# Voltage-Clamp of Cut-end Skeletal Muscle Fibre: a Diffusion Experiment\*

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Abstract. Membrane potential and current were studied in cut end fibres of frog skeletal muscle under current and voltage clamp conditions, by the double sucrose gap technique. Similar action potentials were recorded under current clamp conditions with either the microelectrode or the double sucrose gap techniques. Under voltage clamp conditions, the control of the membrane potential was maintained adequately. The early current was sensitive to both TTX and external Na concentration suggesting that the current was carried by Na ions. Sodium current  $(I_{Na})$  was subsequently analysed using the Hodgkin-Huxley formulae.  $I_{Na}$  half-activation and inactivation occurred at -34 mV and  $-60 \,\mathrm{mV}$ , respectively. Na-rich solution applied internally by diffusion through cut ends produced a reduction of  $I_{Na}$  associated with a shift of the sodium current reversal potential  $(V_{Na})$  towards more negative membrane potentials. This suggested that the sodium electromotive force was reduced by the increase in internal Na content of the fibre. Iodate applied externally changed neither the activation nor the inactivation time courses of  $I_{Na}$ , but reduced the peak current. Conversely, internally applied by diffusion from the cut end of skeletal muscle fibre, iodate slowed down the time course of  $I_{Na}$  inactivation and decreased the current peak. In conclusion, the double sucrose gap technique adapted to cut end frog skeletal muscle fibre allows a satisfactory analysis of  $I_{Na}$ .

**Key words:** Double sucrose gap technique — Cut skeletal muscle fibre — Current and voltage clamp — Sodium channel — Iodate

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# Introduction

The fast sodium current in intact isolated skeletal muscle fibres has been successfully recorded and analysed for more than 10 years (Ildefonse and Rougier 1972; Ildefonse and Roy 1972) by means of the double sucrose gap technique. Using a vaseline gap chamber similar to that used for myelinated nerve fibres Nonner (1969), Hille and Campbell (1976), and several other authors (Collins et al. 1982a; Zachar et al. 1982; Arispe et al. 1984) recorded the sodium current in cut end skeletal muscle fibres. Both the double sucrose gap and the vaseline gap method have been reported to offer good voltage control for early currents. The cut end fibre method seems to allow a better stability of the preparation than the methods working with intact fibres since the former provides the possibility of controlling the intracellular medium (Arispe et al. 1984). Aimed at showing that by changing the internal medium of frog skeletal muscle fibres the Na channel can really be studied by inside manipulations, our experiments were performed with the double sucrose gap technique, which is particularly well adapted to the measurement of ionic currents in skeletal muscle (Ildefonse and Rougier 1972; Duval and Léoty 1978).

### Materials and Methods

### a) Dissection and experimental protocol

Experiments were performed on single muscle fibres isolated from the sartorius muscle of *Rana* esculenta. The dissected muscle was equilibrated at rest for one hour in Ringer solution. Then, single fibres (3 mm in length, 80–120  $\mu$ m in diameter) were mechanically separated using sharpened needles. One muscle fibre fragment was mounted in an experimental chamber (Fig. 1), by positioning the undamaged region across the five partitions of the chamber. Vaseline seals were placed upon the fibre on the divisions separating the compartments. Compartments A and C were filled with internal solution, compartments M1 and M2 (350  $\mu$ m in width) with isotonic mannitol or sucrose solutions; the test compartment B (250  $\mu$ m in width), was perfused with physiological solution. A Peltier cooling device, mounted under the chamber, allowed experiments to be performed at a chosen constant temperature (10°C unless otherwise stated).

### b) Solutions

The Ringer solution had the following composition (mmol/1): NaCl 110.5; KCl 2.5; CaCl<sub>2</sub> 2; MOPS buffer 5 (pH 7.2). The standard internal solution contained (mmol/l): K-Aspartate 120; ATP 5; phosphocreatine 5; MgCl<sub>2</sub> 2; MOPS buffer 5 (pH 7.2). The internal sodium-rich (120 mmol/l) solution was prepared by replacing all K-aspartate with an equivalent amount of Na-acetate. In the external iodate solution, 30 mmol/l NaCl were replaced by 30 mmol/l NaIO<sub>3</sub>. In the internal iodate solution, 30 mmol/l K-Aspartate were replaced by 30 mmol/l KIO<sub>3</sub>. The internal solutions were introduced into the left side pool of the test chamber. In some experiments, tetrodotoxin (TTX;  $5.7 \times 10^{-7}$  mol/l) was used to block the sodium current; choline chloride (atropine 10<sup>-4</sup> mol/l) was

### Voltage-clamp of Cut End Fibre

used as a substitute for sodium ions. The Ringer solution contained tetraethylammonium (TEA; 10 mmol/l) to suppress K current.

