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T- and L-type Ca^{2+} currents in freshly dispersed smooth muscle cells from the human proximal urethra

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The purpose of the present study was to characterise Ca^{2+} currents in smooth muscle cells isolated from biopsy samples taken from the proximal urethra of patients undergoing surgery for bladder or prostate cancer. Cells were studied at 37 °C using the amphotericin B perforated-patch configuration of the patch-clamp technique. Currents were recorded using Cs^+-rich pipette solutions to block K^+ currents. Two components of current, with electrophysiological and pharmacological properties typical of T- and L-type Ca^{2+} currents, were present in these cells. When steady-state inactivation curves for the L current were fitted with a Boltzmann equation, this yielded a $V_{½}$ of −45 ± 5 mV. In contrast, the T current inactivated with a $V_{½}$ of −80 ± 3 mV. The L currents were reduced in a concentration-dependent manner by nifedipine (ED_{50} = 159 ± 54 nM) and Ni^{2+} (ED_{50} = 65 ± 16 μM) but were enhanced when external Ca^{2+} was substituted with Ba^{2+}. The T current was little affected by TTX, reduction in external Na^+, application of nifedipine at concentrations below 300 nM or substitution of external Ca^{2+} with Ba^{2+}, but was reduced by Ni^{2+} with an ED_{50} of 6 ± 1 μM. When cells were stepped from −100 to −30 mV in Ca^{2+}-free conditions, small inward currents could be detected. These were enhanced 40-fold in divalent-cation-free solution and blocked in a concentration-dependent manner by Mg^{2+} with an ED_{50} of 32 ± 16 μM. These data support the idea that human urethral myocytes possess currents with electrophysiological and pharmacological properties typical of T- and L-type Ca^{2+} currents.

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The urethra generates sufficient tone to prevent leakage from the bladder and thus plays an important role in maintaining urinary continence. Despite this central role, relatively little is known about the mechanisms that underlie the generation and modulation of urethral tone, although it can be influenced by a number of factors including blood flow through the lamina propria (Brading *et al.* 1999). There is little doubt that a myogenic mechanism also contributes significantly to urethral tone, since it is unaffected *in vitro* by nerve blockade in a variety of species including pigs (Bridgewater *et al.* 1993), sheep (Thornbury *et al.* 1992), rats (McKeag *et al.* 2001) and humans (Brading *et al.* 1999).

A number of studies have demonstrated that urethral myogenic tone is critically dependent on the influx of Ca^{2+} across the cell membrane, since removal of external Ca^{2+} or inhibition of L-type Ca^{2+} channels reduces tone significantly in rats, humans and pigs *in vitro* (Bridgewater *et al.* 1993; Brading, 1999; Shafei *et al.* 2003). Shafei *et al.* (2003) have demonstrated that application of nifedipine or Ni^{2+} significantly reduces tone in an isolated rat whole urethra preparation, suggesting that Ca^{2+} influx through both T and L channels contributes to urethral tone. Recent studies by Bradley *et al.* (2003) have characterised the Ca^2 currents in isolated rabbit urethral myocytes and demonstrated the existence of currents with biophysical and pharmacological properties typical of L- and T-type Ca^{2+} currents in arterial (Benham *et al.* 1987), venous (Yatani *et al.* 1987) and bladder myocytes (Sui *et al.* 2001).

To date, no study has examined successfully the electrophysiology of human urethral myocytes, presumably because of the poor availability of suitable tissue and the difficulty of obtaining viable cells from small biopsy samples. In this study we provide the first electrophysiological data from freshly dispersed human myocytes obtained from adults undergoing treatment for bladder or prostate cancer. Our results suggest that human urethral myocytes possess Ca^{2+} currents with electrophysiological and pharmacological properties typical of T and L channels (for reviews see Kotlikoff *et al.* 1999; Perez-Reyes, 2003).

Preliminary accounts of this work have been reported to The Physiological Society (Hollywood *et al.* 2001, 2002).

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METHODS

Smooth muscle cells were isolated from transurethral biopsy samples taken from the most proximal 1 cm of the urethra in 21 male and three female patients (mean ± S.E.M. age 57 ± 3 years; range 19–71 years) who gave written, informed consent. The majority of samples were taken from patients undergoing surgery for bladder or prostate cancer. This study was approved by Queen's University Belfast Ethical Committee and is in accordance with the Declaration of Helsinki. Tissue was obtained by either cold-cup biopsy or from open procedures. With cold-cup biopsy forceps, the urethral lumen was visualised through a cystoscope and samples were taken at the internal urethral orifice. During open procedures, an excision biopsy sample was obtained from the area distal to the ureteric orifices and immediately proximal to the prostatic urethra. The tissue was removed, placed in oxygenated Krebs' solution at room temperature and immediately transported to the laboratory.

Cell dispersal

Strips of tissue, 0.5 cm in width, were cut into 1 mm^3 pieces and stored in Hanks' Ca^{2+}-free solution for 30 min prior to cell dispersal. Occasionally, the tissue was stored overnight in Ca^{2+}-free Hanks' solution at 4 °C before 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), 0.5–2 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. These cells were plated in Petri dishes containing 100 μM Ca^{2+} Hanks' solution and stored at 4 °C for use within 8 h.

Solutions

Hanks' solution contained (mM): 129.8 Na^+, 5.8 K^+, 135 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 glucose, 2.9 sucrose and 10 Hepes; the pH was adjusted to 7.4 with NaOH. The Cs^+ perforated-patch pipette solution contained (mM): 133 Cs^+, 135 Cl^-, 1.0 Mg^{2+}, 0.5 EGTA and 10 Hepes; the pH was adjusted to 7.2 with CsOH. The Mg^{2+}-substituted, Ca^{2+}-free Hanks' solution contained (mM): 129.8 Na^+, 5.8 K^+, 135 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 2.7 Mg^{2+}, 0.4 SO_4^{2-}, 10 glucose, 2.9 sucrose, 5 EGTA and 10 Hepes; the pH was adjusted to 7.4 with NaOH. The Ca^{2+}-free/Mg^{2+}-free Hanks' solution comprised (mM): 129.8 Na^+, 5.8 K^+, 130 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 0.4 SO_4^-, 10 glucose, 2.9 sucrose, 5 EGTA and 10 Hepes; the pH was adjusted to 7.4 with NaOH. The Ba^{2+}-substituted Hanks' solution contained (mM): 129.4 Na^+, 5.4 K^+, 135 Cl^-, 4.17 HCO_3^-, 1.8 Ba^{2+}, 0.9 Mg^{2+}, 10 glucose, 2.9 sucrose and 10 Hepes; the pH was adjusted to 7.4 with NaOH. The low-Na^+ Hanks' solution contained (mM): 116.9 *N*-methyl-D-glucamine, 12.9 Na^+, 5.8 K^+, 135 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 glucose, 2.9 sucrose and 10 Hepes; the pH was adjusted to 7.4 with HCl.

WEBMAXC v2.22 (www.stanford.edu/~cpatton/webmaxcSR.htm) was used to calculate the free Mg^{2+} concentration for the divalent-free experiments. Total Mg^{2+} concentrations of 8.1, 2.7, 0.81, 0.27, 0.081 and 0.027 mM yielded free Mg^{2+} concentrations of 5.5 and 1.55 mM, and 429, 139, 41 and 13.7 μM, respectively, in the presence of 5 mM EGTA.

