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Toland, H. M., K. D. McCloskey, K. D. Thornbury, N. G. McHale, and M. A. Hollywood. Ca^{2+}-activated Cl^- current in sheep lymphatic smooth muscle. *Am J Physiol Cell Physiol* 279: C1327–C1335, 2000.—Freshly dispersed sheep mesenteric lymphatic smooth muscle cells were studied at 37°C using the perforated patch-clamp technique with Cs^+- and K^+-filled pipettes. Depolarizing steps evoked currents that consisted of L-type Ca^{2+} [$I_{Ca(L)}$] current and a slowly developing current. The slow current reversed at 1 ± 1.5 mV with symmetrical Cl^- concentrations compared with 23.2 ± 1.2 mV (n = 5) and −34.3 ± 3.5 mV (n = 4) when external Cl^- was substituted with either glutamate (86 mM) or I^- (125 mM). Nifedipine (1 μM) blocked and BAY K 8644 enhanced $I_{Ca(L)}$, the slow-developing sustained current, and the tail current. The Cl^- channel blocker anthracene-9-carboxylic acid (9-AC) reduced only the slowly developing inward and tail currents. Application of caffeine (10 mM) to voltage-clamped cells evoked currents that reversed close to the Cl^- equilibrium potential and were sensitive to 9-AC. Small spontaneous transient depolarizations and larger action potentials were observed in current clamp, and these were blocked by 9-AC. Evoked action potentials were triphasic and had a prominent plateau phase that was selectively blocked by 9-AC. Similarly, fluid output was reduced by 9-AC in doubly cannulated segments of spontaneously pumping sheep lymphatics, suggesting that the Ca^{2+}-activated Cl^- current plays an important role in the electrical activity underlying spontaneous activity in this tissue.

lymphatics; pacemaking; action potentials; spontaneous activity

ALTHOUGH IT IS NOW well accepted that lymphatic vessels produce regular spontaneous contractions that serve to propel fluid from the interstitium back to the general circulation (4, 6, 7, 12, 25, 31), relatively little is known about the ionic mechanisms underlying this spontaneous activity. Van Helden (31) demonstrated that segments of guinea pig mesenteric lymphatic smooth muscle generated small spontaneous transient depolarizations (STDs) and larger action potentials. The initial phase of the action potentials exhibited a similar time course to the rising phase of the STDs, suggesting that STDs either singularly, or through summation, depolarized the membrane sufficiently to initiate action potential firing. Thus STDs were proposed to provide the mechanism for pacemaking in guinea pig mesenteric lymphatics. Van Helden (31) postulated that STDs were generated by the transient activation of a Cl^- conductance, since reducing extracellular Cl^- or chelation of intracellular Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-AM reduced STD amplitude. However, as of yet, such a Cl^- conductance has not been demonstrated or characterized under voltage clamp in lymphatic smooth muscle.

In the present study, we demonstrate for the first time the presence of a Ca^{2+}-activated Cl^- conductance in lymphatics and further suggest that its presence is necessary for normal spontaneous contractions in these vessels.

MATERIALS AND METHODS

Cell isolation. Mesenteric lymphatic vessels were removed from sheep of either sex at a local abattoir within 15 min of slaughter. These were transported to the laboratory in oxygenated Krebs solution at 37°C where they were dissected free from the mesenteric fat and either used immediately or stored at 4°C for use the following day. Several 2- to 3-cm lengths of lymphatic smooth muscle were cut into small pieces (<1 mm^3) and placed in dispersal medium containing [per 5 ml of Ca^{2+}-free Hanks' solution (see *Solutions*)] 15 mg collagenase (Sigma type 1A), 0.5 mg protease (Sigma type XXIV), 5 mg BSA (Sigma), and 15 mg trypsin inhibitor (Sigma) for 25–35 min at 37°C. Tissue was then transferred to Ca^{2+}-free Hanks' solution and stirred for a further 15–30 min to release single relaxed smooth muscle cells. These cells were plated in petri dishes containing 100 μM Ca^{2+} Hanks' solution and were stored at 4°C for use within 8 h.

Solutions. The composition of the solutions used was as follows (in mM): *1*) Hanks' solution: 129.8 Na^+, 5.8 K^+, 135 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 glucose, 2.9 sucrose, and 10 HEPES, pH adjusted to 7.4 with NaOH; *2*) 49 mM Cl^- Hanks' solution: 129.8 Na^+, 5.8 K^+, 86 glutamate, 49 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 dextrose, 2.9 sucrose, and 10 HEPES, pH adjusted to 7.4 with NaOH; *3*) I^--substituted Hanks' solution: 129.8 Na^+, 5.8 K^+,

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125 I^-, 10 Cl^-, 4.17 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 dextrose, 2.9 sucrose, and 10 HEPES, pH adjusted to 7.4 with NaOH; *4*) Cs^+ perforated patch pipette solution: 133 Cs^+, 135 Cl^-, 1.0 Mg^{2+}, 0.5 EGTA, and 10 HEPES, pH adjusted to 7.2 with CsOH; *5*) K^+ perforated patch pipette solution: 133 K^+, 135 Cl^-, 1.0 Mg^{2+}, 0.5 EGTA, and 10 HEPES, pH adjusted to 7.2 with KOH; and *6*) Krebs solution: 146.2 Na^+, 5.9 K^+, 133.3 Cl^-, 25 HCO_3^-, 1.2 $H_2PO_4^-$, 2.5 Ca^{2+}, 1.2 Mg^{2+}, and 11 glucose, pH maintained at 7.4 by bubbling with 95% O_2-5% CO_2.

Perforated patch voltage clamp of single cells. Currents were recorded using the perforated patch configuration of the whole cell patch-clamp technique (15, 22, 30). This circumvented the problem of current rundown encountered using the conventional whole cell configuration. The cell membrane was perforated using the antibiotic amphotericin B (600 μg/ml). Patch pipettes were initially front filled by being dipped in pipette solution and then were back filled with the amphotericin B-containing solution. Pipettes were pulled from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.17 mm inner diameter; Clark Medical Instruments) to a tip diameter of ~1–1.5 μm and resistance of 2–4 MΩ.