### c) Control circuit

The electronic circuitry (Fig. 1) used to control the membrane potential was similar to that developed by Sauviat and Suchaud (1981) for frog heart fibres. The membrane potential of the right side pool, (C) representing the inside of the fibre, was measured with respect to the test compartment (B) on the outside surface of the fibre kept at virtual ground. The different compartments of the chamber were connected to the electronic apparatus by means of calomel electrodes and KCl saturated (3 mol/l) agar glass bridges.



**Fig. 1.** Electronic apparatus used in current and voltage clamp measurements. Membrane voltage  $(V_m)$  is measured as potential difference between compartments B and C by means of a set of voltage monitors  $A_1$ ,  $A_4$  and the differential amplifier  $A_5$ .  $A_2$  is a current/voltage converter. The current flowing through the membrane is recorded at the output of amplifier  $A_3$ . The left compartment was connected to the output of the error amplifier  $A_6$ . IS: selection switch; O: voltage monitor allowing to record resting potential (RP) and action potential (AP); CC: current clamp mode; VC: voltage clamp mode; HP: holding potential; stim: square pulses delivered by a stimulator.

In practice, the resting membrane potential of the sartorius muscle fibres, measured in the Ringer solution in an intact clean muscle by means of intracellular microelectrodes (resistance 15 MΩ; tip potential  $\leq \pm 3$  mV) was  $-84.2 \pm 0.6$  mV (mean  $\pm$  SEM of 40 impalements in four muscles). Thus, the resting membrane potential was -80 mV for both the current and the voltage clamp experiments. Starting from a holding potential (HP) of -80 mV, the potential of the test node was displaced in rectangular steps; positive potentials correspond to depolarizations. The sodium current ( $I_{Na}$ ) was measured as net inward and outward if not otherwise specified. Preparations were stimulated at a rate of 0.2 Hz. Transmembrane potentials and currents were displayed on an oscilloscope (Tektronix 5110) and photographed for analysis. The computer program used to fit the experimental points is written in Basic on Hewlett Packard 9826.

#### d) Passive properties measurements

Several passive properties of the membrane were determined as follow: the series resistance  $(R_{\rm s})$ , the membrane capacity ( $C_{\rm m}$ ) and the capacitive time constant ( $\tau_{\rm c}$ ) were estimated from current records under low amplitude depolarizing pulse (Ildefonse and Rougier 1972; Duval and Léoty 1978). The membrane resistance ( $R_m$ ) was calculated as described by Adrian et al. (1970).  $I_{Na}$  was measured as net Na current after subtracting the peak current  $(I_{peak})$  from the current recorded after TTX  $(5.7 \times 10^{-7} \text{ mol/l})$  treatment. The sodium current reversal potential (V<sub>Na</sub>) was taken as the point of intersection of I-V curves drawn in the TEA-Ringer solution before and after TTX application. The relation between the current and the corresponding conductance described by Hodgkin and Huxley (1952a) is:  $G_{\text{Na}} = I_{\text{Na}}/(V_{\text{m}} - V_{\text{Na}})$ , where  $I_{\text{Na}}$  is the amplitude of the current and  $V_{\text{m}}$  the imposed potential. The maximal Na conductance  $\bar{G}_{Na}$  is a constant and represents the absolute maximum value, independent of the potential that can be reached by  $G_{Na}$ .  $I_{Namax}$  corresponds to the maximum amplitude of the sodium current. This value was calculated using the independence principle (Hodgkin and Huxley 1952b), i.e. without considering the overlap between activation and inactivation (Bezanilla and Armstrong 1977). The membrane surface was calculated from an optical estimation of the diameter and the length of the fibre in the test gap. The average surface of the membrane clamped in the different experiments was  $7 \times 10^{-4} \pm 0.5 \times 10^{-4} \text{ cm}^2$  (17) (apparent external diameter between  $80-120 \,\mu\text{m}$ , and length of the fibre in the test gap between  $160-220 \,\mu\text{m}$ ). Nevertheless, the area estimated from geometrical measurements represents a source of error. The tubular membrane surface has not been taken into account. Thus, the values cannot be considered as accurate.

## Results

### A - Action potential

In current clamp conditions, the action potential (AP) was measured by applying a brief current stimulus. In order to estimate the validity of the method, we recorded the AP of the test node with a glass micropipette electrode impaled in the middle of the fibre and connected to a voltage monitoring system independent of the current clamp system itself. Fig. 2A shows that AP developing in response to suprathreshold constant current pulse application were similar in both recording conditions. The AP amplitude was  $120.3 \pm 3.6 \,\text{mV}$  (10) and  $119.8 \pm 2.9 \,\text{mV}$  (14) (mean  $\pm \text{SEM}$ , (number of fibres)) with the microelectrode and the double sucrose gap technique, respectively.

