Tetrodotoxin-sensitive sodium current in sheep lymphatic smooth muscle

M. A. Hollywood, K. D. Cotton, K. D. Thornbury and N. G. McHale *

Department of Physiology, School of Biomedical Science, Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK

1. Fast inward currents were elicited in freshly isolated sheep lymphatic smooth muscle cells by depolarization from a holding potential of −80 mV using the whole-cell patch-clamp technique. The currents activated at voltages positive to −40 mV and peaked at 0 mV.

2. When sodium chloride in the bathing solution was replaced isosmotically with choline chloride inward currents were abolished at all potentials.

3. These currents were very sensitive to tetrodotoxin (TTX). Peak current was almost abolished at 1 μM with half-maximal inhibition at 17 nM.

4. Examination of the voltage dependence of steady state inactivation showed that more than 90% of the current was available at the normal resting potential of these cells (−60 mV).

5. The time course of recovery from inactivation was studied using a double-pulse protocol and showed that recovery was complete within 100 ms with a time constant of recovery of 20 ms.

6. Under current clamp, action potentials were elicited by depolarizing current pulses. These had a rapid upstroke and a short duration and could be blocked with 1 μM TTX.

7. Spontaneous contractions of isolated rings of sheep mesenteric lymphatic vessels were abolished or significantly depressed by 1 μM TTX.

The walls of lymphatic collecting ducts in sheep have a continuous layer of smooth muscle and have valves at regular intervals along their length. This combination allows them to function as a unique and very effective smooth muscle pump. Their electrical activity shows an interesting pattern that is almost midway between that of cardiac muscle and visceral smooth muscle. Thus, like cardiac muscle, there is a one-to-one relationship between action potential and phasic contraction (Kirkpatrick & McHale, 1977) and contraction is normally complete before another action potential is fired. This is similar to the pattern found in ureter (Shuba, 1977) but contrasts with most other types of smooth muscle such as portal vein, myometrium and taenia coli where contraction is initiated by a burst of action potentials and the contractile response is a fused tetanus rather than a single phasic contraction. Lymphatic smooth muscle has other similarities to cardiac muscle in that it has a very regular pattern of spontaneous activity which can be modulated by the autonomic nervous system and, as we report in the present paper, it has a fast sodium current which contributes to the rising phase of the action potential.

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

METHODS

Cell preparation.

Solutions and drugs

The composition of solutions used was as follows. (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) Physiological salt solution (PSS) (mM): 100 Na^+, 30 TEA^+, 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) K^+ 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) Cs^+ pipette solution (mM): 110 caesium gluconate, 20 CsCl, 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 CsOH.

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* To whom correspondence should be addressed.

Drugs used were TTX (Sigma) and nifedipine (Bayer) and these were made up to final concentration in the appropriate perfusing solution.

In some experiments recordings were made of spontaneous contractions of isolated lymphatic rings. In these cases rings 2 mm in diameter and 8 mm in length were dissected from the main duct, suspended between stainless-steel hooks and placed into a water jacketed organ bath (volume, 5 ml) maintained at 37 °C. The rings were perfused with Krebs solution of composition (mM): 120 NaCl, 25·0 NaHCO$_3$, 5·9 KCl, 1·2 Na$_2$HPO$_4$, 2·5 CaCl$_2$, 1·2 MgCl$_2$ and 11·0 dextrose; gassed with 95% O$_2$–5% CO$_2$. The rings were adjusted to a tension of 2–4 mN and the vessels were allowed to equilibrate for at least 30 min. Isometric tension changes were measured with Dynamometer UF1 transducers and the output from these was written on a Gould 8000S chart recorder. When the vessels developed regular spontaneous activity the effects of changing the bath perfusion solution to one containing TTX were examined.

