Outward currents in smooth muscle cells isolated from sheep mesenteric lymphatics

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1. The patch-clamp technique was used to measure membrane currents in isolated smooth muscle cells dispersed from sheep mesenteric lymphatics. Depolarizing steps positive to −30 mV evoked rapid inward currents followed by noisy outward currents.

2. Nifedipine (1 μM) markedly reduced the outward current, while Bay K 8644 (1 μM) enhanced it. Up to 90% of the outward current was also blocked by iberiotoxin (K_d = 36 nM).

3. Large conductance (304 ± 15 pS, 7 cells), Ca^{2+}- and voltage-sensitive channels were observed during single-channel recordings on inside-out patches using symmetrical 140 mM K^+ solutions (at 37 °C). The voltage required for half-maximal activation of the channels ($V_{½}$) shifted in the hyperpolarizing direction by 146 mV per 10-fold increase in $[Ca^{2+}]_i$.

4. In whole-cell experiments a voltage-dependent outward current remained when the Ca^{2+}-activated current was blocked with penitrem A (100 nM). This current activated at potentials positive to −20 mV and demonstrated the phenomenon of voltage-dependent inactivation ($V_{½}$ = −41 ± 2 mV, slope factor = 18 ± 2 mV, 5 cells).

6. Tetraethylammonium (TEA; 30 mM) reduced the voltage-dependent current by 75% (K_d = 3·3 mM, 5 cells) while a maximal concentration of 4-aminopyridine (4-AP; 10 mM) blocked only 40% of the current. TEA alone had as much effect as TEA and 4-AP together, suggesting that there are at least two components to the voltage-sensitive K^+ current.

7. These results suggest that lymphatic smooth muscle cells generate a Ca^{2+}-activated current, largely mediated by large conductance Ca^{2+}-activated K^+ channels, and several components of voltage-dependent outward current which resemble 'delayed rectifier' currents in other smooth muscle preparations.

We are at an interesting stage in the study of the lymphatic system in that it is now widely accepted that there is an intrinsic pump and that this is important for normal lymph drainage but there is little detailed knowledge of how the lymph pump works. We have, for example, very little information on the basic electrical properties of lymphatic smooth muscle and most of what is known derives from experiments using the sucrose gap (Kirkpatrick & McHale, 1977; Allen, Iggulden & McHale, 1986; McHale, Allen & Iggulden, 1987), but this technique has many limitations. Electrical activity is averaged over many cells making it impossible to resolve accurately the time course of observed changes. Neither can one measure the true value of membrane potential. More recent work by Van Helden (1993) using intracellular recording does provide a better insight into conductance changes underlying excitation but it is clear that these changes could be more precisely studied by voltage clamping single freshly dispersed cells. Apart from a few preliminary studies (McHale, Carl & Sanders, 1989; McHale, 1990) this technique has not been successfully applied to the study of lymphatic smooth muscle largely due to the difficulty of isolating viable smooth muscle cells from the dense collagen matrix in which they are embedded. In the present study we have overcome some of these difficulties and used the whole-cell patch-clamp technique to study membrane currents in isolated smooth muscle cells from sheep mesenteric lymphatics.

A preliminary account of this work has been communicated to The Physiological Society (Cotton, Hollywood, McHale & Thornbury, 1996*a*).

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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 either used immediately, or stored at 4 °C for use the following day. 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 bovine serum albumin (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. (1) Hanks' Ca^{2+}-free solution (mM): 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 (mM): 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) Whole-cell pipette solution (mM): 110 potassium gluconate, 20 KCl, 0·5 $MgCl_2$, 1 K_2ATP, 0·1 Na_2GTP, 2·5 sodium phosphocreatine, 5 Hepes and 1 EGTA; pH adjusted to 7·2 with KOH. (4) Pipette and bath solution (for single-channel experiments) (mM): 140 K^+, 0·9 Ca^{2+}, 142 Cl^-, 10 dextrose, 1 EGTA and 10 Hepes. $CaCl_2$ was added to 1 l of bath solution at 37 °C to give bath [Ca^{2+}] of 1·0, 0·75, 0·5, 0·25 and 0·1 μM (Carl & Sanders, 1990).

The methods used to measure macroscopic membrane currents were similar to those described previously (Cotton, Hollywood, Thornbury & McHale, 1996*b*). Patch pipettes had a resistance of 2–4 MΩ. After gigaseals were obtained and the patch was ruptured the series resistance was usually less than 6 MΩ. Series resistance and the capacitative surge were uncompensated. Data were corrected for a junction potential of −10 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 10 s, to a solution containing a drug.

In the single-channel experiments voltage commands were applied using pCLAMP ramped potentials (Carl & Sanders, 1990). Activation curves were calculated by averaging current responses to fifteen potential ramps and dividing each data point of the averaged current by the single-channel amplitude at that holding potential after leakage current correction. The rate of change of the applied ramp potentials (100 mV s^{-1}) was sufficiently slow for the activation curves not to be distorted by the time constants of activation or deactivation (Carl & Sanders, 1990). This analysis provides a continuous recording of Np_o (N is the number of channels in the patch, p_o is the single-channel open probability) over the entire voltage range. All single-channel and whole-cell experiments were carried out at 37 °C.

The following drugs were used: Bay K 8644 (RBI), nifedipine (Bayer), tetraethylammonium chloride (TEA; Sigma), iberiotoxin (Sigma), 4-aminopyridine (4-AP; Sigma) and penitrem A (Sigma). Solutions containing 4-AP were corrected for pH with HCl. 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, presumably because the ratio of collagen to smooth muscle is relatively high in lymphatic vessels. Nevertheless, there were usually enough relaxed, healthy looking cells to make experiments possible. Resting membrane potential in cells was measured in current-clamp mode and averaged −37 ± 2 mV (9 cells). This is lower than the resting potentials in intact sheep (−60 mV; H. Toland & N. G. McHale, unpublished observations) and bovine mesenteric lymphatics recorded with microelectrodes (Ward, Sanders, Thornbury, McHale, 1991). The reason for this difference is not clear, although it is not uncommon for isolated smooth muscle cells to have lower resting potentials than intact tissue (e.g. Vogalis & Sanders, 1991). Input resistance was estimated in voltage-clamp mode by measuring the passive current responses to a series of hyperpolarizing and depolarizing voltage steps from a holding potential of −70 mV. This was usually within the range 1–3 GΩ and averaged 1·1 ± 0·1 GΩ (9 cells). Cell capacitance averaged 47 ± 4 pF (9 cells), as calculated by integrating the capacitative current evoked by small hyperpolarizing and depolarizing voltage steps, and dividing by the amplitude of the voltage change.

