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SLOW SODIUM CHANNEL INACTIVATION IN RAT FAST-TWITCH MUSCLE

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SUMMARY

1. Voltage-clamp Na^+ currents (I_{Na}) were measured in rat fast-twitch fibres using the loose-patch-clamp technique. Changes in the conditioning membrane potential produced slow changes in the peak I_{Na} elicited by short test depolarizations, due to a slow inactivation process.

2. Inactivation was increased by application of steady depolarizing potentials and was reversed by steady hyperpolarizations. These changes in peak I_{Na} could be well fitted by single-exponential functions with time constants in the range of 1–4 min.

3. The steady-state values of the maximum peak I_{Na} at any potential could be well fitted by a function identical to the one describing the fast inactivation process. This gave a potential of −108 mV at which 50 % of the channels were closed due to slow inactivation.

4. The maximum peak current densities obtained with the slow inactivation fully removed were as large as 20 mA cm^{-2}.

INTRODUCTION

In their studies of the kinetics of ion movements underlying the generation of the nerve impulse, Hodgkin & Huxley (1952) proposed two independent processes to explain the transient increase of Na^+ permeability (P_{Na}): an initial 'activation' followed by an 'inactivation' which develops with depolarization and recovers under repolarization with time constants of only a few milliseconds. However, some features of the potential-dependent variation in P_{Na} are not explained by this description. Evidence that suggested the presence of a slower inactivation process of P_{Na} was first found in lobster giant axon (Narahashi, 1964).

Different methods have been used to investigate the slow inactivation process in a variety of preparations, most of them involving voltage clamping. The axons in lobster (Narahasi, 1964), in squid (Adelman & Palti, 1969; Rudy, 1978) and in *Myxicola* (Schauf, Pencek & Davis, 1976; Rudy, 1981) have been described as having a slow inactivation process for Na^+ currents. A similar process has been described in myelinated nerve fibres (Peganov, Khodorov & Shishikova, 1973; Fox, 1976; Brismar, 1977; Chiu, 1977). Frog skeletal muscle was studied by the Vaseline-gap technique (Collins, Rojas & Suarez-Isla, 1982). More recently, the loose-patch-clamp technique, which allows recording of Na^+ currents over long periods of time under

physiological conditions, has been applied successfully to frog skeletal muscles (Almers, Stanfield & Stühmer, 1983*b*). We used the loose-patch-clamp technique to investigate slow inactivation of Na^+ currents in the extensor digitorum longus (e.d.l.) muscle of the rat. This is the most complete description of the slow inactivation process in a mammalian muscle to date.

METHODS

Preparation and recording

The e.d.l. muscles were removed from male and female Wistar rats (300–400 g) which had been sedated with ether and then decapitated. The muscles were dissected while immersed in either a Tyrode solution or a modified Tyrode solution containing 6 mM-Ca^{2+}. Surface connective tissue was removed as completely as possible by dissection without enzyme treatment. The experiments were performed at 18 ± 1 °C in a small chamber (5 ml) perfused with a steady flow of fresh Tyrode solution gassed vigorously with 95 % O_2–5 % CO_2.

The 'loose-patch' voltage-clamp method described earlier (Stühmer, Roberts & Almers, 1983; Almers, Stanfield & Stühmer, 1983*a*) was used. Na^+ currents were recorded with a fire-polished micropipette pressed against the sarcolemma. The pipette had an internal tip diameter of 14–16 μm and was filled with Tyrode solution. In some experiments, gentle suction (1–1·5 kPa) was applied continuously to the inside of the pipette in order to make the contact between pipette and membrane more stable.

Pipette resistances (R_p) ranged between 0·15 and 0·3 MΩ and seal resistances (R_s) between 0·4 and 1 MΩ. The seal factors ($A = R_s/(R_s + R_p)$) were kept between 0·73 and 0·83. Compensation of the seal resistance was performed automatically during pulse intervals by means of a motor potentiometer. This device balanced the impedance bridge formed by R_p, R_s and their two equivalent resistances by adjusting the resistance equivalent to the seal resistance R_s (for details consult Stühmer *et al.* 1983). This arrangement eliminates any potential drop due to the series resistance of the pipette and guarantees that the potential at the tip of the electrode (V_t) is always under control.