Experimental procedures

Recordings of whole-cell currents were made using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes with a resistance of 2–4 MΩ were used. After gigaseals were obtained and the patch was ruptured the series resistance was usually less than 6 MΩ and was uncompensated. Capacitative transients were removed either by subtracting currents obtained by applying a pulse of the same voltage in sodium-free solutions from those obtained under control conditions or by the $P + P/n$ method described by Benzanilla & Armstrong (1977). Voltage-clamp commands were delivered with an Axopatch 1D patch-clamp amplifier (Axon Instruments) and membrane 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). Junction potentials averaged less than 3 mV and were not compensated. During experiments the Petri dish containing the cells was continuously superfused with PSS at room temperature (20 ± 1 °C, with the

exception of experiments done to show total inward current when the dish was maintained at 35 °C). In addition, the cell under study was continuously superfused by means of a close delivery system consisting of a pipette of tip diameter 200 μm placed approximately 300 μm away. The PSS in the close delivery system could be switched, with a dead space time of 10 s, to a solution containing a drug.

In some experiments recordings were made using the amphotericin B perforated patch method (Rae, Cooper, Gates & Watsky, 1991). Briefly, this consisted of dipping the tips of the patch pipettes in amphotericin-free pipette solution for a few seconds and then backfilling with pipette solution containing 6 μg ml^{-1} of amphotericin B (Sigma). After gigaseals were obtained the series resistance fell over a 10–15 min period to 10–15 MΩ and remained stable for up to 1 h.

Data were presented as means ± s.e.m. and statistical comparisons were made using Student's paired t test taking $P < 0.05$ as significant.

RESULTS

When cells were held at a potential of −60 mV and stepped to 0 mV (at a temperature of 35 °C) a current such as that shown in the lower trace of Fig. 1 was obtained. This consisted of two components: a transient inward current superimposed upon a slowly inactivating component. In the presence of 100 μM Ni^{2+} and 1 μM nifedipine the slowly inactivating current was removed suggesting that it was carried by Ca^{2+}, presumably mainly through L-type channels. The current remaining in the presence of the above blockers inactivated completely within 4 ms suggesting that it was a fast sodium current. To study this in greater detail, all further experiments were conducted at room temperature (20 ± 1 °C) and in the presence of 100 μM Ni^{2+} and 1 μM nifedipine (which are known to block T- and
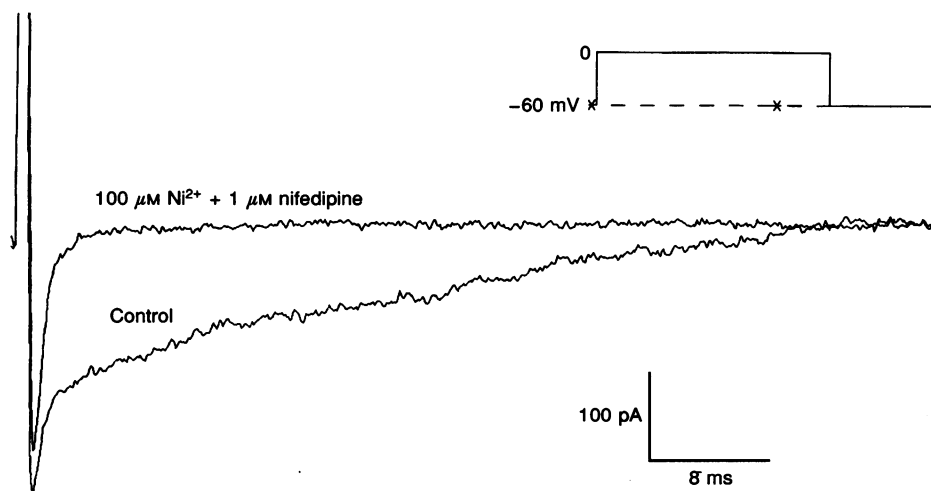

Figure 1. Examples of inward current recorded at 35 °C

Stepping from a holding potential of −60 mV to 0 mV elicited a transient followed by a slowly inactivating current under control conditions. In the presence of the calcium channel blockers Ni^{2+} and nifedipine only the rapidly inactivating component remained. The crosses on the dashed line in the voltage protocol indicate the part of the record displayed.

L-type calcium currents in sheep lymphatic smooth muscle; Hollywood *et al.* 1997). Cs^+ pipette solution was used in all voltage-clamp experiments. K^+ pipette solution was used in current-clamp experiments.