Net membrane currents and Ca^{2+}-activated K^+ current

Net membrane currents were investigated, in the absence of drugs, using voltage stepping protocols where the cells were held at −70 mV and stepped to test potentials ranging from −90 to +50 mV to produce a family of currents (Fig. 1*A*, top panel). At test potentials of −40 to +20 mV, there was an initial inward current which then reversed to give outward currents which often became noisy, especially at the more positive potentials. The Ca^{2+} channel agonist Bay K 8644 (1 μM) enhanced the outward current and also produced a small increase in the amplitude of the inward current (Fig. 1*A*, middle panel and inset). In contrast, nifedipine (1 μM) markedly reduced the outward current and, although it also reduced the inward current, there was also an inward component which was resistant to this drug (Fig. 1*A*, bottom panel). Evidence presented in the following paper (Hollywood, Cotton, Thornbury & McHale, 1997) suggests that this remaining current is carried by Na^+ ions. A mean current–voltage relationship is shown for the peak

outward current in nine cells in Fig. 1*B*. This current was large and activated at around −30 mV. The mean current was enhanced by Bay K 8644 and markedly depressed by nifedipine, suggesting that it was dependent upon an influx of Ca^{2+}. The most obvious candidate for the channel mediating such a current is the large conductance Ca^{2+}-dependent K^+ channel (BK channel). To investigate this possibility the effect of iberiotoxin, a selective blocker of these channels, was examined.

In the experiment shown in Fig. 2*A* outward currents were evoked by stepping from a holding potential of −70 mV to test potentials ranging from −70 to +70 mV. In this, as in some other examples, there was no obvious inward current. The reasons for this are not clear at present (for discussion see later). Another difference between the currents in Fig. 1 and Fig. 2*A* is that the latter did not show any apparent inactivation, while the former did. It seems possible that the inactivation may reflect the inactivation of Ca^{2+} current (e.g. Cole & Sanders, 1989). Iberiotoxin (300 nM) reduced these currents by approximately 90% throughout the voltage range (Fig. 2*A*). The dose–response relationship for iberiotoxin was examined by holding cells at −70 mV and stepping repeatedly to test potentials of +30 mV. It can be seen from the example in Fig. 2*B* that iberiotoxin dose dependently decreased the current, with the maximal effect occurring close to 300 nM. In seven control experiments, performed over a similar time period (3 min), there was no run-down of the outward current seen on stepping to +30 mV (averaging 1395 ± 220 and 1464 ± 231 pA, $P > 0{\cdot}4$, at the beginning and end of the period, respectively). Figure 2*C* is a summary of the effect of iberiotoxin in eight cells where this protocol was followed. The data were fitted with a Langmuir equation of the form:

$$I_{drug}/I_{control} = (1 - C)/(1 + ([drug]/K_d) + C$$

where K_d is the dose for half-maximal effect and C is the residual current at supramaximal concentrations. This gave a K_d of 36 nM, and residual current of 11% of control. We also found that the effect of another BK channel blocker penitrem A was indistinguishable from that of iberiotoxin with its maximal effect occurring at a concentration of 100 nM (Fig. 2*D*). Penitrem A was used in subsequent experiments for reasons of economy.

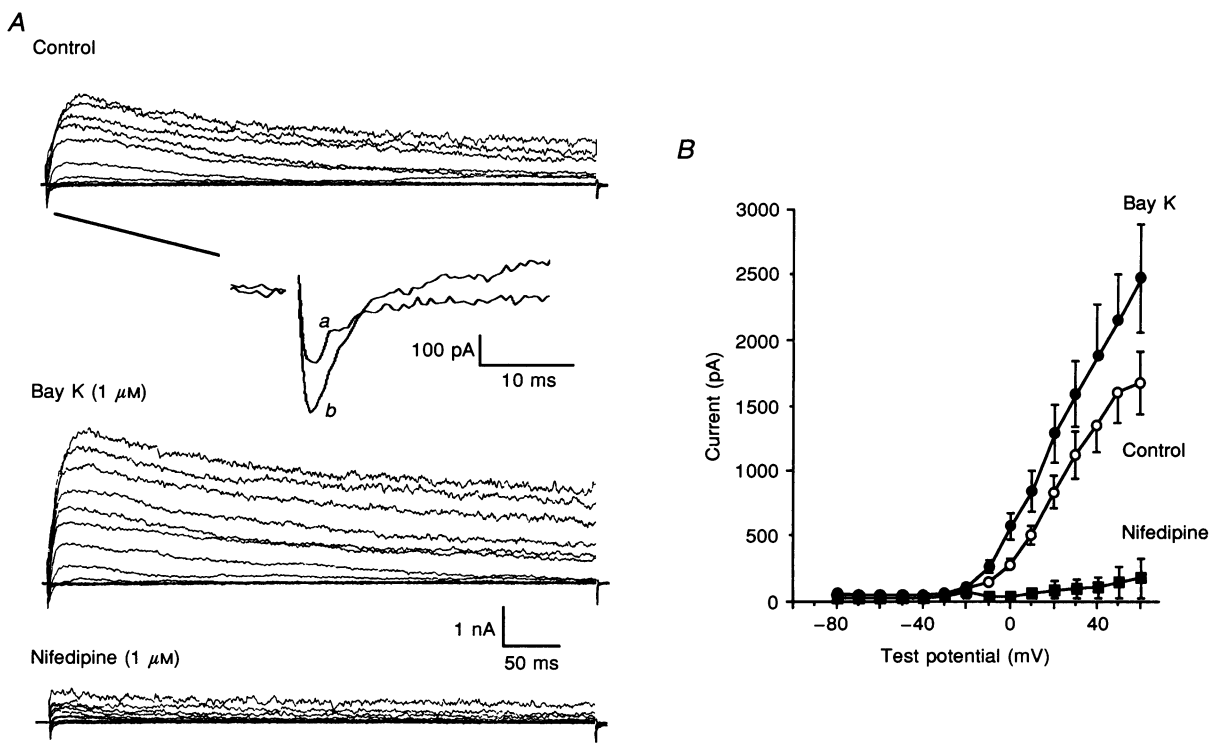

Figure 1. Effects of Bay K 8644 and nifedipine on net membrane currents

A, control currents were elicited in the absence of drugs as described in the text. Bay K 8644 (1 μM, Bay K) enhanced outward currents, while nifedipine (1 μM) depressed them. Inset shows the effect of Bay K 8644 on inward current on an expanded scale: *a*, step to −20 mV before, and *b*, step to −20 mV after, Bay K 8644. *B*, summary of the effects of Bay K 8644 (1 μM) and nifedipine (1 μM) on the current–voltage relationships in 9 cells. The control currents (○) were significantly enhanced by Bay K 8644 (●) and depressed by nifedipine (■) at potentials of −20 mV and above ($P < 0{\cdot}05$).