Perforated-patch recordings from single cells

Currents were recorded using the perforated-patch configuration of the whole-cell patch-clamp technique (Horn & Marty, 1988). This circumvented the problem of current rundown that is encountered using the conventional whole-cell configuration. The cell membrane was perforated using the antibiotic amphotericin B (600 μg ml^{-1}). Patch pipettes were initially front-filled by dipping them 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 a 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 AT-type computer running pCLAMP software. During experiments, the dish containing the cells was continuously perfused with Hanks' solution at 36 ± 1 °C. In addition, the cell under study was continuously superfused by means of a custom-built closed delivery system with a pipette of tip diameter 200 μm placed approximately 300 μm from the cell. The Hanks' solution in the closed delivery system could be switched to a drug-containing solution with a dead-space time of less than 5 s. In all experiments, *n* refers to the number of cells studied and each experimental set usually contained samples from a minimum of three patients. 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 the $P < 0.05$ level as significant.

Drugs used

The following drugs were used: amphotericin B (Sigma), $NiCl_2$ (Sigma) and nifedipine (Tocris).

Stock solutions of $NiCl_2$ (concentration 0.1 M) were made up in water. Nifedipine (1 mM) was made up in ethanol. All drugs were then diluted to their final concentrations in Hanks' solution. Drug vehicles had no effect on the currents studied.

RESULTS

Using our dispersal procedure, relaxed smooth muscle cells could be isolated reliably from human tissue samples. The smooth muscle cells were unbranched and spindle shaped, and contracted in response to either depolarising pulses or application of noradrenaline (10 μM). They had an input resistance of 1.2 ± 0.1 GΩ and a capacitance of 65 ± 6 pF ($n = 30$ cells from 12 patients). Under identical recording conditions to those used in previous studies (Cotton *et al.* 1997; Sergeant *et al.* 2000), more than 80 % of human urethral myocytes possessed both low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca^{2+} currents, but rarely possessed Ca^{2+}-activated Cl^- currents (< 5 %). On the basis of the electrophysiological and pharmacological data presented below, we will henceforth refer to the HVA current as the L current and the LVA current as the T current.

Effect on currents of altering the holding potential

Figure 1*A* shows representative traces taken from an experiment in which a cell was held at a potential of either −60 (left trace) or −100 mV (middle trace) and then depolarised from −70 through to 0 mV for 500 ms in 10 mV steps. When cells were held at −60 mV and then depolarised, inward currents activated at ~−40 mV. In contrast, when the same cell was held at −100 mV (Fig. 1*A*, middle trace) and the voltage steps repeated, inward currents now activated at −60 mV. To isolate the currents that were unmasked by holding the cell at −100 mV, difference currents were obtained by subtracting the currents obtained at holding potentials of −60 mV from those obtained at −100 mV and are shown in the right panel of Fig. 1*A*. The difference currents activated at ~−60 mV, inactivated rapidly, were maximal at −30 mV and reversed at approximately 20 mV. Figure 1*B* shows a summary current–voltage (*I–V*) plot for eight cells obtained from five patients, at holding potentials of −60 (filled circles) and −100 mV (open squares), and the associated difference currents (open circles). Interestingly, the reversal potential of the T current was ~20 mV, whereas that of the L currents was ~45 mV. The T currents also inactivated more rapidly than the L currents. This difference is illustrated by the step to −20 mV seen in Fig. 1*A*, where the L (left panel) and T (right panel) currents are of similar amplitude. These data support the idea that both of these components of inward current are expressed in human myocytes.

Voltage dependence of inactivation

To assess the voltage dependence of inactivation of both currents, a standard double-pulse protocol was employed. Figure 2*A* shows a typical experiment in which a cell was held at conditioning potentials ranging from −110 to 0 mV for 2 s prior to stepping to the test potential of 0 mV for 500 ms to evoke the peak L current. Most inactivation of this current occurred over the potential range of −70 to −30 mV. The filled circles in Fig. 2*B* show steady-state activation curves constructed from the data shown in Fig. 1*B*. The inactivation data were fitted with a Boltzmann equation of the form

$$I/I_{max} = 1/\{1 + \exp[-K(V - V_{½})]\},$$

where K is the slope factor and $V_{½}$ is the voltage at which there is half-maximal inactivation. This yielded a $V_{½}$ of −45 ± 5 mV. Although the data points for inactivation at potentials positive to −50 mV were well fitted by this equation, those at potentials negative to −50 mV deviated from the Boltzmann fit, suggesting that the step to 0 mV evoked contamination of the L current by a small component of the T current.

We next examined the inactivation of the T current by applying the same conditioning potentials as above but stepping to −30 mV to elicit the peak T current. These experiments were carried out on cells that possessed little L-type Ca^{2+} current to ensure that the inactivation data was contaminated as little as possible with L current. Figure 2*C* shows a typical example of the voltage-dependent inactivation obtained using the protocol shown. In contrast to the L current, the T current was almost completely inactivated when conditioning potentials were more

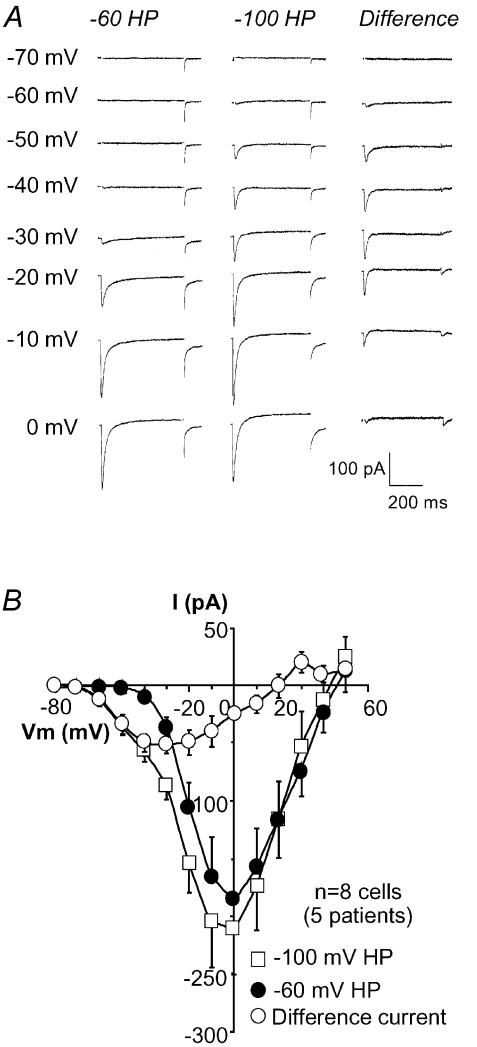

Figure 1. Characteristics of T and L currents in human urethral smooth muscle cells

A, families of currents recorded from a cell held at −60 and −100 mV (−60 HP and −100 HP, respectively). The right panel shows the difference currents obtained by subtraction and demonstrates a negatively activating inward current that peaks at −30 mV. *B*, summary *I–V* plots for eight cells from five patients in which the peak inward currents were plotted when cells were depolarised from holding potentials of −100 mV (open squares) and −60 mV (filled circles). The open circles show the difference currents, which were obtained by subtraction. V_m, membrane potential.

positive than −50 mV. Figure 2*D* (open circles) shows summary data obtained from eight cells (from five patients). When these data were fitted with the aforementioned Boltzmann equation (continuous line), a $V_{½}$ of −80 ± 3 mV was obtained.