The effect of Na_o^+ removal

The suspicion that the rapidly inactivating current observed at 35 °C was a sodium current was reinforced by the results of the experiment shown in Fig. 2. From a holding potential of −80 mV the membrane was stepped to +50 mV in 10 mV steps eliciting a family of inward currents activating at potentials positive to −40 mV (Fig. 2*A*). When sodium chloride in the bathing solution was replaced isosmotically with choline chloride, inward currents were abolished at all potentials (Fig. 2*B*). The difference currents in (Fig. 2*C*) were obtained by subtracting the currents obtained in sodium-free solution (Fig. 2*B*) from those obtained under control conditions (Fig. 2*A*). A summary of the current–voltage relationship in eight such experiments is shown in Fig. 3. Current activated at potentials positive to −40 mV and peaked at 0 mV.

The effect of TTX

To verify that the Na^+ current described above was carried through specific sodium channels the effects of TTX (which is known to be a selective blocker of sodium channels) were examined. Figure 4*A* shows the effect of increasing concentrations of TTX from 1 nM to 1 μM on currents elicited by stepping from a holding potential of −80 mV to 0 mV. The current elicited in the presence of 1 nM TTX was little different from control while inward current was almost abolished at a concentration of 1 μM. Figure 4*B* shows a summary of five such experiments where current, expressed as a percentage of the maximum, is plotted against TTX

Figure 2. The effect of sodium removal

A, 10 mV steps from −80 to +50 mV produced a family of inward currents activating at voltages positive to −40 mV. *B*, when sodium chloride in the perfusate was replaced with choline chloride, inward current was abolished at all voltages leaving only the capacitative transients. *C*, when these were subtracted the sodium-dependent difference currents were revealed.
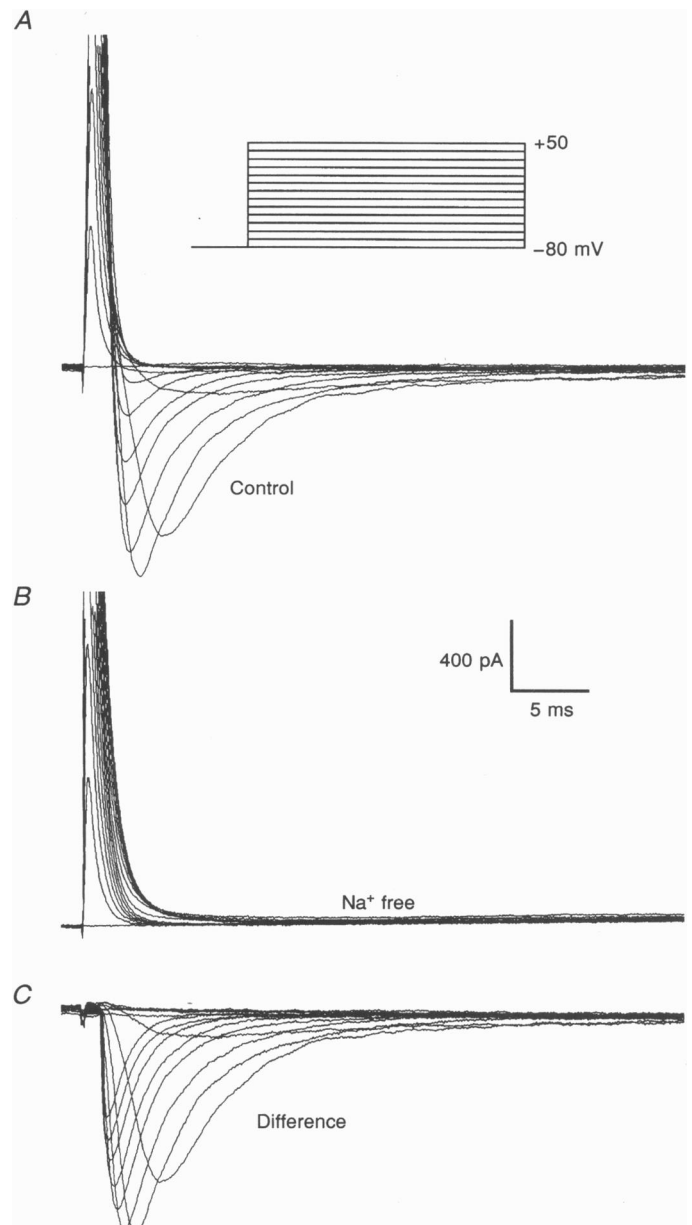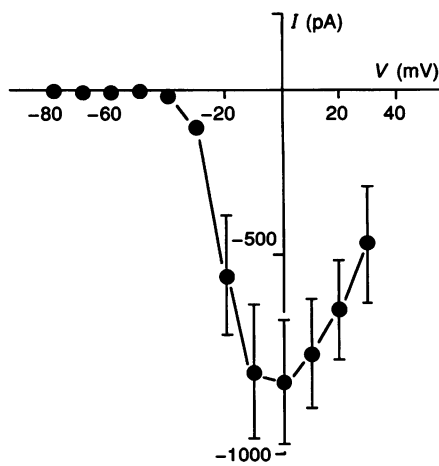
Figure 3. Current–voltage relationship of difference currents