Na^+ currents were recorded through an eight-pole low-pass Bessel filter and sampled at 20 kHz by a 12-bit analog-to-digital converter. The data for each record, including additional information such as time, seal factor, temperature, and amplifier gain were stored on disk and later analysed with a PDF–11/23 computer.

The pulse protocol used to measure the maximum Na^+ current was similar to the one described by Almers *et al.* (1983*b*). It consisted of pulse sequences and pulse intervals. For details regarding the pulse sequence refer to Fig. 1. During the pulse intervals a steady potential was applied to the patch in order to change the average membrane potential (V_m) under the patch and induce the slow changes in Na^+ currents described.

A conventional intracellular electrode (3 M-KCl, 10–20 MΩ) was used to measure the resting potential of the fibres. Resting potentials of several fibres were measured before every experiment. The measurements of the fibre from which the data were taken were performed after the recording using the loose-patch clamp method to avoid loss of potential due to impalement damage. During the experiment, indirect estimates of the resting potential were obtained periodically from the position of the 'h_∞ curve' for conventional, fast Na^+ inactivation (Hodgkin & Huxley, 1952) produced by 20 ms conditioning pre-pulses. These measurements were made whenever the holding potential had remained constant sufficiently long for peak Na^+ conductance to reach a stationary value.

Solutions

The Tyrode solution contained (mM): NaCl, 121; KCl, 3·5; $NaHCO_3$, 25; NaH_2PO_4, 1; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 12. High-Ca^{2+} Tyrode solution contained (mM): NaCl, 115; KCl, 3·5; $NaHCO_3$, 25; NaH_2PO_4, 1; $MgCl_2$, 1; $CaCl_2$, 6. Both solutions were gassed with 95 % O_2–5 % CO_2. Most of the experiments were performed in high-Ca^{2+} Tyrode solution because the seals were more stable, allowing us to record from patches of membrane for several hours. Values are expressed as mean ± S.E. of the mean.

RESULTS

Peak current measurement

Fig. 2*A* shows Na^+ currents recorded during depolarizing pulses of different amplitudes. The inward peaks were fitted by polynomials of third order whose minima defined the peak current at each potential. Groups of six depolarizing pulses in steps of 6 mV were applied periodically to measure the maximum inward Na^+ current. Fig. 2*B* shows a plot of peak current *vs.* potential. The six peak Na^+ currents were

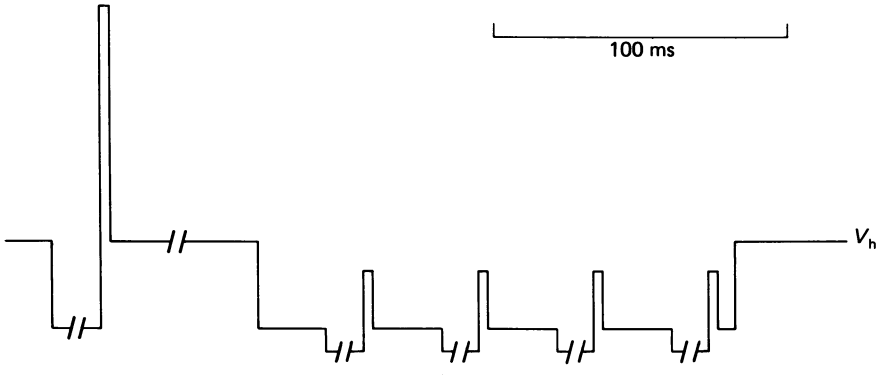

Fig. 1. Pulse sequence for measuring Na^+ currents. The potential across the patch membrane is first changed from the holding potential (V_h) to a depolarized level (test pulse). This test pulse is preceded by −30 mV hyperpolarization to remove fast and intermediate inactivation of Na^+ channels. The duration of this hyperpolarization was varied in some experiments up to 137 ms. After 160 ms four control pulses for leakage compensation purposes follow. They are superimposed on a −30 mV hyperpolarizing potential step in order to ensure that they do not elicit Na^+ currents. These control pulses are scaled in amplitude to one-fourth of the test pulse.

fitted by a parabola and the minimum of this parabola was the maximum inward current. The maximum inward current was used as an assay for the number of functional channels in the patch. The membrane potential that elicited the maximum inward Na^+ current ($V_{I,Na,max}$) was $-23{\cdot}5 \pm 1{\cdot}9$ mV ($n = 16$) in high-Ca^{2+} Tyrode solution.

