Ca^{2+} current and Ca^{2+}-activated chloride current in isolated smooth muscle cells of the sheep urethra

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1. Isolated sheep urethral cells were studied using the perforated patch clamp technique ($T = 37$ °C). Depolarizing steps ranging from −40 to −10 mV evoked an inward current that peaked within 10 ms and a slower inward current. Stepping back to the holding potential of −80 mV evoked large inward tail currents. All three currents were abolished by nifedipine (1 μM). Substitution of external Ca^{2+} with Ba^{2+} resulted in potentiation of the fast inward current and blockade of the slow current and tails.

2. Changing the chloride equilibrium potential (E_{Cl}) from 0 to +27 mV shifted the reversal potential of the tail currents from 1 ± 1 to 27 ± 1 mV (number of cells, $n = 5$). Chloride channel blockers, niflumic acid (10 μM) and anthracene-9-carboxylic acid (9AC, 1 mM), reduced the slow current and tails suggesting that these were Ca^{2+}-activated Cl^- currents, $I_{Cl(Ca)}$.

4. Caffeine (10 mM) induced currents that reversed at E_{Cl} and were blocked by niflumic acid (10 μM).

5. In current clamp mode, some cells developed spontaneous transient depolarizations (STDs) and action potentials. Short exposure to nifedipine blocked the action potentials and unmasked STDs. In contrast, 9AC and niflumic acid reduced the amplitude of the STDs and blocked the action potentials.

6. In conclusion, these cells have both L-type I_{Ca} and $I_{Cl(Ca)}$. The former appears to be responsible for the upstroke of the action potential, while the latter may act as a pacemaker current.

The urethra generates spontaneous myogenic tone which is thought to play an important part in maintaining urinary continence (Bridgewater, MacNeill & Brading, 1993). Although the underlying mechanisms are poorly understood, it has recently been suggested that both Ca^{2+} current (I_{Ca}) and Ca^{2+}-activated chloride currents ($I_{Cl(Ca)}$) are involved in generating electrical 'slow waves' in the rabbit urethra (Hashitani, Van Helden & Suzuki, 1996). $I_{Cl(Ca)}$ has been reported in a wide range of vascular and visceral smooth muscle types, where it is normally thought to mediate the effects of certain excitatory agonists (Large & Wang, 1996). In the urethra and in lymphatic vessels, however, $I_{Cl(Ca)}$ has been proposed to act as a pacemaker current, giving rise to spontaneous transient depolarizations (STDs), which then initiate either 'slow waves' or Ca^{2+} action potentials, respectively (Van Helden, 1993; Hashitani *et al.* 1996). To date, there have been few patch clamp studies of currents of urethral cells, and these have mainly focused on K_{ATP} channels (ATP-sensitive K^+ channels; Teramoto & Brading, 1996, 1997). Therefore, there is still no direct evidence that urethral cells possess $I_{Cl(Ca)}$, and I_{Ca} has yet to be characterized in this tissue. The purpose of the present study was to characterize I_{Ca} and seek evidence for the existence of $I_{Cl(Ca)}$ in urethral cells using the perforated patch clamp technique. We will also present some evidence suggesting that both of these currents are involved in the generation of spontaneous electrical activity in isolated urethral cells.

A preliminary account of this work has been reported to the Physiological Society (Cotton, Hollywood, McHale & Thornbury, 1997).

METHODS

Bladders and urethras of sheep of either sex were obtained from an abattoir approximately 15 min after slaughter and transported to the laboratory in Krebs solution at 20 °C. The most proximal 1 cm of the urethra was removed and 0·5 cm strips of smooth muscle dissected free and cut into 1 mm^3 pieces. The methods for cell isolation were similar to those described previously (Cotton, Hollywood, Thornbury & McHale, 1996). The tissue pieces were stored in Hanks Ca^{2+} free solution (see below) for 30 min after

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which they were incubated in an enzyme medium containing (per 5 ml of Hanks Ca^{2+}-free solution): collagenase, 15 mg (Sigma type 1a); protease, 1 mg (Sigma type XXIV); BSA, 10 mg (Sigma); and trypsin inhibitor, 10 mg (Sigma); for approximately 40 min at 37 °C. They were then placed in Hanks Ca^{2+}-free solution and stirred for a further 15–30 min to release single relaxed smooth muscle cells. These were plated in petri dishes containing Hanks solution (100 μM Ca^{2+}) and stored at 4 °C for use within 8 h. Sometimes, on days when the abattoir was shut, cells were isolated from tissue that had been stored in physiological salt solution (PSS) for 24 h. The solutions used were of the following composition (mM): (1) Hanks Ca^{2+}-free solution: 141 Na^+, 5·8 K^+, 130·3 Cl^-, 15·5 HCO_3^-, 0·34 HPO_4^{2-}, 0·44 $H_2PO_4^-$, 10 dextrose, 2·9 sucrose and 10 Hepes, pH adjusted to 7·4 with NaOH: (2) PSS: 130 Na^+, 5·8 K^+, 135 Cl^-, 4·16 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 dextrose, 2·9 sucrose and 10 Hepes, pH adjusted to 7·4 with NaOH: (3) low chloride solution: 130 Na^+, 86 glutamate, 5·8 K^+, 49 Cl^-, 4·16 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 dextrose, 2·9 sucrose and 10 Hepes, pH adjusted to 7·4 with NaOH: (4) Cs^+ pipette solution: 133 Cs^+, 1 Mg^{2+}, 135 Cl^-, 0·5 EGTA and 10 Hepes, pH adjusted to 7·2 with CsOH: (5) K^+ pipette solution: 123 K^+, 1 Mg^{2+}, 55 Cl^-, 70 gluconate, 0·5 EGTA and 10 Hepes, pH adjusted to 7·2 with KOH. Recordings were made using the amphotericin B perforated patch method (Rae, Cooper, Gates & Watsky, 1991). Briefly, this consisted of dipping the tips of the patch pipettes in amphotericin-free pipette solution for a few seconds and then backfilling with pipette solution containing 6 μg ml^{-1} of amphotericin B (Sigma). After gigaseals were obtained the series resistance fell over a 10–15 min period to 10–15 MΩ and remained stable for up to 1 h. Series resistance and the capacitative surge were usually uncompensated. The maximal voltage error arising from the series resistance during test potentials (given maximal currents of around 200–300 pA) was in the range 2·0–4·5 mV. Although amphotericin pores are cation selective, the permeability for Cl^- is not negligible (Kleinberg & Finkelstein, 1984), so the reversal potential of Cl^- current was determined by the bath and pipette Cl^- concentrations, as described by Horn & Marty (1988).

