Hyperpolarisation-activated inward current in isolated sheep mesenteric lymphatic smooth muscle

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1. Freshly isolated sheep lymphatic smooth muscle cells were studied using the perforated patch-clamp technique. Hyperpolarisation with constant-current pulses caused a time-dependent rectification evident as a depolarising 'sag' followed by an anode-break overshoot at the end of the pulse. Both sag and overshoot were blocked with 1 mM Cs^+.

2. Cells were voltage clamped at −30 mV and stepped to −120 mV in 10 mV steps of 2 s duration. Steps negative to −60 mV evoked a slowly activating, non-inactivating inward current which increased in size and rate of activation with increasing hyperpolarisation.

3. The slowly activating current was reduced in Na^+-free bathing solution but enhanced when the extracellular K^+ concentration was increased to 60 mM. The current was significantly reduced by 1 mM Cs^+ and 1 μM ZD7288 but not by 1·8 mM Ba^{2+}.

4. The steady-state activation curve of the underlying conductance showed a threshold at −50 mV and half-maximal activation at −81 mV. Neither threshold nor half-maximal activation was significantly affected by increasing the external K^+ concentration to 60 mM.

5. The frequency of spontaneous contractions and fluid propulsion in isolated cannulated segments of sheep mesenteric lymphatics were decreased by 1 mM Cs^+ and by 1 μM ZD7288.

6. We conclude that sheep lymphatics have a hyperpolarisation-activated inward current similar to the I_f seen in sinoatrial node cells of the heart. Blockade of this current slows spontaneous pumping in intact lymphatic vessels suggesting that it is important in normal pacemaking.

There is now compelling evidence that lymph is propelled mainly by the spontaneous contractions of collecting lymphatic ducts (Aukland & Reed, 1993). These are thin-walled vessels which have valves at regular intervals along their length and whorls of smooth muscle in their walls. This smooth muscle is capable of very regular and well-co-ordinated phasic contractions which efficiently propel fluid in the direction determined by the orientation of the valves. Each contraction is preceded by a single action potential (Kirkpatrick & McHale, 1977) or by an action potential complex (Ward *et al.* 1991) showing a pattern of excitation and contraction that is more akin to that of the heart than of most smooth muscles. Immediately after the firing of an action potential the cell membrane potential dips to its maximum hyperpolarising level after which it slowly depolarises until it reaches the threshold for firing the next action potential. We still have very little information on the current or currents that underlie this pacemaker potential. In a study using the double sucrose gap technique, Allen & McHale (1988) showed that the voltage deflection in response to a 5 s constant-current hyperpolarising pulse sagged to a less hyperpolarised level during the first two seconds suggesting the switching on of a slowly activating conductance. The fact that this voltage relaxation could be blocked with 10 mM caesium led them to suggest that the developing inward rectification may have been due to activation of a hyperpolarisation-activated inward current similar to the I_f seen in sinoatrial node cells in the heart (DiFrancesco, 1991) or the I_h seen in many neurones (salamander photoreceptor: Bader & Bertrand, 1984; lobster stretch receptor: Edman *et al.* 1987; hippocampal interneurones: Maccaferri & McBain, 1996).

It was not possible to achieve adequate voltage clamp using the sucrose gap technique so a detailed study of the currents underlying pacemaking in lymphatics had to await the successful application of the patch-clamp technique (Hamill *et al.* 1981) to single smooth muscle cells isolated from lymphatic vessels. We have recently begun to study lymphatic electrophysiology using this technique (Hollywood *et al.* 1997*a*; Cotton *et al.* 1997). The purpose of the present investigation was to establish whether or not such a hyperpolarisation-activated inward current is found in lymphatic

smooth muscle. A preliminary account of the study has been communicated to The Physiological Society (Cotton *et al.* 1998).

METHODS

Mesenteric lymphatics of sheep of either sex were obtained from an abattoir approximately 15 min after slaughter and transported to the laboratory in Krebs solution at 37 °C. They were dissected free from the mesenteric fat and used immediately for pumping studies in the intact vessels or for cell dispersal. Some were stored at 4 °C for use the following day.

Cell dispersal

Several 2–3 cm lengths of lymphatics were cut into small (< 1 mm^3) pieces and smooth muscle cells were isolated from these by incubation in a dispersal medium containing (per 5 ml of Hanks' Ca^{2+}-free solution): 15 mg collagenase (Sigma, Type 1a), 0·5 mg protease (Sigma, Type XXIV), 5 mg BSA (Sigma) and 15 mg trypsin inhibitor (Sigma) for approximately 40 min at 35 °C, after which the tissue pieces were 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. 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) external bath solution: 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; (3) high K^+ solution: 75·3 Na^+, 60 K^+, 130·3 Cl^-, 4·7 HCO_3^-, 0·34 HPO_4^{2-}, 0·44 $H_2PO_4^-$, 0·9 Mg^{2+}, 1·8 Ca^{2+}, 0·4 SO_4^{2-}, 10 dextrose, 2·9 sucrose and 10 Hepes, pH adjusted to 7·4 with NaOH; (4) Na^+-free solution: 125 *N*-methyl D-glucamine (NMDG), 10·8 K^+, 130·36 Cl^-, 4·17 HCO_3^-, 0·38 HPO_4^{2-}, 1·8 Ca^{2+}, 0·9 Mg^{2+}, 0·4 SO_4^{2-}, 10 dextrose, 2·9 sucrose and 10 Hepes (the NMDG base was titrated with HCl to a pH of 7·4); (5) control solution for the sodium replacement experiments: 125 Na^+, 10·8 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; (6) pipette solution: 133 K^+, 1 Mg^{2+}, 55 Cl^-, 80 gluconate, 0·5 EGTA and 10 Hepes, pH adjusted to 7·4 with KOH; and (7) Krebs solution for pumping studies: 120 NaCl, 25·0 $NaHCO_3$, 5·9 KCl, 1·2 Na_2HPO_4, 2·5 $CaCl_2$, 1·2 $MgCl_2$ and 11·0 glucose, gassed with 95% O_2–5% CO_2.