When a 30 mV hyperpolarizing holding potential was applied in normal Tyrode solution containing 2 mM-Ca^{2+}, the steady-state voltage dependence of activation and inactivation gating were shifted by about −10 mV. The shift of both activation and fast inactivation characteristics developed with time constants in the range of 1–2 min. In high-Ca^{2+} Tyrode solution, these shifts were reduced to less than 3 mV, even at large hyperpolarizations. Besides eliminating these shifts, the high-Ca^{2+} Tyrode solution made the recordings more stable over extended periods of time, so most experiments were made in that solution.

Determination of h_∞

Fig. 3 plots peak Na^+ current during fixed depolarizing steps against the potential during a 20 ms conditioning pre-pulse. The points trace out the familiar h_∞ curve

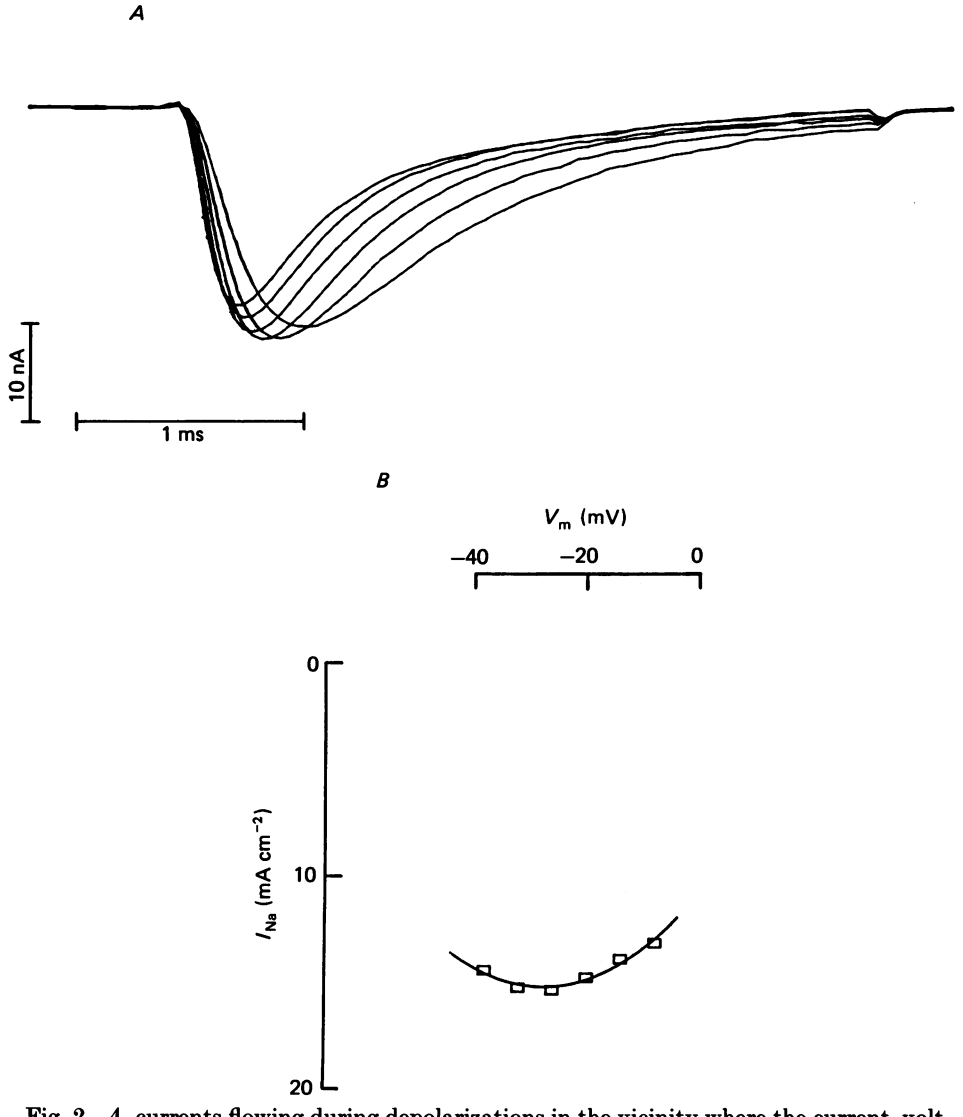

Fig. 2. *A*, currents flowing during depolarizations in the vicinity where the current–voltage relation has maximum currents. The test pulses depolarized the membrane patch from −38 to −8 mV in steps of 6 mV. Temperature 19 °C, pipette resistance 138 kΩ, seal resistance 515 kΩ, pipette tip diameter 14 μm. *B*, plot of the six peak currents as a function of test-pulse potential (V_m). The line through the points is a second-order polynomial fitted to the data points. The minimum corresponds to a current density of 15·3 mA cm^{-2}.

(Hodgkin & Huxley, 1952) and describe the dependence of the fast inactivation process on potential. The line is a least-squares fit of the equation

