# Spontaneous Electrical Activity in Sheep Mesenteric Lymphatics

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## ABSTRACT

*Background:* **It has recently become apparent that the lymph pump is an electrical entity that rivals the heart in complexity. Many interesting currents have been demonstrated by voltage clamping isolated lymphatic smooth muscle cells, but until now the role of these currents in the intact syncitium has not been studied.**

*Methods and Results:* **Intracellular microelectrode recordings were made from smooth muscle of sheep mesenteric lymphatics to investigate the electrophysiological basis of lymphatic pumping. Approximately 50% of the vessels exhibited spontaneous electrical activity, varying from regular oscillations in membrane potential to spike complexes. Spike complexes generally consisted of one or more action potentials superimposed on a slower depolarization or 'plateau' phase and were often preceded by a slow diastolic depolarization or 'pre-potential'. Norepinephrine (5 $\mu M$) induced depolarizing events in quiescent preparations. Both agonist-induced oscillations and spike complexes were attenuated or completely abolished by 2-aminoethoxydiphenyl borate (2-APB); 10–100 $\mu M$). Cesium (1 m$M$) reduced the frequency of spontaneous firing by approximately 30% by flattening the pre-potential phase. In addition to having a negative inotropic effect, 10 m$M$ Cs$^+$ also caused gradual membrane depolarization and prolonged the plateau. 1 $\mu M$ nifedipine abolished spontaneous events while tetrodotoxin (TTX; 0.5–1 $\mu M$) decreased the amplitude and maximum dV/dt of the spike upstroke or stopped activity completely. Spontaneously active segments of lymphatic vessel were inhibited by the chloride channel blocker, anthracene-9-carboxylic acid (9-AC; 250 $\mu M$ −1 m$M$) suggesting that $I_{Cl(Ca)}$ plays a significant role in the generation of spontaneous activity in this tissue. Penitrem-A (0.1 $\mu M$) did not affect resting membrane potential but increased action potential amplitude and prolonged the plateau, suggesting that calcium-activated potassium current does not make a significant contribution to resting membrane conductance but is important in membrane repolarization following calcium influx during the action potential. In contrast 4-aminopyridine (4-AP; 5 $\mu M$) caused significant membrane depolarization, suggesting the existence of an active 4-AP-sensitive current at rest.**

*Conclusions:* **These results demonstrate that the currents found in isolated voltage-clamped cells from sheep mesenteric lymphatics do play a significant role in the shaping of spontaneous electrical activity of the intact syncitium.**

## INTRODUCTION

In recent years the whole-cell and perforated patch clamp techniques have been successfully employed to study membrane currents in isolated smooth muscle cells from sheep mesenteric lymphatics.[1–3] Using these techniques, it has been possible to demonstrate the existence of a number of ionic conductances that are thought to underlie the generation of spontaneous electrical events in sheep lymphatic smooth muscle. For accurate characterization of currents exhibited by the smooth muscle cells, these techniques have proved invaluable. However, these techniques also have their limitations. It is often difficult to assess the functional relevance of a specific ionic conductance from studies involving single cells that have been completely isolated from the muscle syncytium. Spontaneous activity is very rarely seen in smooth muscle cells isolated from lymphatic preparations (observed in less than 1% of cells) and the resting membrane potentials of the cells are significantly depolarized. Thus, due to the lack of spontaneous activity seen in current-clamped smooth muscle cells, it has been difficult to assess the contribution of each ionic conductance to the shape of the action potentials underlying muscle contractions.

This is the first study in which spontaneous electrical activity has been recorded from sheep mesenteric lymphatics using intracellular microelectrodes. This technique allows assessment of the electrical activity underlying contraction in an intact muscle syncytium. Patch clamp investigations and contractile studies[2–5] have suggested that numerous inward currents including hypolarization-activated inward current, $I_f$ (which can be blocked by cesium and is important as a pacemaker current in the heart); calcium-activated chloride current, $I_{Ca(Cl)}$ (in smooth muscle cells which have a high internal chloride content the outward movement of $Cl^-$ causes depolarization and could also contribute to pacemaking); L-type calcium current, $I_{Ca(L)}$; T-type calcium current, $I_{Ca(T)}$; fast sodium current, $I_{Na}$) and at least one outward potassium current (4-AP-sensitive $I_K$,[1] contribute to the generation and modulation of pacemaking in sheep lymphatics. The purpose of this study was to examine the effects of selective blockers of these currents on membrane potential, pacemaker potentials, and action potentials recorded from spontaneously active segments of lymphatic vessel.

## MATERIALS AND METHODS

Mesenteric lymphatics dissected from freshly slaughtered sheep of either sex were transported from the abattoir to the laboratory in warmed oxygenated Krebs solution. After removing fat and connective tissue by sharp dissection, the vessels were cut into 1–2 cm lengths and either used immediately or stored in Krebs solution at 4°C for use the following day. Each separate experiment was carried out on tissue taken from a different animal; thus $n$ values in each case refer to the number of animals studied.

Two fine steel wires were inserted through the intact lumen of the lymphatic vessel segment. The wires were anchored to the sylgard elastomere, Weisbaden, Germany floor of a 35 mm recording chamber using steel pins. An incision approximately 0.5 cm in length was made at one end of the vessel. The opened section of muscle wall was stretched flat using tungsten pins to reveal the lumenal surface. The tissue preparations were perfused with Krebs solution of composition (m$M$): NaCI, 120; NaHCO$_3$, 25.0; KC1, 5.9; Na$_2$HP0$_4$, 1.2; CaCl, 2.5; MgCl$_2$, 1.2; glucose, 11.0; bubbled with 95% 0$_2$, 5% C0$_2$. The vessel segments were stretched to 150–200% of resting tone and allowed to equilibrate for 1–2 h. Smooth muscle cells in the proximal region of the vessel were impaled with glass microelectrodes filled with 3 $M$ KC1 and having resistances of 80–120 M$\Omega$. Transmembrane potentials were measured with a high-impedence electrometer (IE-251; Warner Instrument Corporation, Hamden, CT) and outputs were displayed on a Gould digital recording oscilloscope (Gould Electronics Ltd, Essex, UK). Electrical signals were recorded using a digital tape recorder (DTR 1200 BioLogic, Claix, France). For analysis and figure production, data was transferred onto a computer running Axoscope 8.0 software via a digital analogue converter (Digidata 1200 series; Axon Instruments, CA).

The following drugs (obtained from Sigma-Aldridch, Dorsett, UK, except where indicated) were used: Cesium chloride, anthracene-9-carboxylic acid (9-AC), penitrem A, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), norepinephrine, 2-aminoethoxydiphenyl borate (2-APB), tetrodotoxin (TTX; Tocris Cookson), nifedipine (Bayer, Berkshire, UK). Stock solutions of nifedipine, TTX, and 2-APB were made in ethanol. 9-AC and penitrem A were made up in dimethyl sulphoxide (DMSO; 100 m$M$ stock). Stock solutions of the remaining drugs were made up in distilled water. The stock solutions were then diluted as required in Krebs solution to the final concentrations as stated in the results section. Krebs solution containing 4-AP was titrated to pH 7.4 using 1 $M$ HC1 before use.

Data were expressed as means $\pm$ SEM unless otherwise stated. Differences in the data before and after drug application were evaluated by the Students paired $t$-test; p values $< 0.05$ were taken as statistically significant. The $n$ values reported in the text refer to the number of animals used.