Where stated, data were corrected for junction potentials of −3 mV in PSS and +2 mV in low chloride solution according to the method described by Neher (1992). Voltage clamp commands were delivered with an Axopatch 1D patch-clamp amplifier (Axon Instruments) and currents were recorded by means of a 12-bit AD/DA converter (Labmaster, Scientific Solutions, Solon, OH, USA) interfaced to an AT-type computer running pCLAMP software (Axon Instruments). During experiments the dish containing the cells was superfused

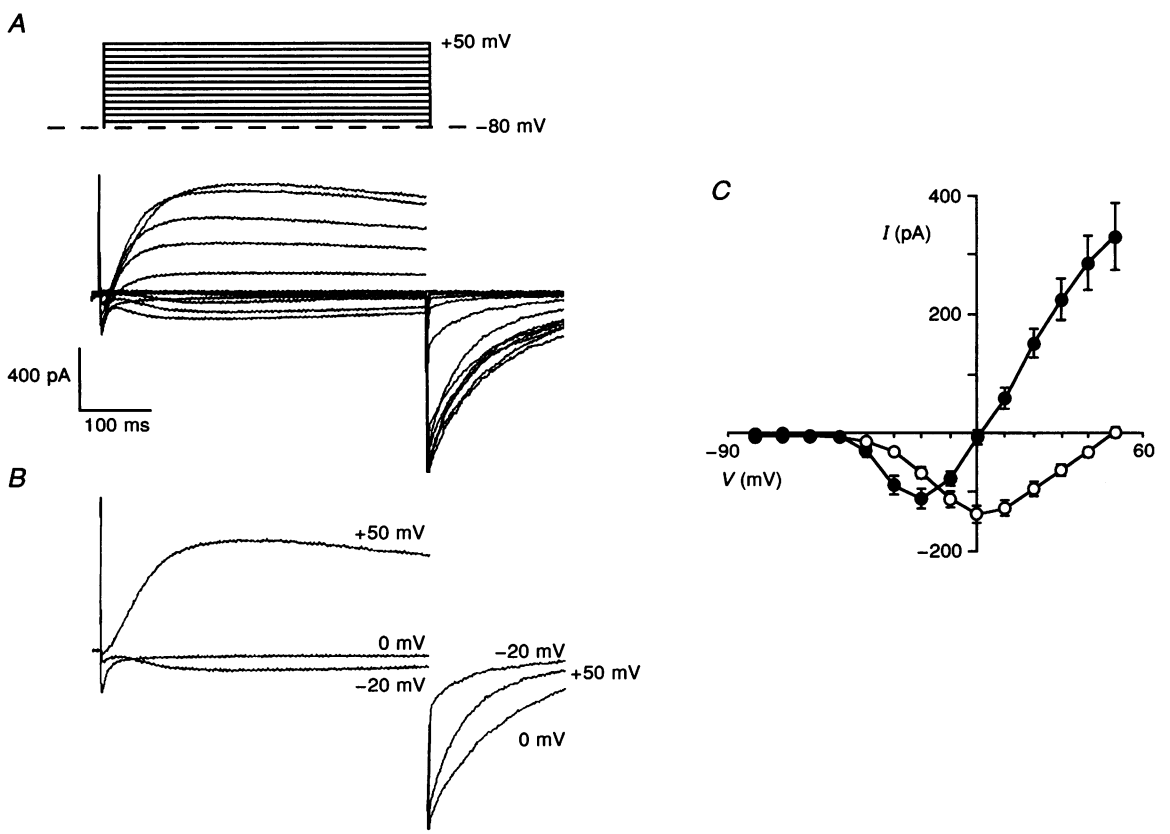

Figure 1. Membrane currents recorded from a urethral cell using the perforated patch technique with Cs^+-filled pipettes

A, family of currents recorded on stepping from −80 mV to potentals of −70 to +50 mV. Inward currents may be resolved into fast and slow components. Outward current was observed on stepping positive to 0 mV and large inward tails occured on returning to −80 mV after the test step. *B*, 3 steps taken from the same record as *A* to show the relationship between the test potential and the size of the tail current. *C*, summary current–voltage plots from 34 cells. ○ shows the fast inward current, measured near the beginning of the 500 ms test potential, and ● shows the slower current, measured near the end of the test potential.

with bath solution. In addition, the cell under study was continuously superfused by means of a closed 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 < 5 s, to a solution containing a drug. All experiments were carried out at 37 °C.

The following drugs were used: nifedipine (Bayer), caffeine (Sigma) niflumic acid (Sigma), anthracene-9-carboxylic acid (9AC; Sigma). Data are presented as means ± S.E.M., and statistical differences were compared using Student's paired t test, taking the $P < 0.05$ level as significant.

RESULTS

Passive properties

In general, the yield of cells following the dispersal procedure was modest, possibly reflecting the high ratio of collagen to smooth muscle in the urethra (Chen, Hu, Fan & Brading, 1993; Cotton, 1997), although the cells were relaxed and appeared healthy. Resting membrane potential was measured in current clamp mode using K^+-filled electrodes in eight cells and averaged −36 ± 2 mV. Input resistance was estimated in the same cells in voltage clamp mode by measuring the passive current responses to a series of hyperpolarizing and depolarizing voltage steps from a holding potential of −80 mV and averaged 1·4 ± 0·2 GΩ. Cell capacitance averaged 66 ± 5 pF (number of cells, $n = 8$), as calculated by integrating the capacitative current evoked by small hyperpolarizing and depolarizing voltage steps, and dividing by the amplitude of the voltage change.

I_{Ca} and $I_{Cl(Ca)}$

Voltage clamp experiments were performed using Cs^+-filled pipettes to characterize the inward currents evoked by depolarization. Cells were held at −80 mV and subjected to 500 ms test pulses ranging from −80 to +50 mV. Figure 1*A* shows an example of the currents evoked by this protocol. Steps to −40, −30, −20 and −10 mV evoked fast inward current (peaking within 10 ms) followed by a more slowly developing inward current. The fast current was maximal at 0 mV and reversed near +50 mV, while the slow current reversed at 0 mV and became a large outward current at positive potentials (Fig. 1*B*). Evidence presented below suggests that the fast current is an L-type Ca^{2+} current (I_{Ca}), while Ca^{2+}-activated Cl^- channels mediate the slowly developing inward and outward currents ($I_{Cl(Ca)}$). In addition to the currents evoked during the test step, large slowly decaying inward tail currents were observed on stepping back to −80 mV. These were less pronounced following the step to +50 mV than the step to 0 mV, suggesting that they were activated by influx of Ca^{2+} (Fig. 1*B*).