$$h_\infty = 1/(1+\exp((V_{h,\frac{1}{2}} - V)/a_h)), \quad (1)$$

where V is the transmembrane potential, $V_{h,\frac{1}{2}}$ is the potential where Na^+ currents are half-maximum and a_h is a parameter determining how steeply h_∞ depends on membrane potential.

Resting potential and fast inactivation measurement

In normal Tyrode solution, the average resting potential was -103 mV± 2 mV ($n = 14$). In the high-Ca^{2+} Tyrode solution it was -109 mV± 2 mV ($n = 22$). The membrane potential at which half of the channels were inactivated by the rapid inactivation process ($V_{h,\frac{1}{2}}$ of eqn. (1)), as measured with an intracellular electrode after measuring fast inactivation, was $-79{\cdot}6\pm 1{\cdot}7$ mV ($n = 14$) and $-76{\cdot}7\pm 0{\cdot}5$ mV

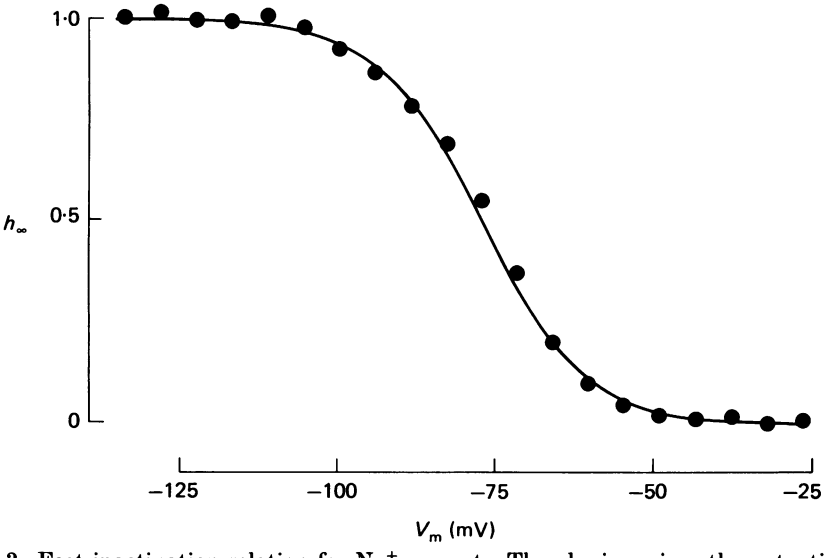

Fig. 3. Fast inactivation relation for Na^+ currents. The abscissa gives the potentials of conditioning pre-pulses. The ordinate gives the normalized peak Na^+ currents obtained when the membrane of the patch was depolarized to -34 mV after a conditioning pre-pulse (h_∞). Half of the currents were fast inactivated by a depolarizing pre-pulse of 56 mV from the holding potential. Assuming that this corresponds to an effective membrane potential of -77 mV, the resulting effective membrane potential was -133 mV under the patch. The continuous line gives a least-squares fit to the function in eqn (1). Temperature 19·1 °C.