Summary of 8 experiments such as that shown in Fig. 2. Current activated at voltages positive to −40 mV and peaked at 0 mV. The vertical bars represent ± 1 s.e.m.

concentration. The data were well fitted by a curve of the form:

$$I_{TTX}/I_{con} = 1/(1 + [TTX]/K_d),$$

where I_{TTX} and I_{con} are the current amplitude in the presence and absence of TTX, respectively, and K_d is the dissociation constant for TTX, assuming a single TTX binding site and one-to-one stoichiometry for TTX binding. This yielded a K_d of 17·3 ± 2·4 nM.

Inactivation of sodium current

The time course of inactivation of sodium current (I_{Na}) was measured by calculating the exponentials that best fitted the decay phase of I_{Na} at a series of voltages from −20 to +50 mV. These were well fitted with single exponentials giving mean τ values of 6·6 ± 1·2 ms at −20 mV; 3·2 ± 0·5 ms at −10 mV; 2·5 ± 0·3 ms at 0 mV; 2·0 ± 0·3 ms at 10 mV; 1·8 ± 0·3 ms at 20 mV; 1·6 ± 0·2 ms at 30 mV; 1·5 ± 0·1 ms at 40 mV; 1·1 ± 0·1 ms at 50 mV (7 cells).

Steady-state availability of I_{Na}

A two-pulse protocol was used to study voltage dependence of steady-state availability of I_{Na}. From a holding potential of −60 mV cells were stepped to a range of potentials from −100 to 0 mV in 10 mV steps for a period of 2 s and then stepped to 0 mV. The open circles in Fig. 5 represent the current available at that voltage expressed as maximum
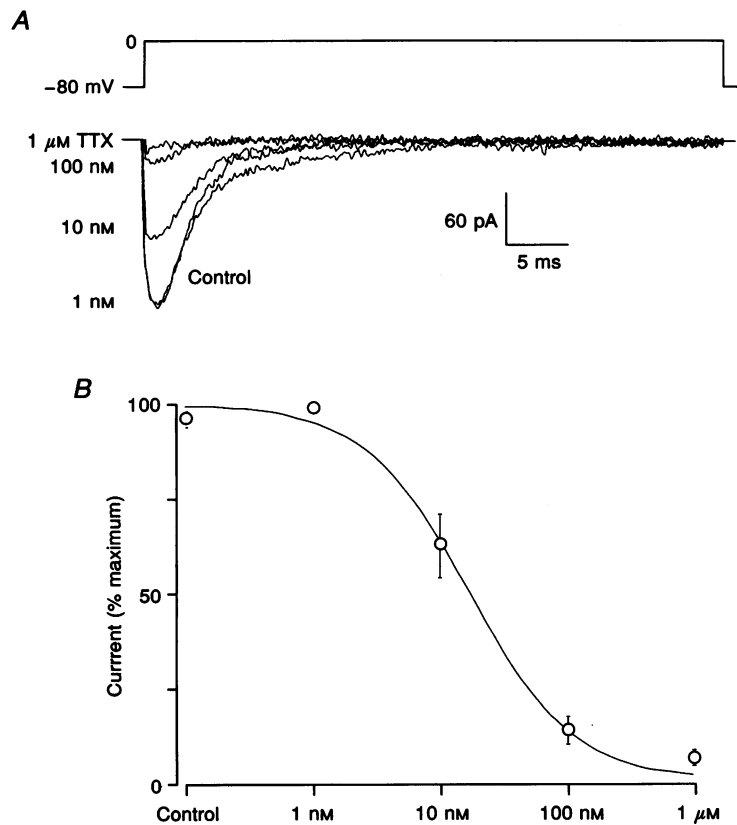

Figure 4. Concentration–effect relationship for TTX

A, the effect of increasing concentrations of TTX on currents elicited by stepping from −80 to 0 mV; 1 nM had little effect while 1 μM almost abolished the current. *B*, plotted values for 5 such experiments. The points were fitted with the Langmuir equation yielding a K_d for TTX of 17·3 nM.