# B — Sodium current

Under voltage clamp conditions, after the surge of the capacitive current, a fast current developed. The average amplitude of the maximal current peak was 5.6  $\pm 2.1 \text{ mA/cm}^2$  (17). A family of fast currents is shown in Fig. 2*B*. The current is net inward from depolarizing pulses ranging from + 30 to + 100 mV and then clearly reverses. Fig. 2*C* shows that the peaks of both the inward and the outward current are strongly sensitive to TTX application. Such TTX-sensiti-

vity indicated that both currents were carried by sodium ions through the fast kinetic channels. Under voltage clamp, a source of error in the membrane potential records arises from a voltage drop outside the fibre during the current flow; indeed, a portion of the current flows through the series resistance  $(R_s)$ . Owing to this, the quality of good voltage clamp control of the membrane was checked during the flow of  $I_{\rm Na}$ , by means of a microelectrode system independent of the voltage clamp system itself. Fig. 2D shows that the membrane potential  $(V_m)$  deviated from the clamp potential  $(V_c)$  by  $2.8 \pm 0.7$  mV (6) during the flow of maximum  $I_{\rm Na}$ .



Fig. 2. A: action potential recorded (1) in current clamp conditions with the double sucrose gap technique and (2) using intracellular microelectrode.  $I_{\rm m}$ , current applied to the membrane. B: family of  $I_{\rm Na}$  elicited in Ringer solution by pulses from -20 to +130 mV (HP -80 mV) in steps of 10 mV. Fibre diameter:  $100 \,\mu$ m. Membrane surface area in the test compartment:  $5 \times 10^{-4} \,{\rm cm}^2$ . C: Effect of  $5.7 \times 10^{-7} \,{\rm mol}/l$  TTX (arrows) on  $I_{\rm Na}$  obtained during  $+70 \,{\rm mV}$  (downward deflection) and  $+130 \,{\rm mV}$  (upward deflection) depolarizing pulses (HP  $-80 \,{\rm mV}$ ). D: control of the membrane potential using the double sucrose gap technique during the flow of  $I_{\rm Na}$  elicited by a 70 mV depolarizing command potential ( $V_c$ ). ( $V_m$ ): transmembrane potential recorded by the intracellular microelectrode system.  $I_m$ : membrane current.  $V_m$  was very close to the holding potential value HP =  $-80 \,{\rm mV}$ . Traces (2) in A and ( $V_m$ ) in D were intentionally time-shifted.

Properties	$\frac{R_{\rm m}}{(\Omega \times {\rm cm}^2)}$ (C	$R_{\rm s}$ 2 × cm <sup>2</sup> )	$(\mu s)^{\tau_c}$	$C_{\rm m}$ ( $\mu { m F/cm^2}$ )	$I_{\text{Na max}}$ (mA/cm <sup>2</sup> )	$ar{G}_{ m Na}$ (mS/cm <sup>2</sup> )	V <sub>Na</sub> (mV)
Values	2650	7	35.7	4.2	5.6	104	52.7
$\pm$ SEM	300	0.5	3.1	0.3	2.1	30	2.6
n	6	10	10	10	17	7	7

Table 1. Electrical properties of muscle preparations

The values were obtained under voltage-clamp conditions at  $10^{\circ}$ C. *n* the number of experiments performed.

The passive properties of the fibre are summarized in Table 1. In physiological solution, the specific membrane resistance  $(R_m)$  and the total membrane capacity  $(C_m)$  per unit surface were respectively  $2650 \pm 300 \Omega \times \text{cm}^2$  (6) and 4.2  $\pm 0.3 \,\mu\text{F/cm}^2$ (6). The value of  $R_m$  determined in our preparations is consistent with that of  $3100 \Omega \times \text{cm}^2$  obtained by Sanchez and Stefani (1978); however, it is more than twice as high as the value reported by Ildefonse and Rougier (1972).



**Fig. 3.** A: Current-voltage relationships of peaks current  $(I_{\text{peak}})$ : ( $\bigcirc$ ) 110.5 mmol/l Na in TEA-Ringer solution; ( $\bigcirc$ ) 55 mmol/l Na in TEA-Ringer solution; ( $\triangle$ ), with TTX (5.7 × 10<sup>-7</sup> mol/l). In *B*, the current-voltage relationships of  $I_{\text{Na}}$  were plotted after leakage subtraction. Fibre diameter: 100 µm. Membrane surface area in the test compartment:  $6 \times 10^{-4} \text{ cm}^2$ .