Electrophysiology

All experiments were carried out at 37 °C. Recordings were made using the amphotericin B perforated patch method (Rae *et al.* 1991). 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 0·6 mg ml^{-1} of amphotericin B (Sigma) dissolved in DMSO, giving a final DMSO concentration of 1%. 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. 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) interfaced to an AT-type computer running pCLAMP software (Axon Instruments). During experiments the dish containing the cells was superfused with bath solution. In addition, the cell under study was continuously superfused by means of a close delivery system consisting of a pipette (tip diameter, 200 μm) placed approximately 300 μm away. This could be switched, with a dead space time of around 5 s, to a solution containing a drug.

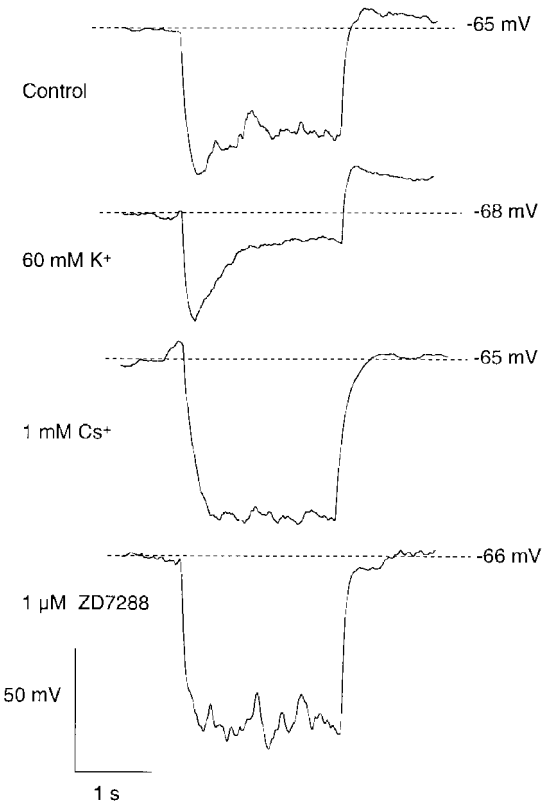

Figure 1. Inward rectification of the electrotonic potential elicited in response to constant-current hyperpolarising pulses

When cells were studied in current-clamp mode they were found to have resting potentials significantly more depolarised that those found in intact tissues. In the example shown resting potential was initially found to be −44 mV. To bring this to a level closer to that found in intact tissue a steady background current was injected which brought the potential to around −65 mV. Hyperpolarising current pulses (50 pA for 2 s) were applied under a variety of conditions. In control solution a voltage 'sag' developed followed by a rebound repolarisation at the end of the pulse. In solutions containing 60 mM K^+ both voltage sag and rebound depolarisation were greatly exaggerated while in 1 mM Cs^+ and 1 μM ZD7288 both sag and rebound depolarisation were abolished.

Pumping experiments

In some experiments we investigated the pumping activity of intact segments of mesenteric lymphatic. Fifty millimetre lengths of vessel were cannulated at both ends and set up in a horizontal organ bath so that, as a result of the spontaneous lymphatic contractions, fluid was pumped from a constant-pressure reservoir at the inflow end through a drop counter on the outflow (McHale & Roddie, 1976). Measurements were made of outflow pressure using a pressure transducer (Statham P23H) and of flow using the drop counter. Drugs were added to the Krebs solution which was continuously perfusing the outside of the vessel.

The following drugs were used: tetraethylammonium chloride (TEA; Sigma), penitrem A (Sigma) and ZD7288 (Tocris Cookson Inc.).

Data are presented as means ± S.E.M., and statistical differences were compared using Student's paired t test, taking the $P < 0{\cdot}05$ level as significant.

RESULTS

Lymphatic vessels have large amounts of collagen and relatively little smooth muscle in their walls so the yield of cells following the dispersal procedure was usually modest. There were, nevertheless, enough relaxed healthy looking cells to make experiments possible. More than 800 cells were studied in total although it was possible to demonstrate a hyperpolarisation-activated current in only 5% of these