Series resistance and the capacitative current were usually uncompensated for in this study since the maximal voltage error arising from the series resistance during test potentials was in the range of 0.5–6.0 mV, given that maximal currents evoked were in the range of 50–300 pA. Voltage-clamp commands were delivered via an Axopatch 1D patch-clamp amplifier (Axon Instruments), and membrane currents were recorded by a 12-bit AD/DA converter (Axodata 1200 or Labmaster; Scientific Solutions) interfaced to an AT-type computer running pClamp software. During experiments, the dish containing the cells was continuously perfused with Hanks' solution (see *Solutions*) at 37 ± 1°C. Additionally, the cell under study was continuously superfused by means of a custom-built close delivery system with a pipette of tip diameter 200 μm placed ~300 μm from the cell. The Hanks' solution in the close delivery system could be switched to a drug-containing solution with a dead space time of <5 s.

Isolated cells were continually perfused with TTX (1 μM) to avoid contamination by the fast sodium current previously described in these cells (21). Cs^+-filled pipettes were used to block K^+ currents (9), and the holding potential was set at −60 mV to inactivate T-type Ca^{2+} current (20). Action potentials were recorded with K^+ pipettes in Hanks' solution in the absence of TTX and were evoked by injecting a depolarizing current of 100 pA for 40 ms. To facilitate study of a Cl^- conductance, Cl^- equilibrium potential (E_{Cl}) was set to 0 mV by using symmetrical Cl^- concentrations (135 mM) across the cell membrane. Where stated, data were corrected for junction potentials of −3 mV in standard Hanks' solution, +2 mV in low-Cl^- Hanks', and +2 mV in I^--substituted Hanks' as measured according to the method described previously (27). Summary data are presented as means ± SE, and statistical comparisons were made using Student's paired *t*-test, taking $P < 0.05$ as significant.

Isolated doubly cannulated lymphatic vessel recordings. Sections of mesenteric lymphatic vessels 4–7 cm long and 1–3 mm in diameter were cannulated at both ends with polythene tubing, mounted horizontally in an open-topped, water-jacketed organ bath, and perfused with Krebs' solution at 37 ± 1°C as described previously (25). The inflow cannula was connected to a pressure reservoir of variable height, and the outflow cannula was connected to a drop counter. The inflow reservoir and outflow cannulas were maintained at the same level so that when the vessel was not contracting the inflow and outflow pressures were equal and there was no movement of fluid through the vessel. However, when the vessel developed contractile activity, any flow that occurred was due to pumping activity of the vessel. The transmural pressure was set to 2–4 cmH_20, and the preparation was allowed to equilibrate for 30 min, during which time spontaneous pumping activity usually developed. Contractions were measured as pressure fluctuations at the outflow end of the lymphatic using a Statham P23H pressure transducer with the output recorded on a Gould 2200 chart recorder. Fluid output was measured by a custom-built drop counter and was recorded simultaneously on the Gould chart recorder. Flow was intermittent, with each contraction resulting in the expulsion of a number of drops. The drop counter was reset at 1-min intervals so that output consisted of a series of ramps, the heights of which were an index of the flow rate. During experiments, the Krebs solution in the organ bath could be completely exchanged for a drug-containing solution within 5 min.

The following drugs were used: anthracene-9-carboxylic acid (9-AC), caffeine (Sigma), BAY K 8644, nifedipine (Bayer), and TTX (Tocris Cookson). Stock solutions of nifedipine, BAY K 8644, and TTX were made up in ethanol (1 mM stock). 9-AC was made up in DMSO (100 mM stock). All other compounds were dissolved directly in the perfusate.

RESULTS

L-type Ca^{2+} current and Ca^{2+}-activated Cl^- current. As noted previously (9), the yield of cells was usually modest, but enough healthy relaxed smooth muscle cells were obtained to make experimentation possible. When cells were held at −60 mV and then stepped to a series of potentials from −80 mV to +50 mV for 500 ms in 10-mV increments, a family of inward, outward, and tail currents (I_{tail}) were evoked and are shown in Fig. 1*A*. Similar responses to voltage steps were found in >70% of cells studied. The inward current was comprised of two components: an initial fast-activating, fast-inactivating current and a more slowly developing, sustained current. After repolarization to −60 mV, a slowly decaying inward I_{tail} was observed. The fast inward current was measured within the first 20 ms of the test pulse, as indicated in Fig. 1*A*, and the slowly developing current was measured at the end of the 500-ms pulse. This procedure was chosen to minimize contamination of the slowly developing current with the rapidly activating and inactivating current. Figure 1*B* shows a plot of the current-voltage relationship for 21 cells. The fast current activated at potentials positive to −40 mV and peaked at −10 mV, characteristics typical of the L-type Ca^{2+} [$I_{Ca(L)}$] current previously described in these cells (20). There was considerable variation in the amount of current expressed between different cells. Thus the mean peak Cl^- current measured at −20 mV was −47 ± 9 pA ($n = 21$) with a range of values from −5 to −178 pA. Similarly, the peak Ca^{2+} current at −10 mV averaged −65 pA ± 18 pA ($n = 21$) and ranged from −19 to −346 pA.

Although the slower-developing current also activated close to −40 mV, it peaked at −20 mV and became a large outward current at potentials positive to 0 mV. This sustained current reversed close to 0 mV, which, under the symmetrical Cl^- conditions of these