The filled circles in Fig. 2*B* and *D* show the summary data points used to construct steady-state activation curves for the L and T current, respectively, obtained from the data in Fig. 1*B*. The T current activation $V_{½}$ was −45 ± 4 mV, and an appreciable window current was apparent at potentials between −80 and −20 mV. The continuous grey lines in Fig. 2*D* show the Boltzmann fits to the activation/inactivation curves for the L current for comparison. In contrast to the T current, the $V_{½}$ of activation for the L current was −21 ± 2 mV and the window current extended from −50 to −10 mV.

Pharmacology of the inward currents

Having demonstrated that two components of inward current could be isolated on the basis of their voltage dependence of activation and inactivation, we next wanted to test if each of these currents possessed a different pharmacological profile. We first wished to exclude the possibility that the negatively activating current was a Na^+ current by examining the effects of (1) TTX and (2) reducing external Na^+ on currents evoked by a step from −100 to −30 mV. Application of TTX (1 μM) failed to alter the amplitude of the current in two cells (two patients, data not shown). When cells were depolarised

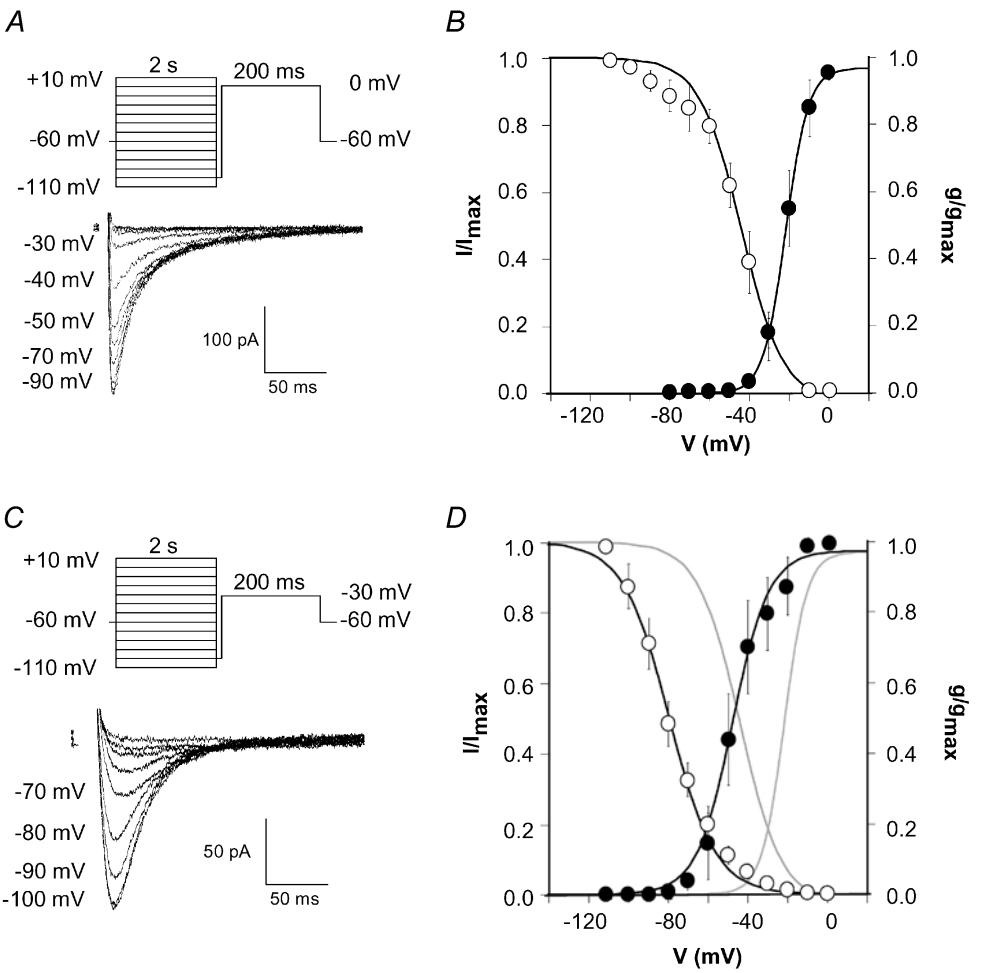

Figure 2. Voltage-dependent inactivation of L and T currents in human urethral smooth muscle cells

A, a typical family of currents obtained by stepping to 0 mV for 200 ms following application of a series of 2 s conditioning potentials from −110 to +10 mV. *B*, summary data demonstrating the voltage dependence of inactivation (open circles) and activation curves (filled circles) of the L current in six cells (*g*, conductance; g_{max}, maximum conductance). The continuous lines represent Boltzmann fits to the data. *C*, a typical inactivation profile for a T current, which was obtained by stepping to −30 mV for 200 ms following application of a series of 2 s conditioning potentials from −110 to +10 mV. *D*, summary data demonstrating the voltage dependence of inactivation (open circles) and activation curves (filled circles) of the T current in eight cells. The continuous black lines represent Boltzmann fits to the data. The continuous grey lines show the fits obtained for the L current from the data in Fig. 2*B* for comparison.

from −100 to −30 mV in 130 mM external Na^+, the peak current was −85 ± 12 pA, and this was slightly reduced to −66 ± 10 pA when external Na^+ was reduced to 13 mM (n = 5 cells, not significant (n.s.)). This small reduction in T current after a 10-fold reduction in external Na^+ may be due to inhibition of contaminant L-type current, since a reduction of external Na^+ will elevate intracellular Ca^{2+} and cause further Ca^{2+}-dependent inactivation of any contaminant L current. Alternatively, the elevation of intracellular Ca^{2+} could reduce the driving force favouring Ca^{2+} influx, and this may account for the observed reduction in T current in low-Na^+ solutions.

Effect of nifedipine

We examined the effect of the L-type Ca^{2+} channel antagonist nifedipine on both components of the inward current. The inset of Fig. 3*A* shows the protocol used to evoke both components of the inward current. Cells were held at −100 mV and stepped to −30 mV for 250 ms to evoke the peak T current, and then depolarised to −50 mV for 500 ms to inactivate any remaining T current. The cell was further depolarised to 0 mV for 250 ms to evoke a peak L-type Ca^{2+} current. Figure 3*A* shows currents obtained before and during application of increasing concentrations of nifedipine. Application of nifedipine at concentrations up to 300 nM decreased the amplitude of the L current by ~90 %, whereas the T current was only reduced by ~30 %. The ability of nifedipine to discriminate between both components of the inward current is reflected in the summary data shown in Fig. 3*B*. The concentration–effect curves illustrating the effects of nifedipine on T (filled circles) and L currents (open circles) were constructed from data obtained from seven cells (four patients). When the data for the L-type current were fitted with a Langmuir equation of the form:

$$I_{drug}/I_{control} = 1/\{1 + ([drug]/ED_{50})\},$$

where the ED_{50} of is the half-maximal effective dose, this yielded an ED_{50} of 159 ± 54 nM. In contrast, the data obtained from a step to −30 mV (filled circles) could not be easily fitted with a Langmuir equation. For example, while 30 nM nifedipine reduced the T current by ~20 %, the next two concentrations had little further effect. The reduction in current evoked by this voltage protocol in the presence of 30 nM nifedipine presumably reflects its effect on a small component of L current present at −30 mV.