## RESULTS

Impaled smooth muscle cells of lymphatic preparations had negative resting membrane potentials in the range $-49$ to $-65$ mV ($-57.5 \pm 4.1$; mean $\pm$ SD). Approximately 50% of the lymphatic segments contracted in a rhythmic fashion after an incubation period of 1–2 h, discharging depolarizing events at frequencies of 2.5–8.0 per min (with a mean of 4.8 and a standard deviation of 1.4 min$^{-1}$, $n = 32$).

A variety of patterns of depolarizing events was recorded, both from cells of the same tissue preparation and from cells of different lymphatic segments (Figs. 1A–1H). Twelve cells displayed regular oscillatory changes in membrane potential (average amplitude = 16.3 $\pm$ 2.0 mV, frequency = 4.3 $\pm$ 0.3 min$^{-1}$; mean $\pm$ SEM, $n = 12$; Figs. 1A and B) but did not generate action potentials. Membrane potential decreased at about 300 mV sec$^{-1}$ (i.e., $dV/dt$ max = 0.33 $\pm$ 0.06 V sec$^{-1}$, $n = 12$) and did not go positive to $-30$ mV. The most typical events were spike complexes, during which one or more action potentials were fired (Figs. 1C–H)

and most commonly these were of the type shown in Figures 1C and 1D. A slow diastolic potential (or 'pre-potential') often preceded the rapid depolarizing upstroke. During the pre-potential phase, the membrane gradually depolarized by 1–5 mV (3.0 $\pm$ 0.3 mV) at a rate of 0.5 $\pm$ 0.1 mV sec$^{-1}$. Often, incomplete depolarization occurred after firing of the first spike, giving the appearance of a plateau phase. Frequently additional spikes were superimposed on the plateau (Fig. 1C). The peak depolarization of spike complexes was 40–60 mV (48.6 $\pm$ 2.4 mV) with the average maximum rate of potential change being 7.8 $\pm$ 0.9 V sec$^{-1}$ Average duration of the spike and plateau phases, measured at half maximal amplitude, were 9.5 $\pm$ 1.3 ms and 630 $\pm$ 95 ms respectively ($n = 10$).

In three exceptional preparations, different patterns were evident (E–H). For example, Figures 1E and 1F show activity, seen in two preparations, in which early spikes of the complex were grossly attenuated with only one action potential being fired. The action potential had fast kinetics (duration measured at half maximal amplitude = 5.8 ms; $dV/dt$ max = 9.8 V sec$^{-1}$; and was superimposed on a relatively slow transient depolarization or 'plateau' phase. In another preparation (Figs. 1G and 1H), the slow pacemaker potential ($dV/dt = 0.41 \pm 0.02$ mV sec$^{-1}$) was followed by a rapid depolarizing spike (amplitude = 56.1 $\pm$ 0.6 mV; $dV/dt$ max 20.2 $\pm$ 1.1 V sec$^{-1}$; means and standard errors were calculated from ten separate spike complexes). This spike event differed from those described previously as, immediately after firing, the membrane fully repolarized to resting membrane potential. The average spike duration measured at half maximal amplitude was 6.6 $\pm$ 0.1 msec. The initial fast spike was followed by a much slower depolarization with an average amplitude of 22.1 $\pm$ 0.2 mV. Repolarization from this secondary component was gradual, with resting potential being reached approximately 2 sec after peak depolarization (Fig. 2G). The most rapid change in membrane potential recorded in any of the preparations investigated was 20.8 Vsec$^{-1}$.

### Norepinephrine-induced electrical activity

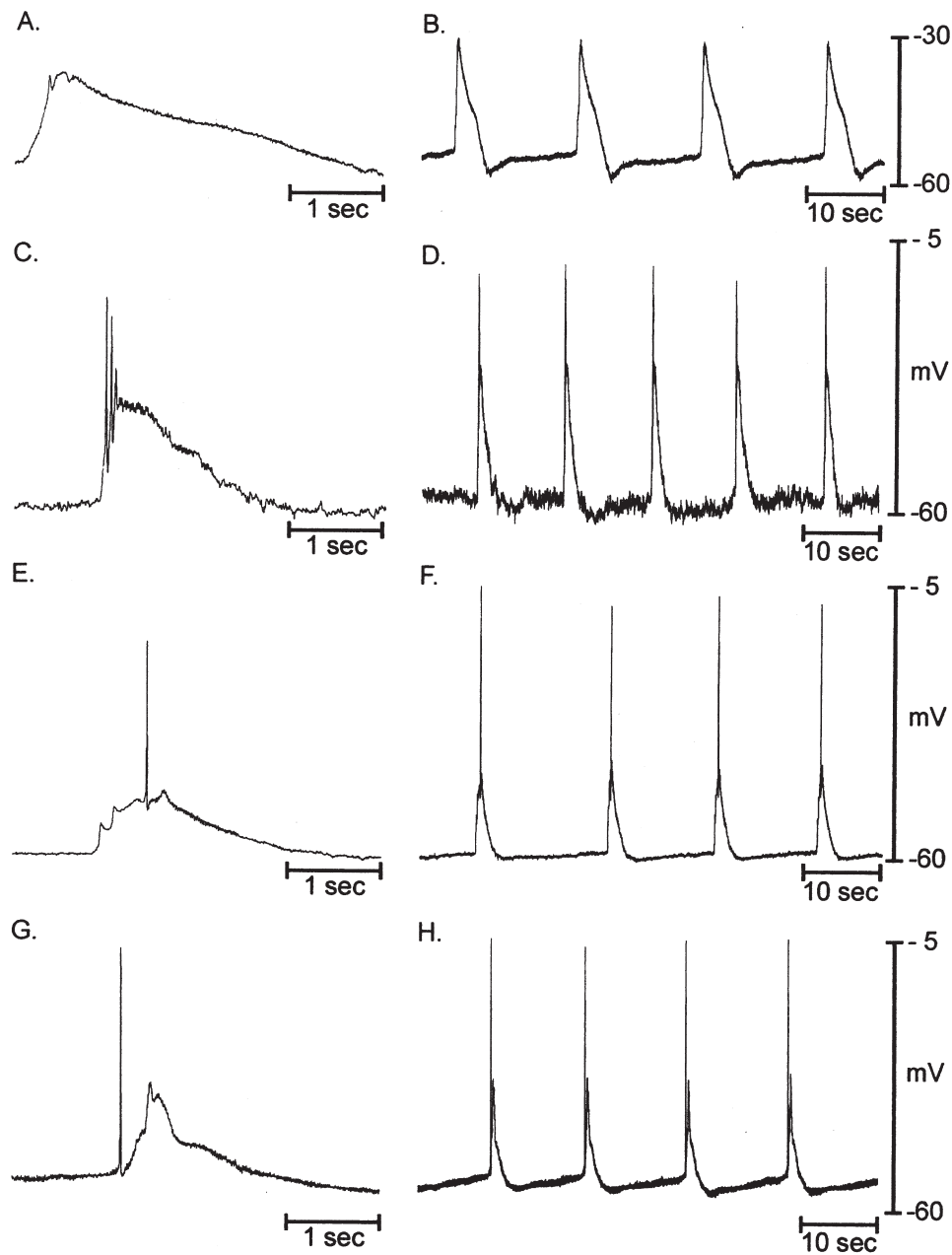Approximately 50% of preparations did not display spontaneous activity after incubation