($n = 36$), respectively in normal and high-Ca^{2+} Tyrode solution. The parameter a_h (eqn. (1)) was $6{\cdot}56\pm 0{\cdot}67$ mV ($n = 36$) in the high-Ca^{2+} solution. We used $V_{h,\frac{1}{2}}$ obtained from fits of eqn. (1) to monitor the effective transmembrane potential. The effective potential seen by the Na^+ channels at different holding potentials in the high-Ca^{2+} Tyrode solution was assumed to be $V_{h,\frac{1}{2}}$ measured at the resting potential minus the applied conditioning potential that was required to inactivate 50 % of I_{Na}. In some cases in which the resting potential measurements were not reliable, $-76{\cdot}7$ mV was assumed for $V_{h,\frac{1}{2}}$. A typical fast inactivation curve for Na^+ currents is shown in Fig. 3. Measurements of this type were made whenever the currents had stopped changing with time.

Slow changes in current densities

Fig. 4 shows slow changes in peak Na^+ current resulting from changes in potential applied to the patch electrode during pulse intervals. When the holding potential was stepped from -106 to -133 mV, maximum I_{Na} doubled over the following 10 min.

I_{Na} slowly decreased during the subsequent depolarizations to −113 and −103 mV. The change in peak current followed a very slow time course, taking several minutes to complete at any given potential. Peak current densities were measured as in Fig. 2. These results indicate that maintained hyperpolarization slowly recruits functional channels from an inactive pool, and that on return to the resting potential most channels return to an inactive state. Slow inactivation is the slow conversion of

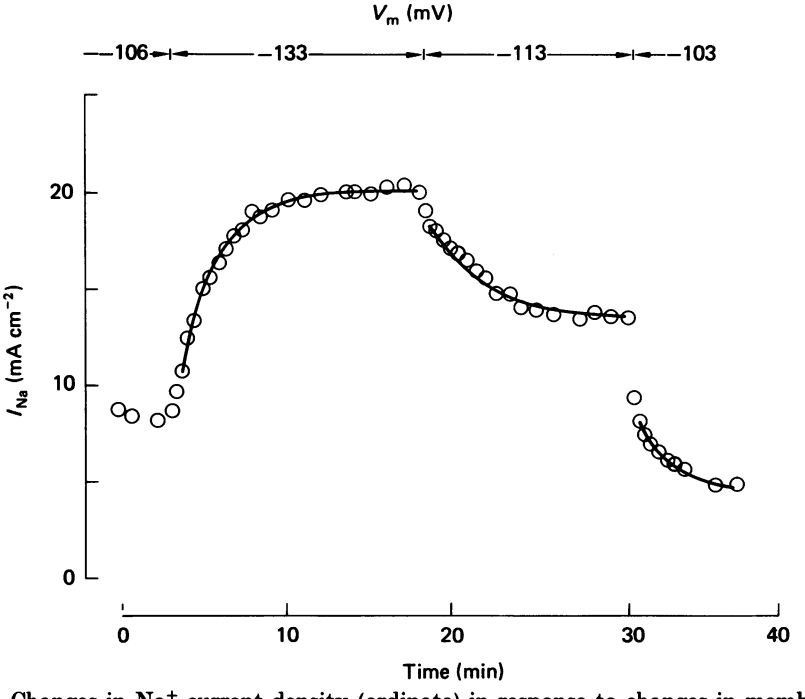

Fig. 4. Changes in Na^+ current density (ordinate) in response to changes in membrane potential during the pulse interval, plotted against time (abscissa). This particular record originated after 13 min of starting the current recording during an experiment lasting 91 min. The top shows the effective potentials seen by the Na^+ channels during the pulse intervals. The continuous lines are drawn through the points to give the best fit to eqn. (2). The pre-pulse duration in this experiment was 20 ms. The fast inactivation relation of Fig. 3 was taken before changing the potential from −133 to −113 mV. Same experimental conditions as in Fig. 3.

channels from an excitable to an inexcitable state with maintained small depolarizations. The steady-state values of Na^+ current and the time course of slow inactivation were obtained by fitting the experimental data to the function:

$$I_{Na}(t) = I_{Na,\infty} - (I_{Na,\infty} - I_{Na,0}) \exp(-t/\tau). \quad (2)$$