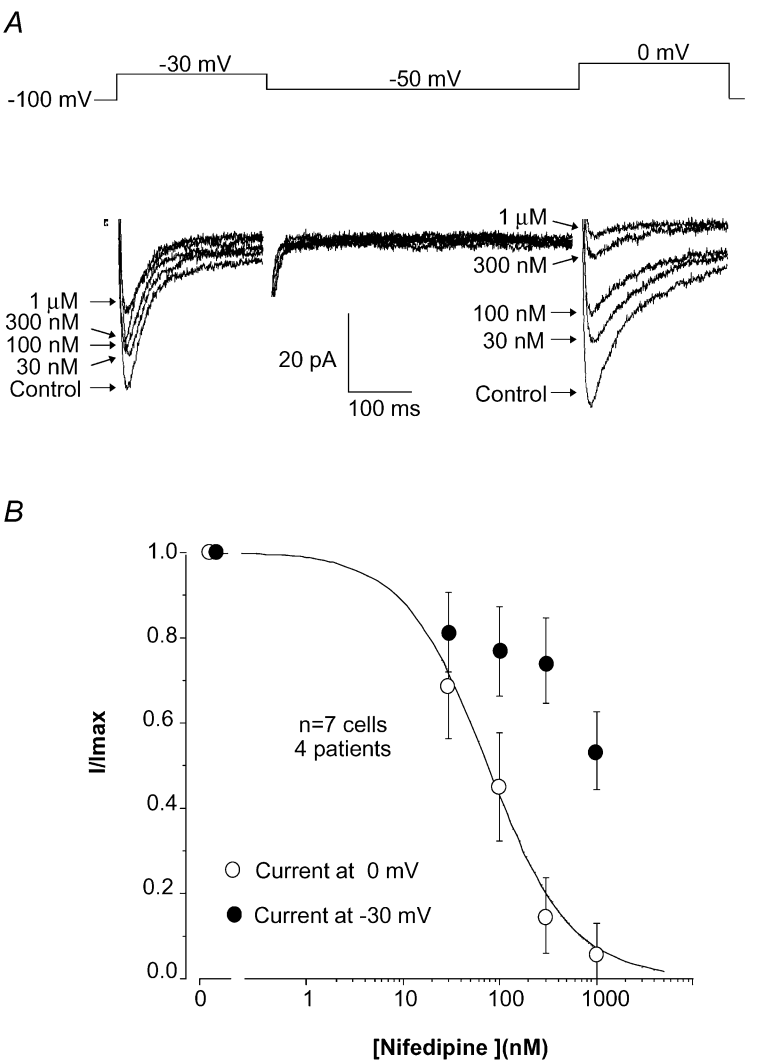

Figure 3. Effect of nifedipine on T and L currents

A, peak T and L currents were evoked by a step from −100 to −30 mV for 250 ms. A step to −50 mV for 500 ms was applied to inactivate the T current, and this was followed by a further step to 0 mV to evoke peak L currents in the same cell. The traces demonstrate the effect of increasing concentrations of nifedipine on the peak T and L current. Note that 300 nM nifedipine practically abolished the L current evoked by a step from −50 to 0 mV, but had less effect on the peak T current evoked by a step from −100 to −30 mV. *B*, a summary of the effects of a variety of nifedipine concentrations on the peak T (filled circles) and L currents (open circles). The L-current data were fitted with a Langmuir equation (continuous line, see text) and yielded an ED_{50} of 159 ± 54 nM. The T-current data were easily fitted with a Langmuir equation.

To test for the possibility that the effects of nifedipine on steps to −30 mV were due to blockade of the contaminant L current rather than an effect on the T current, we next examined the effects of 300 nM nifedipine on the *I–V* relationship. Cells were held at −100 mV and depolarised from −80 through to +50 mV in 10 mV increments. Figure 4*A* shows representative currents from −70 to 0 mV, obtained before (left trace) and during (middle trace) the application of nifedipine. The nifedipine-sensitive difference currents were obtained by subtraction and are shown at the right panel of Fig. 4*A*. A summary of six similar experiments in cells taken from five patients is shown in Fig. 4*B*. Prior to the application of nifedipine (open squares), currents activated at ~−70 mV. Application of nifedipine (filled squares) had little effect on the amplitude of currents evoked at potentials negative to −30 mV, but significantly reduced the currents obtained at potentials positive to −30 mV. The nifedipine-sensitive currents (filled triangles) activated at ~−40 mV, peaked at 0 mV and reversed positive to +40 mV, consistent with the idea that nifedipine blocks mainly the L-type current.

Effect of Ni^{2+}

To test the possibility that the currents activated at negative potentials were due to the activation of T-type Ca^{2+} channels, we next examined the effects of a variety of concentrations of Ni^{2+} on both components of current. The inset of Fig. 5*A* shows the typical double-pulse protocol used to activate peak T and L currents at −30 and 0 mV, respectively. Figure 5*A* shows the effects of increasing concentrations of Ni^{2+} on both currents. Although Ni^{2+} reduced both currents in a concentration-dependent manner, the T current was more sensitive to Ni^{2+} and was reduced by 70 % in the presence 10 μM Ni^{2+}. In contrast, the same concentration of Ni^{2+} reduced the L-type current

