordings from single cells

Currents were recorded using the perforated patch configuration of the whole cell patch clamp technique (Rae *et al.* 1991). The cell membrane was perforated using the antibiotic amphotericin B (600 $\mu g\,ml^{-1}$). 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) and membrane currents were recorded by a 12 bit AD/DA converter (Axodata 1200 or Labmaster, Scientific Solutions) interfaced to an Intel computer running pCLAMP software.

Calcium imaging

Cells were incubated in 2 μM fluo-4 AM (Molecular Probes) in Hanks' solution for 30 min at 37°C. They were then washed in Hanks' solution and allowed to settle in glass-bottomed Petri dishes until they had stuck down. Cells were imaged using either an iXon 887 EMCCD camera (Andor Technology, Belfast; 512 × 512 pixels, pixel size 16 μm × 16 μm) coupled to a Nipkow spinning disk confocal head (CSU22, Yokogawa, Japan) or a MegaXR10 GenIII + ICCD (Stanford Photonics, USA; 1024 × 1000 pixels, pixel size 7 μm × 7 μm) attached to a Nipkow spinning disk confocal head (CSU10, Visitech, UK). 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. Images were usually acquired at 5 or 15 frames s^{-1}. Plots of F/F_0 were obtained by measuring the mean fluorescence intensity of the entire cell over the course of the experiment. Background fluorescence from the camera, obtained using a null frame, was subtracted from these values to obtain 'F'. F_0 was determined as the minimum fluorescence measured between oscillations under control conditions. Plots of $\Delta F/F_0$ were used for measurements of Ca^{2+} transient amplitude.

Mechanical recordings

Circular orientated smooth muscle strips (8 mm × 1 mm × 1 mm) were suspended in water-jacketed organ baths (volume 5 ml) maintained at 37°C. Strips were perfused with Krebs solution and allowed to equilibrate for 1 h. Contractions were measured using Statham UC3 and Dynamometer UF1 transducers, the output of which were recorded on a Grass (model 7400) chart recorder.

Total RNA isolation and RT-PCR

Isolated cells and smooth muscle strips were prepared as above. Elongated spindle-shaped smooth muscle cells (SMC) were collected by applying negative pressure to a micropipette made from boroscillate glass of tip diameter ∼100 μm. Approximately 50 smooth muscle cells were obtained. The same process was employed for branched, non-contractile ICC. In this case 10–20 ICC were collected. Collected cells were expunged into a 0.5 ml

microcentrifuge tube containing cell lysis buffer prior to RNA isolation.

Total RNA was prepared from brain and urethra tissue using the TRIZOL method (Invitrogen) as per manufacturer's instructions and treated with DNAse (Stratagene). Total RNA was prepared from isolated SMC and ICC with the use of a RNeasy Mini Kit (Qiagen). First strand cDNA was prepared from the RNA preparations using the Superscript II RNase H reverse transcriptase (Invitrogen); 200 μg ml^{-1} of random hexamer was used to reverse transcribe the RNA sample. The cDNA reverse transcription product was amplified with specific primers by PCR. PCR was preformed in a 25 μl reaction containing 12.5 μl Amplitaq Gold Mastermix (Applied Biosystems), 8.5 μl of water, 1 μl of sense and antisense primers (at a concentration of 10 μM) and 2 μl of template cDNA. All reactions were preformed in a Techne TC-512 gradient thermal cycler. The amplification profile for all primer pairs were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, with a final extension step at 72°C for 7 min. cDNA from cells were subjected to a second round of amplification. The amplified products were separated by electrophoresis on a 2% agarose–1 × TAE (Tris, acetic acid, EDTA) gel and the DNA bands were subsequently visualized by ethidium bromide staining and documented on an INGENIUS gel documentation system (Syngene Bio Imaging).

Primer design

The following PCR primers were used. In each case the number in parentheses represents the GenBank accession number: NCX1 (U52665): sense nucleotide (nt) 876–893, GCCATCTTCCAAGACTGA and antisense nt 1176–1192, TAGCTTGGTCAGCTGCA. Amplicon, 317 base pairs (bp). NCX2 primers were designed against the published rat sequence (U08141): sense nt 1932–51, GATAGCAGAGATGGGCAAGC and antisense nt 2114–2133, ATCCTCATCCTCCTCCTCGT. Amplicon, 202 bp. NCX3 primers were previously published by Lu *et al.* (2002). (U53420) sense nt 988–1007, CAGACTGCAAGGAGGGTGTC and antisense nt 1307–1326, AATCACCAGCAATGAACCCG. Amplicon, 339 bp.

Solutions

The compositions of the solutions used were as follows (mM): Krebs solution: NaCl (120), KCl (5.9), $NaHCO_3$ (1.2), glucose (5.5) CaCl (12.5), $MgCl_2$ (6); pH maintained at 7.4 by bubbling with 95% O_2–5% CO_2. Ca^{2+}-free Hanks' solution: NaCl (125), KCl (5.36), glucose (10), sucrose (2.9), $NaHCO_3$ (15.5), KH_2PO_4 (0.44), Na_2HPO_4 (0.33), Hepes (10); pH adjusted to 7.4 with NaOH. Hanks' solution: NaCl (125), KCl (5.36), glucose (10), sucrose (2.9), $NaHCO_3$ (4.17), KH_2PO_4 (0.44), Na_2HPO_4 (0.33), $MgSO_4$ (0.4), $MgCl_2$ (0.5), $CaCl_2$ (1.8) and Hepes (10); pH adjusted to 7.4 with NaOH. In experiments using low Na^+ Hanks' solution, NaCl was reduced to 13 mM from 130 mM by replacement with equimolar *N*-methyl D-glucamine (NMDG). Experiments using 0 mM extracellular K^+ were achieved by equimolar substitution with NaCl and omission of KH_2PO_4 from the solution. All patch clamp experiments were performed using Cs^+ perforated patch solution of composition: CsCl (133), $MgCl_2$ (1.0), EGTA (0.5), Hepes (10); pH adjusted to 7.2 with CsOH.