Figure 5. Voltage dependence of steady-state activation and inactivation

Graph showing I/I_{max} measured using a two-pulse protocol (O) and normalized conductance g/g_{max} (□). The continuous lines represent Boltzmann fits of the data in each case. The curves intersected at −30 mV, allowing a small window current between −40 and −20 mV. It is significant that more than 90 % of the current was available at the normal resting potential of these cells (−60 mV).
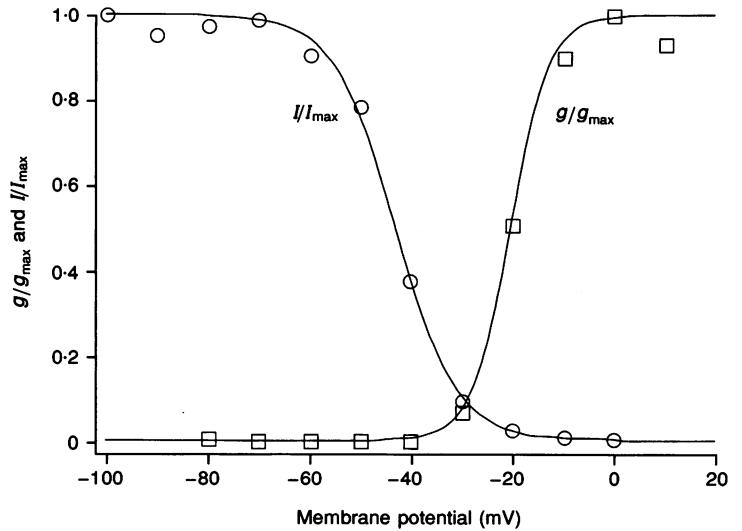
available current. The points were well fitted by a Boltzmann function of the form:

$$I/I_{max} = 1/[1 + \exp(-K(V - V_{\frac{1}{2}}))],$$

where I/I_{max} is the normalized current, $V_{\frac{1}{2}}$ is the membrane potential at which half-inactivation occurs and K^{-1} is the maximum slope factor at $V = V_{\frac{1}{2}}$. For the open circle data in Fig. 5 the half-inactivation voltage was −43 mV and the maximum slope factor was −6 mV. In five such experiments

the mean $V_{\frac{1}{2}}$ was −44·0 ± 2·2 mV and the mean slope factor was −7·2 ± 0·6 mV. Normalized conductance was plotted against membrane potential yielding the activation curve shown in Fig. 5 (squares). The Boltzmann fit of these data yielded a voltage at half-activation of −20 mV and a slope factor of 3·7 mV. The region of overlap between the two curves was in the range of −40 to −20 mV, which would result in a small sustained inward current (window current).