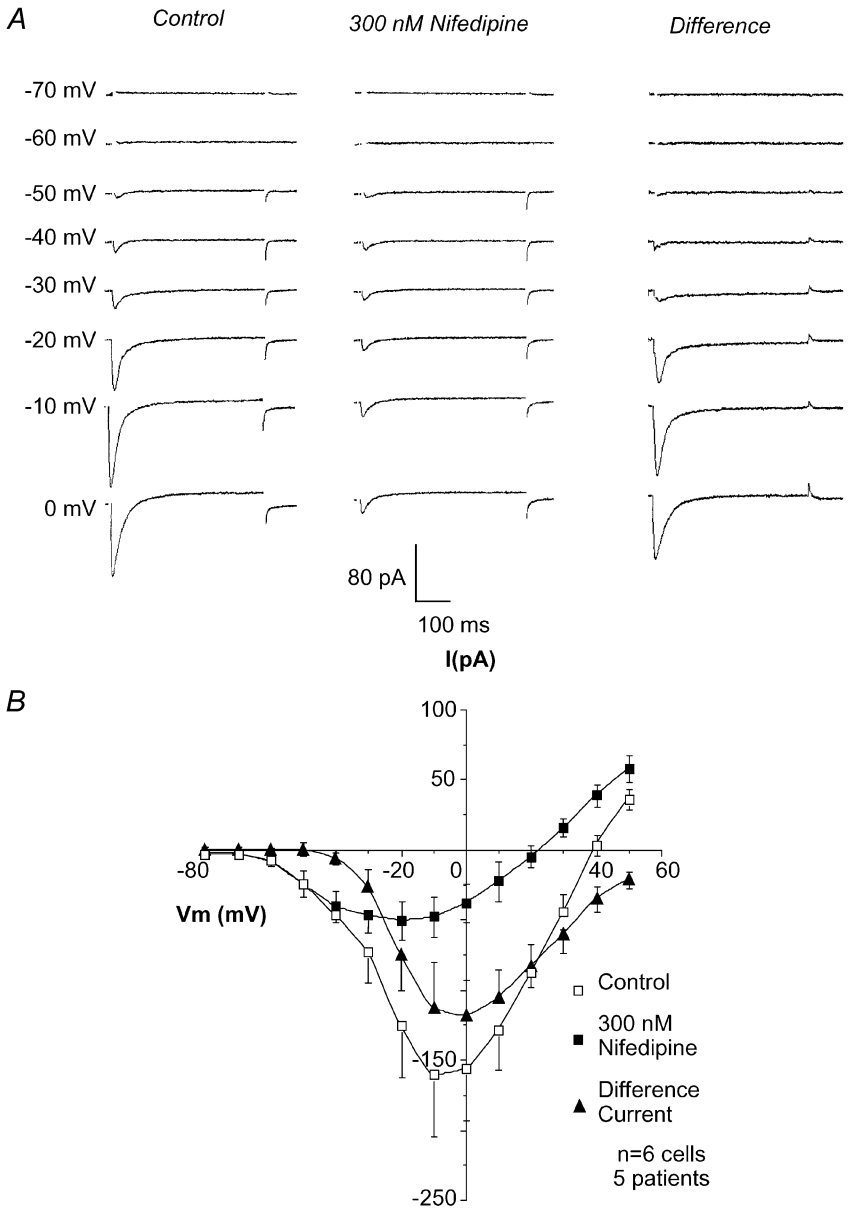

Figure 4. Application of 300 nM nifedipine unmasks the T current

A, currents obtained by depolarising from −70 through to 0 mV from a holding potential of −100 mV under control conditions (left panel) and during application of nifedipine (middle panel). The nifedipine-sensitive current ('Difference', right panel) was obtained by subtraction. *B*, a summary *I–V* curve for six cells obtained from five patients before (open squares) and during (filled squares) the application of 300 nM nifedipine. The nifedipine-sensitive current (filled triangles) was obtained by subtracting the middle panel from the right panel in *A*. This current activated at ~−40 mV and peaked at 0 mV, whereas the nifedipine-resistant current (filled squares) activated at ~−60 mV and peaked at −20 mV.

by ~35 %. Figure 5*B* shows a summary concentration–effect curve for the effect of Ni^{2+} on the T (filled circles) and L currents (open circles) in seven cells taken from four patients. When the data were fitted with Langmuir equations, the ED_{50} for the T current (filled circles) was 5.9 ± 1 μM, whereas that for the L current (open circles) was 65 ± 16 μM.

Effect of equimolar substitution of Ca^{2+} with Ba^{2+}

We next examined the effect of replacing external Ca^{2+} with Ba^{2+}, since a number of studies have demonstrated that Ba^{2+} is equipermeant to Ca^{2+} through T-type Ca^{2+} channels, but more permeant than Ca^{2+} through L-type channels. The trace in Fig. 6*A* shows the results from an experiment in which T and L currents were evoked by steps to −30 and 0 mV, respectively. Under control conditions, in this cell, both currents had similar amplitudes. When Ca^{2+} was substituted with equimolar (1.8 mM) Ba^{2+}, the amplitude of the T current was little affected, but the amplitude of the L current was dramatically increased and its time-dependent inactivation was slowed. Figure 6*B* shows a summary bar chart for six similar cells obtained from four patients. Under control conditions the peak T current was −83 ± 16 pA, while in the presence of 1.8 mM Ba^{2+} it was −91 ± 12 pA (n.s.; $P > 0.05$). In contrast, the peak L current was −141 ± 38 pA in 1.8 mM Ca^{2+}, increasing to −237 ± 44 pA when Ca^{2+} was substituted with Ba^{2+} ($P < 0.05$).

Effect of removing external Ca^{2+}

The data presented so far support the idea that human urethral smooth muscle cells possess both T- and L-type Ca^{2+} channels. We next wanted to observe the effect of Ca^{2+} removal on the amplitude of the peak inward currents evoked by steps to −30 and 0 mV. Figure 7*A* shows that when external Ca^{2+} was substituted with Mg^{2+} (+5 mM EGTA), the amplitude of the currents at −30 and 0 mV was reduced but not abolished. The persistence of this current in the absence of Ca^{2+} is illustrated by the summary data in Fig. 7*B*, which were obtained from seven cells from five patients. Under control conditions, steps to −30 and 0 mV evoked peak T and L currents of −47 ± 7 and −82 ± 11 pA, respectively. Following removal of external Ca^{2+}, the amplitude of the peak T and L currents was significantly reduced to −11 ± 3 and −3 ± 4 pA, respectively ($P < 0.05$). The persistence of the currents in Ca^{2+}-free conditions could imply that the remaining currents were not carried by Ca^{2+}. However, a number of studies have demonstrated that Ca^{2+} channels become permeable to monovalent cations in the absence of external Ca^{2+}, and that this current is blocked in a concentration-dependent manner by Mg^{2+} (Fukushima & Hagiwara, 1985).