The fit was started at least 10 s after changing the potential. This was done in order to avoid possible contamination of the rate constants from any inactivating process with time constants ranging from several hundred milliseconds to seconds (Fox, 1976; Brismar, 1977; Almers *et al.* 1983*b*). In most of the experiments the patch was hyperpolarized by 30 mV from the resting potential for 80 ms to remove rapidly developing inactivation before testing for Na^+ currents. That the data are fitted quite

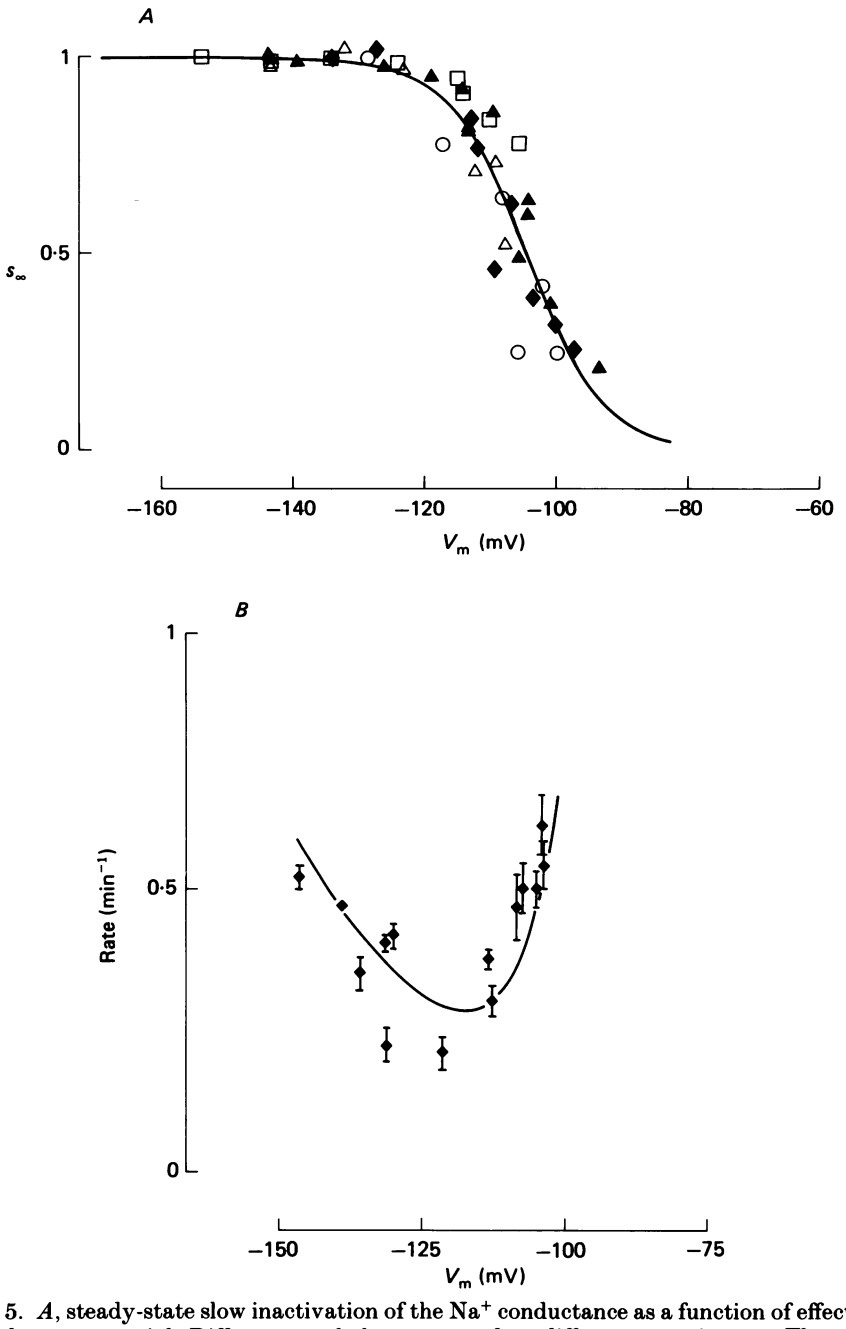

Fig. 5. *A*, steady-state slow inactivation of the Na^+ conductance as a function of effective membrane potential. Different symbols correspond to different experiments. The open circles are from the experiment shown partially in Fig. 4. The line is drawn to give the best fit to eqn. (3). $V_{s,\frac{1}{2}} = -108$ mV and $a_s = 5{\cdot}8$ mV. *B*, rate constants of development and recovery from slow inactivation plotted against membrane potential. The line is drawn to give the best fit to eqn. (3). The parameters $V_{s,\frac{1}{2}}$ and a_s are those of Fig. 5*A*. The best fit was obtained for $R = 0{\cdot}34$ and $K = 0{\cdot}196$ min^{-1}.

well by a single exponential function further suggests that there is only one main process involved in slow inactivation.

Determination of s_∞

Fig. 5*A* illustrates the membrane potential dependence of the slow inactivation process described here. The data from five experiments were combined. The line is a least-squares fit of the equation

$$s_\infty = 1/(1+\exp((V_{s,\frac{1}{2}}-V)/a_s), \tag{3}$$

where s_∞ is the relative value of the maximum I_{Na} at a given membrane potential compared to the maximum I_{Na} at very hyperpolarized potentials, $V_{s,\frac{1}{2}}$ is the potential at which half of the Na^+ channels are inexcitable, and a_s is a parameter determining how steeply s_∞ depended on membrane potential. The maximum Na^+ current density from each patch was $17{\cdot}6 \pm 1{\cdot}4$ mA cm^{-2} ($n = 5$), and $V_{s,\frac{1}{2}}$ was -108 mV.

The relation between the rate constant ($1/\tau$) for loss or recovery of Na^+ currents and the membrane potential is shown in Fig. 5*B*. The rate constants for steps in membrane potential which gave only small changes in current densities were discarded because the amplitude term ($I_{Na,\infty} - I_{Na,0}$) in eqn. (2) was too small to give reliable fits. The smooth line is a fit to the function (eqn. (24) in Benz & Conti, 1981)

$$1/\tau = 2K\cosh(U/2)\exp(-U/(R-1/2)), \tag{4}$$

where $U = (V - V_{s,\frac{1}{2}})/a_s$, K is the equilibrium rate constant in a simple gating model, and R is the fraction of membrane thickness to be overcome by a gating particle to reach the top of the energy barrier. In the case of a symmetrical energy barrier, R would be 0·5. The best fit to our data was obtained for $R = 0{\cdot}34$ and $K = 0{\cdot}196$ min^{-1}.