Figure 6. Time course of recovery from inactivation

A, an experiment where cells were held at −80 mV and then stepped to 0 mV. After a variable delay from 10 to 120 ms the cell was again stepped to 0 mV. In this case recovery was complete within 100 ms. *B*, a summary of 6 such experiments where $1 - I/I_{max}$ is plotted against time. The continuous line is a single exponential fit of the points giving a time constant of recovery from inactivation of 19·8 ms.
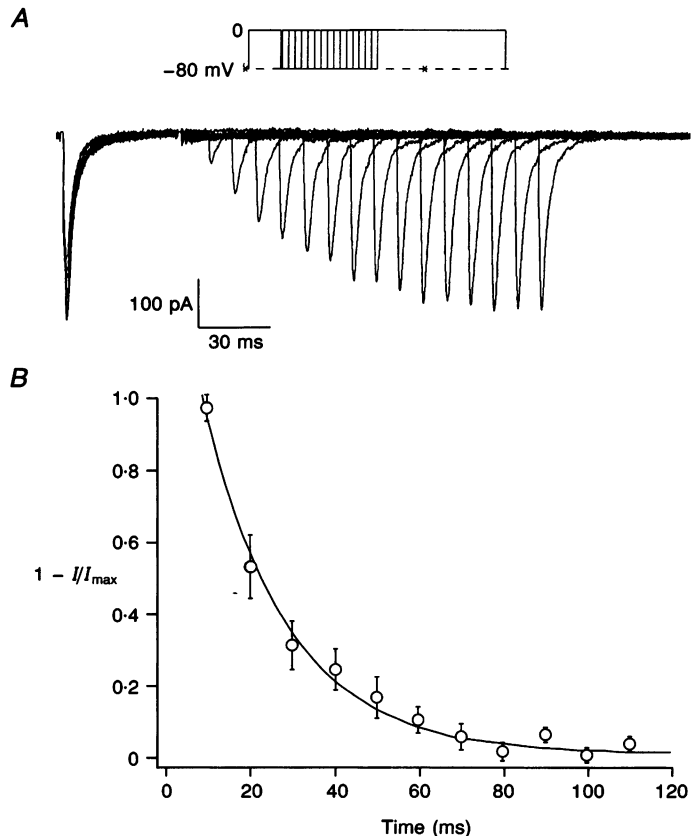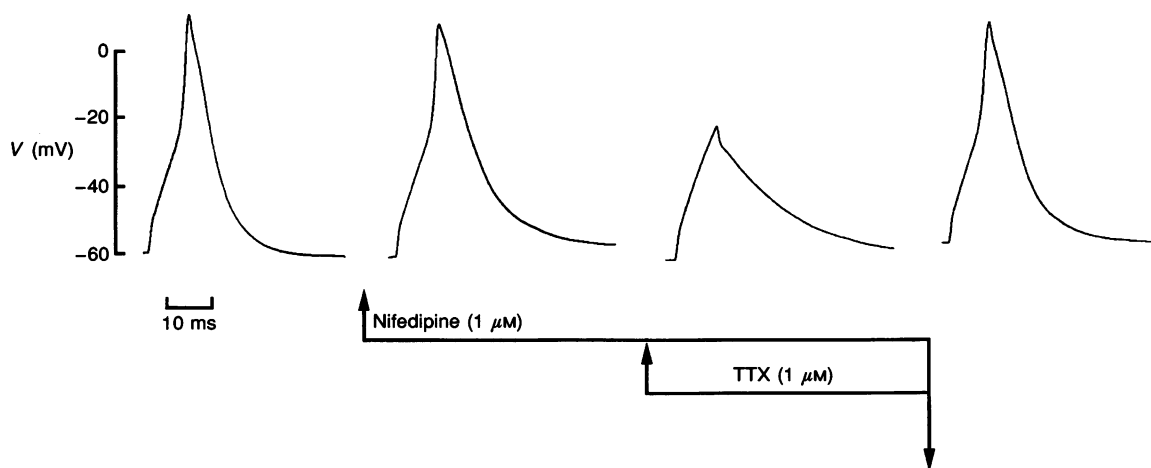
Figure 7. The effect of TTX on an evoked action potential

Action potentials evoked by 60 pA, 10 ms pulses in current-clamp mode (perforated patch method, K^+ pipette solution). The action potential elicited under control conditions was little affected by 1 μM nifedipine but almost abolished when 1 μM TTX was added. After washout the action potential returned to control level.

Recovery from inactivation

The time course of recovery from inactivation was studied using a double-pulse protocol. Cells were held at −80 mV and then stepped to 0 mV. This was followed by a variable delay from 10 to 120 ms whereupon the cells were again stepped to 0 mV (Fig. 6A). Recovery was usually complete after delays greater than 100 ms. Figure 6B shows a summary of six such experiments where normalized current was plotted against time. The data were well fitted by a single exponential giving a time constant for recovery from inactivation of 19·8 ms.