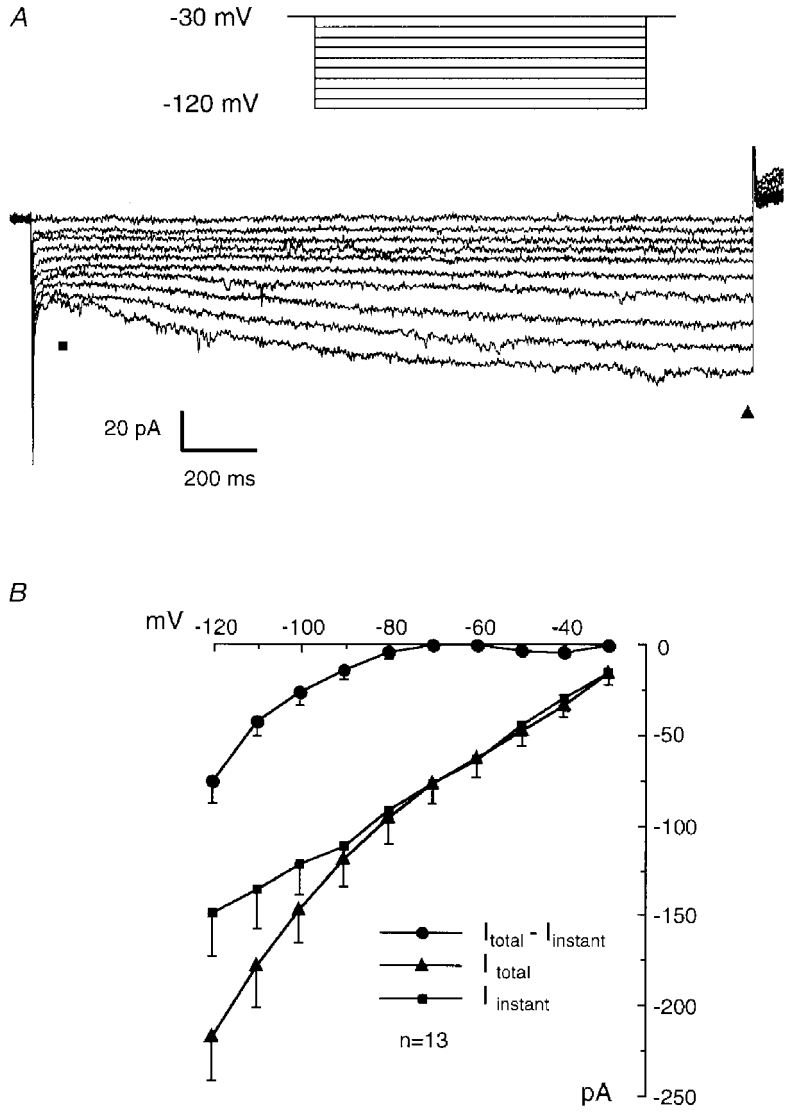

Figure 2. Currents evoked by hyperpolarising steps in voltage-clamp mode

A, the family of currents elicited, in the presence of the K_{Ca} channel blocker penitrem A (100 nM), by stepping from a holding potential of −30 mV to −120 mV in 10 mV steps. Pulses of 2·0 s duration were delivered every 20 s and a total episode duration of 2·1 s was recorded on each occasion. Measurements were made of total current just after the decay of the capacitance transient (approximately 5 ms after the beginning of the pulse (■) and just before the end of the pulse (▲). *B*, a summary of 13 such experiments. Total or steady-state current (▲) shows evidence of inward rectification but this is entirely accounted for by the slow relaxations of the current developing at potentials negative to −70 mV (●) since the instantaneous conductance plot (■) is essentially linear.

even though all of the cells chosen for study had the typical spindle-shaped appearance of healthy lymphatic smooth muscle cells and could be shown to have other inward and outward currents. We have no way of knowing, given the evidence presently available, whether the hyperpolarisation-activated current was confined to a specialised group of pacemaker cells or whether it was absent in the majority of cells because of damage sustained during the dispersal process.

Allen & McHale (1988) reported that when constant-current hyperpolarising pulses were applied to strips of lymphatic smooth muscle in the double sucrose gap the size of the resultant electrotonic potential decreased during the first 2 s of a 5 s pulse. A similar voltage 'sag' was evident when constant-current hyperpolarising pulses were applied to isolated smooth muscle cells as shown in the top panel in Fig. 1. On switching to current-clamp mode the resting potential was originally measured at −44 mV. This was adjusted to approximately −65 mV before each experiment by injecting a steady background current. At the beginning of a 2 s pulse (50 pA) a maximum 60 mV deflection gradually relaxed to a value of 45 mV. Again, as in the sucrose gap study referred to above, termination of the current pulse was accompanied by a rebound depolarisation. Both voltage relaxation and rebound depolarisation were increased by replacing the external solution with one containing 60 mM K^+ (Fig. 1, second panel). In the presence of 1 mM Cs^+ or 1 μM ZD7288 (Fig. 1, third and fourth panels) both voltage relaxation and rebound depolarisation were blocked. These results suggest that the hyperpolarising current pulse was evoking a slowly activating (and deactivating) current which was carried in part by K^+. In voltage-clamp mode such a current could be elicited (Fig. 2*A*). Cells were held at −30 mV and stepped to −120 mV in 10 mV steps in the presence of penitrem A to block calcium-activated potassium current (this was considered unnecessary in subsequent experiments since calcium-activated potassium (K_{Ca}) channels show very little activity at the hyperpolarising potentials required to elicit the slow inward current under study). Steps from −30 to −70 mV yielded (after decay of the capacitance transient) a steady current of between 5 and 20 pA. Larger hyperpolarising steps evoked, in addition to this instantaneous current, a slowly activating inward current which increased with increasing hyperpolarisation, reaching a maximum at −120 mV. In some experiments voltages as high as −140 mV were used but since it was difficult to maintain the seal at these very high voltages and since maximal current was usually elicited at −120 mV this was the most hyperpolarising step used in subsequent experiments. The pattern

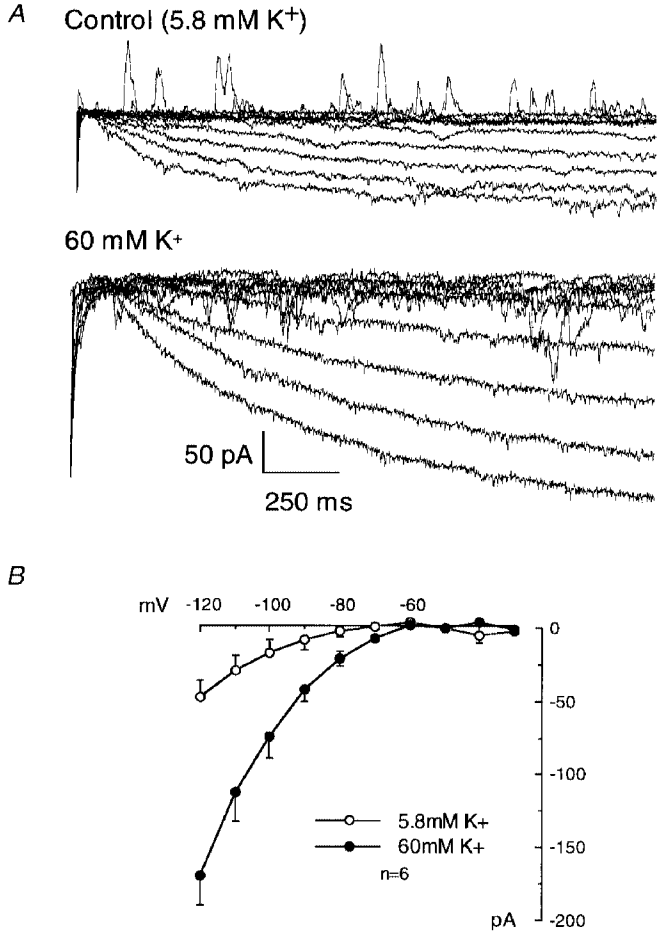

Figure 3. The effect of raising external K^+ concentration

A, increasing the extracellular K^+ concentration to 60 mM (giving a theoretical potassium equilibrium potential, E_K, of −21 mV) more than doubled the slowly activating current without affecting the instantaneous current (which has been subtracted from all the traces illustrated). Six such experiments are summarised in *B*. High K^+ solution significantly increased current at all voltages negative to −60 mV ($P < 0{\cdot}05$). The effect was particularly striking at −120 mV where the mean current of -50 ± 12 pA in 5·8 mM external K^+ was increased more than threefold to -170 ± 22 pA in 60 mM external K^+ solution.