Table 1. Effect of Ca^{2+} on voltage-dependent activation of channels in inside-out patches

$[Ca^{2+}]_i$ (μM)	$V_{½}$ (mV)	K^{-1} (mV)	Number of patches
0·10	131 ± 9	24·6 ± 2·8	6
0·25	87 ± 11	23·3 ± 3·4	4
0·50	71 ± 4	20·4 ± 2·3	6
0·75	26	19·4	2
1·00	−15 ± 9	21·4 ± 4·5	6

Single-channel studies

The above experiments suggested that most of the outward currents were mediated by BK channels. To study this further, single-channel studies were performed using inside-out patches with symmetrical 140 mM K^+ solutions at a temperature of 37 °C. Under these conditions, the predominant channel had properties typical of the BK channel. The single-channel conductance was estimated by stepping to potentials of 20, 40, 60, 80 and 100 mV to obtain the single-channel current–voltage relationship. The unitary currents were plotted against the appropriate potentials, and fitted with a straight line to give a mean conductance of 304 ± 15 pS and reversal potential of −5 ± 2 mV (Fig. 3*A*, 7 cells). Figure 3*B* shows a typical single-channel recording where Ca^{2+} on the inner side of the membrane (i.e. $[Ca^{2+}]_i$) was buffered to 0·5 μM and the potential held at 50 mV. Frequent openings of large conductance channels could be seen during the control period. Penitrem A (100 nM) was added to the bath at the point indicated by the arrow and appeared to transiently activate the channels before completely blocking them. This result was typical of five similar experiments where both the transient activation and complete blockade were seen. Washes of up to 10 min in drug-free solution failed to reverse the blockade.

The voltage profiles of channel activation and Ca^{2+} sensitivity were examined using ramp protocols. The activation curves derived for three different $[Ca^{2+}]_i$ values from such an experiment are shown in Fig. 3*C*, where increasing $[Ca^{2+}]_i$ caused a parallel leftward shift in the

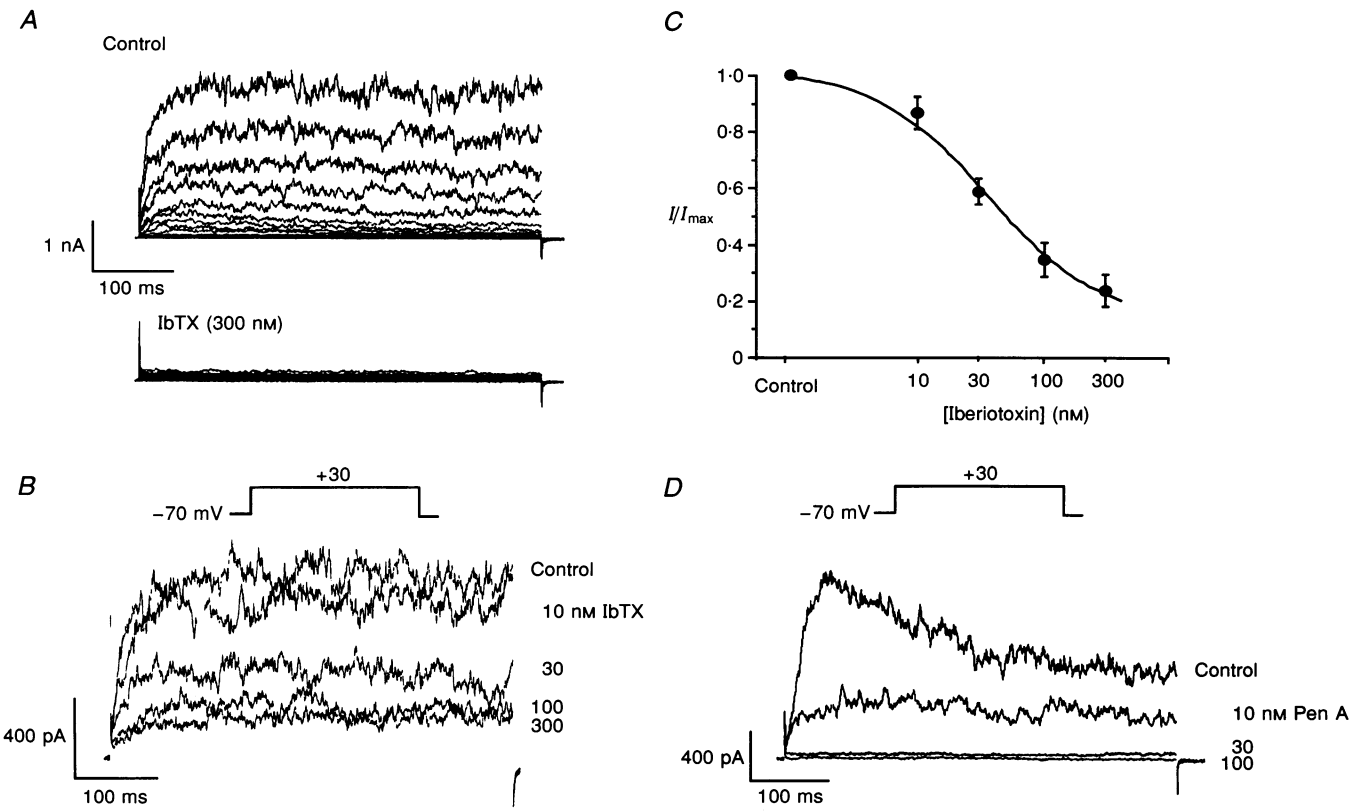

Figure 2. Effects of iberiotoxin and penitrem A on outward currents

A, control currents were recorded in the absence of drugs by stepping from −70 to +70 mV in 10 mV steps. Iberiotoxin (IbTX, 300 nM) greatly reduced the current throughout the potential range. *B*, the dose–response relationship was determined by stepping repeatedly to +30 mV and exposing the cell to increasing doses of iberiotoxin. *C*, summary data from 8 cells where the above dose–response protocol was followed. The current (I) recorded during each dose was normalized by expressing it as a fraction of the current in the absence of iberiotoxin (I_{max}). Means ± S.E.M. are shown and the continuous line represents a fit of the data using the Langmuir equation (see text), giving a K_d of 36 nM. *D*, the dose–response relationship for penitrem A (Pen A), determined as for IbTX in *B*.