Drugs

The following drugs were used: Amphotericin B (Sigma), atropine (BDH laboratories), caffeine (Sigma), guanethidine (Sigma), KB-R7943 (Tocris), noradrenaline (Levophed; Sanofi Winthrop, UK). SEA (2-[4-[(2,5difluorophenyl)methoxy]phenoxy]-5-ethoxy-aniline; SEA0400) was synthesized in Taisho Pharmaceutical (Saitama, Japan). All drugs were made up in the appropriate stock solution before being diluted to their final concentrations in Hanks' solution.

Statistics

In all experiments '*n*' refers to the number of cells studied and each experimental set usually contained samples from a minimum of four animals. Summary data are presented as the mean ± S.E.M. and statistical comparisons were made on raw data using Student's paired *t* test, taking $P < 0.05$ level as significant.

Results

On the basis of a stoichiometry of 3 Na^+ : 1 Ca^{2+} it can be shown that the reversal potential for NCX (E_{NCX}) is described by the equation: $E_{NCX} = 3E_{Na} - 2E_{Ca}$ (Blaustein & Lederer, 1999). In order to calculate E_{NCX} in the present study it was necessary to make an estimate of $[Na]_i$ as it has not yet been measured in urethra ICC. $[Na]_i$ values of 13 mM have been reported in other smooth muscles (Moore & Fay, 1993). If a similar value is true for ICC, E_{NCX} would be ~ -72 mV, assuming $[Ca]_i$ was 100 nM. This means that at potentials positive to -72 mV Ca^{2+} influx via reverse NCX would be expected to occur, given normal extracellular Na^+ $[Na^+]_o$ levels. Figure 1 shows the results of experiments designed to assess the contribution of reverse NCX to Ca^{2+} oscillations in urethra ICC. $[Na^+]_o$ was reduced from 130 mM to 13 mM by replacement with equimolar NMDG, thereby increasing the driving force for Ca^{2+} influx via reverse NCX. ICC produced regularly occurring Ca^{2+} oscillations under control conditions which doubled in frequency when $[Na^+]_o$ was reduced to 13 mM (Fig. 1*A*). Figure 1*A* also shows that reduction of $[Na^+]_o$ to 13 mM elevated basal

Ca^{2+} levels. Summary data for these effects are plotted in Fig. 1*B*, *C* and *D*, respectively. In six cells the mean Ca^{2+} oscillation frequency doubled from 2.6 ± 0.42 min^{-1} under control conditions to 5.2 ± 0.9 min^{-1} in 13 mM $[Na^+]_o$ ($P < 0.05$). The mean amplitude of these events was slightly decreased from 1.4 ± 0.29 to 1.12 ± 0.26 ($\Delta F/F_0$, $P < 0.05$). Basal Ca^{2+} was significantly increased from 0.99 ± 0.01 to 1.23 ± 0.09 F/F_0, ($P < 0.05$). It should also be noted that in some cells (4 out of 6) the amplitude of the Ca^{2+} signals did not return to control levels following exposure to low $[Na^+]_o$ solution.

Effect of reverse NCX inhibitors on Ca^{2+} oscillations in ICC

In this series of experiments we investigated the effect of inhibiting reverse NCX with the selective NCX inhibitors KB-R7943 and SEA0400 on Ca^{2+} oscillations in ICC. KB-R7943 is a novel isothiourea derivative reported to selectively inhibit reverse mode NCX in ventricular cells and fibroblasts transfected with NCX1 (Iwamoto *et al.* 1996; Watano *et al.* 1996). SEA0400 is an inhibitor of NCX (Matsuda *et al.* 2001) that is more effective against reverse mode exchange (Lee *et al.* 2004). Figure 2*A* shows that spontaneous Ca^{2+} oscillations in ICC were

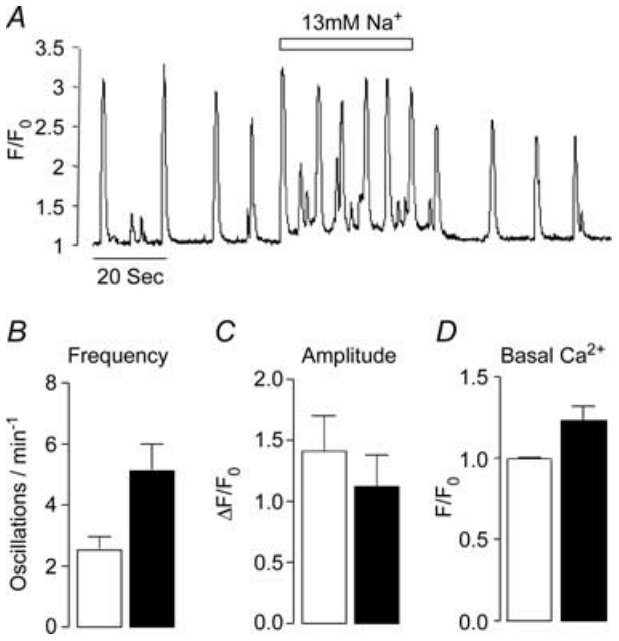

Figure 1. Effect of low $[Na^+]_o$ on spontaneous Ca^{2+} oscillations in ICC
A is a representative recording from an isolated ICC showing that reduction of $[Na^+]_o$ from 130 to 13 mM doubles the frequency of spontaneous Ca^{2+} oscillations in ICC. *B–D* are summary bar charts showing mean frequency and amplitude of Ca^{2+} oscillations as well as the basal Ca^{2+} levels in 130 mM $[Na^+]_o$ (open bars) and 13 mM $[Na^+]_o$ (filled bars). Error bars refer to standard errors of the mean in each example.