of activation appeared to be sigmoidal, i.e. there was an initial delay followed by a slow activation which could be represented by a single exponential of the type $i = i_\infty(1 - e^{-t/\tau})$, where i_∞ is the value of i at steady state and τ is the time constant of activation. The least squares fit of the curve after the initial delay gave a mean τ value of 1622 ± 561 ms at -80 mV ($n = 4$), 1048 ± 203 ms at -100 mV ($n = 4$) and 514 ± 30 ms ($n = 9$) at -120 mV. Both the duration of the delay and the time course of activation were strongly voltage dependent both being shorter at more negative potentials. When instantaneous current was measured just after the capacitance transient (at the point indicated by the filled square) and plotted against voltage a graph such as that shown in Fig. 2*B* was obtained. Total current just before the end of the pulse was also measured (filled triangle). Subtraction of instantaneous current from total current yielded the peak value of the slowly activating component (filled circle). The mean results of 13 such experiments are plotted in Fig. 2*B*. Note that while the total or steady-state current (filled triangles) shows evidence of inward rectification this is entirely accounted for by the slow relaxations of the current developing at potentials negative to -70 mV (filled circles) since the instantaneous conductance plot (filled squares) is essentially linear.

The effect of changing $[K^+]_o$ and $[Na^+]_o$

When the concentration of external K^+ was increased to 60 mM, by equimolar substitution of KCl for NaCl, instantaneous current was unaffected (maximum instantaneous current (at -120 mV) in control solution was $-147 \pm 15{\cdot}7$ pA, in high K^+ solution it was $-120 \pm 13{\cdot}6$ pA, $P > 0{\cdot}29$, paired t test, $n = 6$) but there was a dramatic increase in the slowly activating current. Figure 3*A* shows a family of currents elicited using the same protocol as in Fig. 2*A*, this time with the instantaneous current subtracted to reveal the slowly developing hyperpolarisation-activated current. The lower panel in Fig. 3*A* shows the effect of increasing the K^+ concentration of the bathing solution to 60 mM. Peak inward current at the -120 mV step increased from a control value of -110 pA to -260 pA in the high K^+ solution. Six such experiments are summarised in Fig. 3*B*. High K^+ solution significantly

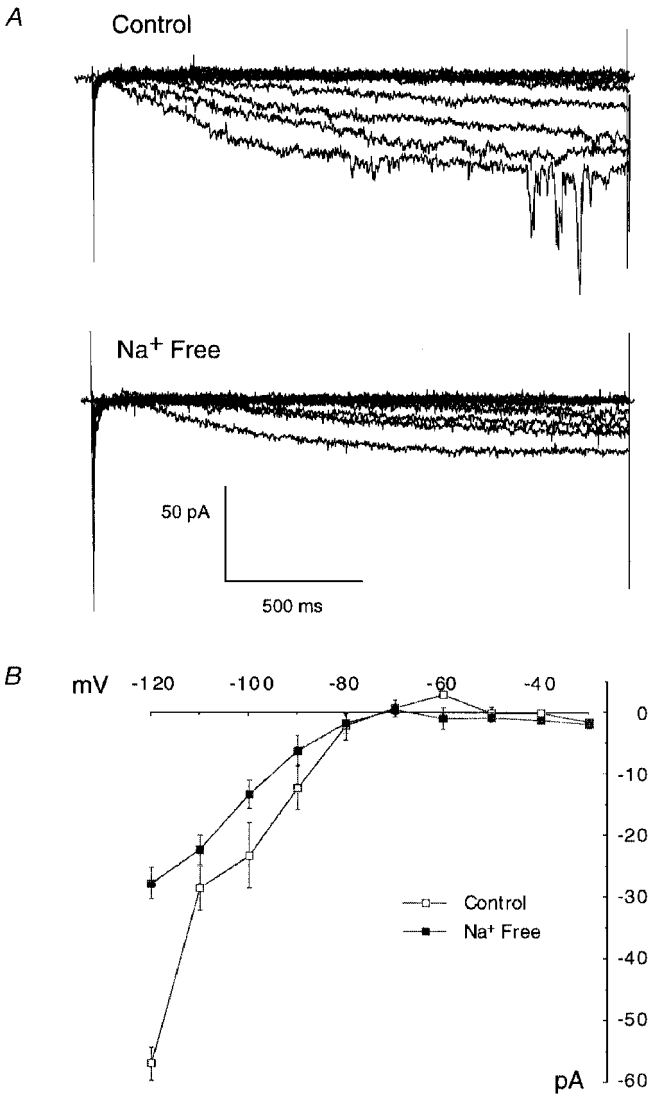

Figure 4. The effect of replacement of external sodium with NMDG

A, in control conditions (upper record) the current measured at the end of the -120 mV pulse (instantaneous currents were subtracted in each case) was approximately -50 pA and this was approximately halved in Na^+-free solution. *B*, a summary of 4 such experiments. Mean current was lower at voltages negative to -80 mV in Na^+-free solution but the difference was significant only at -120 mV ($P < 0{\cdot}05$).

($P < 0{\cdot}05$, paired t test) increased current at all voltages negative to −60 mV. This was particularly striking at −120 mV where the mean current of -50 ± 12 pA in 5·8 mM external K^+ was increased more than threefold to -170 ± 22 pA in 60 mM external K^+ solution. In a separate set of experiments a plot of fully activated current gave a straight line with a slope of 1·72 nS and a reversal potential of −28·7 mV. Raising the external K^+ concentration to 60 mM increased the conductance to 2·65 nS and shifted the reversal potential to −15·1 mV (not shown).

The effects of substituting NMDG (chloride) for external sodium are shown in Fig. 4. Figure 4*A* shows a family of currents elicited before and after replacement of sodium in the bath solution. Current at the end of the −120 mV step was reduced by about 50%. A summary of four such experiments is plotted in Fig. 4*B*. Mean values of peak current were lower at all voltages negative to −80 mV in Na^+-free solution but the difference was significant only for the −120 mV step ($P < 0{\cdot}05$, paired t test).