As far as  $C_m$  is concerned, Hille and Campbell (1976) found an effective capacity of 11.3  $\mu$ F/cm<sup>2</sup> at 5°C, and Collins et al. (1982a) 3—6  $\mu$ F/cm<sup>2</sup> at 15°C. It should be stressed that the values refering to membrane surface area were calculated without considering the tubular membrane surface system, which contributes to a considerable extent to the total capacity of the muscle, and only little to the ionic current detected in compartment B (Hille and Campbell 1976). The decay of the capacitive current gave an average time constant of 35.7 ± 3.1  $\mu$ s (10). Our estimation of  $R_s$  (7 $\Omega \times cm^2$ ) was comparable to 17.5 $\Omega \times cm^2$  reported by Ildefonse and Rougier (1972) and of 5.9 $\Omega \times cm^2$  obtained by Hille and Campbell (1976).

Another way how to check the adequateness of the membrane potential control consists in reducing Na concentration in TEA-Ringer solution. The current-voltage relationships (Fig. 3A) plotted for  $I_{\text{Na}}$  show that Na reduction in the control solution by 50% produced a decrease of  $I_{\text{Na}}$  amplitude at all the potentials tested. Further TTX application entirely inhibited the remaining current. The net ionic current ( $I_{\text{Na}}$ ) is obtained by subtracting the leak current (i.e. after TTX treatment) from the peak current ( $I_{\text{peak}}$ ) (Fig. 3B). Upon reducing sodium concentration, the reversal potential was shifted by 17 mV towards more negative values. This is identical to the value calculated from the Nernst equation. The average values of  $V_{\text{Na}}$  were  $+52.7 \pm 2.6 \text{ mV}$  (6) and  $+32 \pm 2.5 \text{ mV}$  (6) in 100% and 50% Na solutions, respectively. Calculations of the maximal Na conductance ( $\bar{G}_{\text{Na}}$ ) from the I-V curves gave a mean value of  $104 \pm 30 \text{ mS/cm}^2$  (7).

# a) Activation and inactivation parameters

Hodgkin and Huxley (1952a, b) formulae, describing Na conductance ( $G_{Na}$ ), were used to study activation and inactivation parameters. According to this model, the Na conductance is described by

$$G_{\rm Na} = \bar{G}_{\rm Na} \times m^3 \times h \tag{1}$$

where *m* and *h* represent Na time-dependent activation and inactivation, respectively, and  $\bar{G}_{Na}$  the maximal Na conductance.

In Fig. 4 the activation parameters have been plotted as a function of voltage. The steady-state parameter for activation  $(m_{\infty})$  was calculated as a function of membrane potential  $(V_{\rm m})$  from the peak conductance measured during each pulse and normalized to  $\bar{G}_{\rm Na}$  obtained in the whole family of pulses as

$$m_{\infty} = (G_{\rm Na}/\bar{G}_{\rm Na})^{1/3} \tag{2}$$

Fig 4A shows a series of  $m_{\infty} - V_{\rm m}$  experimental points for  $I_{\rm Na}$  plotted within a range of membrane potentials from -40 to +20 mV. The mid-point of the

activation voltage was  $-34.2 \pm 2.1 \text{ mV}$  (5) (Fig. 4*A*). The values of  $\tau_{\rm m}$  shown in Fig. 4*B* were obtained either 1) from equation:

$$I_{Na} = I'_{Na} (1 - \exp(-t/\tau_m))^3 (\exp(-t/\tau_h))$$
(3)

where  $I'_{Na}$  represents the value that  $I_{Na}$  would be able to reach if inactivation was maintained at its resting level (in absence of inactivation);  $I'_{Na}$  was obtained by plotting the value of  $I_{Na}$  during  $I_{Na}$  inactivation phase as a function of time on a semi-logarithmic scale. The intersection of the straight line with the ordinate at the origin corresponds to the value of  $I'_{Na}$ ; or 2) from the exponential decay of Na current tails recorded at the end of short depolarizing pulses (Meves 1984). Fig. 4*B* illustrates the relationship between  $\tau_m^{-1}$  and  $V_m$ . The maximum of  $\tau_m$  is reached at -30 mV.



**Fig. 4.** *A*, *B*, *C*, *D*: Steady-state and kinetic parameters of  $I_{Na}$  activation. Continuous curves were obtained by fitting experimental points by the following equations:  $m_{\infty} = \alpha_m/(\alpha_m + \beta_m)$ ,  $\tau_m = 1/(\alpha_m + \beta_m)$ , (6) and (7), respectively. (Symbols  $\bigcirc, \bigoplus, \square, \blacksquare$  represent the same points for the different figures *A*, *B*, *C* and *D*).