Data were pooled from control experiments described below, giving the summary current–voltage (I–V) relationships for I_{Ca} and $I_{Cl(Ca)}$ presented in Fig. 1*C* ($n = 34$). In these experiments, I_{Ca} was measured as the peak current that

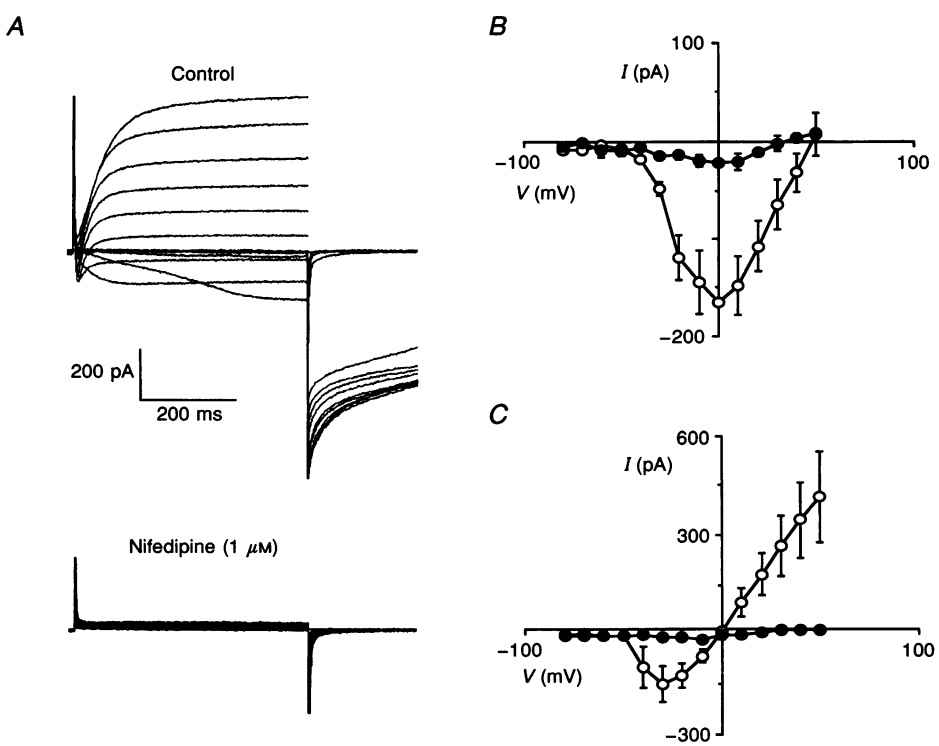

Figure 2. Effect of nifedipine

A, currents recorded using the same protocol as in Fig. 1 before (Control) and after nifedipine (1 μM). *B*, summary I–V plots ($n = 7$) of the fast inward current before (○) and after nifedipine (●). *C*, summary I–V plots ($n = 7$) of the slow current, measured near the end of the test potential before (○) and after nifedipine (●).

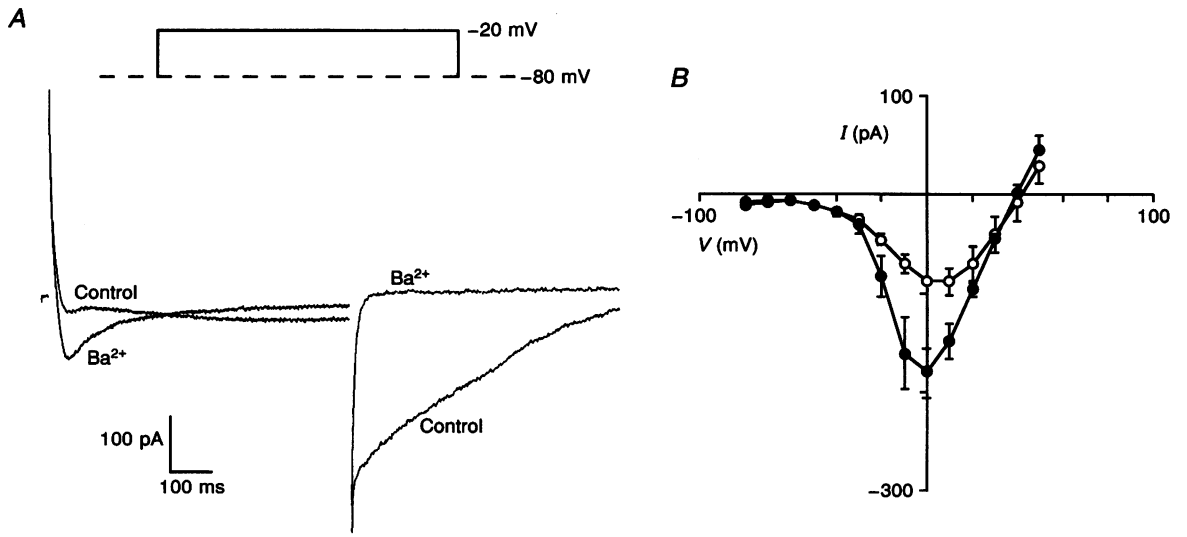

Figure 3. Substitution of external Ca^{2+} with Ba^{2+}

A, control recording was made in normal Ca^{2+} (1·8 mM). Replacement of Ca^{2+} with Ba^{2+} resulted in potentiation of the fast inward current, reduction of the slow current and abolition of the inward tail current. *B*, *I–V* plots of the fast inward current in 4 cells in 1·8 mM Ca^{2+} (○) and Ba^{2+} (●).

developed within the first 10 ms of the test potential, while $I_{Cl(Ca)}$ was measured at the end of the 500 ms test potential. This procedure was chosen so as to minimize contamination of $I_{Cl(Ca)}$ with I_{Ca}, which had almost fully inactivated by this time. However, it slightly underestimated $I_{Cl(Ca)}$ in some cases where $I_{Cl(Ca)}$ had already started to decline before the end of the 500 ms period. Figure 1*C* shows that I_{Ca} was maximal at 0 mV and reversed close to +50 mV, while maximal $I_{Cl(Ca)}$ occurred near −20 mV and reversed close to 0 mV, the calculated value for the Cl^- equilibrium potental (E_{Cl}).

Effect of nifedipine and Ba^{2+} substitution

The effect of nifedipine (1 μM) was examined on currents evoked by stepping from −80 mV to test potentials of −80 to +50 mV (Fig. 2*A*). In control conditions this yielded typical *I–V* relationships for I_{Ca} and $I_{Cl(Ca)}$ as described above. Application of nifedipine blocked all of the currents evoked under these conditions, including the tail currents (Fig. 2*A*, lower panel). Summaries of the effects of nifedipine on $I_{Cl(Ca)}$ and I_{Ca} in seven experiments are shown in Fig. 2*B* and *C*, where it is clear that nifedipine virtually abolished both currents.

Ba^{2+} is known to be a better charge carrier for L-type Ca^{2+} current than Ca^{2+} itself, but it did not activate $I_{Cl(Ca)}$ in rabbit oesophageal smooth muscle (Akbarali & Giles, 1993). The effect of substituting external Ba^{2+} for Ca^{2+} is shown in Fig. 3*A* and *B*, where it is clear that Ba^{2+} enhanced I_{Ca}, reduced the sustained inward current, and abolished the tails. Summary *I–V* relations for four experiments are shown in Fig. 3*B*, where it is clear that Ba^{2+} enhanced I_{Ca}.