abolished by application of 5 μM KB-R7943 and basal Ca^{2+} was also substantially reduced. Summary data from nine cells show that the mean frequency of Ca^{2+} oscillations was significantly reduced from 5.6 ± 0.9 to 0.13 ± 0.13 min^{-1} in the presence of KB-R7943 ($P < 0.05$, Fig. 2*B*). Likewise, mean amplitude was also significantly reduced from 1.5 ± 0.27 to 0.33 ± 0.33 $\Delta F/F_0$ ($P < 0.05$, Fig. 2*C*) and basal Ca^{2+} was decreased from 1 ± 0.01 F/F_0 under control conditions to 0.8 ± 0.08 F/F_0 in the presence of the drug ($P < 0.05$, Fig. 2*D*). SEA0400 (1 μM) produced similar effects to KB-R7943. Figure 2*E* is a representative example which shows that the frequency of Ca^{2+} oscillations and basal Ca^{2+} levels were reduced by SEA0400. Summary data plotted in Fig. 2*F*, *G* and *H* show that the mean frequency of Ca^{2+} oscillations was significantly reduced from 5.8 ± 1.5 to 1.19 ± 0.45 min^{-1} (Fig. 2*F*, $P < 0.05$, $n = 10$) and amplitude was decreased from 1.18 ± 0.2 to 0.64 ± 0.24 $\Delta F/F_0$ ($P < 0.05$, Fig. 2*G*). Basal Ca^{2+} was reduced from 1 ± 0.008 to 0.85 ± 0.06 F/F_0 (Fig. 2*H*, $P < 0.05$).

We next tested the effect of SEA0400 on Ca^{2+} oscillations induced by low $[Na^+]_o$ in order to ensure that this activity was similar in nature to that recorded under control conditions. Figure 3*A* is a representative example showing that low $[Na^+]_o$ increases the frequency of spontaneous Ca^{2+} oscillations as shown in Fig. 1. Figure 3*B* shows that in the presence of SEA0400 reapplication of solution containing 13 mM $[Na^+]_o$ to the same cell no longer causes an increase in the frequency of Ca^{2+} oscillations. These data are summarized in Fig. 3*C* and show that in the absence of SEA0400 the mean frequency of Ca^{2+} oscillations induced by low $[Na^+]_o$ was 10.5 ± 4.3 min^{-1} *versus* 3.3 ± 1 min^{-1} in the presence of SEA0400 ($P < 0.05$, $n = 6$).

Effect of reduced $[Na^+]_o$ on spontaneous transient inward currents in ICC

Figure 4 shows the effect of low $[Na^+]_o$ on STICs recorded from urethral ICC voltage clamped at −60 mV. Reduction of $[Na^+]_o$ caused an increase in both the frequency and amplitude of STICs as shown in the example in Fig. 4*A*. Data from nine similar experiments are plotted in Fig. 4*B* and show that mean amplitude and frequency of STICS increased from -195.1 ± 70.9 pA and 4.8 ± 1.6 STICs min^{-1} under control conditions to -412.9 ± 130.2 pA and 8.5 ± 3.1 STICs min^{-1}, respectively, when $[Na^+]_o$ was reduced to 13 mM ($P < 0.05$, $n = 9$). These effects were also accompanied by a significant decrease in holding current by 49.2 ± 4.9 pA ($n = 9$, $P < 0.05$).

Effect of 0 mM $[K^+]_o$ on spontaneous Ca^{2+} oscillations in ICC

An alternative method for enhancing reverse mode NCX is to increase $[Na^+]_i$ by inhibiting the Na^+–K^+ pump.

Figure 5 shows that inhibition of the Na^+–K^+ pump by removal of external K^+ from the bath solution increased the frequency of spontaneous Ca^{2+} waves. This effect was observed in 9 out of 14 cells. The mean frequency of Ca^{2+} waves increased from $5.86 \pm 0.9\ min^{-1}$ under control conditions to $7.15 \pm 0.7\ min^{-1}$ in 0 mM $[K^+]_o$. This effect was statistically significant ($P < 0.05$, $n = 14$).

Effect of reverse NCX inhibitors on spontaneous transient inward currents in ICC

Experiments were then performed to compare the effects of KB-R7943 and SEA0400 on STICs recorded at −60 mV (positive to the predicted E_{NCX} favouring reverse mode NCX) and −100 mV (negative to the predicted E_{NCX} favouring forward mode NCX). KB-R7943 greatly attenuated STICs at −60 mV (Fig. 6*A*). Summary data for these effects are shown in Fig. 6*B*. In 14 cells application of KB-R7943 significantly reduced mean STIC amplitude from -336 ± 57 pA to -20 ± 22 pA ($P < 0.05$) and frequency from $11 \pm 2.6\ min^{-1}$ to $1.6 \pm 0.8\ min^{-1}$ ($P < 0.05$). However, at −100 mV STICs were unaffected by KB-R7943 (Fig. 6*C*). Summary data plotted in Fig. 6*D* show that mean STIC amplitude was -484 ± 146 pA in the absence of drugs and reduced to -370 ± 96 pA in the presence of KB-R7943. This effect was not statistically significant ($P > 0.05$, $n = 9$). The frequency of STICs at −100 mV was also not significantly affected by KB-R7943. For example, under control conditions mean STIC frequency was $6.3 \pm 0.7\ min^{-1}$ *versus* $6.9 \pm 1.1\ min^{-1}$ in KB-R7943 ($P > 0.05$). Figure 6*E* and *G* are representative recordings showing the effect of SEA0400 on STICs recorded at −60 and −100 mV, respectively. At −60 mV the mean amplitude of STICs was reduced from -193 ± 24 pA to -56 ± 19 pA by application of SEA0400 ($P < 0.05$, $n = 12$, Fig. 6*F*). SEA0400 also reduced the mean frequency of STICs at −60 mV from $8 \pm 1.2\ min^{-1}$ to $1.3 \pm 0.4\ min^{-1}$ ($P < 0.05$). In contrast, at −100 mV STICs were not significantly affected by SEA0400 (Fig. 6*H*). Under control conditions mean STIC amplitude and frequency were -348 ± 95 pA and $13 \pm 3\ min^{-1}$, respectively, compared with -368 ± 88 pA and $10 \pm 2.8\ min^{-1}$ in the presence of SEA0400 ($P > 0.05$, $n = 6$).