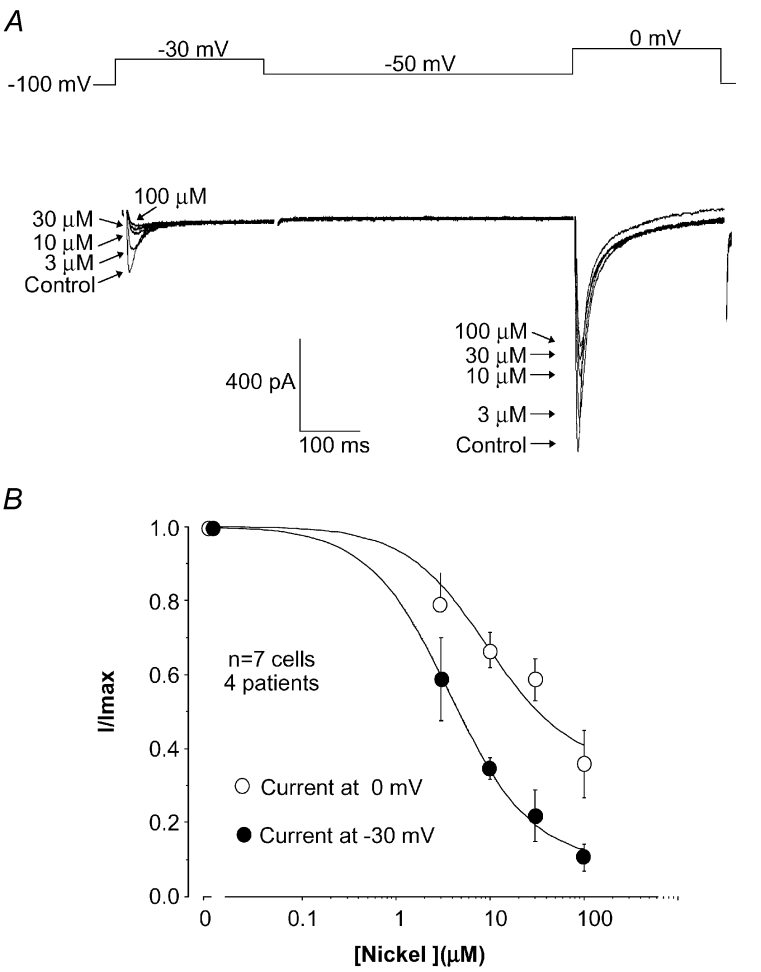

Figure 5. Effects of Ni^{2+} on T and L currents

A, peak T and L currents were evoked using the protocol described in Fig. 3*A*. Application of 10 μM Ni^{2+} practically abolished the T current, but reduced the L current by only 30 %. *B*, summary concentration–effect curves, with data obtained from seven cells (four patients). When the data obtained for T currents (filled circles) and L currents (open circles) were fitted with the Langmuir equation (continuous lines), this yielded an ED_{50} of 5.9 ± 1 μM and 65 ± 16 μM, respectively.

Effect of divalent-free solutions

To test the aforementioned possibility that Mg^{2+} is able to block the T current in a concentration-dependent manner, we examined the effects of removing external Mg^{2+} from Ca^{2+}-free solutions on the amplitude of the T current. All experiments were carried out in the presence of 300 nM nifedipine to block the L current. Figure 8*A* shows a typical example in which T currents were evoked by a step from −100 to −30 mV in Ca^{2+}-free solution before and during the removal of external Mg^{2+}. In six cells (from three patients), removal of external Mg^{2+} increased the amplitude of the current from −18 to −779 pA ($P < 0.05$). Figure 8*B* shows a summary *I–V* plot of the currents after removal of external Mg^{2+} (filled circles) from the bathing solution. Under these conditions, the current amplitude increased dramatically at all potentials and the peak current was shifted to approximately −40 mV. Although the currents peaked at more negative potentials than those recorded in normal Hanks' solution (cf. Fig. 4*B*), this was not associated with any alteration in the voltage dependence of inactivation. In five cells taken from three patients, the $V_{½}$ of inactivation was −79 ± 6 mV, a value that is not significantly different from that obtained in normal Hanks' solution (−80 ± 3 mV, $n = 8$ cells, five patients). These data are consistent with the idea that the currents observed in divalent-free Hanks' solution reflect the flow of monovalent cations through T channels.

We next quantified the effects of reintroducing Mg^{2+} to the bathing solution on the amplitude of the currents evoked by a step from −100 to −30 mV. Figure 8*C* shows a typical experiment in which a cell was repeatedly depolarised to −30 mV in the absence of divalent cations and then in the presence of increasing concentrations of Mg^{2+}. Re-introduction of Mg^{2+} caused a concentration-dependent decrease in the amplitude of the currents recorded in Ca^{2+}-

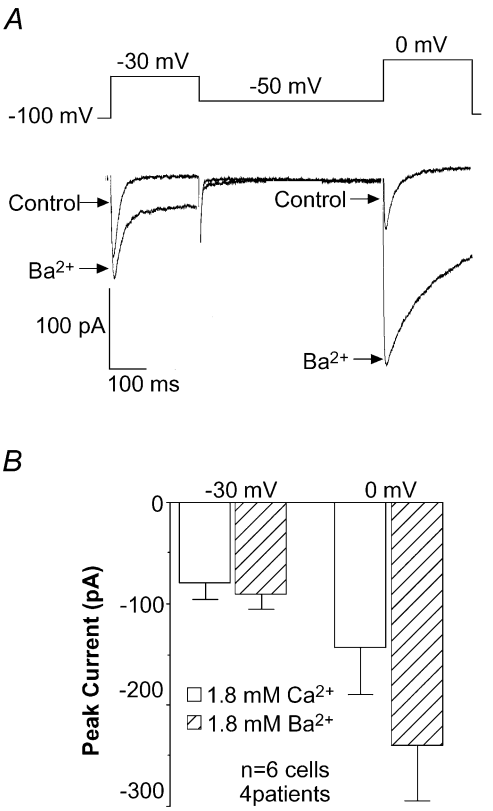

Figure 6. Effects of Ba^{2+} on T and L currents

A, T and L currents were elicited using the protocol described in Fig. 3*A*. Substitution of external Ca^{2+} with equimolar Ba^{2+} increased the amplitude and slowed the decay of the current evoked by a step from −50 to 0 mV, but had little effect on the amplitude of the current evoked by a depolarisation to −30 mV from a holding potential of −100 mV. *B*, a summary bar chart for six cells obtained from four patients. The bars show peak T and L currents obtained before (open bars) and during (hatched bars) equimolar substitution of external Ca^{2+} with Ba^{2+}.

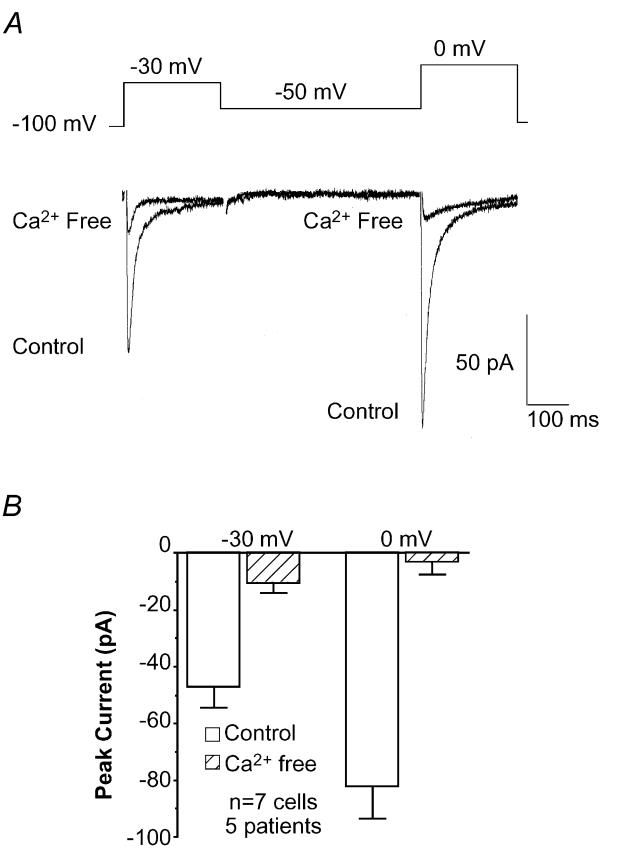

Figure 7. Persistence of inward currents after removal of external Ca^{2+}

A, typical T and L currents evoked by the protocol described in Fig. 3*A*. When external Ca^{2+} in the bathing solution was substituted with equimolar Mg^{2+} (+5 mM EGTA), the amplitudes of the currents were reduced at both potentials, but not abolished. *B*, a summary bar chart from seven cells (five patients) of the peak T and L currents before (open bars) and after (hatched bars) removal of external Ca^{2+}.

free bathing solution, consistent with the idea that Mg^{2+} blocks the flow of monovalent cations through T channels. Figure 8*D* shows a summary concentration–effect curve that was produced using the data obtained in six cells from three patients. When these data were fitted with the Langmuir equation, this yielded an ED_{50} of 36 ± 16 μM.