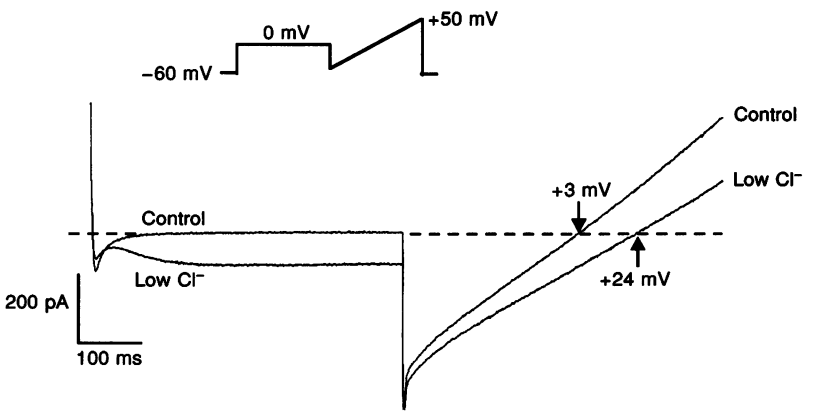

Figure 4. Reduction of external Cl^-

Cell was held at −60 mV and stepped to 0 mV to Ca^{2+} load. It was then ramped from −50 to +50 mV to record the reversal potential of the tail current. Recordings were made in 135 mM Cl^- (Control) and 49 mM Cl^- (low Cl^-).

Summary data for $I_{Cl(Ca)}$ is not shown as the effect on the inward component of this current was difficult to quantify because of sustained Ba^{2+} current. In each of the four experiments, however, tail currents were abolished and outward currents were greatly reduced by Ba^{2+}.

The nifedipine and Ba^{2+} results suggest that the fast current is mediated by L-type Ca^{2+} channels, and the slow current is activated by Ca^{2+} influx.

Effect of low Cl^- solutions and Cl^- channel blockers

To determine whether the slow current was carried by Cl^- ions, the effect of reducing the bath Cl^- concentration on the reversal potential of the tail current was studied. The protocol involved Ca^{2+} loading the cell by means of a step to 0 mV and then applying a voltage ramp from −50 to +50 mV (Fig. 4). This gave a tail current which reversed at +3 mV (0 mV when corrected for a −3 mV junction potential), which is equivalent to the calculated value for E_{Cl} (0 mV). The procedure was then repeated a few seconds after reducing external Cl^- to 49 mM (calculated $E_{Cl} = +27$ mV). This had the effect, not only of shifting the reversal potential of the tail current to +24 mV (+26 mV when corrected for the new junction potential, +2 mV), but also of inducing a slow inward current during the step to 0 mV. These observations suggest that both the slow inward current and the tail current were mediated by Cl^- channels. In a total of five experiments changing to the low chloride solution caused the appearance of the slow inward current at 0 mV, and shifted the reversal potential of the tail currents from 1 ± 1 to 27 ± 1 mV ($P < 0.05$).

The effects of several chloride channel blockers were also examined. Figure 5*A* and *B* shows the effect of niflumic acid (10 and 100 μM) on currents evoked by the stepping protocol described above. It is clear that this drug reduced both the inward and outward components of $I_{Cl(Ca)}$ while, in this example, having little effect on I_{Ca}. The tail currents recorded on stepping back to −80 mV were also reduced in amplitude, but their rate of decay was decreased. Niflumic acid caused a similar reduction in amplitude and prolongation of spontaneous transient inward currents (STICs) and tail currents in rabbit portal vein (Hogg, Wang & Large, 1994*a*,*b*; Greenwood & Large, 1996). A summary of the effect of niflumic acid (10 μM) on $I_{Cl(Ca)}$ in seven cells is shown in Fig. 5*B*, where it reduced the current throughout the potential range ($P < 0.05$). Also, in three out of the seven experiments niflumic acid (10 μM) had no effect on I_{Ca}, but in the other four experiments a variable degree of depression was seen. Overall, the mean I_{Ca} at 0 mV was −217 ± 72 pA before and −166 ± 49 pA after niflumic acid ($P < 0.05$; data not shown). In a separate series of experiments niflumic acid (10 μM) reduced the Ba^{2+} current evoked at 0 mV from −180 ± 22 to 134 ± 17 pA ($n = 6$, $P < 0.05$). 9AC (1 mM), another Cl^- channel blocker (Large & Wang, 1996), also reduced $I_{Cl(Ca)}$ during the test pulse, but only slightly reduced the tails on stepping back to −80 mV (Fig. 6*A*). This small effect on the tails may be

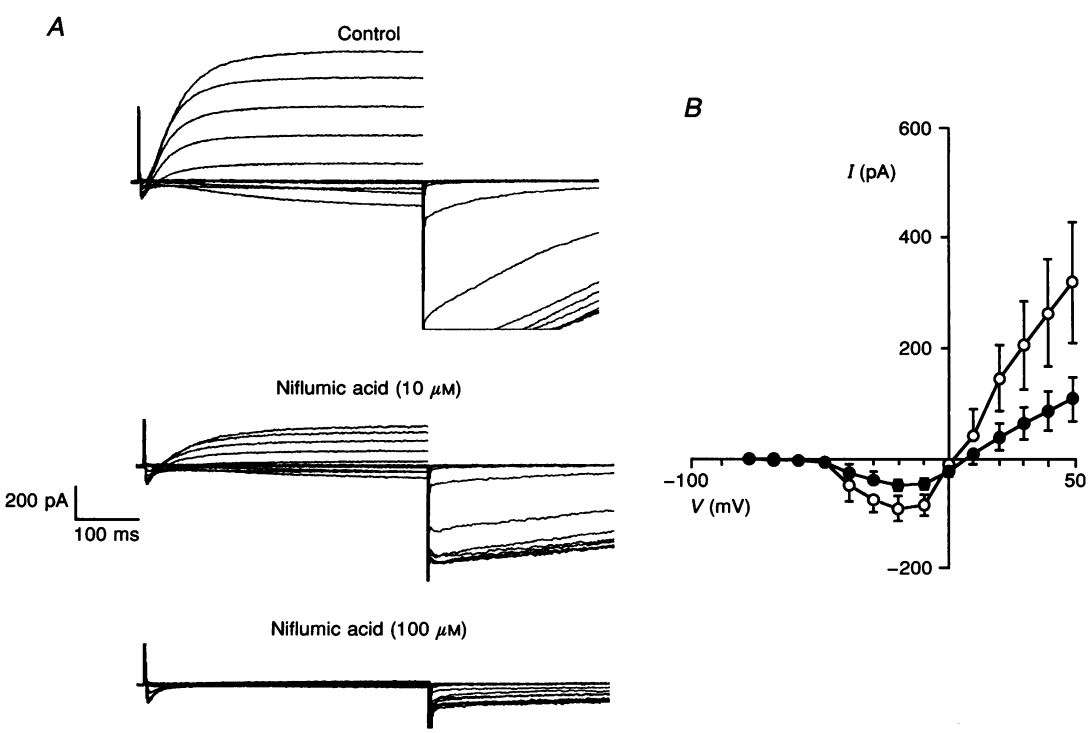

Figure 5. Effect of niflumic acid

A, families of currents recorded on stepping from −80 mV to test potentials of −70 to +50 mV in the absence of drugs (Control) and following exposure to 10 μM and 100 μM niflumic acid. *B*, summary *I–V* plots from 7 cells before (○) and after (●) 10 μM niflumic acid.

accounted for by the fact that 9AC blockade is voltage dependent and less effective at negative potentials (Hogg *et al.* 1994*a*). A summary of the effect of 9AC on $I_{Cl(Ca)}$ is shown in Fig. 6*B* ($n = 6$), where it blocked both inward and outward components of the current ($P < 0{\cdot}05$), although it was more effective on the latter. 9AC was without effect on I_{Ca} throughout the potential range (Fig. 6*C*).