Effect of reverse NCX inhibitors on caffeine and noradrenaline evoked I_{ClCa} in IC

These experiments were designed to test if KB-R7943 or SEA0400 blocked Ca^{2+}-activated Cl^- channels or Ca^{2+} release from IP_3- or ryanodine-sensitive Ca^{2+} stores. Application of caffeine (10 mM) or noradrenaline (10 μM) for 10 s to ICC voltage clamped at −60 mV resulted in activation of transient I_{ClCa} due to release

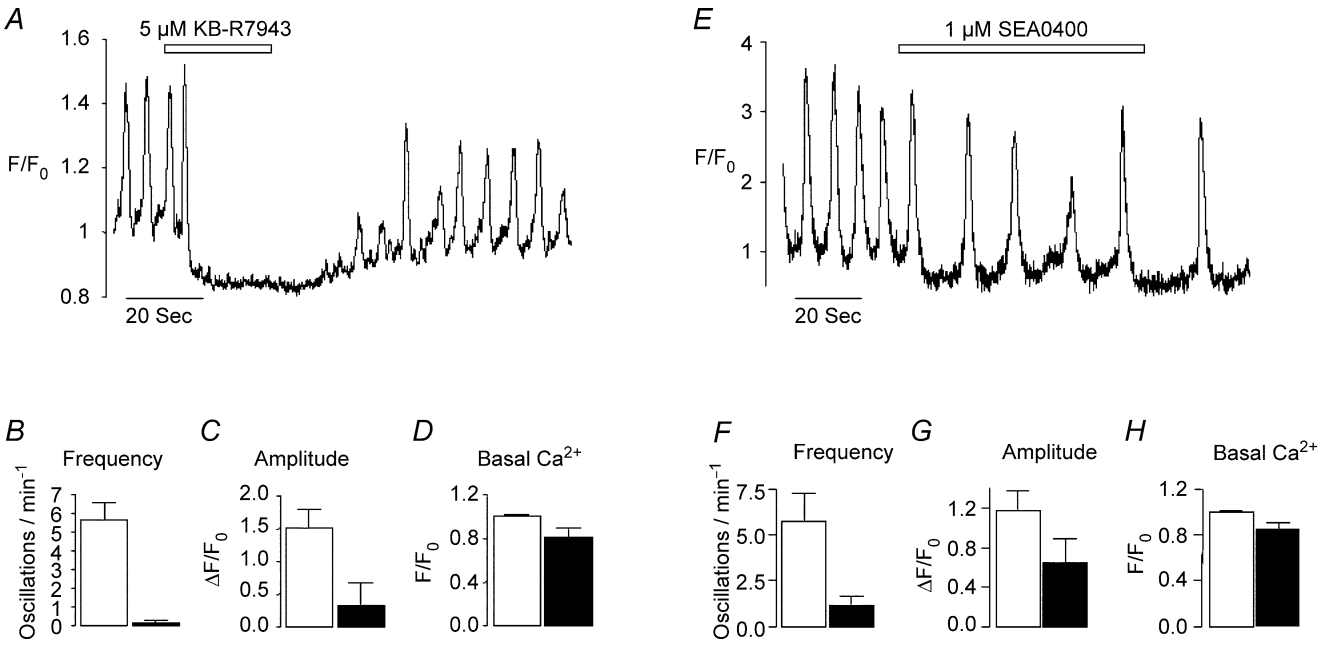

Figure 2. Effect of KB-R7943 and SEA0400 on spontaneous Ca^{2+} oscillations in ICC
The reverse NCX inhibitor KB-R7943 abolished Ca^{2+} oscillations and lowered basal Ca^{2+} levels in urethral ICC (*A*). *B–D*, summary bar charts showing mean frequency and amplitude of Ca^{2+} oscillations and basal Ca^{2+} levels in the absence (open bars) and presence (filled bars) of KB-R7943. The effect of SEA0400 was not as dramatic as KB-R7943 but it also greatly reduced the frequency of Ca^{2+} oscillations and lowered basal Ca^{2+} levels in ICC as shown in the representative example in *E* and summary data plotted in *F–H*.

of Ca^{2+} from ryanodine- and IP_3-sensitive Ca^{2+} stores, respectively. These responses were reproducible if repeated at 80 s intervals. We investigated if these responses were affected by KB-R7943 and SEA0400. Figure 7*A* and *C* are representative current traces which show that caffeine- and noradrenaline-evoked I_{ClCa} are not inhibited by KB-R7943. Bar charts plotting the peak amplitude of the caffeine and noradrenaline currents in the absence, presence and following washout of KB-R7943 are illustrated in Fig. 7*B* and *D*, respectively. In seven cells the mean amplitude of the caffeine-induced current was -661 ± 194 pA in the absence of KB-R7943 and -495 ± 184 pA in its presence ($P > 0.05$, Fig. 7*B*). Figure 7*D* shows that the mean amplitude of noradrenaline-induced inward currents in four cells was -610 ± 110 pA in the absence and -518 ± 106 pA in KB-R7943 ($P > 0.05$). Caffeine and noradrenaline responses were also not inhibited by SEA0400 (Fig. 7*E* and *G*). The summary bar chart plotted in Fig. 7*F* shows that the mean amplitude of the caffeine-evoked current was -483 ± 41 pA before SEA0400 application and -466 ± 50 pA in the presence