**FIG. 1.** Different patterns of electrical activity recorded from spontaneously active segments of sheep mesenteric lymphatic vessels. (**A, B**) Slow oscillations of membrane potential which typically did not exceed $-30$ mV. (**C, D**) Spike complexes consisting of a rapid upstroke followed by additional spikes superimposed on a plateau phase. (**E, F**) In some cells, early spikes of the complexes were attenuated, with often only one action potential being fired during each plateau phase. (**G, H**) In this cell, complete membrane repolarization occurred after the initial spike event. This was followed by a secondary depolarization with slower kinetics. Many cells displayed obvious pacemaker potentials such as those seen in (**H**).

in Krebs solution. To establish whether this quiescence was due to the vessels being non-viable, norepinephrine was added. Varying degrees of electrical activity could be evoked in approximately 70% of quiescent tissues by bath perfusion with norepinephrine (5 $\mu M$). Activ-ity ranged from voltage oscillations of 10 to 31 mV (17.6 $\pm$ 2.0 mV, Figs. 2A and 2B) with no superimposed spikes to those with one or more spikes (C–F). The average frequency of discharge was 6.8 $\pm$ 1.1 min$^{-1}$ ($n$ = 18). Norepi-nephrine-induced spike complexes were simi-
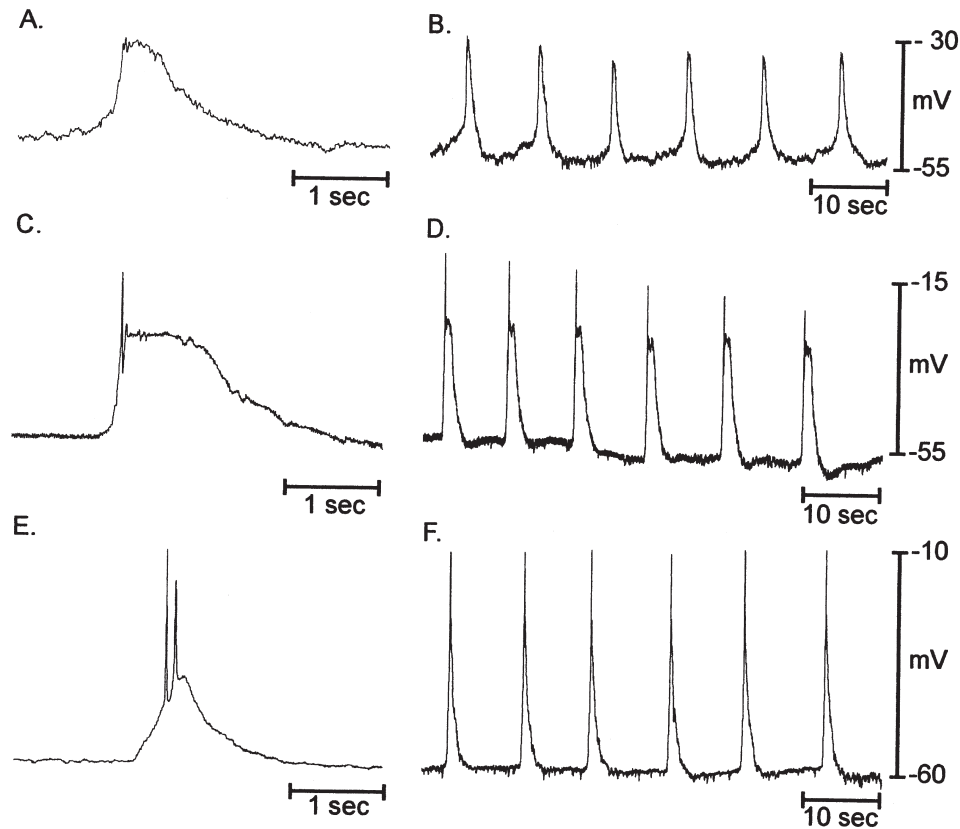
**FIG. 2.** Different patterns of electrical activity induced by norepinephrine (5 $\mu M$). (**A, B**) Oscillations of membrane potential characterized by a relatively slow depolarizing upstroke, followed by gradual repolarization back to resting potential. Oscillations such as these typically did not exceed $-30$ mV. (**C–F**) Spike complexes recorded in the presence of norepinephrine often exhibited a prolonged plateau phase (**C, D**), and in some cells multiple spikes were superimposed on the plateau (**E, F**).

lar to those described previously, comprising of single or multiple action potentials superimposed on a plateau phase. However, the average duration of the plateau phase was longer in the presence of norepinephrine (933 ± 158 ms; $n = 7$) when compared to control traces (680 ± 95 ms, $n = 10$). Norepinephrine-induced events were either attenuated or completely abolished by 2-APB (records not shown). 10 $\mu M$ 2-APB reduced frequency and amplitude of agonist-induced events (i.e., frequency reduced by 44%, from 6.8 ± 1.0 min$^{-1}$ to 3.8 ± 0.5 min$^{-1}$, amplitude reduced by 52% from 19.3 ± 2.8 mV to 9.3 ± 2.3 mV; p < 0.05; $n = 3$). The effect of 2-APB was dose-dependent as higher concentrations (30–100 $\mu M$) completely abolished all activity within 10 min of drug addition ($n = 3$). It should be noted that 2-APB also abolished spontaneous electrical activity in vessels that had not been pretreated with norepinephrine.