The rate constants  $\alpha_m$  and  $\beta_m$  were calculated as follows:

$$\alpha_{\rm m} = m_{\infty} / \tau_{\rm m} \tag{4}$$

$$\beta_{\rm m} = (1 - m_{\infty})/\tau_{\rm m} \tag{5}$$

The values of  $\alpha_m$  and  $\beta_m$  (Fig. 4C and D) were fitted according to equations:

$$a_{\rm m} = 0.63(V_{\rm m} + 17.5)/(1 + \exp\left(-(V_{\rm m} + 17.5)/10\right)) \tag{6}$$

$$\beta_{\rm m} = 0.95 \exp - (V_{\rm m} + 17.5) \tag{7}$$

where the numerical values given for squid giant axon in equations (20) and (21) by Hodgkin and Huxley (1952b) were specifically substituted. The curve in Fig. 4D represents the least square fit of a single exponential (equation (8)) to  $\beta_{\rm m} - V_{\rm m}$ . The parameters  $\alpha_{\rm m}$  and  $\beta_{\rm m}$  (determined as a function of  $V_{\rm m}$ ) of the fit were used to reconstruct the curves  $m_{\infty} - V_{\rm m}$  and  $\tau_{\rm m}^{-1} - V_{\rm m}$  shown in Fig. 4A and B as follows

$$m_{\infty} = \alpha_{\rm m} / (\alpha_{\rm m} + \beta_{\rm m}) \tag{8}$$

$$\tau_{\rm m} = 1/(\alpha_{\rm m} + \beta_{\rm m}) \tag{9}$$

The fit experimental data (using equations (8)) indicates that the voltage dependence of  $I_{Na}$  activation follows the known sigmoid shape.



Fig. 5. A, B, C, D: Steady-state and kinetic parameters of  $I_{Na}$  inactivation. Continuous curves were obtained by fitting the experimental points by the following equations:  $h_{\infty} = a_h/(a_h + \beta_h)$ ,  $\tau_h = 1/((a_h + \beta_h), (12))$  and (13), respectively. (Symbols  $\bigcirc, \bigoplus, \square, \blacksquare$  represent the same points for A, B, C and D).

Fig. 5A illustrates the steady-state inactivation of the Na system  $(h_{\infty})$  as a function of the membrane potential using a double pulse arrangement (Hodgkin and Huxley 1952b). The potential of the test pulse  $(V_{i})$  was kept constant at 0 mV, applied from a HP of -100 mV for 40 ms, and the peak current associated with V, was determined as a function of the initial conditioning pulse. The sodium system was fully operative ( $h_{\infty} = 1$ ) at  $V_{\rm m} = -90 \,{\rm mV}$  and entirely inactivated at  $V_{\rm m} = -40 \,\mathrm{mV}$ . Half-inactivation occurred at  $V_{\rm m} = -59.8 \pm 1.2 \,\mathrm{mV}$  (5) in Ringer solution. The measurement of the inactivation time constant ( $\tau_{\rm h}$ ) was determined either from the decay of  $I_{Na}$  during a depolarizing pulse by plotting the logarithm of the falling phase of  $I_{Na}$  vs. time, or, in order to extend the voltage axis in the negative potential direction, by means of a double pulse protocol which consists in a conditioning pulse of increasing duration (t = 0.5 to 40 ms) followed by a test pulse  $(V_1)$  elicited by 100 mV depolarizing pulse from HP = -100 mV for 15 ms.  $I_{\text{Na}}$  elicited by  $V_t$ ,  $(I_{\text{Na}})_V$ , was determined as a function of time (t) for each voltage level, by calculating the ratio  $(I_{Na})_V/(I_{Na})_o$ ,  $(I_{Na})_o$ being the current recorded without pre-potential at 0 mV (HP = -100 mV). The fitted curves are least square fits of a single exponential to the points, and the time constant is assumed to be  $\tau_{\rm h}$ . Fig. 5B illustrates the relationship between  $\tau_{\rm h}^{-1}$  and  $V_{\rm m}$ . The maximum value of  $\tau_{\rm h}$  was reached at  $V_{\rm m}=-58\,{\rm mV}$ . The rate constants  $\alpha_h$  and  $\beta_h$  were calculated as follows:

$$\alpha_{\rm h} = h_{\infty} / \tau_{\rm h} \tag{10}$$

$$\beta_{\rm h} = (1 - h_{\infty})/\tau_{\rm h} \tag{11}$$

The values of  $\alpha_h$  and  $\beta_h$  presented in Fig. 5C and 5D were fitted, respectively, by the following equations:

$$\alpha_{\rm h} = 0.00078 \exp - (V_{\rm m} + 17.5)/8.5 \tag{12}$$

$$\beta_{\rm h} = 13/(1 + \exp\left(-(V_{\rm m} + 17.5)/7.6\right)) \tag{13}$$

where the numerical values given for the squid giant axon in equations (23) and (24) by Hodgkin and Huxley (1952 b) were specifically replaced.