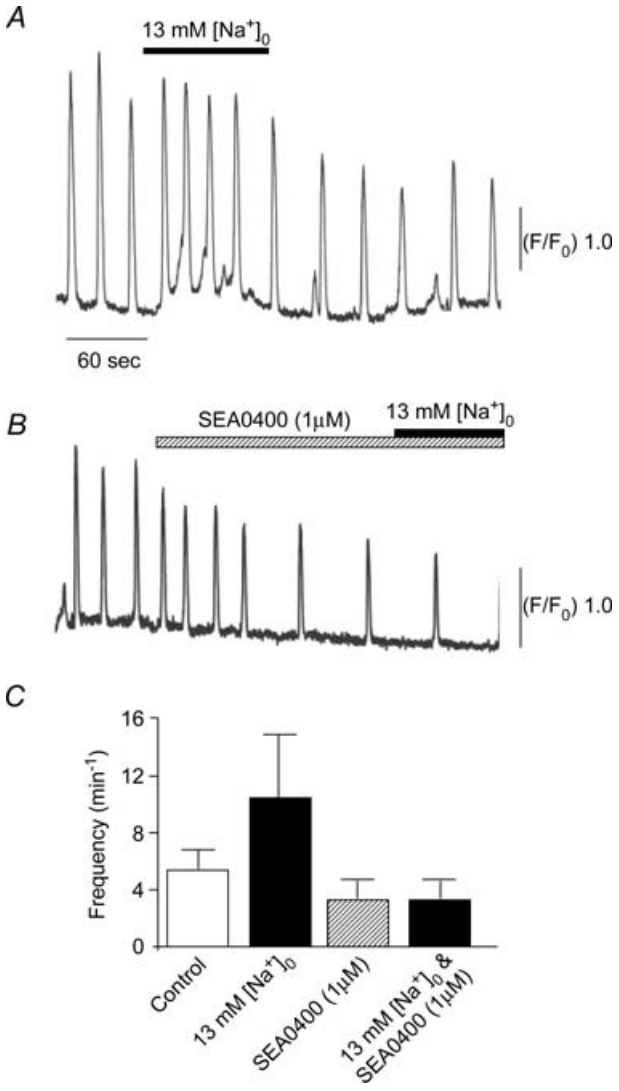

Figure 3. Effect of SEA0400 on Ca^{2+} oscillations induced by $[Na^+]_o$
A and *B*, representative recordings which show the effect of 13 mM $[Na^+]_o$ on spontaneous Ca^{2+} oscillations in the absence (*A*) and presence (*B*) of SEA0400, respectively. *C*, summary bar chart which plots the mean frequency of Ca^{2+} oscillations under control conditions, in the presence of low $[Na^+]_o$, in the presence of SEA0400 and in the presence of SEA0400 and low $[Na^+]_o$ together.

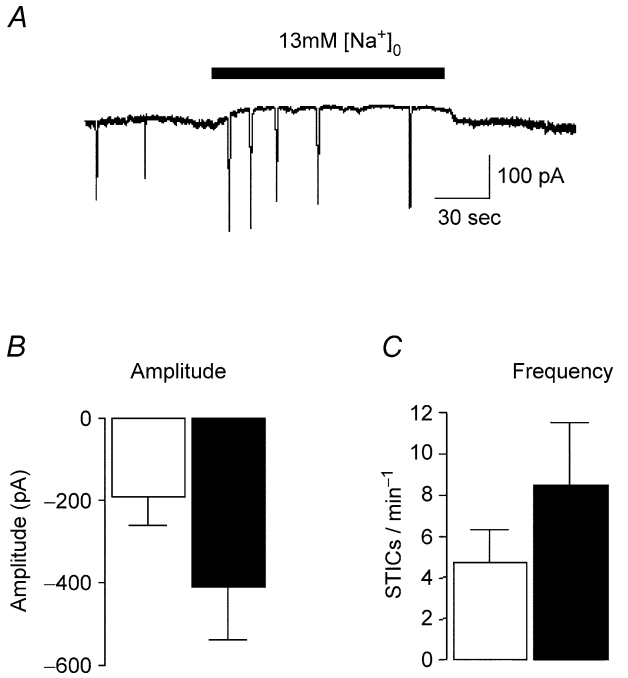

Figure 4. Effect of low $[Na^+]_o$ on spontaneous Ca^{2+} oscillations in ICC
A, representative recording from an isolated ICC showing that reduction of $[Na^+]_o$ from 130 to 13 mM increases the frequency of STICs in ICC. *B* and *C*, summary bar charts showing the effect of 13 mM $[Na^+]_o$ on mean frequency and amplitude of STICs.

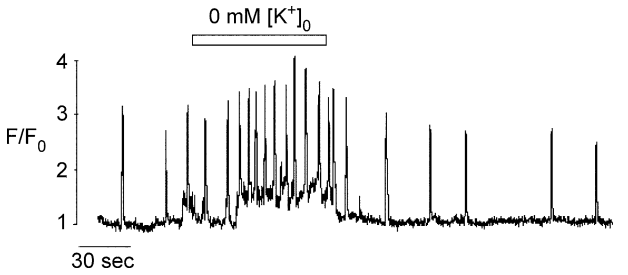

Figure 5. Effect of 0 mM $[K^+]_o$ on spontaneous Ca^{2+} oscillations in ICC
Representative trace showing that removal of K^+ from the bath solution to inhibit the Na^+–K^+ pump causes an increase in the frequency of spontaneous Ca^{2+} oscillations in ICC.

of the drug ($P > 0.05$, $n = 7$). The mean amplitude of the peak noradrenaline-evoked current was -389 ± 87 pA before and -252 ± 79 pA during SEA0400 application (Fig. 7*H*, $P > 0.05$, $n = 6$).

Effect of SEA0400 on spontaneous myogenic tone in rabbit urethra smooth muscle

As the previous single cell experiments indicated that spontaneous activity in individual ICC depends on reverse NCX, experiments were performed to assess the overall contribution of reverse NCX to spontaneous myogenic tone in isolated strips of urethra smooth muscle. Strips were mounted in 5 ml organ baths and allowed to develop spontaneous myogenic tone as described earlier. Figure 8*A* and *B* are typical recordings showing that 1 and 10 μM SEA0400 inhibited urethral tone in a concentration-dependent fashion. Summary data demonstrating that 1 and 10 μM SEA0400 reduced tone in four preparations by 20.3 ± 3.4 and 73.3 ± 8.9 mg of tension, respectively, are plotted in Fig. 8*C*. In two of these experiments we also measured the maximal relaxations produced by EFS (1 Hz for 1 min) of the inhibitory nerves in the presence of atropine and guanethidine. The relaxations induced by 10 μM SEA0400 were ~40% of the amplitude of these responses.