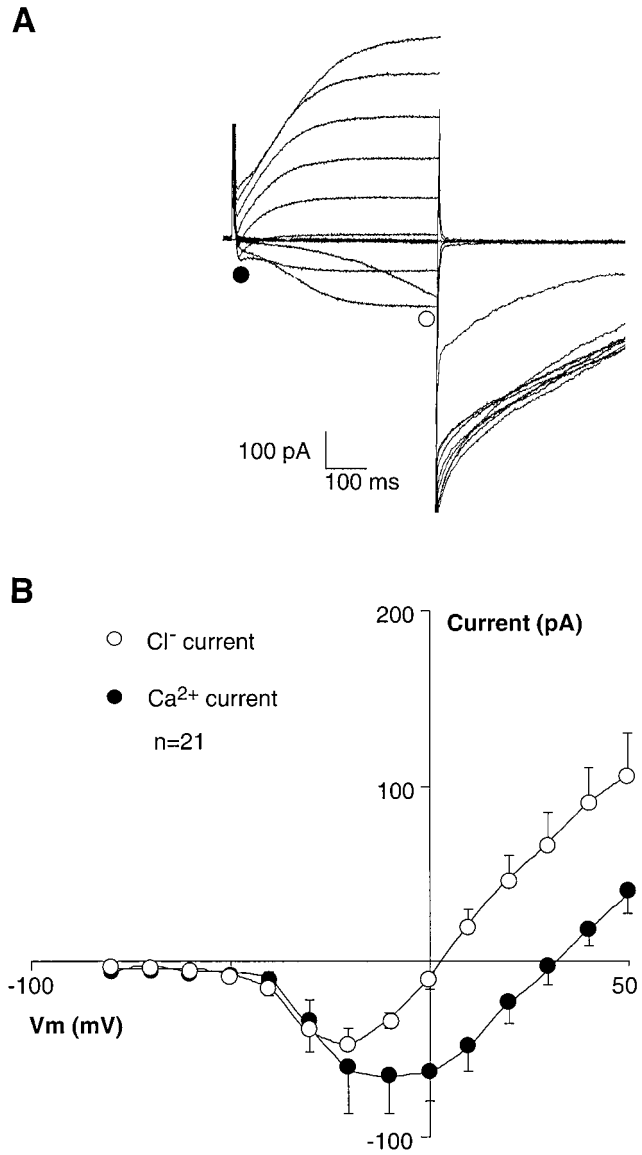

Fig. 1. L-type Ca^{2+} and Ca^{2+}-activated Cl^- current in sheep lymphatic smooth muscle cells. *A*: representative family of currents evoked in a lymphatic smooth muscle cell. Membrane potential was held at −60 mV and was stepped from −80 to +50 mV in 10-mV increments for 500 ms. Note initial fast-activating, fast-inactivating component of inward current (●) followed by a more slowly developing, sustained current (○) and also tail current on stepping from a test potential to a holding potential. *B*: summary of the current-voltage (*I-V*) relationship of the fast-activating current and slower, sustained current evoked in 21 cells using the protocol described in *A*. Currents were leak subtracted before plotting. ●, Mean current measured at beginning of 500-ms pulse; ○, mean current measured at end of the test pulse. Vertical lines show 1 SE.

experiments, is close to the calculated reversal potential (E_{rev}) for a Cl^- conductance.

Effect of nifedipine and BAY K 8644. If the slowly activating inward currents were dependent on the influx of Ca^{2+} through L-type Ca^{2+} channels then it should be possible to block these currents with nifedipine and enhance them with the L-type Ca^{2+} channel agonist BAY K 8644. The following experiments suggested that this was indeed the case. Figure 2*A* shows a current evoked by a step from −60 to −20 mV before and during exposure to the L-type Ca^{2+} channel antagonist nifedipine (1 μM). In the absence of nifedipine, the fast and slow components of inward current could be clearly resolved. Upon repolarization to −60 mV a slow inward I_{tail} was also apparent. After blockade of the $I_{Ca(L)}$, all three components of inward current were abolished. Figure 2*B* shows a summary current voltage relationship for the slowly activating current (measured at the end of the 500-ms pulse) in six similar experiments in the absence and presence of nifedipine. The slowly developing current was significantly reduced by nifedipine ($P < 0.05$). To further investigate the Ca^{2+} dependence of the sustained current, the effect of BAY K 8644 (1 μM), an agent known to enhance $I_{Ca(L)}$, was examined. Figure 2*C* shows the currents evoked in a cell in response to a voltage step from −60 to −20 mV. The rapidly and slowly activating components and the I_{tail} were clearly resolved in the absence of the L-type channel agonist. Application of BAY K 8644 enhanced all three components of inward current. Figure 2*D* shows a summary from seven cells in which the effect of BAY K 8644 was examined across a wide voltage range. The slowly developing current was significantly enhanced by BAY K 8644, with peak current at −20 mV increasing from −48 ± 16 to −100 ± 8 pA ($P < 0.05$). These results suggest that the slowly activating inward current and the I_{tail} evoked by a depolarizing step are dependent on the influx of Ca^{2+} through L-type Ca^{2+} channels.

Effect of ion substitution on the slowly activating current. Under the symmetrical Cl^- conditions used in the experiments described above, the slowly developing inward current reversed close to the E_{Cl} (0 mV). To determine if the slowly activating current was carried by Cl^-, the effect of substituting Cl^- with different anions was examined. We first examined the effect of reducing external Cl^- from 135 to 49 mM by isosmotic substitution with glutamate. The protocol used is shown in Fig. 3, *top*. A cell was stepped from a holding potential of −60 to 0 mV for 500 ms. This was followed by a voltage ramp from −50 to +50 mV for 200 ms. Under symmetrical Cl^- conditions (135 mM extracellular Cl^- concentration), the step to 0 mV evoked only the $I_{Ca(L)}$, and the I_{tail} reversed at +2 mV. This was close to the E_{Cl} of 0 mV predicted by the Nernst equation. The external Cl^- concentration was then reduced to 49 mM by substitution with glutamate (see *Solutions*), giving a new calculated value for E_{Cl} of +27 mV. Under these conditions, stepping to 0 mV not only evoked $I_{Ca(L)}$ but also a slowly developing inward current indicative of a positive shift in the E_{rev} of the slowly developing current. The I_{tail} E_{rev} moved to +22 mV, close to the new E_{Cl}. The effect of reducing external Cl^- concentration on I_{tail} E_{rev} was examined in four other cells and resulted in a mean shift in E_{rev} from 1 ± 1.5 mV in symmetrical Cl^- solutions to 23 ± 1.2 mV (data corrected for junction potentials of −3 and +2 mV in normal and reduced external Cl^- solutions, respectively). Conversely, when the external Cl^- were substituted with I^-, the E_{rev} of the slowly activating