Steady-state activation curve

The voltage dependence of steady-state activation of the conductance underlying I_f was investigated using the two-step protocol shown in Fig. 5*A*. This protocol was based on the observation that the current activated more slowly at lower voltage steps and was maximally activated within 2 s only at the −120 mV step. Thus from a holding potential of −30 mV I_f was elicited by a series of test potentials of gradually increasing duration from 2 s at −120 mV to 10 s at −40 mV. The current was then fully activated by stepping to −120 mV for a further 2 s. The activation plot was obtained by measuring the current (after leak subtraction) at the end of the test potential and this was expressed as a proportion of the maximum current elicited in the subsequent step to −120 mV. The mean results of four such experiments under control conditions (filled circles) and five experiments in bath solution containing 60 mM K^+ (open circles) are shown in Fig. 5*B*. The two sets of results were almost superimposable suggesting that raising external

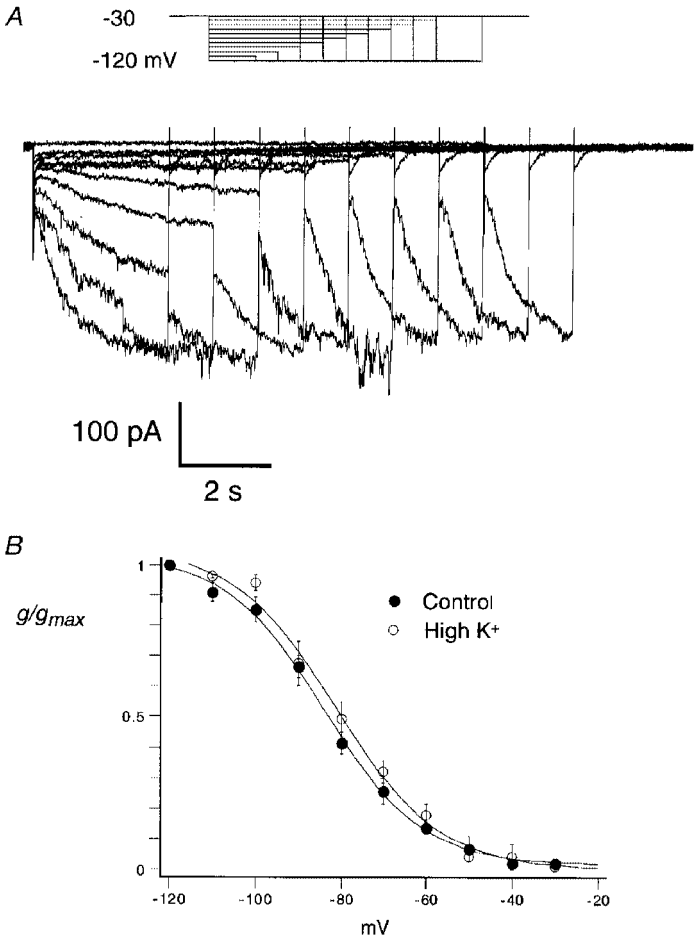

Figure 5. Voltage dependence of steady-state activation

A, from a holding potential of −30 mV currents were elicited by a series of test potentials of gradually increasing duration. The current was then fully activated by stepping to −120 mV for a further 2 s. The activation plot was obtained by expressing the current measured at the end of the test potential as a proportion of the maximum current elicited in the subsequent step to −120 mV. The mean results of 4 such experiments under control conditions and 5 experiments in bath solution containing 60 mM K^+ are shown in *B*. The two sets of results were almost superimposable suggesting that raising external K^+ did not affect the voltage dependence of activation.

K^+ did not affect the voltage dependence of activation of I_f. The mean results were in each case fitted with a Boltzmann function of the form:

$$g/g_{max} = 1/[1 + \exp(-k(V - V_{½}))],$$

where g/g_{max} is the normalised conductance, $V_{½}$ is the membrane potential at which half-maximal activation occurs and k^{-1} is the maximum slope factor at $V = V_{½}$, yielding a $V_{½}$ of $-81 \pm 1{\cdot}8$ mV and a slope factor of $12{\cdot}3 \pm 1{\cdot}8$ mV for the control curve and $-83 \pm 9{\cdot}8$ mV and $11{\cdot}3 \pm 0{\cdot}98$ mV, respectively, for the high K^+ curve.

Effects of Ba^{2+} and Cs^+

Barium and caesium ions are both known to block inward rectification in a variety of neuronal and muscle preparations (DiFrancesco *et al.* 1986; Edman *et al.* 1987; McCormick & Pape, 1990). In Fig. 6*A* the effects of these ions on the hyperpolarisation-activated current in lymphatic smooth muscle are shown. Barium (1·8 mM) slightly reduced the maximum current and made the traces less noisy but its overall effects on the form of the current were small. In contrast 1 mM caesium virtually abolished the current at all voltages, an effect that was reversed on washout. A