Expression of NCX subtypes in rabbit urethra

The data presented thus far indicate that spontaneous activity in urethra ICC is influenced by the activity of plasmalemmal sodium–calcium exchangers. It is now known that there are three mammalian isoforms of the sodium–calcium exchanger encoded by three different genes (NCX1, NCX2 and NCX3; Blaustein & Lederer, 1999). We performed RT-PCR experiments to examine the expression of these isoforms in strips of rabbit urethra smooth muscle as well as individual SMC and ICC. cDNA derived from rabbit brain was used as a positive control for the NCX primers. The gel shown in Fig. 9 demonstrates that signal for NCX1, 2 and 3 were present in rabbit brain and rabbit urethra smooth muscle strips. After two rounds of amplification only signal for NCX3 could be detected in isolated urethra ICC compared with NCX1 and NCX3 in SMC. PCR was also performed on reaction mixtures lacking cDNA (no-template controls) for each primer to test for contamination and non-specific amplification. No amplification product was detected from these reactions (data not shown).

Discussion

Pacemaker activity in urethral ICC is thought to occur due to a combination of Ca^{2+} release from intracellular stores

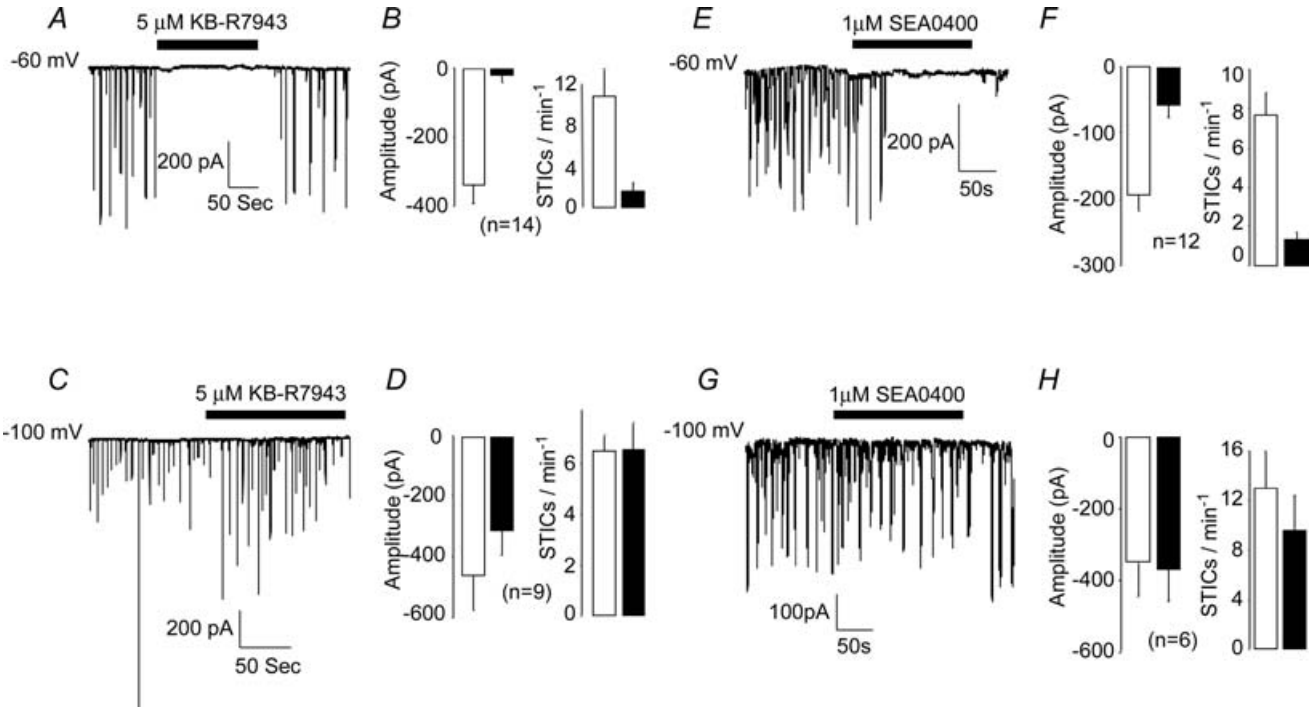

Figure 6. Effect of KB-R7943 and SEA0400 on STICs in ICC
KB-R7943 (5 μM) and SEA0400 (1 μM) abolished STICs in ICC at −60 mV but not at −100 mV. Representative examples of the effects of KB-R7943 at each potential are shown in *A* and *C*. Summary data plotting mean STIC amplitude and frequency at −60 and −100 mV in the absence and presence of KB-R7943 are plotted in *B* and *D*, respectively. Typical records showing the effects of SEA0400 at −60 and −100 mV are shown in *E* and *G*. *F* and *H* are summary bar charts which show the effect of SEA0400 on the amplitude and frequency of STICs at each potential.

and Ca^{2+} influx from the extracellular milieu. The intracellular Ca^{2+} release mechanisms were clarified in a recent study by Johnston *et al.* (2005). They demonstrated that spontaneous global Ca^{2+} oscillations in ICC arose from the conversion of localized Ca^{2+} release from ryanodine receptors (RyRs) to propagating Ca^{2+} waves by activation of IP_3 receptors (IP_3Rs). As release of Ca^{2+} from RyRs was the initial event in this sequence, these events were referred to as the 'prime oscillators'. Johnston *et al.* (2005) also established the importance of Ca^{2+} influx to the process, although the identity and role of the Ca^{2+} entry pathways involved were not determined. Earlier investigations by Sergeant *et al.* (2001) found that STICs at −60 mV were not affected by nifedipine suggesting L-type Ca^{2+} channels were not involved. Also, Bradley *et al.* (2005) concluded that pacemaker activity was not driven by CCE and pointed to the involvement of a non-CCE pathway as a means for sustaining pacemaker activity in ICC. The results of the present study suggest that reverse NCX could fulfil this role.