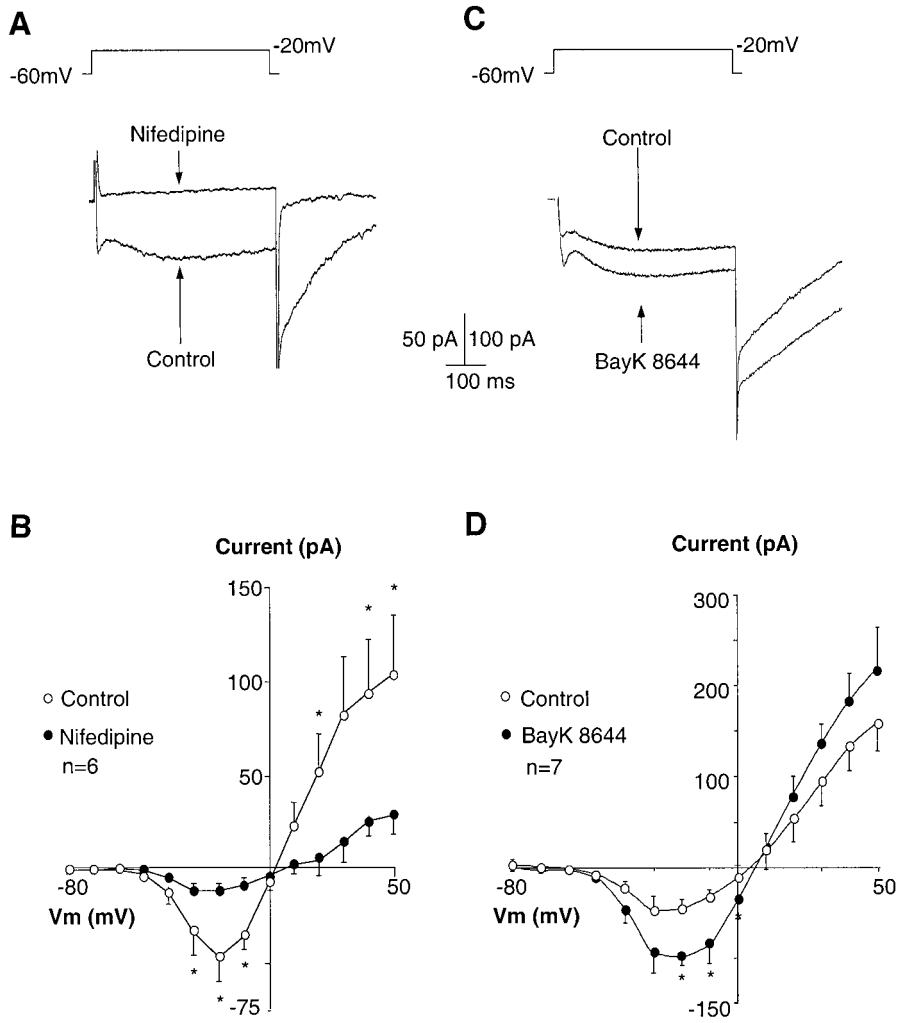

Fig. 2. Effect of nifedipine and BAY K 8644 on inward currents. *A*: inward currents recorded in a cell stepped from a holding potential of −60 to −20 mV for 500 ms before and during exposure to 1 μM nifedipine. The initial fast current, slower inward current, and tail current were blocked by nifedipine. *B*: summary *I-V* relationship of sustained inward current evoked using the protocol described in Fig. 1. Current was measured at end of 500-ms pulse in 6 cells before (○) and during (●) exposure to 1 μM nifedipine. Vertical lines show 1 SE. *C*: inward currents recorded in a cell held at −60 mV and stepped to −20 mV for 500 ms before and during exposure to 1 μM BAY K 8644. The initial fast current and slower inward and tail currents are enhanced in the presence of BAY K 8644. *D*: summary *I-V* relationship of the sustained inward current in 7 cells evoked by the protocol described in Fig. 1 before (○) and during (●) exposure to 1 μM BAY K 8644. *Significant enhancement of sustained inward current ($P < 0.05$). Vertical lines show 1 SE.

inward current shifted negatively, as demonstrated in a number of other studies (11, 14, 23). In four cells, replacement of external Cl^- with I^- shifted the E_{rev} from −4.5 ± 1.6 to −34.3 ± 3.5 mV (data corrected for junctions potentials). These results suggest that the slowly activating current and I_{tail} were predominantly carried by Cl^-.

Effect of 9-AC on currents. The data presented so far support the idea that the slowly activating current is a Ca^{2+}-activated Cl^- current [$I_{Cl(Ca)}$]. If this is the case, then the current should be reduced by Cl^- channel blockers such as 9-AC. The experiment shown in Fig. 4*A* suggested this was the case. In this experiment, the cell was depolarized from −60 to −20 and 0 mV for 500 ms. Under control conditions, stepping to 0 mV evoked $I_{Ca(L)}$ and I_{tail}, while stepping to −20 mV evoked $I_{Ca(L)}$, the slowly developing current, and I_{tail}. During application of 1 mM 9-AC, stepping to 0 mV still activated $I_{Ca(L)}$, but the I_{tail} was clearly reduced. Furthermore, when the cell was depolarized to −20 mV, both the sustained current and the I_{tail} were abolished. Figure 4*B* shows a summary for nine cells in which the effect of 9-AC was examined across the voltage range −80 to +50 mV. 9-AC significantly reduced the sustained current ($P < 0.05$). Although 9-AC significantly inhibited the Cl^- current it did not significantly reduce the $I_{Ca(L)}$ (peak current at 0 mV was −63 ± 16 pA before compared with −48 ± 11 pA in 9-AC, $n = 11$, $P = 0.28$), suggesting that the inhibitory effect of 9-AC on Cl^- current was not due to a decrease in Ca^{2+} influx.