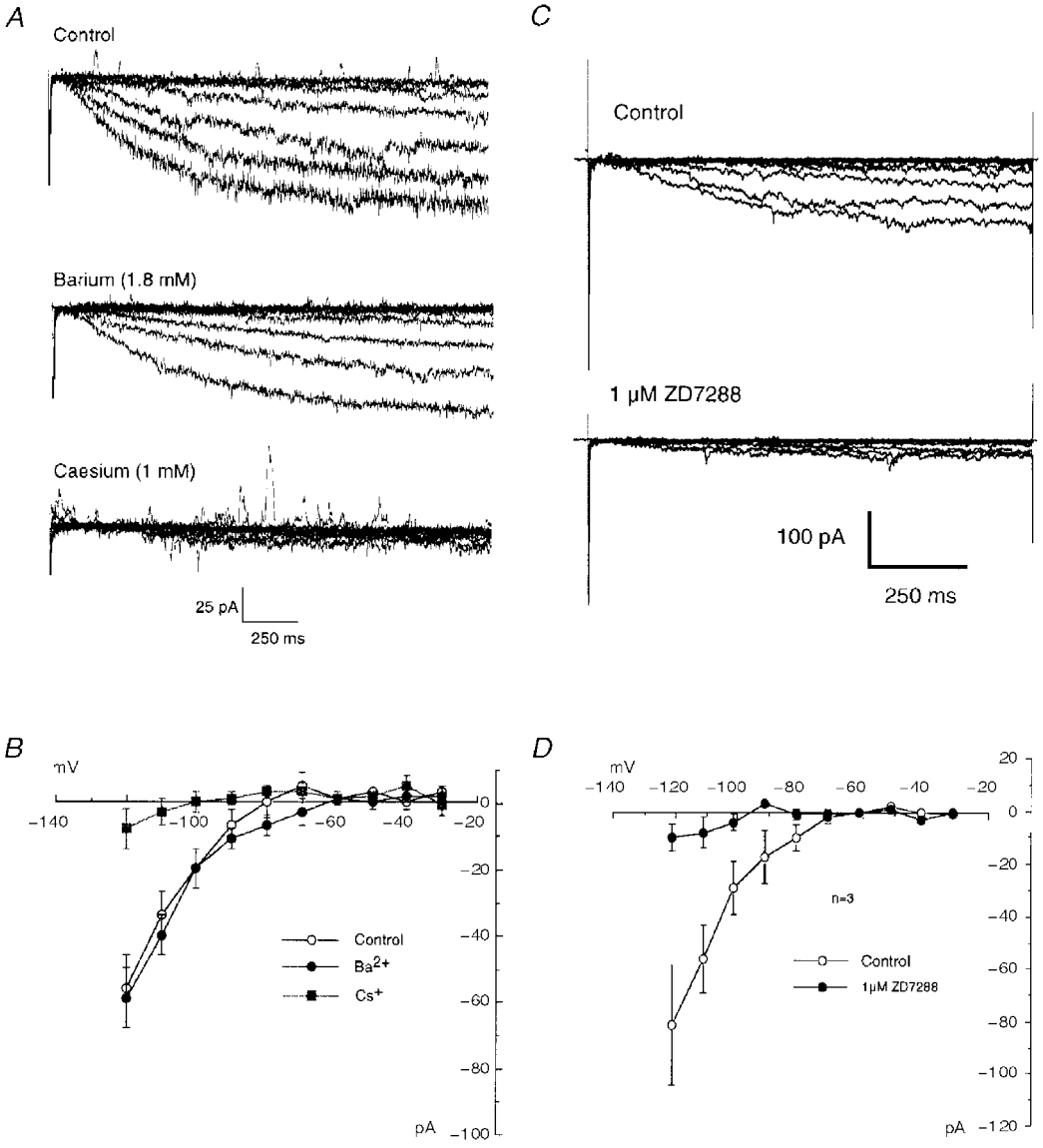

Figure 6. The effects of blockers of inward rectification

A, barium (1·8 mM, 30 s exposure) slightly reduced the maximum current (measured just before the end of the pulse and shown after subtraction of the instantaneous current in each case) and made the traces less noisy but its overall effects were small. In contrast a 30 s exposure to 1 mM Cs^+ virtually abolished the current at all voltages. A summary of 6 such experiments is shown in *B*. The control current–voltage relationship and that in the presence of 1·8 mM Ba^{2+} virtually overlap whereas 1 mM Cs^+ almost abolished the current at all voltages. *C*, the effects of 1 μM ZD7288 (after 20 min exposure) on the family of currents elicited by the same protocol as in *A*. ZD7288 almost abolished currents at all voltages. *D*, a summary of 3 such experiments shows that mean current was significantly blocked at voltages negative to −80 mV ($P < 0{\cdot}05$).

summary of six such experiments is shown in Fig. 6*B*. The control current–voltage relationship and that in the presence of 1·8 mM Ba^{2+} virtually overlap. In contrast the effect of 1 mM Cs^+ was to almost abolish the current at all voltages. This effect of Cs^+ on I_f is in contrast to its lack of effect on other inward and outward currents in lymphatic smooth muscle. For example the peak value of fast sodium current (-387 ± 107 pA before to -392 ± 114 pA in the presence of Cs^+, $n = 5$, n.s.), L-type calcium current (-86 ± 16 pA before to -84 ± 15 pA in the presence of Cs^+, $n = 4$, n.s.), calcium-activated chloride current (-59 ± 20 pA before to -62 ± 26 pA in the presence of Cs^+, $n = 4$, n.s.), total outward current (989 ± 209 pA before to 997 ± 213 pA in the presence of Cs^+, $n = 6$, n.s.) and penitrem-sensitive current, i.e. K_{Ca} (470 ± 107 pA before to 456 ± 102 pA, $n = 6$, n.s.) were virtually unaffected by 1 mM Cs^+.

Recently a drug (Zeneca ZD7288) has become available that is thought to be a specific inhibitor of I_f (BoSmith *et al.* 1993). We thought it would be of interest to examine its effects on the hyperpolarisation-activated current in lymphatics. Unlike Ba^{2+} and Cs^+ which exert their effects within 30 s ZD7288 takes up to 20 min to have its maximal effect. The experiments of Fig. 6*C* show the effects of 1 μM ZD7288 on the family of currents elicited by the same protocol as in Fig. 6*A*. The lower panel in Fig. 6*C* shows that currents were almost abolished at all voltages after a 20 min exposure to ZD7288. These effects are exemplified in a summary of three such experiments in Fig. 6*D*.

Effect of I_f blockers on spontaneous pumping in intact lymphatic vessels

When segments of mesenteric lymphatic were cannulated at both ends and set up so that they could spontaneously pump fluid from an inflow reservoir through an outflow drop counter a record such as that shown in Fig. 7*A* was obtained. Each time the vessel contracted there was an increase in outflow pressure followed by the expulsion of a drop or drops through the drop counter. This was reset at intervals of 1 min so that the height of the resulting ramps indicated the flow generated by the intrinsic pumping of the lymphatic.