Ca^{2+} influx via reverse NCX does not appear to affect refilling of Ca^{2+} stores in ICC under normal circumstances as neither noradrenaline nor caffeine responses were affected by KB-R7943 or SEA0400. This is in contrast

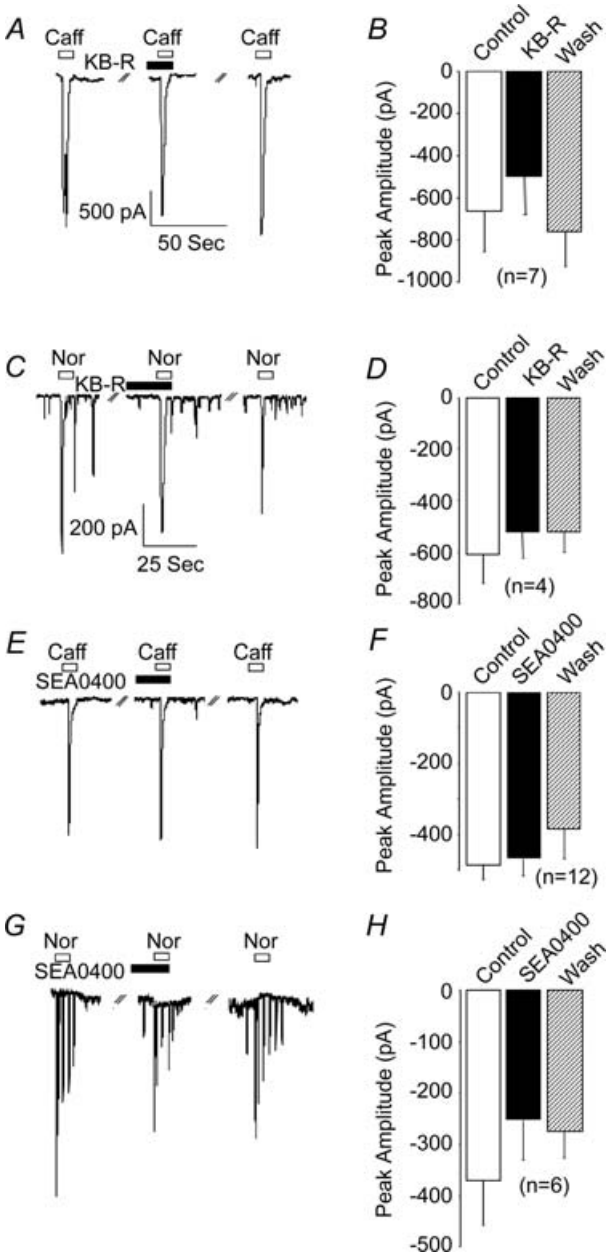

Figure 7. Effect of KB-R7943 and SEA0400 on caffeine- and noradrenaline-induced inward currents in ICC
Application of caffeine (10 mM) to ICC voltage clamped at −60 mV resulted in transient inward currents. These responses were not affected by KB-R7943 as indicated by the representative example shown in *A* and summary data plotting the peak amplitude of the caffeine-evoked current before, during and following washout of KB-R7943 in *B*. Noradrenaline (10 μM) also evoked a transient inward current in ICC. These responses were also not significantly affected by KB-R9743 (*C* and *D*). *E* and *F* show that SEA0400 did also not significantly inhibit caffeine responses. Noradrenaline-induced currents were slightly reduced in amplitude as shown in the example in *G* and summary data in *H*.

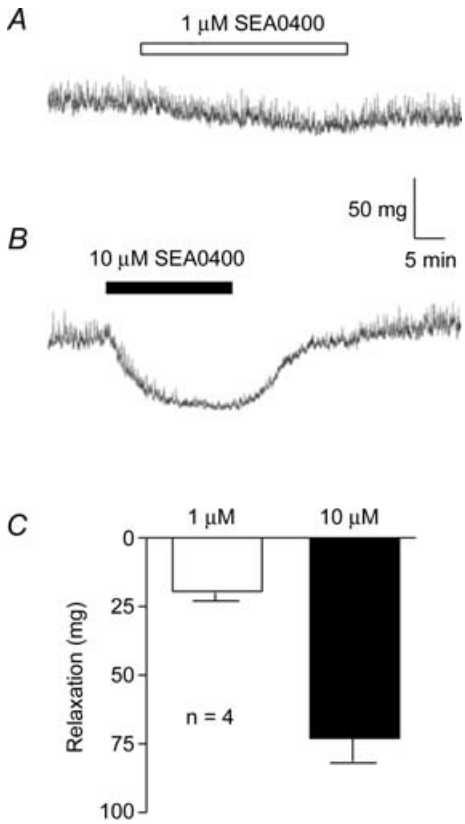

Figure 8. Effect of SEA0400 on spontaneous myogenic tone of rabbit urethra smooth muscle
A and *B*, representative examples showing the effects of 1 and 10 μM SEA0400 on spontaneous myogenic tone in rabbit urethra smooth muscle. *C*, summary bar chart plotting the mean amplitude of the relaxations produced by SEA0400.

to the findings of Wu & Fry (2001) which showed that caffeine-induced Ca^{2+} release was enhanced by Ca^{2+} influx via reverse NCX in guinea pig detrusor smooth muscle cells. In other tissues such as cardiac myocytes, Ca^{2+} influx via reverse NCX acts as a trigger mechanism to stimulate Ca^{2+} release at RyRs (Leblanc & Hume, 1990). Several studies have also demonstrated that Ca^{2+} entry via reverse NCX causes Ca^{2+} release at IP_3Rs in smooth muscle (Ohata *et al.* 1996; Hisamitsu *et al.* 2001). It is possible that reverse NCX may induce CICR at IP_3Rs in ICC; however, we believe this is unlikely as reverse NCX inhibitors produced quite different effects to IP_3R inhibitors. For example, Johnston *et al.* (2005) showed that inhibition of IP_3Rs with 2-APB elevated basal Ca^{2+} levels and decreased the amplitude and spread of Ca^{2+} waves but not frequency, whereas KB-R7943 and SEA0400 decreased basal Ca^{2+} levels and reduced the frequency or else completely abolished the activity. These observations were more reminiscent of the effects of tetracaine or removal of Ca^{2+} from the bathing media (Johnston *et al.* 2005) suggesting that Ca^{2+} influx via reverse NCX may increase basal Ca^{2+} levels and stimulate Ca^{2+} release from RyRs. Other studies have also shown that a key role for reverse NCX is to maintain basal Ca^{2+} levels. For example, Rebolledo *et al.* (2006) showed that $[Ca^{2+}]_i$ in human umbilical artery smooth muscle cells was reduced by application of KB-R7943 and enhanced by reductions in $[Na^+]_o$.