Effect of Caffeine

Caffeine was used to determine whether Ca^{2+} release from intracellular stores could activate the Cl^- current. Application of caffeine (10 mM) for 10 s evoked an inward current when the cell was held at negative potentials and an outward current when the cell was held at positive potentials, with little current seen at 0 mV (Fig. 7*A*). The current decayed within 4–6 s of the onset application, despite the continued presence of caffeine. Caffeine responses were repeatable, provided 20 s was left between applications, otherwise reduced responses were obtained (data not shown). Figure 7*B* shows a summary *I–V* relationship for the caffeine-evoked current in three cells. The current reversed close to 0 mV, the calculated value for E_{Cl}, and showed little rectification. Niflumic acid (10 μM) reduced the caffeine-induced current (cell held at −40 mV), suggesting that it largely consisted of $I_{Cl(Ca)}$ (Fig. 7*C*). In four experiments, niflumic acid (10 μM) reduced the caffeine-induced current from -48 ± 12 to -12 ± 7 pA ($P < 0{\cdot}05$).

Inactivation of I_{Ca}

The voltage dependence of the inactivation of I_{Ca} was studied by subjecting cells to 2 s conditioning potentials ranging from −100 to 0 mV, before stepping to test potentials of 0 mV. The test step of 0 mV was chosen as (i) it was the potential where maximal I_{Ca} was observed, and (ii) under the conditions of these experiments 0 mV was the reversal potential of $I_{Cl(Ca)}$, therefore contamination from this current was negligible. Figure 8*A* shows a typical record where it is clear that the current available during the test step depended on the previous conditioning potential, and more than half of the current was inactivated following a conditioning potential of −30 mV. Figure 8*B* is a summary of seven such experiments where the normalized

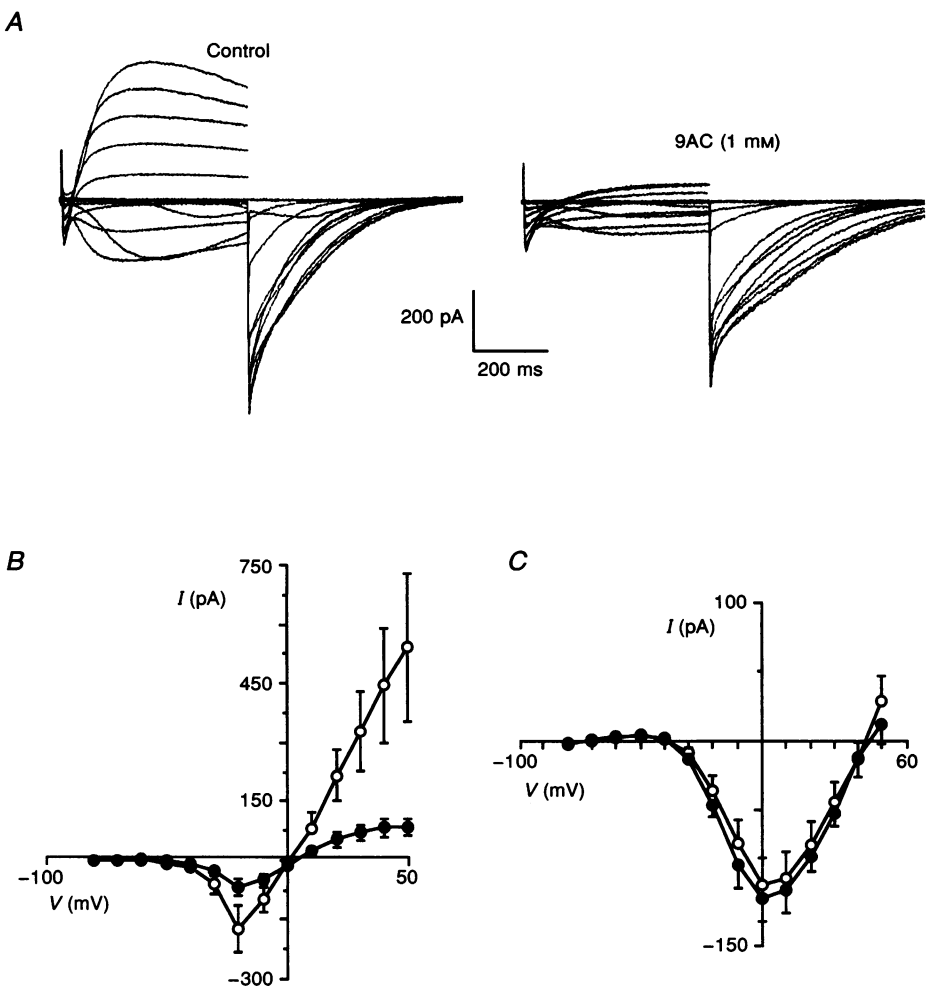

Figure 6. Effect of 9AC

A, families of currents recorded as in Fig. 5 before (Control) and after exposure to 9AC. *B* and *C*, summary *I–V* plots of $I_{Cl(Ca)}$ and I_{Ca} from 6 cells before (○) and after (●) 1 mM 9AC.

peak current during the test step is plotted against the previous conditioning potential. The data was fitted (continuous line) with a Boltzmann equation of the form:

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

where K^{-1} is the slope factor and $V_{½}$ is the voltage at which there is half-maximal inactivation. This yielded values of $-30{\cdot}0 \pm 2{\cdot}5$ mV for $V_{½}$ and $-7{\cdot}3 \pm 2{\cdot}2$ mV for K^{-1}. To study the voltage dependence of activation, the normalized conductance, obtained from the I–V relationship for I_{Ca} in the same seven cells, was plotted against the test potential to give the activation curve shown in Fig. 8*B*. This was fitted with the above Boltzmann function to give a voltage for half-maximum activation, $V_{½}$, of $-13{\cdot}4 \pm 3{\cdot}9$ mV and a slope factor of $7{\cdot}0 \pm 1{\cdot}3$ mV. The region of overlap between the two curves would result in a small sustained Ca^{2+} current (window current) in the range −40 to 0 mV.