Neither the time constants nor the steady-state currents showed any obvious dependence on the direction of the potential change. They only depended on the final potential value, as is the case with fast inactivation.

DISCUSSION

We believe that the changes in peak Na^+ current that accompany changes in holding potential in this preparation are due to a slow inactivation process, as described by several other authors in a variety of different excitable membranes. To reach this conclusion we must exclude other possible interpretations. Electrodiffusion of Na^+ channels in and out of the voltage-clamped area could produce increases or decreases of Na^+ currents, depending on the size of the voltage displacement from rest conditions. The potential applied to the pipette causes not only a potential change of the membrane under the patch, but also a potential gradient under the rim of the electrode tip forming the seal. This gradient will cause electrodiffusion of charged and mobile molecules under the electrode rim. Are Na^+ channels also moved by this lateral field? The best way to answer this question would be to control the lateral and the transmembrane electric fields independently. In this preparation it was difficult to achieve this condition, which requires insertion of at least one intracellular electrode into the fibre to establish a transmembrane voltage clamp. This has to be done close to the loose-patch electrode due to the short space constant for

mammalian muscle fibres (Ruff, Stühmer & Almers, 1982). In almost all cases in which this was attempted the seal between the sarcolemma and the loose-patch electrode was destroyed or became unstable. However, we have several other lines of evidence which seem to exclude the possibility that Na^+ channels undergoing electrodiffusion produced the slow changes in maximum Na^+ current density. First, changes in the Ca^{2+} concentration modified the voltage dependence of Na^+ channel gating without affecting the slow changes in current densities. Secondly, the Na^+ channels are probably immobile. The current densities recorded from the same fibre varied from patch to patch, a finding not compatible with the assumption of laterally mobile channels. In frog sartorious muscle, Stühmer & Almers (1982) found the Na^+ channels to be immobile to within resolution limits. Also, in the same preparation similar results to those in this work were obtained under conditions that prevented Na^+ channel electrodiffusion (Almers *et al.* 1983*b*). The significant regional variations in Na^+ current density in frog (Almers *et al.* 1983*b*), snake (Beam, Caldwell & Campbell, 1985) and rat (Almers, Roberts & Ruff, 1984; Beam *et al.* 1985) muscle fibres are inconsistent with Na^+ channels being freely diffusible.