Role of I_{Na} in the generation of the lymphatic action potential

It is clear from the inactivation curve shown in Fig. 5 that 90% of the fast sodium current is available at the normal resting potential of these vessels (−60 mV, H. Toland & N. G. McHale, unpublished observations). It was of interest

therefore to examine the properties of action potentials that could be elicited in the isolated cells in current-clamp using K^+ pipette solution. In these experiments the perforated patch technique was used to prevent run-down of any calcium current that might be contributing to the action potential. Figure 7 shows an experiment where a cell was depolarized by a 60 pA, 10 ms pulse from a resting potential of −60 mV. This was sufficient to exceed firing threshold and an action potential was elicited. This had a maximum rate of rise of 15 V s^{-1} and an overshoot of 11 mV. When the stimulus was repeated in the presence of 1 μM nifedipine the action potential was little affected. However, in the presence of both nifedipine and TTX (1 μM) the action potential was almost abolished leaving little more than a passive membrane response. It would appear from this result that the upstroke of the action potential in this cell is dependent almost completely upon the fast sodium current. Six of eight cells studied in this way possessed very
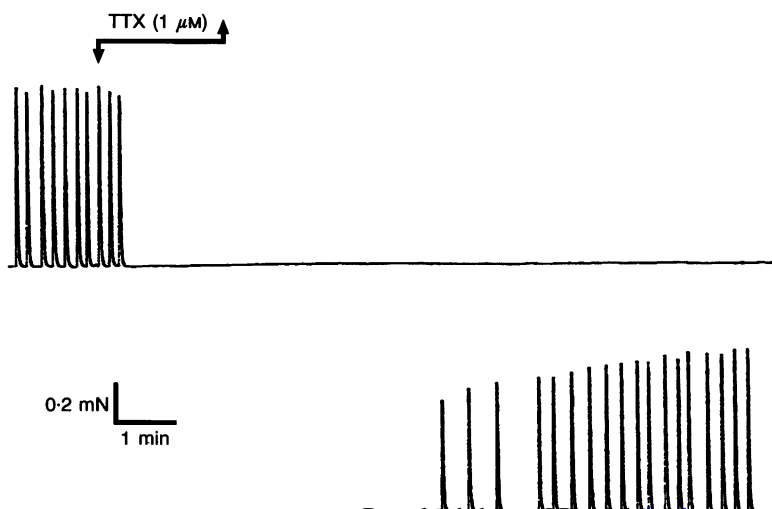

Figure 8. The effect of TTX on spontaneous contractions in isolated rings

Prior to TTX addition the ring was contracting regularly at a frequency of 5 min^{-1}. Within half a minute of drug application contractions had ceased (upper trace). When contractions resumed nearly 20 min later they were initially smaller in amplitude and lower in frequency before returning to control levels (lower trace).

little L-type calcium current and had action potentials that could readily be blocked with TTX. The other two cells had L-type calcium currents and very little sodium current. Thus it appears that there may be two subtypes of lymphatic smooth muscle cell. It would be of interest to investigate this further but it is beyond the scope of the present investigation.

Effect of TTX on spontaneous contractions in isolated lymphatic rings

Figure 8 shows the effect of a 2 min application of 1 μM TTX on spontaneous contractions. Within half a minute of drug application contractions ceased entirely and did not return for nearly 20 min. In seven such experiments the results were variable. Some rings were very sensitive to TTX (such as the one shown) while others showed only a depression of 40% in force of contraction with this dose. The mean depression of force in seven experiments was $64 \pm 12\%$. When the amplitude of contraction in control conditions was compared with that in the presence of TTX using Student's paired t test it was found that 1 μM TTX significantly depressed force of contraction ($P < 0.001$). It is impossible to draw any firm conclusions as to the role of the fast sodium current from experiments such as these but it is clear that the efficiency of contraction is impaired by TTX. This may be due to a decrease in the rate of rise of the action potential resulting in slower and less efficient propagation. This could result in the active response dying out over a short distance which would decrease the number of cells contracting synchronously and thus cause a depression in the force of contraction.