Effect of Ca^{2+} release from intracellular stores. To establish if the release of Ca^{2+} from intracellular stores could activate the Cl^- current in lymphatic smooth muscle cells, the effect of caffeine was examined at a variety of different voltages. Cells were held at a range of values from −80 to +60 mV, and caffeine (10 mM) was administered for 10 s at each potential. Approximately 90 s were allowed between consecutive applications of caffeine to ensure repeatable responses. Application of caffeine at negative potentials evoked inward currents that reversed when the cell was held at potentials positive to E_{Cl} (0 mV; Fig. 5*A*). The caffeine-evoked current decayed within 4–8 s of onset despite the continued presence of caffeine. Figure 5*B* shows a plot of the peak caffeine-evoked current against a holding potential in seven cells. The caffeine-evoked current was inward at potentials negative to 0 mV and reversed close to 0 mV, a finding consistent

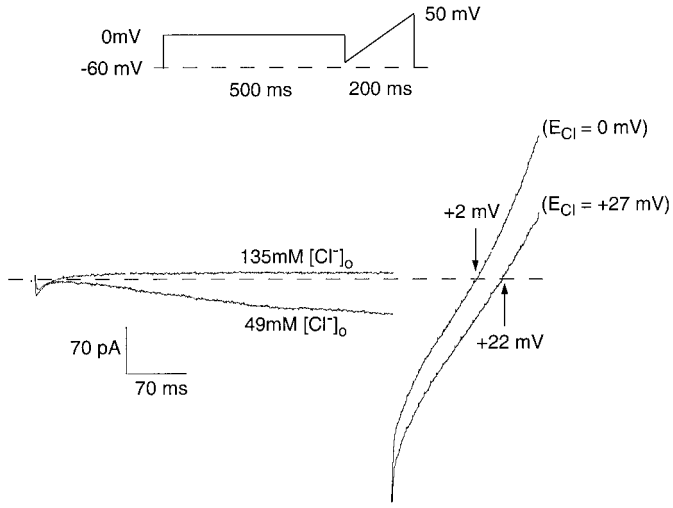

Fig. 3. Effect of ion substitution on the slowly activating inward current and tail current. Inward and tail currents were evoked using the protocol illustrated. A cell was held at −60 mV and stepped to 0 mV for 500 ms. A voltage ramp from −50 to +50 mV was subsequently applied. In standard Hanks' solution (135 mM Cl^-), the step to 0 mV evoked the Ca^{2+} current (I_{Ca}) only, and the tail current (I_{tail}) reversed at +2 mV. Dashed line represents zero current. Under reduced external Cl^- conditions (49 mM Cl^-) the step to 0 mV evoked both the L-type Ca^{2+} current [$I_{Ca(L)}$] and the slower developing inward current, and the I_{tail} reversed at +22 mV. E_{Cl}, Cl^- equilibrium potential; $[Cl^-]_o$, extracellular Cl^- concentration.

with the activation of a Cl^- conductance under the symmetrical Cl^- conditions of the experiments. There was also evidence of outward rectification at the more positive potential, suggesting that this current may show some voltage sensitivity. The Cl^- current showed a similar pharmacology to the slowly activating Cl^- current evoked by a depolarizing step and thus was reversibly blocked by 9-AC (Fig. 5*C*). The effect of 1 mM 9-AC on caffeine-evoked current at −80 mV in four cells is illustrated in Fig. 5*D*. 9-AC significantly reduced the inward current from −117 ± 15 to −21 ± 11 pA ($P < 0.05$).

Effect of 9-AC on spontaneous activity in single cells. In an attempt to examine the possible physiological role of such a current in lymphatic smooth muscle, we examined electrical activity under current-clamp mode using Cs^+ pipettes. In these experiments, it was necessary to inject a steady background current to bring the membrane potential to −60 mV. In more than 1,000 cells examined in the course of this and another related project (24), spontaneous activity was observed in <1% of cells. Spontaneously active cells exhibited transient depolarizations of variable amplitude (9.8 ± 0.8 mV, $n = 7$ cells), a rise time of 292 ± 28 ms ($n = 7$ cells), and were occasionally large enough to raise the membrane potential to the threshold value (approximately −40 mV) from which an action potential was fired. Figure 6 shows typical examples of STDs and action potentials recorded from a lymphatic smooth muscle cell using a Cs^+-filled pipette. Figure 6*A*, *top*, shows recordings from a cell in which both small transient depolarizations and action potentials were present. Figure 6*A*, *bottom*, shows an action potential and STD displayed on an expanded time scale. The action potential was biphasic, consisting of a small depolarization that clearly preceded and initiated the more rapid depolarization. When the STD marked with the asterisk was scaled in amplitude, it was apparent that the time course of activation for the STDs and the initial part of the action potential were very similar.

The pattern of spontaneous activity observed in single cells was very similar to that in guinea pig lymphatics (31), so it was of interest to test the idea that it was due to activation of a Cl^- conductance. Figure 6*B* shows a continuous recording of spontaneous activity before, during, and after application of the Cl^- channel blocker 9-AC (1 mM). When 9-AC was applied, the