DISCUSSION

To the best of our knowledge, this study is the first to examine the pharmacology and electrophysiology of freshly dispersed smooth muscle cells from the human proximal urethra. Our data support the idea that the currents share characteristics typical of T- and L-type Ca^{2+} currents. It is unlikely that the LVA currents in urethral myocytes were due to fast Na^{+} channels (Muraki *et al.* 1991; Hollywood *et al.* 1997) or the novel voltage-activated non-specific cation current described recently (Koh *et al.* 2001), since neither application of TTX nor reduction in external Na^{+} significantly reduced the currents.

We believe that the LVA and HVA currents in human myocytes represent the activation of T and L channels, similar to those described in a variety of cells including smooth muscle (for review see Janssen, 1997; Klockner *et al.* 1999; Kotlikoff *et al.* 1999; Bradley *et al.* 2003). Voltage-dependent Ca^{2+} channels play a central role in the rapid entry of Ca^{2+} into smooth muscle, and thus act as critical

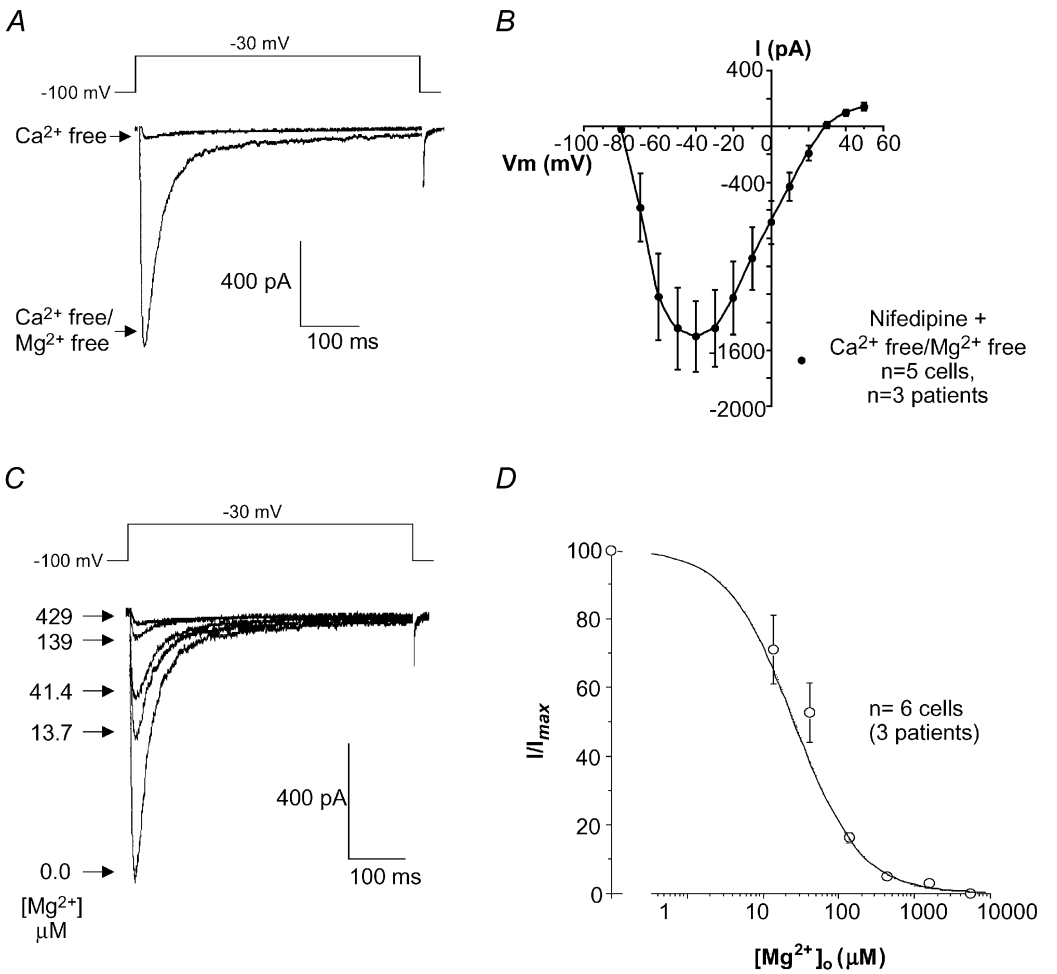

Figure 8. Characteristics of monovalent current through T channels

A, currents elicited by a step to −30 mV from a holding potential of −100 mV in the absence of external Ca^{2+}. All experiments were carried out in the presence of 300 nM nifedipine to block any contaminant L current. Removal of divalent cations from the bathing solution dramatically enhanced the amplitude of the inward current. *B*, a summary *I–V* curve of currents from five cells (three patients) recorded in the absence of divalent cations (filled circles). Under these conditions, the amplitude of the current was dramatically enhanced and the peak current was shifted to ~−40 mV. *C*, the concentration-dependent reduction of divalent current following the reintroduction of Mg^{2+} into the bathing solution. *D*, the concentration-dependent inhibition of divalent current by Mg^{2+} obtained from six cells (three patients). The continuous line represents a Langmuir fit to the data, which yielded an ED_{50} of 36 ± 16 μM.

determinants of cellular excitability. T currents can be readily discriminated from L currents on the basis of their rapid inactivation, activation at relatively low voltages (between −80 and −60 mV), inactivation over negative voltage ranges ($V_{½}$ between −90 and −70 mV), equal permeability to Ca^{2+} and Ba^{2+} and their insensitivity to dihydropyridines (Kotlikoff *et al.* 1999; Perez-Reyes, 2003). The T currents that could be evoked in human urethra cells at potentials positive to −70 mV were rapidly inactivating with a $V_{½}$ of −78 mV. In contrast, the L currents were activated at ~−40 mV and inactivated more slowly with a $V_{½}$ of −45 mV. Although the reversal potentials of the T and L currents were different (T current ~20 mV, L current ~45 mV), this has been noted in other studies (e.g. Staes *et al.* 2001). Fukushima & Hagiwara (1985) demonstrated that T channels pass significant outward K^+ currents at potentials positive to +20 mV in the presence of normal external Ca^{2+}. Although we have not assessed whether our T currents conduct K^+ at these potentials, it is possible that such a mechanism may help to explain the observed difference in reversal potentials of the T and L currents in human urethral myocytes.

In the present study we chose a voltage protocol that stepped from −100 to −30 mV, and then stepped from −50 to 0 mV to elicit T and L currents, respectively. We have chosen to interpret these currents as L-type and T-type Ca^{2+} currents, respectively. However, it is clear from the data presented in Figs 1 and 2 that L and T currents could contaminate the currents observed at −30 and 0 mV, respectively. We estimate that up to 30 % of the current observed at −30 mV may be due to contaminant L current. In contrast, a step from −50 mV to 0 mV would evoke an L current where less than 15 % of the current would be due to contaminant T current.