The parameters  $\alpha_h$  and  $\beta_h$  of the fit were used to reconstruct the curves  $h_{\infty} - V_m$  and  $\tau_h^{-1} - V_m$  shown in Fig. 5A and B:

$$h_{\infty} = \alpha_{\rm h} / (\alpha_{\rm h} + \beta_{\rm h}) \tag{14}$$

$$\tau_{\rm h} = 1/(\alpha_{\rm h} + \beta_{\rm h}) \tag{15}$$

# b) Diffusion experiments

Two series of experiments were carried out to demonstrate the reliability of the diffusion experiments in our preparations.

First, we checked changes in  $V_{\text{Na}}$  due to changes in internal Na concentration in the internal pool ([Na]<sub>i</sub>). Families of sodium currents were recorded at membrane potentials ranging from +10 to +80 mV (HP = -80 mV) in 10 mV steps. In standard internal solution (Fig. 6Aa), the current is inward from +10 to +40 mV and reverses at about +50 mV. The preparations were kept in these conditions and submitted to repetitive pulsation for 10 min with no modification



Fig. 6. A: superimposed traces of Na currents in Ringer solution elicited by depolarizing pulses ranging from +90 to +160 mV in 10 mV steps (HP = -80 mV); a) in standard internal solution; b) after 11 min in sodium-rich (120 mmol/l) internal solution; c) current-voltage relationships for  $I_{\text{Na}}$  to show the shift of  $V_{\text{Na}}$  induced by Na diffusion (11 min): (O) standard internal solution; ( $\bullet$ ) sodium-rich internal solution.  $I_{\text{Na}}$  was obtained by subtracting the current from  $I_{\text{peak}}$  recorded after TTX application. B: a) superimposed records of  $I_{\text{Na}}$  recorded in Ringer solution (1) and with iodate (30 mmol/l) applied externally (2); b) superimposed records of  $I_{\text{Na}}$  recorded in Ringer solution (1) and with iodate (30 mmol/l) applied internally (2). In a and b,  $I_{\text{Na}}$  was recorded under a 70 mV depolarizing pulse (HP = -100 mV). Fibre diameter and membrane surface area: in A, 120 µm;  $8 \times 10^{-4}$  cm<sup>2</sup> and in B, 110 µm;  $7.6 \times 10^{-4}$  cm<sup>2</sup>.

of  $V_{\rm Na}$ . Then, internal sodium-rich solution (120 mmol/l Na) was applied through cut ends. After 11 min exposure to the internal sodium-rich solution (Fig. 6*Ab*), the maximum amplitude of the inward current was reduced by about 25% whereas the current reversed at around +40 mV. Current-voltage relationships plotted in Fig. 6*Ac* for  $I_{\rm Na}$  indicate that  $V_{\rm Na}$  was shifted by 11 mV towards more negative potentials. The average value of  $V_{\rm Na}$  was +48.3 ± 1.7 mV (6) and +36.3 ± 3.0 mV (6) in standard and sodium-rich internal solutions, respectively. This suggests that Na ions diffused through cut ends and modified [Na]<sub>i</sub>. The mean value of [Na]<sub>i</sub> calculated according to the Nernst equation at 10°C was 15.3 ± 0.8 mmol/l (6) and 25.2 ± 2.0 mmol/l (6) in standard and sodium-rich internal solutions, respectively.

In another series of experiments (Fig. 6B), we tested iodate molecule which, when applied internally, is reported to act specifically on the  $I_{Na}$  inactivation process in the node of Ranvier (Stämpfli 1974). Fig. 6Ba illustrates the effects of external applications of iodate. In normal Ringer solution (Fig. 6Ba, trace 1),  $I_{Na}$  recorded during a 70 mV depolarizing step is rapidly activated with a slower inactivation. After 20 min of external exposure to iodate (30 mmol/l), the  $I_{Na}$ activation and inactivation time course remained unchanged while the peak current was decreased by 12.5%; the average reduction was by 14%  $\pm 2\%$  (6). In contrast, after 25 min internal iodate (30 mmol/l) application, the peak current was irreversibly reduced by 25% and the time course of inactivation was slowed down (Fig. 6B, trace 2). The falling phase of the current (traces 1 and 2) was best fitted by a single exponential.  $\tau_{\rm h}$  increased from 0.58 ms to 1.2 ms in the presence of iodate. The average values of  $\tau_{\rm h}$  from 5 fibres under control solution and after internal iodate application were  $0.51 \pm 0.05$  ms and  $1.33 \pm 0.11$  ms, respectively, and the peak current reduction was  $23 \pm 1.5\%$ . In accordance to Schmidtmayer et al. (1982), the effects of internal iodate application on  $I_{Na}$  could not be reversed and were not significantly different regardless of the iodate concentration applied to the side pool.