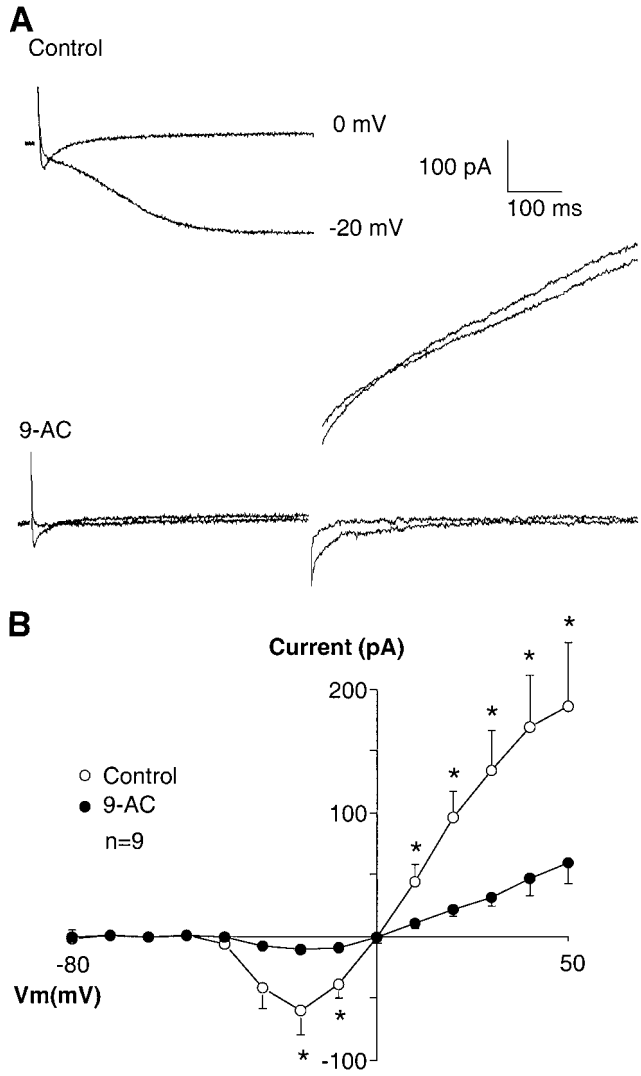

Fig. 4. Blockade of the slow inward current by the Cl^- channel blocker anthracene-9-carboxylic acid (9-AC). *A*: inward currents recorded in a cell held at −60 mV and stepped to −20 and 0 mV for 500 ms before (*top*) and during (*bottom*) exposure to 1 mM 9-AC. The initial fast current was unaffected by 9-AC exposure, whereas the slower developing and tail currents were blocked by 9-AC. *B*: summary *I-V* relationship of the sustained current measured at the end of the 500-ms pulse using the protocol described in Fig. 1 in 9 cells before (○) and during (●) exposure to 1 mM 9-AC. *Significant block of inward and outward currents ($P < 0.05$). Vertical lines show 1 SE.

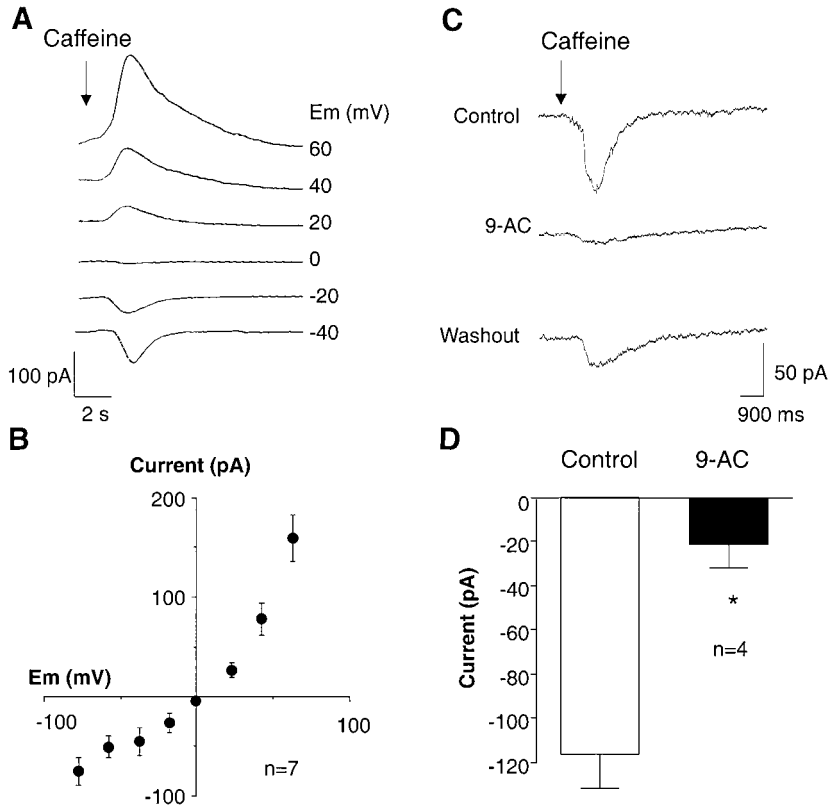

Fig. 5. Caffeine evokes Cl^- current in lymphatic smooth muscle cells. *A*: inward and outward currents evoked by 10-s pulses of 10 mM caffeine applied to a cell held at range of potentials from −40 to +60 mV. E_m, membrane potential. *B*: mean caffeine-evoked current in 7 cells plotted against holding potential. The current reversed close to 0 mV under symmetrical Cl^- conditions. Vertical lines show 1 SE. *C*: inward currents evoked by 10-s applications of 10 mM caffeine to a cell held at −80 mV. Caffeine-evoked inward currents were reversibly blocked by 1 mM 9-AC. *D*: summary of the effect of 9-AC on caffeine-evoked Cl^- currents in 4 cells. *9-AC significantly reduced the caffeine-evoked current ($P < 0.05$). Vertical lines show 1 SE.

action potentials ceased immediately, and the amplitude of the STDs decreased (compare events in *trace 3* with *trace 1*). Upon washout of 9-AC the STDs gradually increased in amplitude before action potentials were again fired. The inhibition of spontaneous activity by Cl^- channel blockers was observed in three cells.

Effect of 9-AC on evoked action potentials in single cells. Having demonstrated that spontaneous activity was abolished when Cl^- channels were blocked, we next examined the effect of 9-AC on evoked action potentials using K^+ pipettes. Figure 7 shows a typical experiment in which action potentials were evoked every 20 s by the injection of depolarizing current (100 pA, 40-ms duration). The action potentials were triphasic in nature, consisting of an upstroke, repolarization, and slowing after depolarization or plateau. Application of 9-AC (1 mM) had no effect on either the upstroke or repolarization but reduced the amplitude of the plateau, suggesting that this phase was dependent on $I_{Cl(Ca)}$. Similar effects were observed in three cells.

Effect of 9-AC on spontaneous contractile activity of whole tissue. The results from the above experiments on single cells suggest that the Cl^- current contributes to both the generation of spontaneous activity and the plateau phase of the action potential. To establish a role for $I_{Cl(Ca)}$ in the regular spontaneous contractile activity of lymphatic smooth muscle, the effect of blocking Cl^- current was examined on a spontaneously pumping segment of lymphatic smooth muscle. Figure 8 shows a recording of fluid output and outflow pressure before, during, and after application of 9-AC (1 mM). Before application of any drugs, the lymphatic contracted at a frequency of 6/min, and ~300 μl of fluid were expelled per minute. Application of 9-AC (1 mM) at first slowed and then reversibly abolished spontaneous contractions. The decrease in spontaneous contractions was associated with a cessation of fluid output. This inhibitory effect of 9-AC was observed in a total of five preparations where contraction frequency was reduced from 6.2 ± 0.8/min before compared with 2.7 ± 1.7/min in the presence of 9-AC. Similarly, flow was reduced from 225 ± 40 to 58 ± 25 μl/min in the presence of 9-AC ($P < 0.02$).