The results of the present study show that urethra ICC only expressed NCX3 whereas SMC collected from the same preparation expressed both NCX1 and NCX3. NCX1 is widely distributed in most cells; however, expression of NCX3 was previously thought to be restricted to brain and skeletal muscle (Nicol *et al.* 1996) though transcripts have also been detected in lung, liver and heart (Lindgren *et al.* 2005). NCX3 is known to be a key molecular determinant of reverse NCX in skeletal muscle. Sokolow *et al.* (2004) showed that in wild-type ($Ncx^{+/+}$) mice, application of solution with reduced $[Na^+]_o$ to skeletal muscle fibres induced a transient rise in $[Ca^{2+}]_i$ due to reverse NCX. However, in NCX3-deficient ($Ncx^{-/-}$) mice these responses were absent. Previous investigations (Iwamoto *et al.* 2004) have reported that NCX3 is insensitive to SEA0400 and three times more sensitive to KBR-7943 compared with NCX1, whereas Linck *et al.* (1998) did not observe any pharmacological differences among the different NCX subtypes and reported that the NCX subtypes had very similar properties. In the present study we found that spontaneous activity was sensitive to SEA0400 and since NCX3 was the only NCX isoform detected in ICC the implication is that NCX3 activity in these cells is sensitive to SEA0400. However, more detailed experiments would be required to confirm this conclusion and we cannot rule out the possibility that SEA0400 could act by an as yet unidentified pathway.

ICC in the urethra are thought to be analogous to ICC in the gut (Sanders, 1996). The cellular basis of pacemaker activity in ICC in the GI tract is slightly different to that in the urethra; however, it is also brought about by a complex interplay between intracellular Ca^{2+} handling and Ca^{2+} influx. Torihashi *et al.* (2002) showed that pacemaker activity in ICC clusters requires extracellular Ca^{2+}, as spontaneous Ca^{2+} oscillations were inhibited by Ca^{2+}-free bath solution and Mn^{2+}. It is currently unclear if pacemaker activity in individual ICC is directly reliant on Ca^{2+} influx per se. However, it is known that pacemaker activity can be entrained by Ca^{2+} influx. For example, Ca^{2+} entry during the inter slow wave period is thought to enhance the excitability of individual ICC by increasing the sensitivity of IP_3Rs to further changes in $[Ca^{2+}]_i$ or IP_3 (Sanders *et al.* 2004). A recent study by Cho & Daniel (2005) found that NCX1 was colocalized with ICC in the jejunum and lower oesophageal sphincter. However, it appears unlikely that reverse NCX is involved in pacemaker activity in the gut as SEA0400 failed to inhibit spontaneous contractions (Boddy *et al.* 2004). An alternative possibility is that pacemaker activity in the GI tract is regulated by forward mode NCX as Prosser (1995) showed that the frequency of contractions in frog and toad stomach were dependent on the rate of Ca^{2+} removal.

Reverse NCX can only occur when V_m is positive to E_{NCX}, therefore as KB-R7943 and SEA0400 inhibited STICs at −60 mV but not at −100 mV, it suggests that E_{NCX} in ICC lies between these values. An alternative explanation for these observations is that the inhibitory effects of

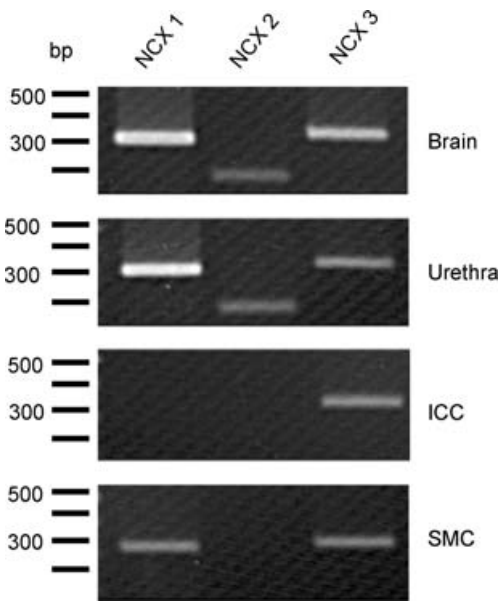

Figure 9. Expression of NCX in the rabbit urethra
A representative gel displaying amplification products from RNA derived from rabbit brain, urethra and urethra SMC and ICC using gene-specific primers for NCX1, NCX2 and NCX3.

KB-R7943 and SEA0400 are voltage dependent and are ineffective at −100 mV. Thus far, however, we have found no evidence that this is the case and studies by Kimura *et al.* (1999) and Lu *et al.* (2002) reported that the inhibitory effects of these agents are not voltage dependent. The results of the present study also show that caffeine and noradrenaline responses were not blocked by either drug demonstrating that they do not inhibit Ca^{2+}-activated Cl^- channels, prevent uptake or release of Ca^{2+} from intracellular stores, or block α_1-adrenoceptors. Therefore, the inhibitory effects of these agents in the present study cannot be attributed to an indirect effect on any of these targets. These data also raise the question of which influx pathways sustain spontaneous activity at −100 mV in the absence of Ca^{2+} influx via reverse NCX. At present we cannot provide a definitive answer to this question but speculate that Ca^{2+} leak across the plasma membrane will be augmented due to the increased driving force for Ca^{2+} entry under these conditions.

A crucial element of the present study was the finding that isolated strips of rabbit urethra smooth muscle generate spontaneous myogenic tone. These data are in direct contrast to an earlier report by Waldeck *et al.* (1998) which stated that 'rabbit urethral strip preparations do not develop a spontaneous tone'. Tone generated by the urethra smooth muscle strips in the present study was sensitive to the NCX inhibitor SEA0400 and therefore suggests that NCX activity contributes to the development of spontaneous myogenic tone. In summary, the results of the present study suggest that pacemaker activity in urethra ICC is regulated via reverse NCX. We speculate that Ca^{2+} entry via this pathway affects Ca^{2+} release, possibly at RyRs. If this is the case it opens up the exciting possibility that tone in the urethra can be modulated by Ca^{2+} influx via reverse NCX in ICC.

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