One surprising finding is that at an effective membrane potential of -108 mV half of the Na^+ current is not available. In frog muscle slow inactivation has little effect on the Na^+ current density at the resting potential. The membrane potential had to be depolarized to -76 mV to obtain a 50 % reduction in current amplitude by slow inactivation in frog skeletal muscle (Almers *et al.* 1983*b*). The slow inactivation process in rat fast-twitch muscle greatly reduced Na^+ current density, even for cells with very negative resting potentials. Ruff *et al.* (1982) needed a hyperpolarizing potential in order to record Na^+ currents in rat fast-twitch muscle fibres with an average resting potential of -87 mV. The value of $V_{h,\frac{1}{2}} = -76{\cdot}7$ mV for fast inactivation in high-Ca^{2+} Tyrode was similar to that found in rat fast-twitch muscle in prior loose-patch-clamp studies using normal Ca^{2+} Tyrode solution. In experiments performed at room temperature, Ruff *et al.* (1982) reported $V_{h,\frac{1}{2}}$ to be -71 mV and Almers *et al.* (1984) found it to be -76 mV. Using different voltage-clamp techniques to study the fast inactivation process Adrian & Marshall (1977) found $V_{h,\frac{1}{2}}$ to be -71 mV and Duval & Léoty (1978) reported values of -73 mV, and -67 mV in a later paper (Duval & Léoty, 1980).

The resting potentials we measured were more negative than the ones reported by other authors. *In vivo* studies suggest that the physiological mean resting potential of rat fast-twitch hind-limb muscle is about -95 mV (Bolte & Lüderitz, 1968; Ruff *et al.* 1982). We carefully checked this point and believe the discrepancy to be due to the relatively low K^+ concentration in the Tyrode solution used. In addition, we discarded muscles which did not allow us to record Na^+ currents at the resting potential. In muscles with lower average resting potentials, currents could be recovered only by long-lasting hyperpolarization.

The physiological role of slow inactivation is not yet clear, but this process is certainly important in long-term regulation of the availability of Na^+ currents. Slow inactivation may play a prominent role in diseases associated with abnormalities in sarcolemmal excitability. The periodic paralyses are a group of disorders in which the sarcolemma becomes inexcitable in association with relatively small depolarizations (Ruff & Gordon, 1986). The prominent effect of slow inactivation, namely the

drastic reduction of maximum excitable Na^+ currents, may explain why membrane depolarizations, which are insufficient to rapidly inactivate Na^+ channels, can make the sarcolemma electrically inexcitable. In addition, the slow recovery from this inactivation can explain why excitability does not return until the cells have been repolarized for many minutes. The failure of neuromuscular transmission in human and experimental myasthenia gravis is due, in part, to an immune-mediated blockade of acetylcholine receptors, accelerated receptor degradation, and complement mediated destruction of the end-plate sarcolemma (Drachman, 1981). The end-plate membrane damage may further reduce the efficiency of neuromuscular transmission by focally depolarizing the membrane, which should result in a lower maximum Na^+ current due to slow inactivation (Ruff, Simoncini & Stühmer, 1986). Slow inactivation may limit the ability of skeletal muscle fibres to generate repetitive action potentials for extended periods. Reduction in the extent of slow inactivation present at the resting potential could increase sarcolemmal excitability, and slow inactivation processes probably help to terminate myotonic discharges (Rüdel & Lehmann-Horn, 1985).

In summary, our experiments show that slow inactivation of Na^+ conductance is present in fast-twitch rat muscle fibres. Since slow inactivation is prominent at the resting potential, changes in the resting potential that do not detectably affect the fast inactivation will none the less alter membrane excitability.

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