*Effect of cesium*

A role for a hyperpolarization-activated current similar to that described in heart sinoatrial node cells I$_f$,[6] in lymphatic vessel pacemaking has been suggested.[3] In order to investigate this hypothesis further, the effect of Cs$^+$ on electrical activity recorded from spontaneously active lymphatic segments was assessed. Perfusion with Cs$^+$ (1 mM and 10 mM) reduced the rate at which spontaneous spike events were discharged. Typical effects of 1 mM and 10 mM Cs$^+$ are shown in Figures 3A–C and 3E–G. The lower concentration of Cs$^+$ decreased frequency by 30% from 5.0 ± 0.9 to 3.5 ± 0.6 min$^{-1}$ (n = 3; p < 0.05) but did not significantly alter spike shape (Fig. 3A–D). The rate at which the voltage changed during the prepotential phase was reduced from 0.5 ± 0.1 mV sec$^{-1}$ in control conditions to 0.1 ± 0.1 mV sec$^{-1}$ in the presence of 1 mM Cs$^+$ (*p* < 0.05;
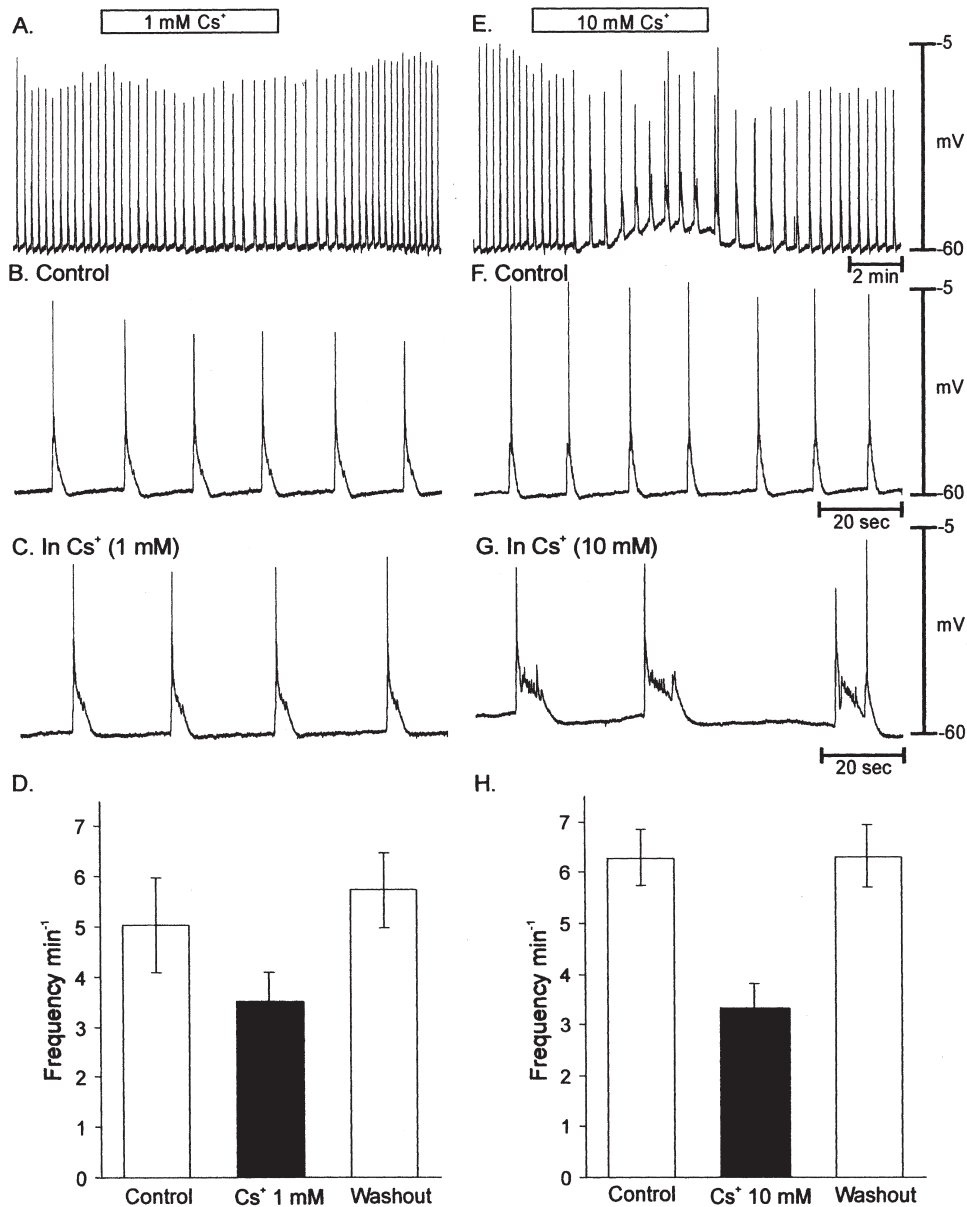
**FIG. 3.** Effect of $Cs^+$ (1 and 10 m$M$) on spontaneous electrical activity. (**A**) $Cs^+$ (1 m$M$) decreased the frequency of spike firing from 3.5 $min^{-1}$ to 2.5 $min^{-1}$. (**B, C**) Spontaneous events recorded in control conditions (**B**) can be compared on the same time scale with those recorded in 1 m$M$ $Cs^+$ (**C**). (**D**) Summary of three experiments using 1 m$M$ $Cs^+$. (**E**) $Cs^+$ (10 m$M$) decreased spike frequency from 4.2 $min^{-1}$ to 1.8 $min^{-1}$. (**F, G**) Spontaneous events recorded in control conditions (**F**) are shown on the same time scale as those recorded in 10 m$M$ $Cs^+$ (**G**). (**H**) Summary of six experiments using 10 m$M$ $Cs^+$. Frequency of firing was significantly reduced by both concentrations of $Cs^+$ ($p < 0.05$). Scale bars shown in **E, F,** and **G** also correspond with traces **B** and **C**, respectively.

$n = 3$). 10 m$M$ $Cs^+$ reduced firing frequency by 45% from 6.3 ± 0.5 to 3.3 ± 0.5 $min^{-1}$ ($n = 6$; $p < 0.05$) and caused a small but significant depolarization (from −57.0 ± 1.0 mV to −55.2 ± 1.5 mV; $n = 6$; $p < 0.05$). $Cs^+$ also increased the plateau duration and caused additional spikes to be fired (Figs. 3E and 3G).

*Effects of calcium channel blockers*

Results from single cell experiments using the patch clamp technique have suggested that lymphatic smooth muscle cells possess two distinct types of calcium current. The pharmacology and inactivation characteristics of these in-

ward currents are typical of L- and T-type calcium currents.[4] In the present study, the effects of L-type channel blockade using nifedipine (1 $\mu M$) and T-type channel blockade using $Ni^{2+}$ (100 $\mu M$) on spontaneous electrical activity were assessed.

Figure 4A shows the effect of nifedipine (1 $\mu M$) on spontaneous electrical activity. Within 30 sec of drug application, action potential firing was disrupted, with some spike events being markedly reduced in amplitude (i.e., from 45 mV to 10–12 mV). Spontaneous activity was completely abolished within 2 min of drug ad-

dition and did not return before the impalement was lost, 40 min after drug washout started. The experiment was repeated twice, using spontaneously active lymphatic segments isolated from different animals, and a similar pattern of events was observed. In all three experiments, no reduction in firing frequency was evident before activity ceased. In two of the three preparations, nifedipine caused slight membrane depolarization (1–2 mV). $Ni^{2+}$ (100 $\mu M$) had a dramatic effect upon spontaneous action potential firing. The change in spontaneous activity was evident almost im-
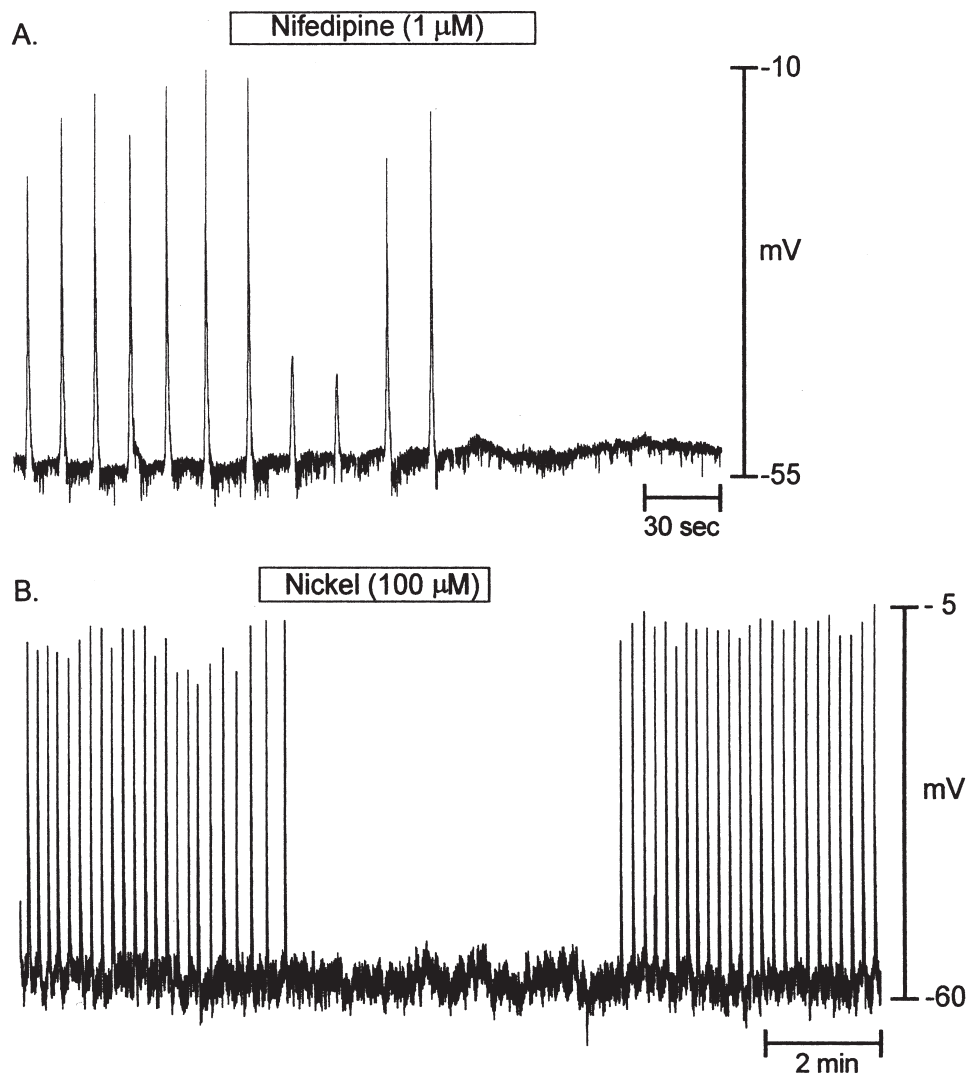