activation curve. To analyse this further, these data were fitted with a Boltzmann function (continuous lines Fig. 3*C*) of the form:

$$Np_o = n/\{1 + \exp[-K(V - V_{½})]\}$$

where N is the number of channels in the patch, p_o is the single-channel open probability, n is the maximal Np_o, K^{-1} is the steepness of the voltage-dependent activation (change in potential necessary to cause an e-fold increase in activation) and $V_{½}$ is the voltage at which there is half-maximal activation. Table 1 lists $V_{½}$ and K^{-1} for different values of $[Ca^{2+}]_i$ obtained from a number of patches. The results suggest that, while changing $[Ca^{2+}]_i$ had little effect on K^{-1} (i.e. there were parallel shifts in the activation curves), the $V_{½}$ was shifted in the hyperpolarizing direction with increasing $[Ca^{2+}]_i$.

Voltage-dependent K^+ currents

Under the conditions of the above experiments, the predominant outward current appeared to be mediated by BK channels. However, there was also another, small component of outward current which persisted in the presence of iberiotoxin or penitrem A. The nature of this current was further investigated in experiments throughout this section with the BK current blocked with penitrem A (100 nM), and the inward currents blocked with TTX (1 μM), nifedipine (1 μM) and Ni^{2+} (100 μM). This current demonstrated the property of voltage-dependent inactivation which was studied by holding the cell at conditioning potentials ranging from −110 to −10 mV for 2 s, before stepping to a test potential of +30 mV (Fig. 4*A*). The example shows that after holding at the more negative potentials the step to +30 mV evoked a current which

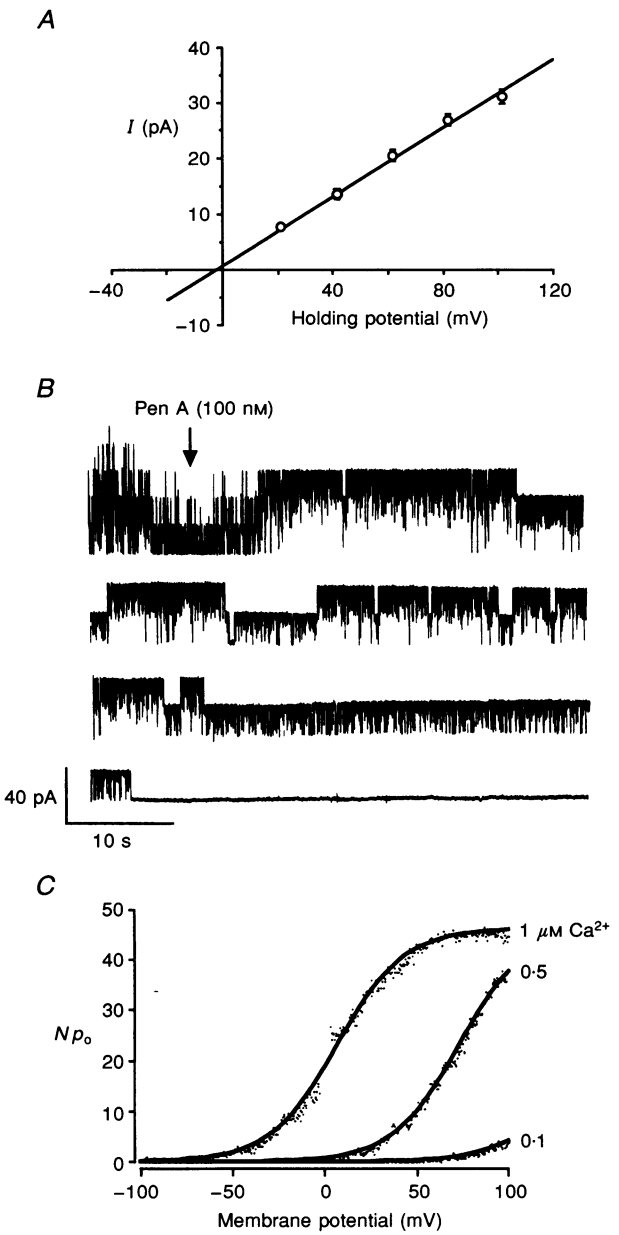

Figure 3. Single-channel recordings
A, mean single-channel current–voltage relationship for 7 patches. *B*, continuous single-channel recordings made in an inside-out patch at +50 mV using symmetrical 140 mM KCl solutions; $[Ca^{2+}]_i = 0{\cdot}5$ μM; temperature, 37 °C. Channel openings are shown in the upward direction. Penitrem A (100 nM) was added to the bath at the point indicated. *C*, Ca^{2+} and voltage dependence of single-channel open probability. Activation curves were derived from an inside-out patch where the membrane potential was ramped from −100 to +100 mV. $[Ca^{2+}]_i$ was buffered to 0·1, 0·5 and 1 μM as indicated. Each data point represents the averaged response from 15 ramps. The continuous lines show Boltzmann fits of the data, giving $V_{½}$ values of 145, 73 and 7 mV.