Spontaneously active cells

Preliminary experiments indicated that some cells developed 9AC-sensitive spontaneous transient inward currents in voltage clamp mode (Cotton, 1997). To investigate the spontaneous activity further, cells were studied in current clamp mode using Cs^{2+} pipette solution, under which conditions they remained depolarized (resting potentials −11 to −3 mV). On injection of a steady background current to bring their potentials down to around −60 mV, approximately 10% of cells then fired regular action potentials as in the example in Fig. 9, and a further 20–30% fired irregularly. The action potentials consisted of a relatively fast upstroke and overshoot, followed by a plateau. Mean values for six cells were: maximum rate of rise, $1{\cdot}5 \pm 0{\cdot}1$ V s^{-1}; upstroke amplitude, $73{\cdot}1 \pm 1$ mV; duration, $2{\cdot}9 \pm 0{\cdot}1$ s; and frequency $8{\cdot}4 \pm 1{\cdot}8$ min^{-1}. When examined on an expanded time scale (Fig. 9*B*) the upstroke was seen to consist of an initially slower component, followed by the fast component, with the point of inflexion occurring at around −40 mV. Nifedipine (1 μM) abolished the fast component of the upstroke and the plateau, and unmasked an underlying depolarization, which resembled the STDs recorded from syncytial preparations of urethra using intracellular microelectrodes (Hashitani *et al.* 1996).

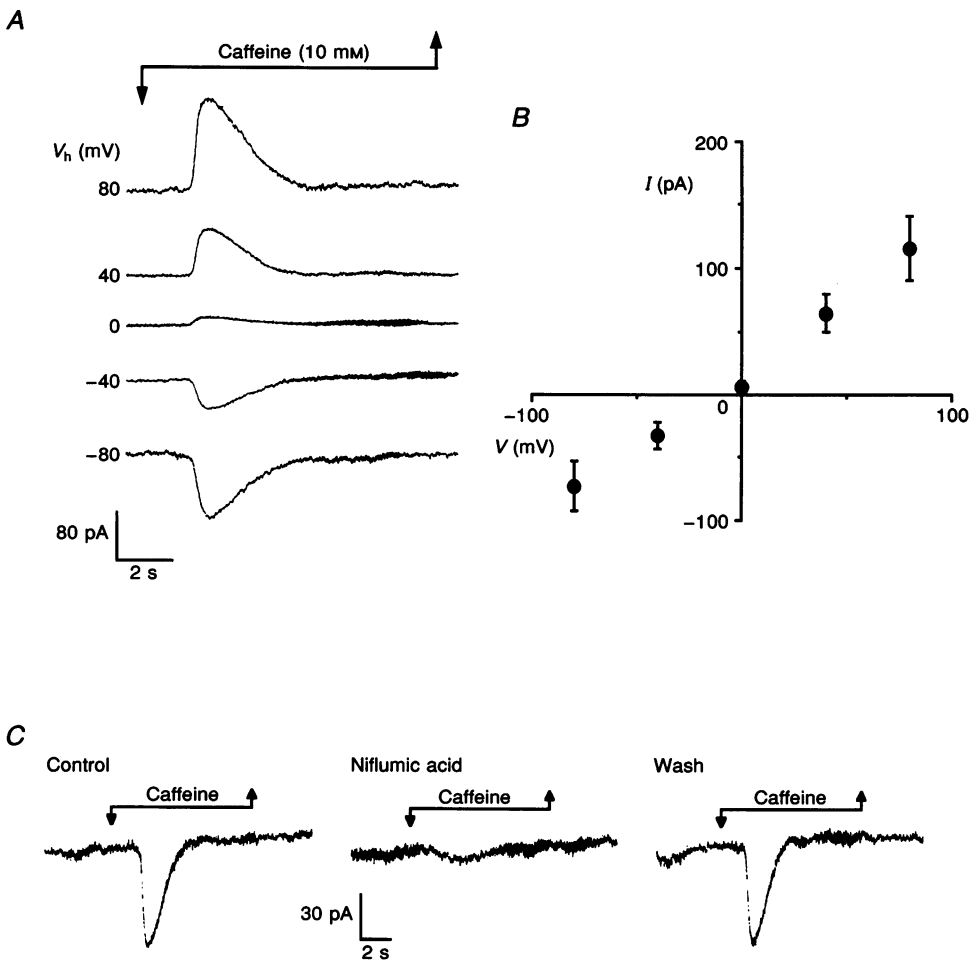

Figure 7. Effect of caffeine

A, caffeine (10 mM) was applied for 10 s periods to a cell held at a range of potentials (V_h). *B*, I–V plot of the caffeine-induced current in 3 cells. *C*, effect of caffeine (10 mM) alone (Control), in the presence of 10 μM niflumic acid, and after washout of niflumic acid (Wash).

The superimposed records in Fig. 9*B* suggest that the amplitude and rate of rise of the STD was unaffected by nifedipine. Similar effects of nifedipine were observed in a total of four cells.

Chloride channel blockers also blocked the action potential but, in contrast to nifedipine, they also clearly reduced the amplitude of the STDs. An example is shown in Fig. 10, where the cell was firing action potentials at a rate of 18 min^{-1} and, on addition of 9AC (1 mM), these rapidly disappeared leaving small STDs. A comparison before and after addition of the drug is shown in Fig. 10*B*, where it is clear that the amplitude of the STD was markedly reduced. Similar results were obtained with 9AC in a total of four cells, and in a further two cells where niflumic acid (10 μM) was used.

DISCUSSION

The smooth muscle of the urethra generates spontaneous tone which is enhanced by excitatory input from noradrenergic and cholinergic nerves (Awad & Downie, 1976; Thornbury, Hollywood & McHale, 1992; Bridgewater *et al.* 1993). Despite an earlier view that urinary continence is maintained mainly by striated muscle (Kuru, 1965), many people now believe that urethral smooth muscle normally makes the greater contribution (e.g. Awad & Downie, 1976; Bridgewater *et al.* 1993). It is of considerable interest, therefore, to understand the mechanisms underlying contraction of this muscle. In this paper we have described two currents which are likely to play an important part in generating urethral tone. Firstly, there is an L-type Ca^{2+} current which in many respects resembles the Ca^{2+} currents found in other smooth muscle preparations. This current activated at around −40 mV, had an inactivation $V_{½}$ of −30 mV, was blocked by nifedipine and was enhanced when Ba^{2+} was substituted for Ca^{2+} as the charge carrier. L-type Ca^{2+} currents are thought to play a part in raising cytosolic Ca^{2+} both directly by Ca^{2+} influx, and indirectly by causing Ca^{2+}-induced Ca^{2+} release. Our evidence suggests that the second current was mediated by Ca^{2+}-activated Cl^- channels as: (i) it reversed close to E_{Cl}; (ii) it was reduced by 9AC and niflumic acid, drugs which block $I_{Cl(Ca)}$ in other smooth muscle preparations (Large & Wang, 1996); (iii) it was blocked if I_{Ca} was blocked by nifedipine or if Ba^{2+} was substituted for Ca^{2+} (Janssen & Sims, 1995; Akbarali & Giles, 1993); and (iv) it could be activated by doses of caffeine which are known to release Ca^{2+} from intracellular stores (Mironneau, Arnaudeau, Macrez-Lepretre & Boittin, 1996). The sensitivity of the current to niflumic acid was within the range found in other smooth muscles (Large & Wang, 1996), although it is interesting to note that the specificity differed slightly in our case in that it appeared to slightly reduce I_{Ca}, whereas in rabbit oesphagus it showed no tendency to block I_{Ca} (Akbarali & Giles, 1993). The block of $I_{Cl(Ca)}$ by niflumic acid and 9AC appeared to be voltage dependent, being less effective at negative membrane potentials. This was particularly noticeable for 9AC, which, although significantly reducing the inward Cl^- currents recorded at −40 to −20 mV, had little effect on the inward tails recorded at −80 mV (Fig. 6). This is consistent with