**FIG. 4.** Effect of calcium channel blocking agents on spontaneous electrical activity. (**A**) Spike firing was completely abolished within 2 min of perfusion with the L-type calcium channel antagonist nifedipine (1 $\mu M$). (**B**) Prior to perfusion with $Ni^+$ (100 $\mu M$), spike complexes occurred spontaneously at a rate of 5.5 events $min^{-1}$. Within 30 sec of drug application, electrical activity was abolished. The effect of $Ni^+$ was reversible, with activity returning to control levels within 5 min of washout.

mediately after $Ni^{2+}$ entered the perfusion chamber. The duration of the pre-potential phase of each action potential was lengthened, thus decreasing the frequency of firing. No decrement of action potential amplitude occurred before activity ceased 1 min after drug addition. The effect of $N^{2+}$ was more readily reversible than that of nifedipine, with activity resuming to control levels 2–3 min after drug washout.

*Effect of TTX*

Blockade of fast sodium channels using depolarizing tetrodotoxin (TTX; 0.3–1 $\mu M$) had a marked effect on action potential generation in spontaneously active lymphatic preparations. Figure 5A shows the typical effect of 1 $\mu M$ TTX, causing blockade of all electrical activity within 3 min of drug delivery. Before electrical quiescence was reached, it was the amplitude, and not the frequency, of spontaneous events that was affected. In addition to generally attenuating the amplitude of any action potentials fired, TTX prevented alternate depolarizing events from reaching threshold for spike firing (Fig. 5A). Action potential firing returned with drug washout. The effect of 1 $\mu M$ TTX was found to be reproducible when repeated using tissue from a different animal. A concentration of 0.5 $\mu M$ TTX gradually decreased the amplitude of spike complexes without reducing frequency (panel B). By the fourth minute after drug addition, an 'alternating' pattern of small spikes (amplitude: 30 mV) and oscillations (amplitude: 8 mV") was observed (panel D). This pattern of activity continued for more than 8 min without further reduction in event amplitude. The maximum rate of rise of the action potential was decreased substantially by 0.5 $\mu M$ TTX from a control value of $14.8 \pm 0.09$ to $1.1 \pm 0.07$ V sec$^{-1}$. Approximately 12 min after perfusion with TTX was started, nifedipine (1 $\mu M$) was added to the bath solution and this abolished all remaining electrical activity in approximately 4.5 min (Figs. 5B and 5E). The response to TTX was variable, however, since in some preparations a concentration of 0.5 $\mu M$ was sufficient to completely abolish all electrical activity within 2 min of drug addition.

*Effect of anthracene-9-carboxylic acid (9-AC)*

Figure 6 shows the gradual reduction in amplitude and frequency of spontaneous oscillations brought about by the chloride channel antagonist anthracene-9-carboxylic acid (9-AC; 250 $\mu M$). Prior to 9-AC, spontaneous oscillations with average amplitude of 24 mV were occurring at a rate of 4.8 min$^{-1}$. 10 minutes after drug addition, amplitude and frequency were reduced to 14 mV and 4.0 min$^{-1}$, respectively. Interestingly, after washout, oscillation amplitude exceeded control values by approximately 5 mV (Fig. 6B). A series of five experiments was performed on spontaneously active lymphatic preparations using 500 $\mu M$ 9-AC. In all five animals, 9-AC reduced both amplitude and frequency of spontaneous events, but the results were extremely variable. Percentage reduction of amplitude from control values measured between the fourth and fifth minutes after drug addition ranged from 11% to 30% whilst reductions in frequency ranged from 13% to 55%. In four of the five preparations, activity was completely abolished by the tenth minute after drug addition. Enhancement of amplitude beyond control values was seen after drug washout in three of the five preparations (mean increase from control amplitudes: $13.5 \pm 2.0$ mV; $n = 3$).

The effect of 1 m$M$ 9-AC is shown in Figure 6C. This concentration of 9-AC gradually decreased both amplitude and frequency so that activity was completely blocked 4 min after drug addition. An almost identical effect was observed when this experiment was repeated using lymphatic tissue isolated from a different animal. In both preparations activity resumed with washout, however amplitude of depolarising events did not exceed control values (Fig. 6D).