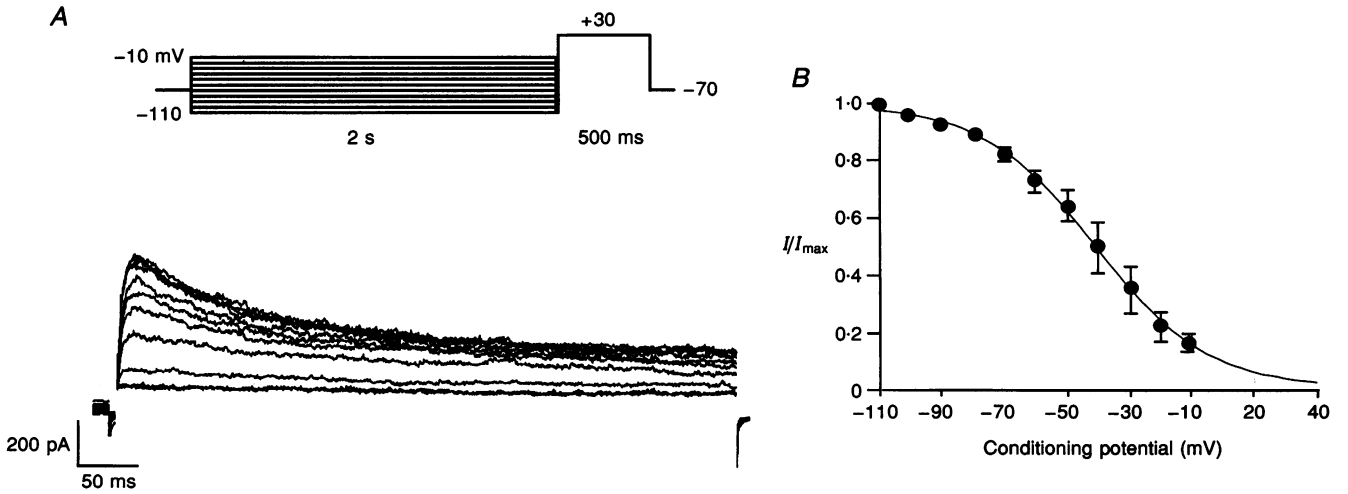

Figure 4. Voltage-dependent inactivation of the voltage-dependent outward current
A, recordings made in penitrem A (100 nM), TTX (1 μM), nifedipine (1 μM) and Ni^{2+} (100 μM). The cell was held at conditioning potentials ranging from −110 to −10 mV for 2 s, before stepping to a test potential of +30 mV. As the cell was held at more positive conditioning potentials, the outward current evoked at +30 mV was reduced. *B*, the mean voltage-dependent inactivation curve for 5 cells. ●, means ± S.E.M. of the normalized peak current (I/I_{max}) evoked following each conditioning potential. The continuous line shows a fit using the Boltzmann equation, giving a $V_{½}$ of −41 mV and slope factor of 18 mV.

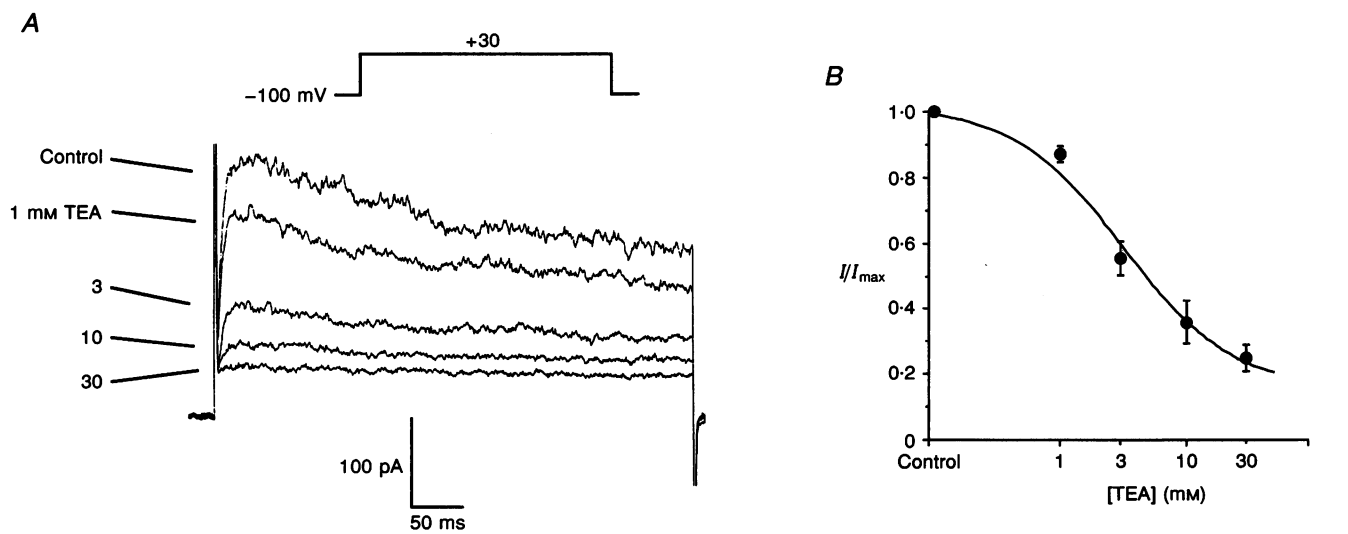

Figure 5. Dose–response relationship of TEA on voltage-dependent outward current
A, cell was stepped repeatedly to +30 mV from a holding potential of −100 mV, and exposed to increasing doses of TEA. Penitrem A (100 nM), TTX (1 μM), nifedipine (1 μM) and Ni^{2+} (100 μM) were present throughout. *B*, summary data from 5 cells where the above dose–response protocol was followed. The current (I) recorded during each dose was normalized by expressing it as a fraction of the current in the absence of TEA (I_{max}). Means ± S.E.M. are shown and the continuous line represents a fit of the data using the Langmuir equation, giving a K_d of 3·3 mM.

relaxed incompletely over the 500 ms period of the test potential (upper traces, Fig. 4*A*). As the cell was progressively held at more positive conditioning potentials, the outward current evoked at +30 mV was reduced. Figure 4*B* shows the voltage-dependent inactivation curve obtained by plotting the normalized peak current evoked at +30 mV against the previous conditioning potential in five cells. The current, which inactivated over the full range of potentials, was fitted with a Boltzmann function:

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

to give a $V_{½}$ of −41 ± 2 mV, a slope factor of 18 ± 2 mV, and the residual (non-inactivating) fraction of current, C, was close to zero. The time-dependent relaxation of the evoked current was examined in nine experiments, stepping from 2 s conditioning potentials of −100 mV. At test potentials below 20 mV the decay was variable and generally difficult to fit. At potentials of 30, 40, 50, 60 and 70 mV the decay could be well fitted with single exponentials, giving mean time constants of 138 ± 21, 114 ± 6, 116 ± 22, 101 ± 11 and 105 ± 18 ms, respectively, suggesting that it was only weakly voltage dependent.