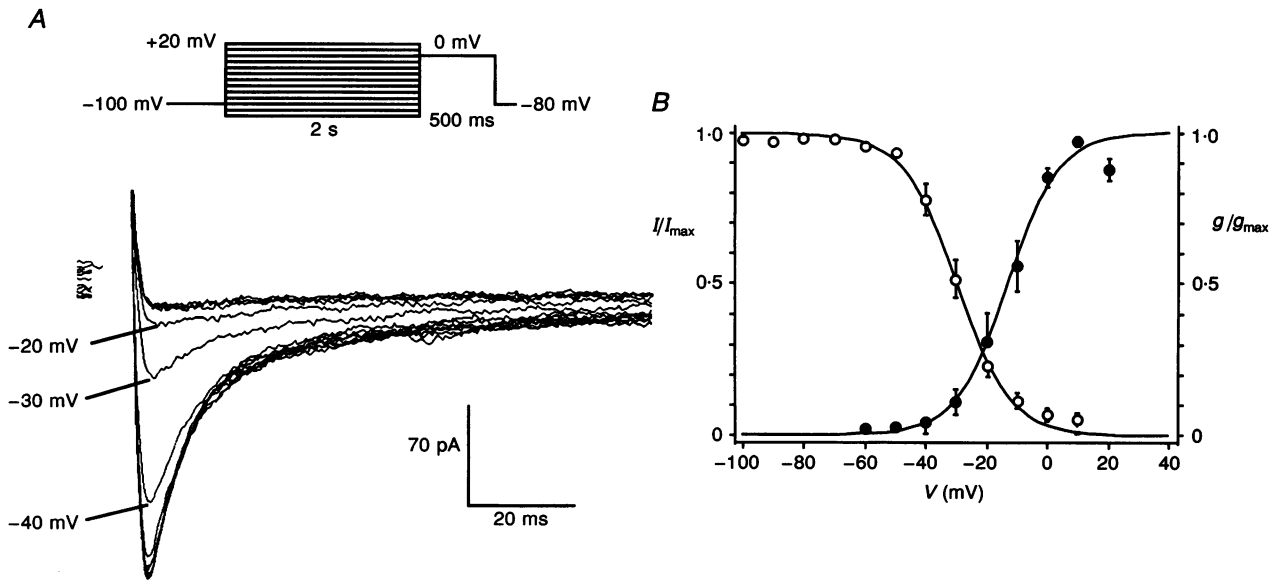

Figure 8. Voltage dependence of steady-state inactivation and activation of Ca^{2+} current

A, inactivation protocol where the cell was stepped to conditioning potentials ranging from −100 to +20 mV before stepping to a test potental of 0 mV. Current recorded during the test steps are shown, and the responses following conditioning potentials of −40, −30 and −20 mV are labelled. *B*, graph showing I/I_{max} measured using the above two-step protocol (○) and the normalized conductance g/g_{max} (●) obtained from the *I–V* relationship in 7 cells. Continuous lines represent Boltzmann fits of the data in each case. The curves intersected at −20 mV allowing a window current between −40 and 0 mV.

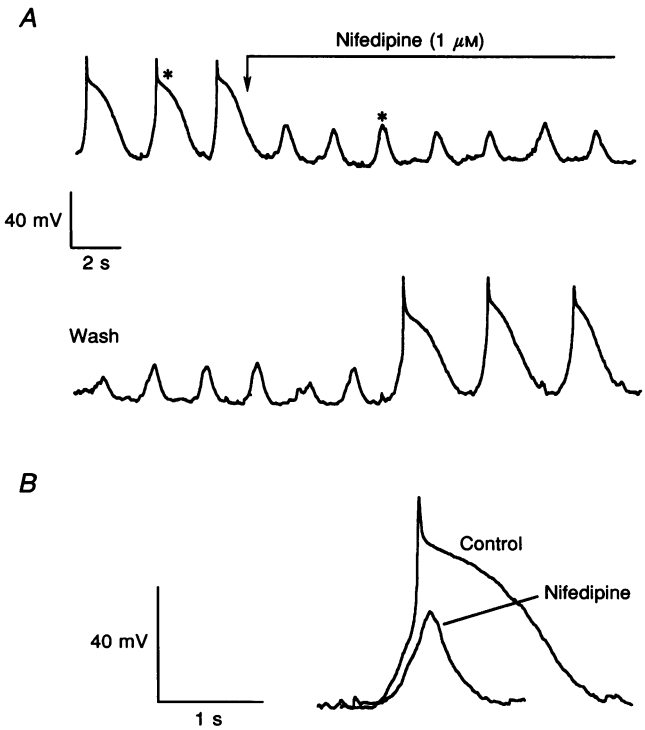

Figure 9. Effect of nifedipine on spontaneous electrical activity

A, recordings made in current clamp using Cs^+-filled pipettes. Cell was firing spontaneous action potentials at a frequency of 30 min^{-1}. Nifedipine (1 μM) blocked the action potentials to leave smaller spontaneous transient depolarizations (STDs). *B*, expanded action potential and STD taken from *A* (*) before and during nifedipine, respectively.

data from portal vein, where the block by 9AC was shown to be more voltage dependent than niflumic acid (Hogg *et al.* 1994*a*, *b*).