The effect of a variety of pharmacological interventions further supported our contention that both T and L currents were present, and also helped us to discriminate between each component of the current. Thus, application of 300 nM nifedipine reduced the L current by ~90 %, but reduced the current evoked by a step to −30 mV by ~30 %. This effect of nifedipine at −30 mV is consistent with blockade of some contaminant L current at −30 mV rather than a direct effect on the T current itself, since it had little effect on the currents evoked at potentials negative to −30 mV (Fig. 3*B*)

A number of studies have demonstrated that Ni^{2+} can block T-type channels (Sui *et al.* 2001; Bradley *et al.* 2003), but can also inhibit L-type channels at higher concentrations (> 100 μM; Hobai *et al.* 2000; Petkov *et al.* 2001). In our cells, application of 10 μM Ni^{2+} reduced the peak T current by ~70 % but also decreased the L current by ~30 %. When concentration–effect curves were obtained, there was only a 10-fold difference in the ED_{50} of Ni^{2+} for T *versus* L currents, suggesting that Ni^{2+} is a rather poor discriminator between both channel types in these human urethral cells. Although the selectivity of Ni^{2+} was poor, it was interesting that such low concentrations of Ni^{2+} were able to block the T current. Recent studies on cloned T channels (Klockner *et al.* 1999) have demonstrated in a variety of cells that at least three different $\alpha 1$ subunits (termed $\alpha 1_G$, $\alpha 1_H$ and $\alpha 1_i$) may underlie the T currents. Although each of the subunits has similar steady-state activation/inactivation characteristics when expressed in oocytes, the $\alpha 1_H$ subunit is approximately 10-fold more sensitive to Ni^{2+} (ED_{50} ~14 μM, Lee *et al.* 1999). It is appealing to speculate that the main pore-forming $\alpha 1$ subunits in human urethral myocytes may by the $\alpha 1_H$ subunits, although this will require confirmation.

We also examined the effects of substituting external Ca^{2+} with Ba^{2+} on both currents. Hess & Tsien (1984) demonstrated that Ba^{2+} permeates L channels more easily than does Ca^{2+}, whereas Ba^{2+} is thought to be as permeant as Ca^{2+} through T channels (Bean, 1985; Fukushima & Hagiwara, 1985). When both currents were examined in the presence of Ba^{2+}, the amplitude of the T current was only increased by ~10 %, an effect that is presumably due to the small amount of L current activated at this potential. In contrast, the L current amplitude was enhanced by ~100 % and the rate of decay was dramatically slowed. These data provide further evidence to suggest that the T current was different to the cation current demonstrated by Koh *et al.* (2001) in the murine colon, since those currents were abolished in the presence of Ba^{2+}.

When we examined the effects of Ca^{2+} removal on our inward currents, we found that a small component of the inward current remained. However, in the absence of external Ca^{2+}, both T- and L-type Ca^{2+} channels become 'promiscuous' by conducting monovalent cations, and this conductance is blocked by low concentrations of divalent cations (Fukushima & Hagiwara, 1985). When experiments were conducted in the absence of Ca^{2+} and in the presence of nifedipine to block L currents, small T currents could be evoked by a step to −30 mV. These were enhanced by ~40-fold in divalent-cation-free conditions, an effect similar to that shown previously in chick sensory neurones (Lux *et al.* 1990) and B lymphocytes (Fukushima & Hagiwara, 1985). Since the conduction of monovalent cations through Ca^{2+} channels is blocked in a concentration-dependant manner by external Mg^{2+} (Fukushima & Hagiwara, 1985; Lux *et al.* 1990), we next quantified its effects on the T current. We found that the monovalent current through T channels was blocked with an ED_{50} of 26 μM, which is similar to the figures of 30 and 25 μM that were reported by Fukushima & Hagiwara (1985) and Lux *et al.* (1990), respectively.

Previous studies have demonstrated the presence of T- and L-type Ca^{2+} currents in rabbit urethral myocytes as well as guinea-pig and human detrusor smooth muscle cells (Gallegos & Fry, 1994; Sui *et al.* 2001; Kajioka *et al.* 2002; Bradley *et al.* 2003). Although in the present study, the electrophysiological characteristics of the T currents in human urethral myocytes were quite similar to those of the rabbit urethra (Bradley *et al.* 2003), they differed significantly from the T and L currents recorded in human detrusor myocytes (Gallegos & Fry, 1994; Sui *et al.* 2001; Kajioka *et al.* 2002). In particular, the $V_{½}$ of activation and inactivation for both T and L currents in human and rabbit urethral cells appear to be approximately 15 mV more negative than those recorded in the human detrusor myocytes (Sui *et al.* 2001). Whether this reflects genuine differences in channel characteristics between the detrusor and urethral smooth muscle or is simply due to different recording conditions is unclear at present. However, it is interesting to note that the sensitivity of L and T channels to nifedipine and Ni^{2+} differ in detrusor myocytes. Kajioka *et al.* (2002) have recently demonstrated in human detrusor cells that the L current is inhibited by nifedipine with an ED_{50} of ~22 nM, which is much lower than that reported for human urethral myocytes (~160 nM). Similarly, the T current in the urethra is almost completely blocked by 10 μM Ni^{2+}, whereas detrusor myocytes require 100 μM Ni^{2+} to block the T current (C. H. Fry, personal communication). Such a difference in the pharmacology of both channel types in the urethra and bladder may reflect important differences in the ion channels in both tissues, and may allow the design of therapeutic interventions that are selective for the bladder or urethra.

The physiological role of both T and L currents in the human urethral myocytes is, as yet, unknown. Sui *et al.* (2001) have suggested that the T current in detrusor myocytes from the guinea-pig and human play a role in refilling Ca^{2+} stores, or perhaps permit the generation of oscillatory Ca^{2+} spikes in these cells. The degree of overlap between the T and L window currents demonstrated in the present study certainly suggests that these currents could contribute to the generation of oscillatory Ca^{2+} spikes. The T window current extended from approximately −80 to −20 mV, with the maximum current occurring at −60 mV. In contrast, the L-type window current extended from −50 to −10 mV, and the maximal overlap occurred at ~−30 mV. Consequently, relatively small depolarisations would be required to elevate the membrane potential sufficiently to activate T currents, bring the membrane potential into the threshold range for L-current activation and thus set off a series of oscillatory Ca^{2+} spikes. Conversely, inhibition of the T current would necessitate larger depolarisations to shift the membrane potential into the L current window range necessary for action potential generation. Bradley *et al.* (2003) used intracellular microelectrode recording techniques to demonstrate that pharmacological inhibition of the T current reduced the frequency of spontaneous action potentials in strips of rabbit urethra. This reduction is consistent with the aforementioned mechanism and it is of interest to speculate that the T current in human urethral myocytes may play a similar role. However, the exact role of the Ca^{2+} currents will only be clarified when the effects of pharmacological blockade of each current type is performed using intracellular recordings from human proximal urethra.

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