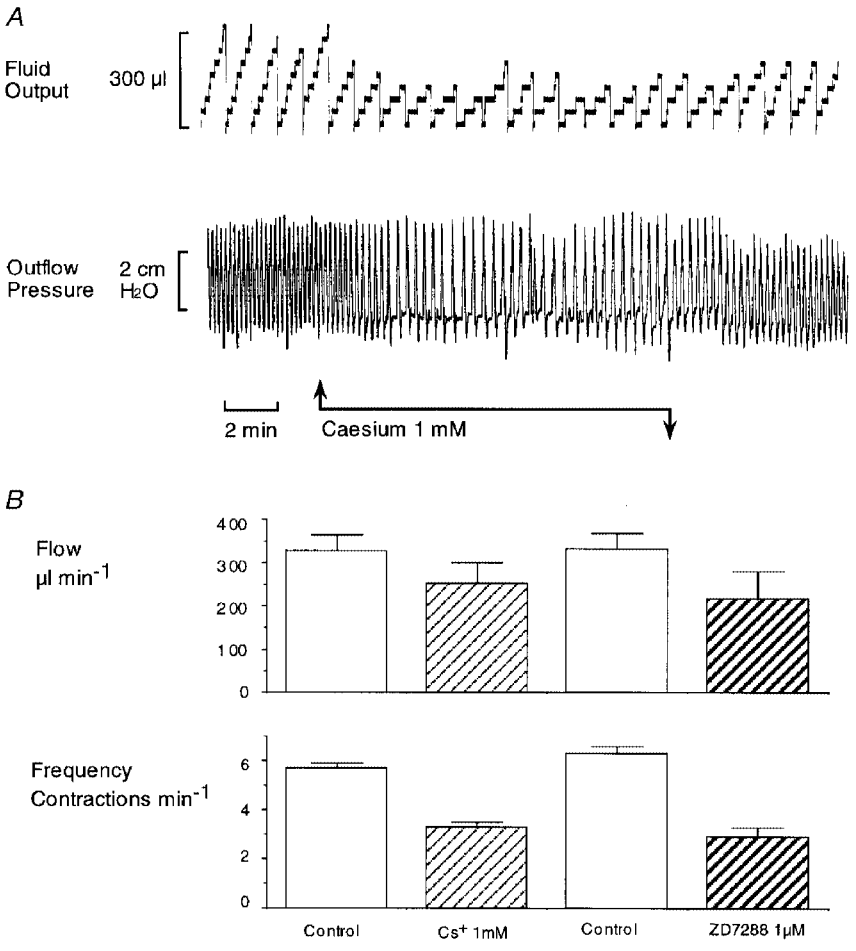

Figure 7. Effect of I_f blockers on spontaneous pumping in intact lymphatic vessels

A, spontaneous contractions of an isolated segment of lymph duct caused phasic increases in outflow pressure (lower record) and these resulted in fluid being pumped through the drop counter (upper record). The drop counter was reset every minute so the height of the ramps is an index of flow. Cs^+ (1 mM) decreased the frequency of contraction from 5·3 to 2·7 contractions min^{-1}. *B*, a summary of 6 experiments in which Cs^+ or ZD7288 were added to the fluid perfusing the outside of the pumping lymphatic. In both cases the frequency of contraction was significantly decreased ($P < 0·05$).

When 1 mM Cs^+ was added to the external bath solution the frequency of spontaneous contractions decreased from the control value of 5·3 to 2·7 contractions min^{-1}. This effect was reversible and frequency and flow returned to control levels within 15 min of washout. A summary of six experiments in which the effects of 1 mM Cs^+ and 1 μM ZD7288 on pumping frequency and flow were tested is shown in Fig. 7*B*. Cs^+ significantly ($P < 0·05$, paired t test) decreased the frequency of lymphatic contraction from a control value of $5·7 \pm 0·2$ to $3·3 \pm 0·2$ contractions min^{-1} with a corresponding decrease in flow while ZD7288 similarly depressed frequency from a control value of $6·3 \pm 0·3$ to $2·9 \pm 0·4$ contractions min^{-1} ($P < 0·05$) again with a concomitant decrease in flow.

DISCUSSION

In this study we have demonstrated the existence of a hyperpolarisation-activated inward current in about 5% of the cells studied. The current had much in common with the slow inward currents activated on hyperpolarisation that are seen in other tissues, such as I_h in many different types of neurones (salamander photoreceptor: Bader & Bertrand, 1984; lobster stretch receptor: Edman *et al.* 1987; hippocampal interneurones: Maccaferri & McBain, 1996) and I_f in sinoatrial nodal cells of the heart (Seyama, 1976; Brown *et al.* 1979; DiFrancesco *et al.* 1986). Thus it had the same characteristic kinetics of activation typified by a sigmoidal time course, i.e. a delay followed by a slow activation that could be well fitted by a single exponential. The resulting time constant of activation (τ) was very voltage dependent varying from a mean of 1622 ± 561 ms ($n = 4$) at −80 mV to 514 ± 30 ms ($n = 9$) at −120 mV. These values are similar to those found for I_h in guinea-pig thalamic neurones (McCormick & Pape, 1990) and I_f in sinoatrial node cells (DiFrancesco *et al.* 1986). Similarly the potential at which the current reversed in control conditions (−28·7 mV) was comparable to the values found by Benham *et al.* (1987) in smooth muscle (−25·6 mV), by Solomon & Nerbonne (1993) in rat superior colliculus (−33 mV) and by DiFrancesco *et al.* (1986) in sinoatrial node cells (−20 mV) under broadly similar conditions. These other hyperpolarisation-activated currents also showed remarkable similarity to the results of the present study in their voltage dependence of activation. Thus activation in general began at potentials negative to −60 mV reaching half-maximal activation at voltages ranging from −75 to −86 mV (Mayer & Westbrook, 1983; DiFrancesco *et al.* 1986; Edman *et al.* 1987; McCormick & Pape, 1990; Solomon & Nerbonne, 1993) as compared to the $V_{½}$ for lymphatic smooth muscle of $-81 \pm 1·8$ mV.