*Effect of potassium channel blockers*

In voltage-clamped single cells isolated from sheep mesenteric lymphatics, BK current accounted for more than 90% of the outward current measured,[1] so it was of interest to assess its role in the intact syncitium. To do this, the effects of the specific blocker penitrem-A[1,7] were examined. Prior to drug addition (0.1 $\mu M$), regular spontaneous events consisting of
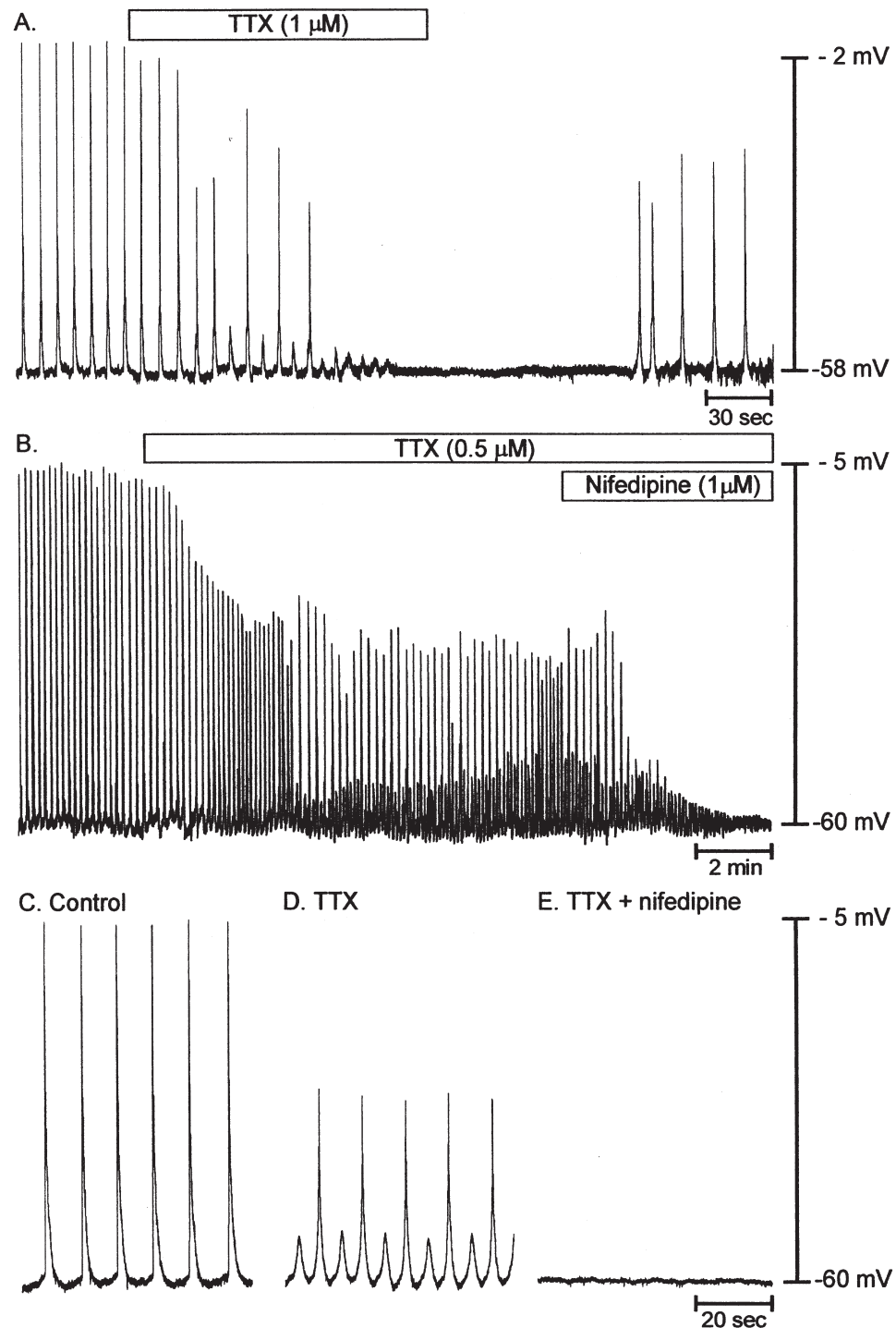
**FIG. 5.** Effect of TTX on spontaneous action potential firing. (**A**) Blockade of fast sodium channels with TTX (1 $\mu M$) abolished spontaneously generated action potentials within 2 min of drug perfusion. The effect of TTX was reversible upon washout. However, in one preparation, oscillations in membrane potential remained after 8 min of perfusion with 0.5 $\mu M$ TTX (**B**, **D**). The oscillating events, which persisted after sodium channel blockade (**D**), were abolished by nifedipine (1 $\mu M$) (**B**, **E**). Electrical events recorded before TTX perfusion are shown in **C**.

a spike, an initial repolarization and an after depolarization (or 'plateau') were discharged at a rate of 1.5 min$^{-1}$ (Fig. 7A). Spike amplitude was consistently around 29 mV and the

peak plateau amplitude about 17 mV. Plateau duration, measured at half maximum amplitude was 245 ms. Penitrem-A dramatically enhanced spike amplitude (from 29 to 75 mV),
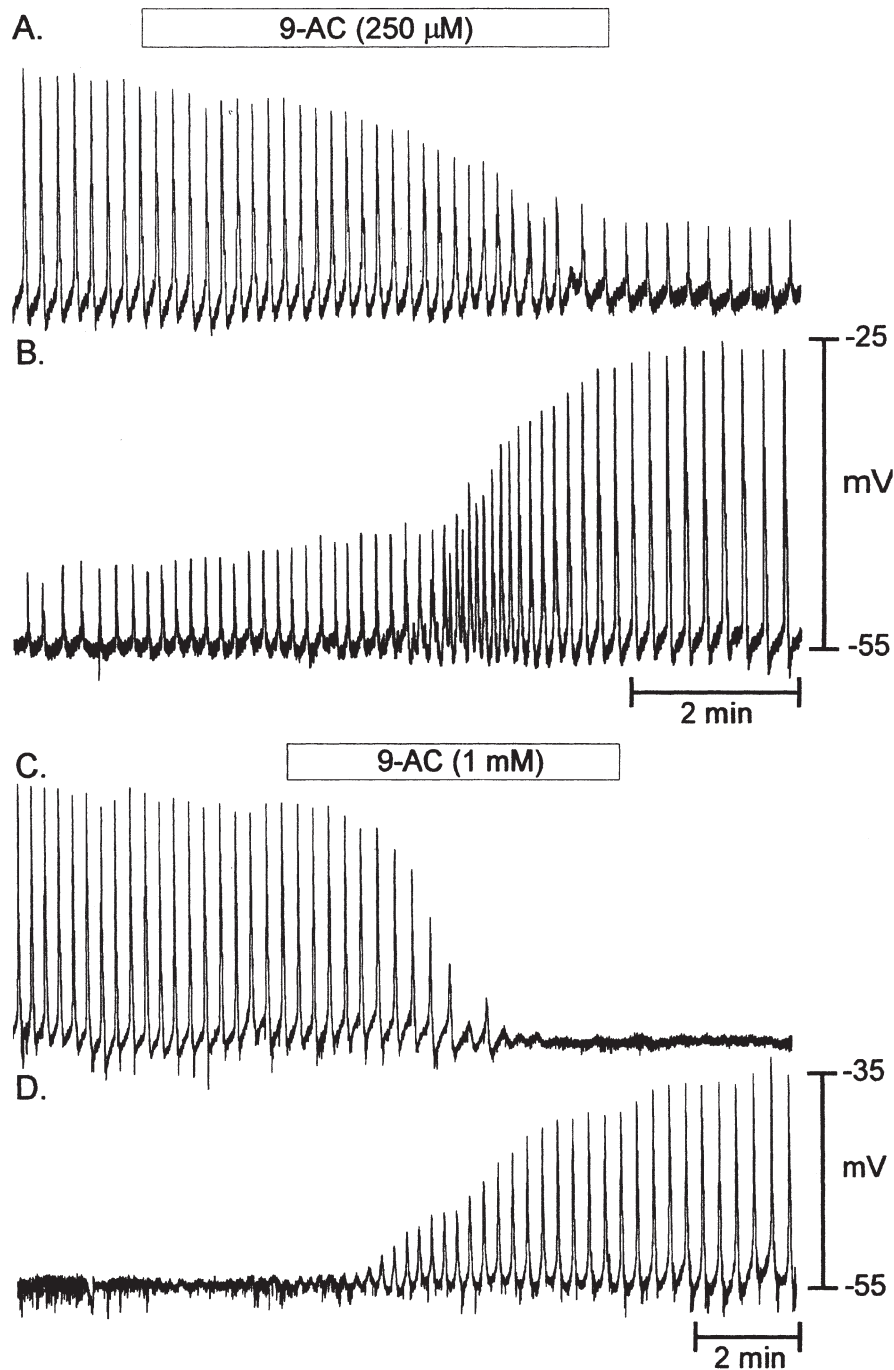
**FIG. 6.** Effect of 9-AC on spontaneous electrical activity. (**A**) Prior to perfusion with 9-AC, the frequency and amplitude of spontaneous events were 4.8 min$^{-1}$ and 24.4 mV, respectively. 9-AC (250 $\mu M$) caused marked attenuation of oscillation amplitude to a value of 14 mV, whilst reducing frequency to 4.0 min$^{-1}$. (**B**) The effect of 9-AC (250 $\mu M$) was reversible on washout. (**C**) 1 m$M$ 9-AC completely abolished spontaneous oscillations within 4 min of drug addition. (**D**) When oscillatory events resumed after drug washout, they were initially smaller in amplitude and lower in frequency before returning to control values. Scale bars for panels **B** and **D** also apply to panels **A** and **C**.