DISCUSSION

Although it is now well established that lymphatics produce regular spontaneous contractions in a variety of species (4, 6, 7, 12, 25, 31), little is known about the mechanisms underlying this rhythmic activity. The demonstration of STDs in guinea pig lymphatics suggests that a Cl^- conductance may act as the pacemaker current in that tissue (31). The E_{Cl} in smooth muscle is thought to be between −30 and −20 mV because of the relatively high intracellular Cl^- concentration in these cells (1). Consequently, activation of a Cl^- current will tend to depolarize cells and may elicit action potentials. However, to date, no study has demonstrated the presence of such a current in lymphatic smooth muscle under voltage-clamp conditions.

Several pieces of evidence suggest that the slowly activating current in sheep lymphatic myocytes is similar to the $I_{Cl(Ca)}$ found in a variety of smooth muscles (reviewed in Ref. 23). The E_{rev} of the current in sheep

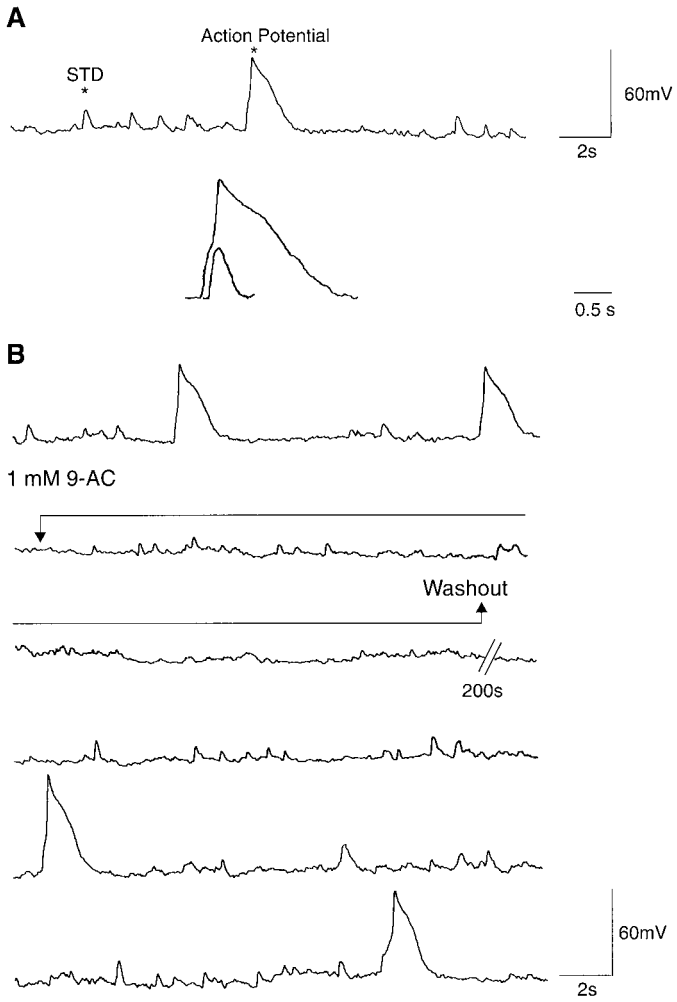

Fig. 6. Effect of 9-AC on spontaneously active smooth muscle cells. *A, top*: spontaneous transient depolarizations (STDs) and action potentials recorded from a lymphatic smooth muscle cell in current-clamp mode using a Cs^+-filled pipette. *Inset* shows an expanded action potential and STD taken from *top* (*). *B*: addition of 1 mM 9-AC reduced the amplitude of the STDs and abolished the action potentials. After washout of 9-AC STDs, action potentials returned. Sweeps are consecutive with exception of a 200-s gap indicated at end of *sweep 3*.

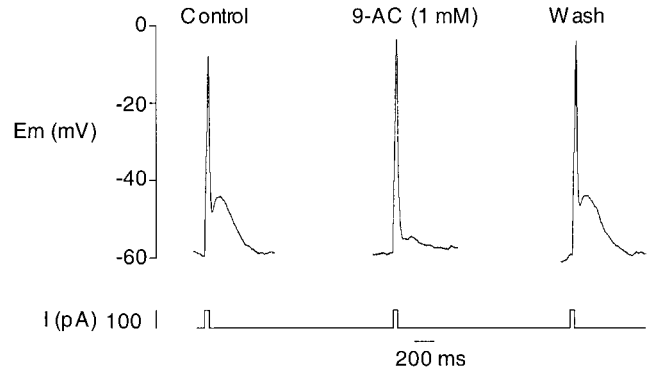

Fig. 7. Cl^- channel blockade abolishes the plateau phase of the action potential. Action potentials were elicited every 20 s by injecting depolarizing current for 40 ms in cells studied with K^+ pipettes. The action potentials were reproducible and triphasic in nature. 9-AC (1 mM) reversibly abolished the plateau phase of the action potential.

lymphatics was close to the calculated E_{rev} for Cl^-, changed in a predictable manner when the Cl^- were substituted with either glutamate or I^-, was blocked by the Cl^- channel blocker 9-AC, and was activated by either Ca^{2+} influx through L-type Ca^{2+} channels or the release of Ca^{2+} from intracellular stores.

To assess the relative permeability of the slowly activating inward current to different anions, the shift in E_{rev} of the current was measured in response to substitution of the Cl^- with either glutamate or I^-. Replacement of external Cl^- with glutamate, an anion thought to be relatively impermeant, shifted E_{rev} positively to a similar extent to that found in urethral and gastrointestinal smooth muscle (8, 29). In contrast, replacement of external Cl^- with I^- shifted E_{rev} negatively, as observed in other smooth muscle preparations (3, 14, 28). When the permeability ratios of these three anions were calculated using the Goldman-Hodgkin-Katz equation (13, 18), values for the permeability of I^- vs. Cl^- were equal to 3.2 and those for the permeability of glutamate vs. Cl^- were equal to 0.11; these values were obtained to give a permeability sequence of $I^- > Cl^- >$ glutamate. These values are in agreement with those found in rat lacrimal gland cells (11), rabbit portal vein myocytes (14), and in other smooth muscle preparations (23). These data are consistent with the view that the slowly activating current is carried mainly by Cl^- through channels with similar permeability properties to $I_{Cl(Ca)}$ in other types of tissue.