Time-dependent decreases in the size of the electrotonic potentials in response to hyperpolarising currents have been recorded in many smooth muscle types (guinea-pig taenia caeci: Tomita, 1966; Bülbring & Tomita, 1967; longitudinal muscle of the guinea-pig ileum: Bolton, 1972; single cells of the toad stomach: Sims *et al.* 1985) but there have been only a few studies of the underlying conductance (Benham *et al.* 1987; Hisada *et al.* 1991; Green *et al.* 1996). The earliest of these in rabbit jejunal smooth muscle cells (Benham *et al.* 1987) described a current which was very like that found in lymphatic smooth muscle. Thus the authors found a similar threshold of activation and $V_{½}$, and a reversal potential of $-24 \pm 3·5$ mV which was shifted in the positive direction in high $[K^+]_o$, blocked by 1 mM Cs^+ and little affected by Ba^{2+}. They also found that raising the extracellular K^+ concentration increased the current without shifting the steady-state activation curve. They concluded by suggesting that the role of the hyperpolarisation-activated current in the rabbit jejunum might be to provide a focus for pacemaking in the jejunal wall.

Low resting membrane potential of isolated lymphatic smooth muscle cells

Many investigators have observed that the resting membrane potential recorded in isolated smooth muscle cells was significantly lower than that measured in the intact tissue. Thus Singer & Walsh (1980) found the mean resting membrane potential in isolated toad stomach smooth muscle cells to be -41 ± 10 mV as compared to −65 mV in intact tissue, while Vogalis & Sanders (1991) found it to be -45 ± 2 mV in isolated dog pyloric cells as compared to −65 mV in the intact tissue. Similarly Veronica *et al.* (1999) reported a value of -41 ± 2 mV in isolated human saphenous venous smooth muscle cells compared to values of greater than −60 mV measured in intact veins. Vogalis & Sanders (1991) suggested that this discrepancy might be due to differences in transmembrane ionic gradients in isolated cells as compared to those in the intact tissue or to a decrease in Na^+,K^+-ATPase activity in isolated cells with a consequent decrease in the electrogenic component of resting membrane potential which is believed to be important in smooth muscle cells. Alternatively Veronica *et al.* (1999) suggested that the discrepancy might be due to a decreased contribution to resting membrane potential of outward current carried via the K_{Ca} channel due to a decrease in subsarcolemmal calcium concentration. Whatever the explanation of the difference in resting membrane potential between isolated cells and intact tissue it undoubtedly helps to explain the absence of spontaneous activity one frequently finds in isolated smooth muscle cells. Thus in the present study none of the 800 cells studied exhibited rhythmic electrical activity although such activity is consistently found in intact lymphatic ducts. Cotton *et al.* (1997) showed that mean resting membrane potential in isolated sheep mesenteric lymphatic smooth muscle was -37 ± 2 mV. At this potential the two currents that have been shown to be responsible for the upstroke of the action potential in lymphatic smooth muscle (L-type calcium and fast sodium) are largely inactivated (Hollywood *et al.* 1997*b*). It would be ideal if we could have examined the effects of modulators of I_f on pacemaking activity in current-clamped cells as has been done in cells isolated from the rabbit sinoatrial node (van Ginneken & Giles, 1991) but the absence of spontaneous activity in isolated lymphatic smooth muscle cells made this

impossible. Isolated sinoatrial cells (unlike isolated smooth muscle cells) have a mean resting potential greater than −60 mV (van Ginneken & Giles, 1991) which is close to the value recorded in intact tissue.

Role of I_f in pacemaking in lymphatic vessels

Just as the role of I_f in pacemaking in the sinoatrial node of the heart is subject to debate (Yanagihara & Irisawa, 1980) it is arguable that a current which activates at such negative potentials is unlikely to play a part in pacemaking in lymphatic vessels. Values of resting membrane potential (or maximum diastolic potential) recorded using intracellular microelectrodes range from −66·4 mV (± 1·9 mV, mean ± S.E.M., $n = 16$) in guinea-pig mesenteric lymphatics (Van Helden, 1993), to −61 mV (± 5·7 mV, mean ± S.D., $n = 46$) in bovine mesenteric lymphatics (Ward *et al.* 1989). Thus it could be argued that, since the maximum diastolic potential of lymphatic vessels lies just inside the threshold of activation of I_f, one might expect this current to make very little contribution to pacemaking. There are, however, several lines of argument which go counter to this assertion. The first is that it is necessary when making intracellular recordings from vigorously contractile smooth muscle preparations to immobilise them by pinning them out in a stretched condition (Ward *et al.* 1989). Since stretch is known to depolarise smooth muscle (Bülbring, 1955) it is likely that recorded values of membrane potential are more depolarised than if the vessel were in its natural condition. Secondly Hagiwara & Irisawa (1989) have shown that I_f activation depends critically on internal Ca^{2+} concentration. One might expect therefore that the influx of Ca^{2+} during the lymphatic action potential would shift the activation curve for I_f in a depolarising direction just when the action potential had ended. Thus I_f could make its maximum depolarising contribution to the beginning of the next pacemaker potential. The third argument worthy of note is the fact that in small cells with a high input impedance (1–3 GΩ) and low capacitance (< 50 pF) not much current would be required to cause the slow depolarisation found in the lymphatic pacemaker potential. The fourth argument for a role of I_f in pacemaking in lymphatics is the most compelling. Substances that are thought to be fairly specific I_f blockers in other tissues and which have been shown in the present study to block the current in isolated lymphatic smooth muscle cells do have a significant effect on pacemaking in spontaneously pumping lymphatic vessels. These isolated cannulated vessels are known to behave in a very similar fashion to the same vessels in the living animal (McHale & Thornbury, 1986) and are thus as close to their normal state as it is possible to get in an isolated preparation. The fact that both Cs^+ and ZD7288 almost halve the rate of spontaneous contraction in these vessels argues strongly for a role of I_f in pacemaking in lymphatic vessels. This is not to suggest, however, that I_f is the only current involved in pacemaking in lymphatic vessels. The results reported above showed that when I_f was blocked by Cs^+ and ZD7288 pacemaking was slowed but not blocked entirely. Thus it would appear that other mechanisms are available to carry on generating the spontaneous rhythm in the absence of I_f. We have already identified several other candidates for this role. These include a T-type calcium current (Hollywood *et al.* 1997*b*) and a calcium-activated chloride current (Hollywood *et al.* 1997*c*).

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