Generally, $I_{Cl(Ca)}$ and I_{Ca} are believed to work together to excite smooth muscle (Large & Wang, 1996). Pumping mechanisms are believed to maintain a high intracellular Cl^- concentration and E_{Cl} in the range −30 to −20 mV, resulting in membrane depolarization when Cl^- channels are activated (Aickin & Brading, 1982). The depolarization may then activate voltage-dependent Ca^{2+} channels with resultant Ca^{2+} influx and contraction. In many instances this sequence of events appears to be initiated by agonists causing Ca^{2+} release from intracellular stores (Large & Wang, 1996). Spontaneous release of Ca^{2+} from stores may also activate $I_{Cl(Ca)}$ and generate STICs in some smooth muscles (for review see Large & Wang, 1996). A similar mechanism is thought to result in STDs in the absence of agonists in mesenteric vein (Van Helden, 1991). While their role

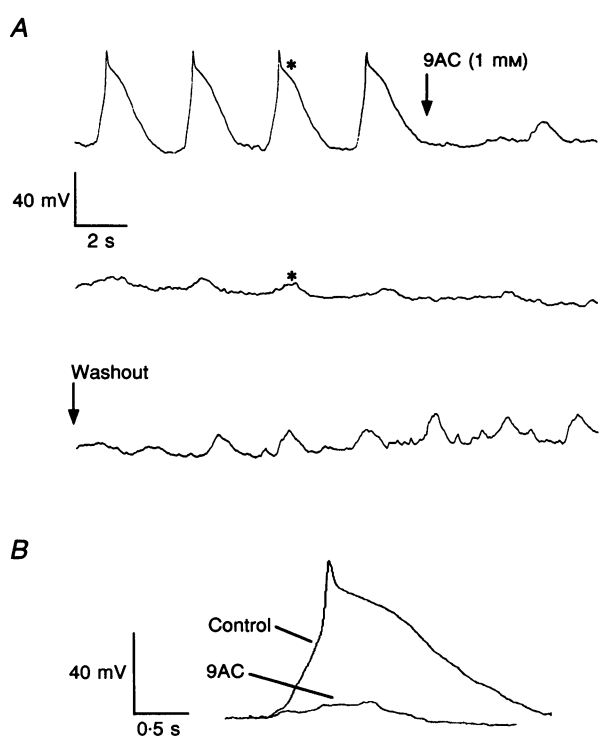

Figure 10. Effect of 9AC on spontaneous electrical activity

A, top panel shows recordings made in current clamp using a Cs^+-filled pipette before addition of 9AC (1 mM). The top two traces are a continuous recording. *B*, events marked by * in *A* are compared on an expanded scale, showing blockade of the action potential and reduced amplitude of the STD.

remains unclear, it has been proposed that multiple asynchronous STDs may contribute to resting membrane noise and overall depolarization (Large & Wang, 1996). Localized release of Ca^{2+} (Ca^{2+} sparks) has also been argued to activate large conductance Ca^{2+}-activated K^+ channels in rat cerebral arteries where the effect would be to cause an overall hyperpolarization and vasodilatation (Nelson *et al.* 1995). Co-localization of the sarcoplasmic reticulum Ca^{2+} release sites and Cl^- or K^+ channels in the membrane in each case is proposed as a requirement for activation of the currents by small elementary release events such as sparks (Fay, 1995; Large & Wang, 1996). On this basis, one possible mechanism for the generation of urethral tone, might be asynchronous spark activity throughout the tissue activating Cl^- channels at multiple sites, resulting in overall depolarization and Ca^{2+} influx. An element of positive feedback could also result in that Ca^{2+} influx would be likely to increase the spark frequency. It is possible to envisage a scenario where this may result in the membrane becoming 'clamped' within the voltage range of the window current for I_{Ca}.

Microelectrode recordings from rabbit urethra, however, suggest that a slightly different mechanism may operate (Hashitani *et al.* 1996). In this tissue both STDs and longer duration 'slow waves' were recorded. The STDs were attributed to $I_{Cl(Ca)}$ activated by Ca^{2+} release from the sarcoplasmic reticulum, while slow waves were thought to be due to activation of I_{Ca} by the STDs. We have observed events similar to these STDs in isolated urethral smooth muscle cells. Often, superimposed on the STDs, there were action potentials consisting of a fast upstroke and a plateau. The STDs were unaffected by short-term exposure to nifedipine, but were reduced in amplitude by niflumic acid and 9AC, suggesting an underlying Cl^- current. The action potentals, on the other hand, were immediately blocked by nifedipine, suggesting that they were largely due to activation of L-type Ca^{2+} channels. In these experiments conditions were ideally set up to facilitate this mechanism in that E_{Cl} was set well above the resting membrane potential and K^+ channels were blocked by internal Cs^+. Further work will be aimed at studying this phenomenon under more physiological conditions.

STDs have also been recorded in guinea-pig mesenteric lymphatic vessels, where they appear to be able to activate Ca^{2+} action potentials (Van Helden, 1993; Van Helden, von der Weid & Crow, 1995). These small vessels are morphologically arranged in small discontinuous units called 'lymphangions' which behave as short isopotential electrical cables, similar to the single cells of the present study. In lymphangions, single STDs were proposed by the authors to be 'quantal' events due to local release of Ca^{2+}. In a short cable, these events were believed to be large enough to evoke Ca^{2+} action potentials. However, in large lymphatic vessels, which are electrically well coupled, it was necessary to propose the synchronous generation of STDs within a group of cells in order to produce depolarizations large enough to evoke action potentials. Recruitment of STDs within smooth muscle bundles was also suggested to account for the slow waves recorded from urethra (Hashitani *et al.* 1996). Here, the upstroke of the slow wave appeared to be the result of summation of STDs as it was slow, was not blocked by nifedipine and was reduced by niflumic acid. The plateau phase of the slow wave was, however, blocked by nifedipine.

Spontaneous transient outward currents (STOCs) are believed to be due to activation of Ca^{2+}-activated K^+ currents by elemental events such as Ca^{2+} sparks or macrosparks (Nelson *et al.* 1995; Fay, 1995; Mironneau *et al.* 1996). The time course of an STD, however, is 10-fold greater than a spark or STOC, suggesting that it is unlikely to be generated by a small localized release of Ca^{2+}. In a recent study on portal vein, Mironneau *et al.* (1996) suggested that, while STOCs could be evoked by single sparks, activation of $I_{Cl(Ca)}$ required the more widespread release of Ca^{2+} seen during Ca^{2+} waves similar to those observed in cardiac myocytes. It therefore seems possible that Ca^{2+} waves may have generated the STDs described here. It is also possible that the spontaneous activity observed in single cells may be an artefact produced by Ca^{2+} overload and, indeed, such conditions are required for the generation of Ca^{2+} waves in cardiac myocytes (Cheng, Lederer, Lederer & Cannell, 1996). However, tonically contracted smooth muscles may behave differently in that they have a high $[Ca^{2+}]_i$ (Large & Wang, 1996), which would be likely to facilitate the generation of Ca^{2+} waves.

In conclusion, we have described a Ca^{2+} inward current and Ca^{2+}-activated Cl^- current in urethral cells. Under the conditions of our experiments these could combine to produce STDs and action potentials which, in some respects, resembled the electrical activity recorded from syncytial urethral preparations. We are not yet in a position to say whether the mechanisms underlying the activity in single cells are similar to those in whole tissue, although it seems likely that the currents we describe are involved, to some extent, in both cases.

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