A number of agents that block Cl^- channels, such as 9-AC, in smooth muscle have been identified (2, 3, 5, 19, 23). These agents vary in their potency and selectivity in different smooth muscle types. 9-AC has been used to block agonist- and voltage step-induced $I_{Cl(Ca)}$ in rabbit portal vein and esophagus and in rat portal vein, with IC_{50} values of 120–650 μM (2, 5, 19, 23). In the present study, 9-AC significantly blocked the depolarization-induced current, I_{tail}, and caffeine-evoked currents at a concentration within the range used in

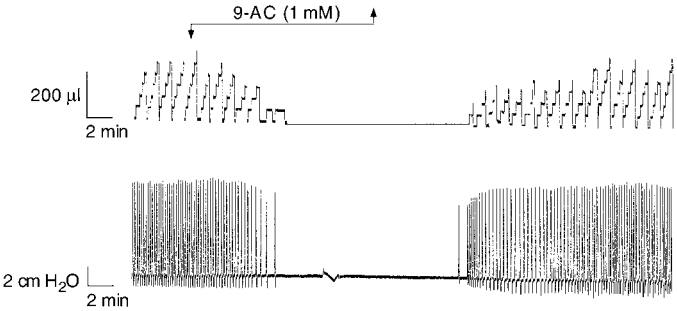

Fig. 8. Inhibition of spontaneous pumping in sheep lymphatic smooth muscle. *Top*: record of fluid output from a double-cannulated segment of lymphatic smooth muscle; *bottom*: record of outflow pressure from a doubly cannulated segment of lymphatic smooth muscle. Application of 9-AC at first slowed the frequency of contractions and then abolished both contractions and fluid output. On washout of 9-AC, both contraction frequency and fluid output returned to control values.

other studies. These data provide further evidence to suggest that the slowly activating current and caffeine-evoked currents were $I_{Cl(Ca)}$. When the effect of 9-AC was investigated on $I_{Ca(L)}$, it did reduce the Ca^{2+} current in some cells, but this effect was not significant and therefore was taken not to account for blockade of $I_{Cl(Ca)}$.

Under the conditions of our experiments we found that the majority of lymphatic smooth muscle cells (~70%) possessed Cl^- currents of variable amplitude although <1% showed regular spontaneous activity. Although this was a surprising finding, it is not without precedent. A recent study on $I_{Cl(Ca)}$ in sheep urethral smooth muscle cells demonstrated that regular spontaneous activity was only observed in ~10% of their cells, even though the majority of cells possessed Cl^- current (8). The reason why so few isolated lymphatic cells were spontaneously active is puzzling given that recording conditions were optimized to allow spontaneous activity to occur (E_{Cl} set at 0 mV, K^+ currents blocked with Cs^+). One possibility is that the isolated smooth muscle cells are damaged in some way during the dispersal procedure, although all of the cells studied were healthy looking relaxed smooth muscle cells. A more likely explanation for the lack of spontaneous activity in our experiments is that lymphatic smooth muscle consists of a heterogeneous population of cells. A recent study on isolated sheep mesenteric lymphatic cells has demonstrated the presence of a hyperpolarization-activated inward current in only 5% of cells, suggesting that such heterogeneity does exist between cells in this tissue (24). It is therefore possible that a minority of cells differ in some way to the bulk of the smooth muscle cells and are capable of firing spontaneous action potentials. However, it was interesting to note that there were no obvious gross morphological differences between electrically quiescent cells and spontaneously active cells using light microscopy in this study.

When spontaneous electrical activity was observed in sheep lymphatic myocytes, it was remarkably similar to that recorded with intracellular microelectrodes in guinea pig mesenteric lymphatics. Thus the sheep lymphatics produced STDs that had a mean amplitude of ~10 mV and a rise time of ~300 ms, values that are similar to the time course and amplitude of STDs in guinea pig lymphatics (31). The action potentials in both guinea pig and sheep lymphatics also appeared to have a large degree of overlap since they were biphasic, and the rise time of the initial "bump" at the beginning of the action potential was very similar to that of the STDs. The above results support the idea that a Ca^{2+}-activated Cl^- conductance underlies the STDs and initiates the action potentials in our single cell experiments. Similar STDs and action potentials have also been recorded in the urethra of sheep and rabbits (8, 16, 17). In both of these tissues, it has been suggested that Ca^{2+} release from the sarcoplasmic reticulum is responsible for the activation of the Cl^- current that in turn depolarizes the membrane and elicits Ca^{2+} action potentials. Whether a similar mechanism acts as the pacemaking current in sheep lymphatics is not clear from the present study, although there is little doubt that release of Ca^{2+} from intracellular stores can activate the Cl^- current and blockade of this current significantly decreases lymphatic contractility. It is interesting that the Cl^- current can also be activated by Ca^{2+} influx through L-type Ca^{2+} channels. If this were to happen during an action potential, then the Cl^- current could maintain Ca^{2+} influx by clamping the membrane potential in the window current for $I_{Ca(L)}$, as has been proposed in the sheep urethra (8). The results shown in Fig. 7 suggest that elicited action potentials in sheep lymphatic smooth muscle have a plateau phase that is sensitive to the Cl^- channel blocker 9-AC. Consequently, the observed inhibitory effects of 9-AC on lymphatic pumping could be explained by either the inhibition of STDs or the inhibition of Ca^{2+} influx during the plateau phase of the action potential.

To suggest that one specific current acts as the pacemaking current in lymphatic smooth muscle is, undoubtedly, an oversimplification. To date, a variety of potential depolarizing currents have been demonstrated in sheep lymphatic smooth muscle (20, 21, 24). We have previously shown that blockade of either the T-type Ca^{2+} current (7) or hyperpolarization-activated inward current (24) slows spontaneous contractions, suggesting that these currents contribute to pacemaking in sheep lymphatics. Thus it is likely that pacemaking in lymphatic vessels is generated not by a single current but relies on complex interactions between a number of currents as is the case in the heart (10). The exact details of how these currents interact with each other in lymphatic vessels are, as yet, far from clear. Future studies may focus on examining how the $I_{Ca(Ca)}$ and other currents act together to produce the regular rhythmic contractions typical of lymphatic smooth muscle.

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