In an attempt to dissect out different components of the voltage-dependent current, the effects of the K^+ channel blockers TEA and 4-AP were examined. The dose–response relationship for TEA was investigated by examining its effect on current evoked at test potentials of +30 mV (from 2 s conditioning potentials of −100 mV). A typical example is shown in Fig. 5*A* where it is clear that 1–30 mM TEA caused a dose-dependent decrease in the current. Summary data from five experiments is shown in Fig. 5*B* where the data was fitted using the Langmuir equation (see above) to give a K_d of 3·3 mM. The effect of TEA was seen throughout the range of potentials over which the voltage-dependent outward current was evoked.

Dose–response experiments were also carried out with 4-AP, where it was found that the threshold dose was 0·3 mM, and the maximal effect occurred in the range 3–10 mM (3 cells, example Fig. 6*A*). Also, as with TEA, the

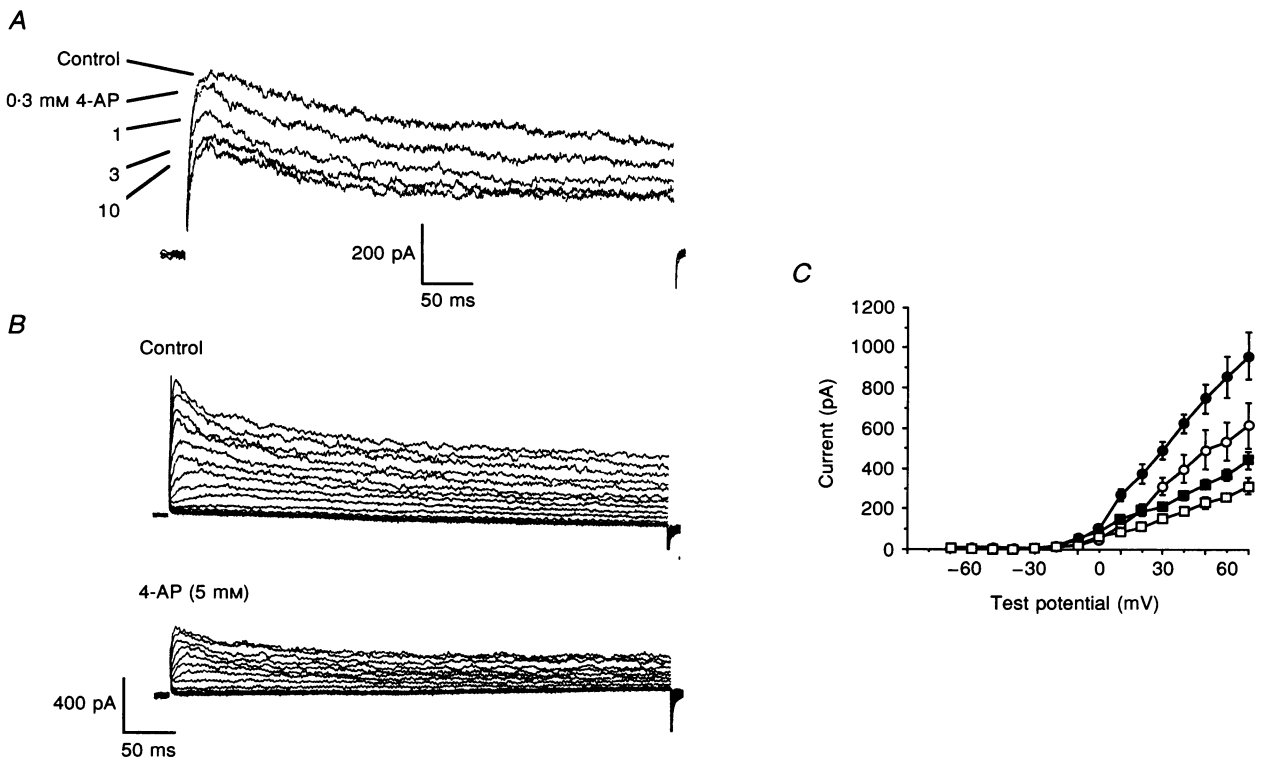

Figure 6. Effect of 4-AP on voltage-dependent outward current

A, cell was stepped repeatedly to +30 mV from a holding potential of −100 mV, and exposed to increasing doses of 4-aminopyridine (4-AP). Penitrem A (100 nM), TTX (1 μM), nifedipine (1 μM) and Ni^{2+} (100 μM) were present throughout. *B*, effect of 4-AP on the current–voltage relationship. Control currents were evoked by stepping to potentials ranging from −70 to +70 mV from a holding potential of −100 mV. The protocol was then repeated during exposure to 4-AP (5 mM). *C*, summary current–voltage relationships before (filled symbols) and after 5 mM 4-AP (open symbols) in 4 cells. Circles indicate peak currents (measured at beginning of test pulses), and squares indicate sustained currents (measured at the end of the test pulses). Mean ± S.E.M. shown in each case. Currents were significantly depressed at potentials of −10 mV and above ($P < 0·05$).

effect was seen throughout the full range of potentials (Fig. 6*B* and *C*), leaving open the possibility that 4-AP and TEA were blocking the same current. However, in contrast to TEA, a maximal dose of 4-AP only reduced the current by an average of around 40% (example Fig. 6*A*) suggesting that 4-AP and TEA may have been blocking different components of the outward current. Experiments conducted to test this idea are shown in Fig. 7. In Fig. 7*A* currents were evoked by stepping to +30 mV from a conditioning potential of −100 mV. The current was reduced by approximately 40% on exposure to a near-maximal dose of 4-AP (3 mM), and further reduced by a near-maximal dose of TEA (10 mM). The drugs were then washed out and the current returned to its previous level (Fig. 7*B*, control trace, same cell). The cell was then exposed to TEA (10 mM), which was as effective alone as both drugs previously were together. Furthermore, addition of 3 mM 4-AP after TEA had little further effect. Summary data of similar experiments are shown in Fig. 7*C* and *D* where it is clear that, although 4-AP had an effect when used alone, TEA alone had as much effect as TEA and 4-AP together. These results suggest that there are at least two components to the voltage-sensitive K^+ current, one of which is both TEA and 4-AP sensitive, while the other is TEA sensitive and 4-AP insensitive. Neither 4-AP nor TEA had any significant effect on the holding current in the range −70 to −40 mV (data not shown), suggesting that neither component of current was activated within this potential range.