DISCUSSION

The sodium current described in the present study should not be confused with the non-specific currents carried by sodium ions through Ca^{2+} channels in low calcium solutions both in heart (Garnier, Rougier, Gargouil & Coraboeuf, 1969) and in smooth muscle (Jmari, Mironneau & Mironneau, 1987). The currents described by Garnier *et al.* (1969) and Jmari *et al.* (1987) were slow to inactivate (inactivation took more than 200 ms), insensitive to TTX and blocked by Ni^{2+} and nifedipine. Rather the properties of the sodium current detailed in the present study, namely fast inactivation kinetics ($\tau < 20$ ms), high sensitivity to TTX ($K_d < 20$ nM) and insensitivity to Ni^{2+} and nifedipine, are more like the I_{Na} found in nerve and skeletal muscle. For many years smooth muscle cells were thought not to possess a fast sodium current. In those tissues that were capable of generating action potentials the charge carrier was thought to be Ca^{2+} (Nonomura, Hotta & Ohashi, 1966; Brading, Bülbring & Tomita, 1969). In early voltage-clamp studies using the double sucrose-gap technique, such as those of Daemers-Lambert (1976) on the portal vein or Vassort (1975) on uterus, no evidence of I_{Na} was detected while Bury & Shuba (1976) found that TTX had no effect on inward current in guinea-pig ureter. However, in recent

times with the application of the whole-cell patch-clamp technique, I_{Na} has been demonstrated in all three of these tissues (Amedée, Renaud, Jmari, Lombet, Mironneau & Lazdunski, 1986; Ohya & Sperelakis, 1989; Mironneau *et al.* 1990; Muraki, Imaizumi & Watanabe, 1991). This suggests either that the limitations of the sucrose-gap technique were too great to enable demonstration of the current (although the technique was used successfully to demonstrate I_{Na} in the heart; Rougier, Vassort & Stämfli, 1968) or that these tissues are heterogeneous with some cells possessing the current while others do not. This latter suggestion gains support from the observation by Muraki *et al.* (1991) that less that 20% of ureter cells had TTX-sensitive I_{Na}.

The present investigation is the first published account of inward current studied under voltage-clamp conditions in lymphatic smooth muscle. We were rather surprised to find a fast sodium current in these cells for the reasons noted above. Its existence, however, helped to explain some puzzling observations we had made in the past while investigating the innervation and pumping activity of sheep lymphatic vessels. The first of these was that when we used TTX to block the effects of field stimulation of intramural nerves in ring preparations the force of spontaneous contractions was significantly reduced or contractions were completely abolished. This meant that we had to use ω-conotoxin to block the effects of intramural nerve stimulation (Hollywood & McHale, 1994) whereas other investigators have used TTX for this purpose in many other smooth muscle preparations (Brock & Cunnane, 1992). The second observation, which fits very well with the existence of a fast sodium current in these vessels, is that cannulated preparations set up to study pumping show remarkably good co-ordination of contraction, with lengths of 6 cm or more contracting almost synchronously, suggesting an efficient propagation of the action potential without significant decrement (Convery, Hollywood, Cotton, Thornbury & McHale, 1997). The fast sodium current is likely to be important in the normal physiology of sheep mesenteric lymphatics for a number of reasons: (1) the current was found in more than 80% of the cells studied; (2) the magnitude of the currents recorded were more than adequate to charge the membrane capacitance and elicit an action potential. Although the results of the present study may not provide an accurate quantitative account of such a fast current because of inadequacies of voltage control even at room temperature, the magnitudes of currents, particularly at more positive voltages, are likely to be under- rather than overestimated; (3) action potentials elicited in current-clamp could be completely blocked by TTX without, apparently, any requirement for I_{Ca}, though as noted above this was not true of all cells studied. Some cells had L-type calcium current and no sodium current and in these the action potential was unaffected by TTX.

The sodium current found in lymphatic vessels belongs to a group with other smooth muscles such as uterus (Sperelakis, Inoue & Ohya, 1992), ureter (Muraki *et al.* 1991) and

pulmonary artery (Okabe, Kitamura & Kuriyama, 1988) which have fast kinetics and high TTX sensitivity. Where it differs from these is in the proportion of the current available at the cell's resting potential. Thus more than 90% of current is available at the normal resting potential of sheep lymphatic cells (−60 mV) as compared with less than 50% in the other types of cell. This may help explain the greater contribution of sodium current to the upstroke of the action potential in sheep lymphatic vessels as compared with other smooth muscle types and the disruption of normal activity produced by TTX, which again is in contrast to results found in other smooth muscle.

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Author's email address

N. McHale: n.mchale@qub.ac.uk