abolished the initial repolarization phase, and decreased frequency of spike firing (from 1.5 to 0.7 min$^{-1}$). Maximum rate of potential change during spike firing was increased from 2.67 ± 0.04 V sec$^{-1}$ in control conditions to 6.74 ± 0.07 V sec$^{-1}$ in penitrem-A. Plateau duration increased from 292 ± 14 to 440 ± 28 ms (mean of five events ± SD). In contrast to the effects of
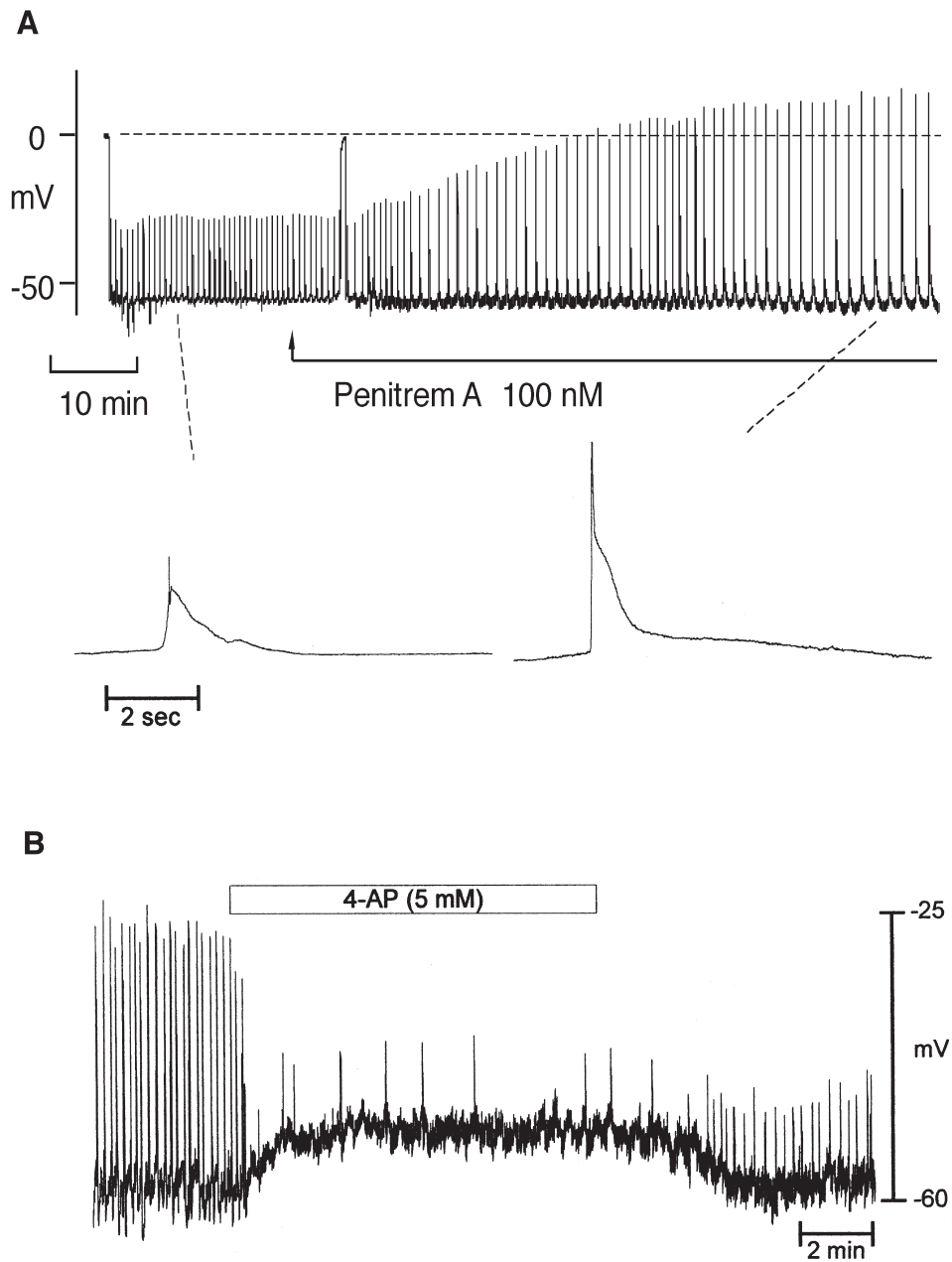
**A**



**B**



**FIG. 7.** Effect of potassium channel blocking agents. (**A**) Spike events, consisting of a rapid upstroke, an equally rapid repolarization followed by a plateau phase, occurred regularly in control conditions. When 100 n$M$ penitrem-A was added, there was no change in resting membrane potential, but the amplitude of the spike event increased to the point that it overshot into the positive range by more than 10 mV. This was accompanied by a decrease in frequency of firing of spontaneous events. Careful inspection of the event on an expanded time scale shows that the rate of repolarization of the initial spike was much reduced. (**B**) In contrast to the effects of penitrem-A, 4-aminopyridine produced a dramatic depolarization and increased the frequency of firing of spontaneous events. 5 m$M$ 4-AP caused membrane depolarization from $-57.0 \pm 1.5$ mV to $-47.0$ mV $\pm$ 1.2 mV ($p < 0.05$; $n = 3$), and reduced the amplitude of spontaneous depolarizations. When the drug was removed, membrane potential gradually repolarized and activity resumed to control values after approximately 30 min, but the amplitude remained depressed for many minutes after washout.

penitrem-A, which decreased firing frequency and had no effect on resting membrane potential, 4-aminopyridine (4-AP) produced a dramatic depolarization and increased the fre-

quency of spontaneous events. The typical effect of 5 m$M$ 4-AP is shown in Figure 7B. 4-AP caused membrane depolarization from $-57.0 \pm 1.5$ mV to $-47.0$ mV $\pm$ 1.2 mV ($p <$

0.05; $n = 3$), and reduced the amplitude of spontaneous depolarizations. In two preparations, spontaneous electrical activity was completely abolished after 10 min perfusion with 4-AP (5 m$M$, data not shown). With drug washout, membrane potential gradually repolarized and activity returned to control values after approximately 30 min.