DISCUSSION

So far as we are aware there are no other published accounts of membrane currents in isolated lymphatic smooth muscle cells, a deficiency which probably reflects the fact that these vessels are small and fragile and contain relatively little smooth muscle compared with many other preparations. Nevertheless, they produce co-ordinated spontaneous activity which results in efficient propulsion of fluid (Hall, Morris & Woolley, 1965; McHale & Roddie, 1976; Olszewski & Engeset, 1980). Our approach in the past has been to study the lymph vessels of large ruminants, which are well developed and more robust than the vessels of smaller species, making them suitable for mechanical studies of pumping activity (McHale & Roddie, 1976; McHale & Thornbury, 1986; McGeown, McHale & Thornbury, 1987). The mesenteric vessels of the sheep, when subjected to a

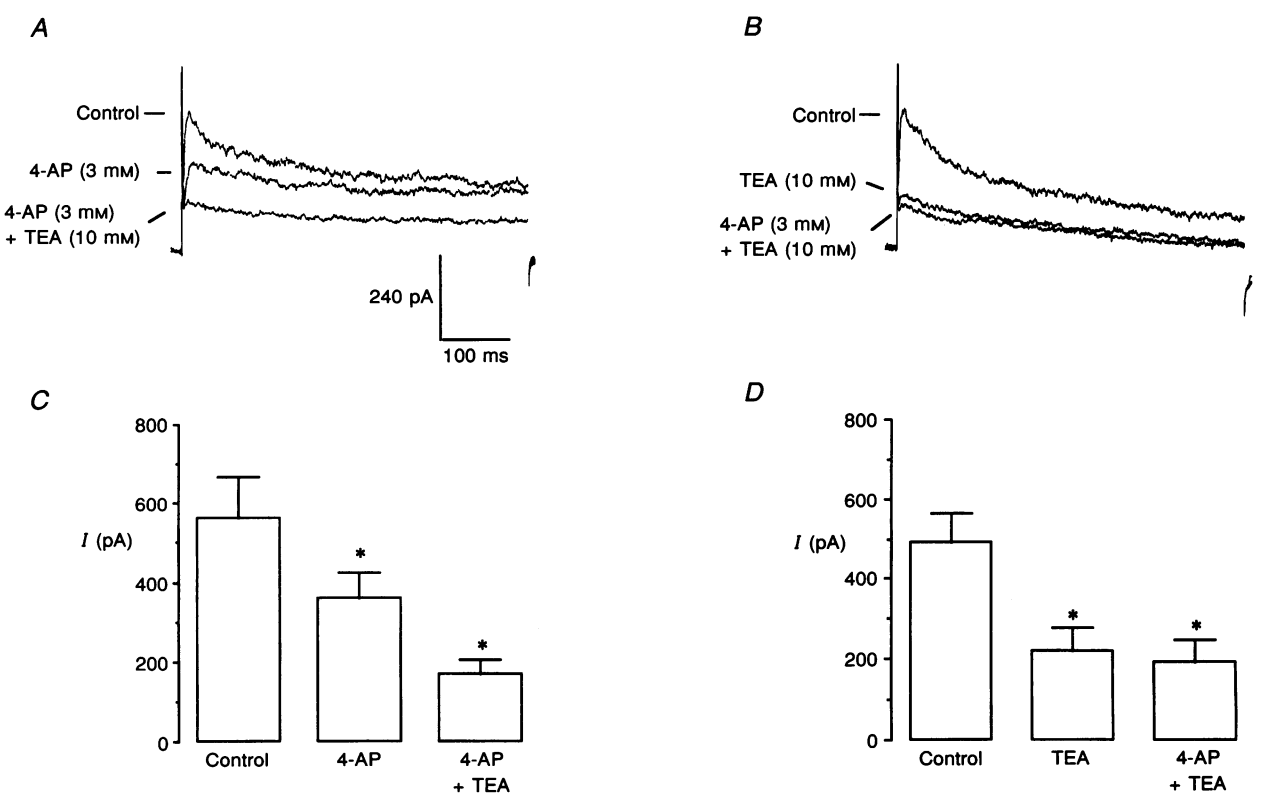

Figure 7. Differential effects of 4-AP and TEA on voltage-dependent outward current

A, currents were evoked by stepping to +30 mV from a conditioning potential of − 100 mV. The current was reduced by approximately 40% on exposure to 4-AP (3 mM), and further reduced by TEA (10 mM). *B*, same cell after drugs were washed out. The cell was then exposed to TEA (10 mM), which reduced the current by 70%, but subsequent addition of 3 mM 4-AP had little further effect. *C* and *D*, summary data from 6 experiments similar to those presented in *A* and *B*. Mean + S.E.M. of the peak current is shown in each case (* significantly different from control, $P < 0{\cdot}05$).

fairly long (40 min) digestion procedure, have now proved a useful source of single lymphatic smooth muscle cells for patch-clamp studies. Although our cell yields are low, as might be expected from preparations in which the muscle layer is some 3–4 cells thick (unpublished observations) it has proved possible to obtain enough cells to allow a characterization of their membrane currents.

We have demonstrated that these cells contain several inward currents (Hollywood *et al.* 1997) and a variety of outward currents, including a Ca^{2+}-sensitive current, and a voltage-dependent current which itself probably consists of more than one component. The Ca^{2+}-sensitive current observed under the conditions of our experiments appears to be mediated mainly by large conductance Ca^{2+}-activated K^+ channels (BK channels) as (1) it was sensitive to both iberiotoxin and penitrem A, which are thought to be highly selective blockers of these channels (Garcia, Galvez, Garcia-Calvo, King, Vazquez & Kaczorowski, 1991; Knaus *et al.* 1994), and (2) large channels with the characteristics of BK channels were present in inside-out patches derived from lymphatic cell membranes and, indeed, were by far the most prominent channel observed. The average conductance of 304 pS is at the upper end of the conductances described for other BK channels, which typically range from 200 to 300 pS (Carl, Lee & Sanders, 1996). This can probably be explained by the fact that our recordings were made at 37 °C, rather than room temperature, since temperature is known to affect the conductance of these channels (Barrett, Magleby & Pallotta, 1982). We have previously described a 298 pS Ca^{2+}-activated K^+ channel in sheep bladder muscle cells under similar recording conditions, suggesting that the BK channels are similar in both of these tissues (Cotton *et al.* 1996*b*).