### Discussion

The main contribution of the present paper is that cut end fibres of frog sartorius muscles can be voltage clamped in a satisfactory manner by means of the double sucrose gap technique and used to analyse  $I_{\rm Na}$ . Futhermore, diffusion experiments through the cut ends can be performed, permitting to study the effects of chemical compounds applied to the sarcoplasmic side of the membrane. Under current clamp conditions, similar AP configurations were obtained with both the microelectrode and the sucrose gap technique. Under voltage clamp conditions with a microelectrode impaled in the fibre,  $V_{\rm m}$  deviation produced by the

current flow through  $R_s$  was rather small in some experiments. The inhibition of the current by TTX and the lowering of Na concentration confirmed that the fast inward current is carried by Na ions. However, as suggested by Arispe et al. (1984), some precautions were required with the internal solution. These authors observed that during long lasting experiments  $V_{Na}$  could not be stabilized without some energetic compounds (ATP and phosphocreatine), and particularly without a suitable anion such as aspartate. In order to avoid shifts in  $V_{Na}$ , we used standard internal solution of the same composition as described by these authors. This modified internal solution allowed to keep the structure of the transport apparatus intact and thus to restore the sodium pump. Our measurements of passive electrical properties reported in the present work are in agreement with results of previous studies performed in both intact and cut end muscle fibres. The kinetic properties of  $I_{Na}$  were thereafter analysed in terms of the Hodgkin-Huxley formulae. Indeed, the  $I_{Na}$  half-activation voltage (-32 mV in our experiments) is close to mid-point values (from -30 to -50 mV at 15°C) reported by Adrian et al. (1970), Ildefonse and Roy (1972), Hille and Campbell (1976), Zachar et al. (1982) and Collins et al. (1982a). With respect to the Na inactivation system, the mid-point of the inactivation-voltage curves varies from -55 to -70 mV. The respective maximal time constants of activation and inactivation,  $\tau_{\rm m}$  and  $\tau_{\rm h}$ , measured in our experimental conditions (10°C) were 310 µs and 12 ms. To be able to compare our results with previous reports, we extrapolated the values of  $\tau_{\rm m}$  and  $\tau_{\rm h}$  to 15°C, using  $Q_{10}$  of 2 for  $\tau_{\rm m}$  and  $Q_{10}$  of 3 for  $\tau_{\rm h}$  (Collins et al. 1982a, b); this gave  $\tau_{\rm m} = 220\,\mu {\rm s}$  and  $\tau_{\rm h} = 6.9\,{\rm ms}$ . These values are comparable to those obtained by other authors: 150–430  $\mu$ s for  $\tau_m$ and  $0.9 - 7.2 \,\mathrm{ms}$  for  $\tau_{\rm h}$  (Collins et al. 1982a, b).

One advantage of cut end preparations is the possibility to apply substances to the cut ends and to study their effects on the conductance after their diffusion along the fibre. With this in view, two kinds of experiments were performed. First, we checked changes in Na reversal potentials  $(V_{Na})$  resulting from Na concentration increase in the internal pool. A shift of V<sub>Na</sub> towards more negative potentials could be produced by reducing  $I_{Na}$ . It is obvious that Na ions diffuse through the cut ends and raise the internal Na concentration of the fibre, thus weakening the electromotive force. An average reduction of  $V_{\rm Na}$  by  $12 \pm 0.7 \,\mathrm{mV}$ (6) was observed in this type of experiments. Then, an anion known to have different effects when applied externally and internally was used. Iodate seemed appropriate in this respect (Stämpfli 1974). Superfusion with iodate had no striking effect on  $I_{Na}$ , while diffusion of iodate through the cut ends produced a delay in I<sub>Na</sub> inactivation. Our results are in agreement with previous observations on different tissues. Keana and Stämpfli (1974) did not observe changes in action potential, Na, K or leak currents in single myelinated nerve fibres after external iodate application. However, internal iodate diffusion from a cut end

induced a delay in inactivation in the nodal membrane (Stämpfli 1974; Conti et al. 1976; Neumcke et al. 1980; Schmidtmayer et al. 1983). Similarly, Nonner et al. (1980) observed a parallel effect upon inactivation in frog muscle using the vaseline gap technique. According to Keana and Stämpfli (1974), the chemical agent probably reacts with amino acid groups involved in the gating process of inactivation on the sarcoplasmic side of the membrane (iodate is known to oxidize cystine to two molecules of the corresponding sulphonic acid and disulphide bonds in insulin).