## CONCLUSIONS

The most striking aspect of these results was the great variability in the spontaneous electrical events recorded. This is to be expected, to some extent, since the shape of electrical events will be determined both by the voltage changes responsible for their generation and those involved in their propagation. Thus, any given electrical event will be influenced by the site of recording in relation to the site of generation of the impulse. Recorded activity ranged from a spike complex with several spikes superimposed on a slower plateau to a plateau alone with no spikes. However close inspection reveals similarities between the various patterns. For example, in all cases there was a plateau of some kind whether this occurred after spike firing (Fig. 1G, and to some extent Fig. 1C), in the absence of spikes (Fig. 1A) or where a spike occurred towards the end of the plateau (Fig. 1E). Even in those plateaux where there were no clear-cut spikes, there was evidence of small rapid depolarizations or "abortive spikes." This may have been due to the filtering effect of the syncitium where the impaled cell was remote from the site of spike generation, and the intervening cells were acting as a low-pass filter. Evidence that spike propagation can decrement in this fashion has been seen in many smooth muscle types.[8–11] We could not be certain that we were recording from smooth muscle cells but it is unlikely that we were recording from either endothelial cells (they are very thin and are not known to exhibit spontaneous activity of the type described) or from pacemaker cells since there are very few of these (although we could not exclude this possibility and indeed this may account in part for the variability we observed).

*Origin of pacemaking*

Van Helden[12] suggested that spontaneous transient depolarizations (STDs) resulting from the oscillatory release of calcium from intracellular stores were responsible for pacemaking in guinea pig mesenteric lymphatics. Toland *et al.*[5] observed similar transient depolarizations in single sheep mesenteric lymphatic cells under current clamp and demonstrated that these were due to the opening of calcium-activated chloride channels. The STDs had similar shape and kinetics to the initial phase of the action potential, so it is plausible that activation of $I_{Cl(Ca)}$ is an important component of pacemaking in sheep lymphatics. The results of the present study would add weight to that speculation. Thus the calcium-activated chloride channel blocker 9-AC in a dose of 1 m$M$ completely abolished spontaneous electrical activity within 3 min. It is unlikely, however, that pacemaking in sheep mesenteric lymph ducts depends only on this mechanism since other currents that are important in pacemaking in the heart such as $I_f$ and $I_{Ca(T)}$[6] have also been found in lymphatics.[3] Again, intracellular recordings in the present study reinforce the results of voltage clamp experiments done on single cells. Thus the $I_f$ current blocker, $Cs^+$, flattened the slope of the prepotential and decreased the frequency of action potential firing in a concentration-dependent fashion. Similarly 100 $\mu M$ nickel completely abolished action potential firing within seconds of its addition. 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.[6]

*Propagation of the contractile wave*

Mesenteric lymphatics act as very efficient lymph pumps,[13,14] and such activity depends on effective coordination of the contractile wave. At first sight the arrangement of smooth muscle within the vessel wall would not seem ideal for this purpose. Thus, the smooth muscle cells are arranged in a mainly circumferential orientation with bundles which interlock and interdigitate in a basket-weave fashion.[15] Such an arrangement would not seem optimal

for the longitudinal propagation of the contractile wave and yet there is clear experimental evidence that such propagation does occur.[13,16] It may be, however, that the smooth muscle cells are not responsible for longitudinal propagation of the electrical impulse. McCloskey et al.[15] have identified a subset of cells which lie just under the endothelium and are arranged in a longitudinal orientation. These cells are kit-positive and vimentin positive and do not contain smooth muscle myosin. It is possible, but as yet unproven, that these cells act as both pacemakers and conductors of the electrical wave in sheep mesenteric lymphatics. It may be that these cells have the fast sodium current[2] that is responsible for the very fast (by smooth muscle standards) spikes recorded in the present study and that these are essential for the very good coordination of contractile activity seen in the intact pumping preparation. This would accord with the observations of Convery et al.[16] that coordination of the contractile wave was disrupted by TTX.

*Role of outward currents*

A calcium-activated potassium current, or BK current, has been identified and characterized in isolated lymphatic smooth muscle cells.[1] This current appears to account for up to 90% of all outward current present in these cells, and therefore we were interested to determine what effect its blockade would have on spontaneous action potential firing. The tremorigenic indole alkaloid, penitrem-A, is considered to be a highly selective blocker of BK channels,[7] therefore we used this agent to assess the effect of BK blockade. The increase in action potential amplitude and blockade of early repolarization suggests strongly that BK current is involved in the initial repolarization of the action potential. It was particularly noteworthy that the action potential consistently showed an overshoot into the positive voltage range in the presence of penitrem-A, although this was never observed in control conditions. This would suggest that the BK current activates very rapidly in response to calcium influx during the upstroke of the action potential. The enhancement of spike amplitude and

elongation of the plateau phase may also be due, in part, to alterations in membrane resistance that would result from inhibition of BK channel opening. In the presence of penitrem-A, frequency of action potential firing was reduced. This suggests that under control conditions, the BK current is involved in repolarizing the membrane into a sufficiently negative range for activation of the $I_f$ pacemaker current. When all of the BK current is blocked in isolated lymphatic smooth muscle cells, a voltage-dependent outward current remains. This current is reduced by both tetraethylammonium (TEA) and 4-aminopyridine (4-AP) and is thus likely to be carried by $K^+$ ions.[1] The depolarization of the membrane induced by 4-AP suggests that a 4-AP-sensitive outward current is active at rest and is at least partially responsible for setting the membrane potential close to $E_K$. This is an interesting observation, given that there is no evidence from patch-clamp investigations of a 4-AP sensitive current in isolated cells held at the normal resting potential of −60 mV.[1] This apparent paradox could be explained if, like the $I_f$ pacemaker current, the 4-AP sensitive current is only found in a small subpopulation of lymphatic cells. If this is the case, the likelihood of selecting a dispersed cell that possessed a 4-AP sensitive current might have been slight.

The mean membrane depolarization caused by 4-AP in three tissues was 10 mV (i.e., from 57.0 ± 1.5 mV to −47.0 ± 1.2 mV). Depolarization of the membrane to this extent will reduce the amount of the voltage-dependent $I$Ca(L) and $I_{Na}$ available.[1,2,4] As discussed previously, these two currents appear to be primarily responsible for the upstroke of the action potential. The reduced availability of these currents is likely to explain why the depolarization induced by 4-AP was accompanied a reduction or complete cessation of spontaneous firing. Consideration of the cable properties of smooth muscle, as described by Abe and Tomita,[17] also helps to explain why prolonged depolarization (as caused by 4-AP) hampers action potential propagation. Depolarization of the smooth muscle cells will promote opening of $Cl^-$ channels, which have been shown to be slowly deactivating.[1,18] As a result the resistance pro-

vided by the membrane will be decreased and the length constant reduced. Consequently, signal transmission at gap junctions and propagation through the muscle syncytium will be hampered.

## SUMMARY

These intracellular recordings have revealed that the pattern of spontaneous electrical activity in sheep lymphatic smooth muscle is somewhat similar to that seen in larger bovine mesenteric vessels.[19] In both species, spontaneous spike complexes are regularly discharged and consist of rapid firing action potentials superimposed on a transient depolarization or 'plateau' phase. This intracellular study has provided further support for many of the hypotheses proposed from single cell investigations. In conjunction with the findings of Convery et al.,[16] this study provides strong evidence for the existence of a fast sodium, TTX-sensitive, action potential[2] in this muscle type. The critical importance of an L-type calcium current in the upstroke of the action potential has been reaffirmed, whilst further support has been provided for the involvement of a hyperpolarization-activated inward current in lymphatic pacing. From the results of this study we can be more confident that a calcium-activated chloride ($I_{Cl(Ca)}$) current works in conjunction with $I_{Ca(L)}$ to cause sufficient membrane depolarization for action potential firing, and that a calcium-activated BK current promotes repolarization of the action potential.

## ACKNOWLEDGMENTS

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