The BK current predominated in whole-cell experiments, accounting for up to 90% of the outward current, even though the pipette solution contained 1 mM EGTA. The ability to see this current in several other preparations depended on using lower pipette EGTA concentrations (e.g. Thornbury, Ward & Sanders, 1992*a*), but it has also been observed in smooth muscle cells using concentrations similar to ours (Beech & Bolton, 1989*b*). These differences may reflect differences in subcellular structure (such as coupling of Ca^{2+} and BK channels or accessibility of the submembrane space to EGTA) or could conceivably depend upon differences in the sensitivity of the BK channels to Ca^{2+}. The Ca^{2+} sensitivity may be defined in terms of the shift in $V_{½}$ per 10-fold change in $[Ca^{2+}]_i$ ('$\Delta V_{½}$'; Carl *et al.* 1996). In our experiments $\Delta V_{½}$ was 146 mV, which places lymphatic BK channels amongst the most Ca^{2+} sensitive (Carl *et al.* 1996), although this may also partly reflect the fact that the experiments were carried out at 37 °C.

When all of the BK current was blocked, there was a remaining voltage-dependent outward current. This was absent in cells which were dialysed with Cs^+, instead of K^+ (Hollywood *et al.* 1997), and was reduced by TEA and 4-AP. It is highly likely, therefore, that this current was carried by K^+ ions. Voltage-dependent K^+ currents in smooth muscle fall into two broad groups, (1) 'transient outward' currents characterized by rapid activation and inactivation kinetics, steep voltage-dependent inactivation ($V_{½}$ negative to −60 mV), sensitivity to 4-AP and resistance to TEA, and (2) 'delayed rectifier' currents which inactivate more slowly, inactivate over a more positive voltage range ($V_{½} = -40$ mV) and are variably sensitive to blockade by TEA and 4-AP. Examples of the former are found in guinea-pig ureter (Lang, 1989; Imaizumi, Muraki & Watanabe, 1990), rabbit portal vein (Beech & Bolton, 1989*a*), rat ileum (Smirnov, Zholos & Shuba, 1992) and guinea-pig proximal colon (Vogalis, Lang, Bywater & Taylor, 1993), while examples of the latter are found in rabbit pulmonary artery (Okabe, Kitamura & Kuriyama, 1987), rabbit portal vein (Beech & Bolton, 1989*b*), and canine proximal colon (Thornbury *et al.* 1992*a*; Thornbury, Ward & Sanders, 1992*b*; Carl, 1995). The current described in the present study falls more readily into the second group as it inactivated with a time constant of around 100 ms and had an inactivation $V_{½}$ of −41 mV. In most of our experiments 4-AP had little differential effect on peak current at the beginning of the 500 ms test pulse compared with the sustained current at the end of the pulse (Fig. 6*A–C*), thus providing little evidence of selective blockade of a transient current (Lang, 1989).

The delayed rectifier current in lymphatic cells may be mediated by several K^+ channel subtypes, as TEA and 4-AP appeared to block different components of current. One was sensitive to blockade by both TEA and 4-AP, and another was TEA sensitive but 4-AP insensitive. There was also a small residual current after application of 30 mM TEA, which may possibly represent a third component. Multiple components of delayed rectifier current have also been proposed in canine colon, where a detailed analysis has suggested that there is a fast-activating 4-AP-sensitive current and a slower-activating TEA-sensitive current (Carl, 1995).

In studies such as this, where tissue samples are collected from an abattoir, it is not possible to control either the source of the material or the previous treatment of the experimental animals. Thus individual variation might be expected to be greater than when pure bred strains of animals are used under better controlled conditions. Such factors could have accounted for some of the variation we have observed between cells. For example, the absolute amplitudes of the currents varied somewhat, as did the proportion of BK current to voltage-dependent current. Similarly, the expression of inward currents varied from cell to cell. To some extent the cells in which the voltage-dependent outward currents were studied represent a subgroup, as there was a tendency to select those cells which expressed measurable currents under conditions where the BK current was blocked. It is also possible that the cells dispersed from individual lymphatic vessels are heterogeneous, expressing different currents depending on

their role (e.g. whether they are pacemakers etc.) or location within the vessel wall. On this basis it would be unwise to exclude the possibility that certain currents (such as the transient outward current) exist within a subpopulation of lymphatic cells.

We are at too early a stage to propose definite roles for the currents which have been described, as there is little information regarding the nature of lymphatic spontaneous electrical activity. Sucrose gap recordings in large bovine mesenteric lymphatics (diameter, 2–4 mm) have suggested that each spontaneous contraction is preceded by a single action potential (Kirkpatrick & McHale, 1977; Allen, McHale & Rooney, 1983), while intracellular recordings in small (diameter, < 200 μm) mesenteric lymphatics of the guinea-pig also point to a one-to-one relationship (Van Helden, 1993). Preliminary intracellular recordings in the larger bovine mesenteric vessels, however, revealed the occurrence of spontaneous spike complexes, which are presumably related to contractions (Ward, McHale & Sanders, 1989; Ward *et al.* 1991). It is possible that both the voltage-dependent current and the BK current could play a part in repolarizing lymphatic action potentials. In double sucrose gap studies on bovine mesenteric lymphatics, 10 mM TEA increased the amplitude and duration of the evoked action potential (Allen, Iggulden & McHale, 1986; Allen & McHale, 1988). Since TEA is a potent blocker of BK channels (see Carl *et al.* 1996) as well as the voltage-dependent K^+ current, its effect on the action potential is difficult to interpret. Interestingly, 4-AP had little effect on evoked action potentials in bovine lymphatics (Allen & McHale, 1988), although it depolarized them and increased their firing rate (Ward *et al.* 1991). This is difficult to reconcile with the finding that 4-AP blocked a component of delayed rectifier current, but did not affect the holding current at potentials near the resting potential (−60 mV). Further investigation will be necessary to determine whether this discrepancy may be explained by species differences or by other factors such as different experimental conditions.

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