The present work leads to the conclusion that, using the double sucrose gap technique, it is possible to analyse the sodium channel behaviour of cut end fibres of frog skeletal muscle during inside manipulation by modifying the composition of the internal solution.

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# References

- Adrian R. H., Chandler W. K., Hodgkin A. L. (1970): Voltage clamp experiments in striated muscle fibres. J. Physiol. (London) 208, 607–644
- Arispe N. J., Argibay J. A., Rojas L. V. (1984): Sodium currents in skeletal muscle fibres from the toad *Bufo marinus*. Quart. J. Exp. Physiol. 69, 507–519
- Bezanilla F., Armstrong C. M. (1977): Inactivation of the sodium channel I. Sodium current experiments. J. Gen. Physiol. 70, 549—566
- Collins C. A., Rojas E., Suarez-Isla B. A. (1982a): Activation and inactivation characteristics of the sodium permeability in muscle fibres from *Rana temporaria*. J. Physiol. (London) **324**, 297 —318
- Collins C. A., Rojas E., Suarez-Isla B. A. (1982b): Fast charge movements in skeletal muscle fibres from *Rana temporaria*. J. Physiol. (London) 324, 319–345
- Conti F., Hille B., Neumcke B., Nonner W., Stämpfli R. (1976): Conductance of the sodium channel in myelinated nerve fibres with modified sodium inactivation. J. Physiol. (London) 262, 729-742
- Duval A., Léoty C. (1978): Ionic currents in mammalian fast skeletal muscle. J. Physiol. (London) 278, 403–423
- Hille B., Campbell D. (1976): An improved vaseline gap voltage clamp for skeletal muscle fibres. J. Gen. Physiol. 67, 265—293
- Hodgkin A. L., Huxley A. F. (1952a): Current carried by sodium and potassium ions through the membrane of giant axon of *Loligo*. J. Physiol. (London) 116, 449–472
- Hodgkin A. L., Huxley A. F. (1952b): A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. (London) 117, 500-544
- Ildefonse M., Rougier O. (1972): Voltage clamp analysis of the early current in frog skeletal muscle fibre using the double sucrose gap method. J. Physiol. (London) **222**, 373–395
- Ildefonse M., Roy G. (1972): Kinetic properties of the sodium current in striated muscle fibres on the basis of the Hodgkin-Huxley theory. J. Physiol. (London) 227, 419-431
- Keana JF. W., Stämpfli R. (1974): Effect of several "specific" chemical reagents on the Na<sup>+</sup>, K<sup>+</sup>

and leakage currents in voltage-clamped single nodes of Ranvier. Biochim. Biophys. Acta. 373, 18-33

- Meves H. (1984): Hodgkin-Huxley: Thirty years after. In: Current Topics in Membranes and Transport, Volume 22 (Ed. Arnost Kleinzeller), pp. 279-329, Academic Press, Inc, London
- Neumcke B., Schwartz W., Stämpfli R. (1980): Modification of sodium inactivation in myelinated nerve by *Anemonia* toxin II and iodate. Analysis of current fluctuations and current relaxations. Biochim. Biophys. Acta. 600, 456—466
- Nonner W. (1969): A new voltage clamp method for Ranvier nodes. Pflügers Arch. 309, 176-192
- Nonner W., Spalding B., Hille B. (1980); Low intracellular pH and chemical agents slow inactivation gating in sodium channels of muscle. Nature, **284**, 360-363
- Sanchez J. A., Stefani E. (1978): Inward calcium current in twitch muscle fibres of the frog. J. Physiol. (London) 283, 197-209
- Sauviat MP., Suchaud M. (1981): Effect of RP 30356 on the fast inward Na current in frog atrial fibres. Eur. J. Pharmacol. 71, 185–189
- Schmidtmayer J., Stoye-Herzog M., Ulbricht W. (1982): Combinated action of intraaxonal iodate and external sea anemone toxin ATX II on sodium channel inactivation of frog nerve fiber. Pflügers. Arch. 398, 204–209

Stämpfli R. (1974): Intraaxonal iodate inhibits sodium inactivation. Experientia 30, 505-508

Zachar J., Zacharová D., Henček M., Nasledov G. A., Hladký M. (1982): Voltage clamp experiments in denervated frog tonic muscle fibres. Gen. Physiol. Biophys. 1, 385–402

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