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SODIUM INFLUXES IN INTERNALLY PERFUSED SQUID GIANT AXON DURING VOLTAGE CLAMP

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SUMMARY

1. An experimental method for measuring ionic influxes during voltage clamp in the giant axon of *Dosidicus* is described; the technique combines intracellular perfusion with a method for controlling membrane potential.

2. Sodium influx determinations were carried out while applying rectangular pulses of membrane depolarization. The ratio 'measured sodium influx/computed ionic flux during the early current' is $0{\cdot}92 \pm 0{\cdot}12$.

3. Plots of measured sodium influx and computed ionic flux during the early current against membrane potential are very similar. There was evidence that the membrane potential at which the sodium influx vanishes is the potential at which the early current reverses.

INTRODUCTION

The experimental analysis of Hodgkin & Huxley (1952*a–c*) of the ionic currents which flow during a membrane potential step in a voltage clamp arrangement resolved the total ionic current into an early transient inward current independent of a later sustained outward current. The method which led to this separation of the current was the replacement of the sodium of the sea water by choline, resulting in the disappearance of the early current from the current records. This experiment developed a basic notion which identifies the early current with a net movement of sodium. This early transient current has not been correlated directly with sodium movements in any preparation (Mullins, Adelman & Sjodin, 1962). However, the later outward current has been shown to be directly related to outward potassium movement (Hodgkin & Huxley, 1953; Brinley & Mullins, 1965).

In order to test the sodium hypothesis (which is based on the Hodgkin

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& Huxley (1952*a–d*) work), the measured net sodium movements due to a propagated impulse and the sodium movement predicted by the Hodgkin & Huxley (1952*d*) formulation have been compared (Keynes, 1951; Shanes & Berman, 1955; Rojas & Canessa-Fischer, 1968). Although the agreement could not have been better (Keynes, 1951; Rojas & Canessa-Fischer, 1968), there are some uncertainties which have arisen from these indirect comparisons. The aim of the present research has been to measure directly the net movement of sodium during the early transient currents in a voltage clamp experiment and to compare this sodium flux with that calculated after integration of the ionic current, in order to provide unequivocal experimental evidence for the sodium hypothesis.

First, early and later currents were separated by blocking the early current with tetrodotoxin (Rojas & Atwater, 1967) and subtracting the remaining later current from the total ionic current. Then the early current was integrated to give the ionic flux. It was found that for different membrane potential steps both the computed ionic flux and the measured sodium flux were similar; thus, the transference number for sodium is very close to one. This result strongly supports the sodium hypothesis by chemically identifying the early transient current.

METHODS

Internally perfused squid giant axons from *Dosidicus gigas* were used in this research. A detailed description of the combined perfusion technique and voltage clamp procedure used can be found in earlier publications from this laboratory (Rojas & Ehrenstein, 1965; Rojas & Atwater, 1967; Rojas & Canessa-Fischer, 1968). However, there are some relevant aspects of the experimental procedure which need to be described here.

Figure 1*A* illustrates diagrammatically the system used and shows that the entire length of the axon fibre in sea water (*ca.* 2·5 cm) was internally perfused.

Measurement of membrane current, $I_m(t)$. Currents were radially applied from the axial wire (100 μm platinum wire coated with platinum black) to a pair of external electrodes (platinum plates coated with platinum black) which were electrically connected together and then connected to earth through 10 Ω. The voltage drop in this resistance was amplified during each membrane potential step and recorded on film. Thus, this system of external plates measures

$$I_m(t) = \int_0^l \frac{\partial I_m}{\partial x}\, dx, \tag{1}$$

where l is the total length of the fibre bathed in sea water and equals the length of the external plates (*ca.* 2·5 cm).

The uniformity of the current density, $\frac{\partial I_m}{\partial x}$, was controlled with a pair of differential C-shaped electrodes which recorded the voltage drop in the resistance of the external sea water and which could be moved along the fibre axis (Chandler & Meves, 1965). As sodium flux is directly dependent upon the area of ^{22}Na exchange, care was taken to determine the percentage of the axon membrane extending beyond the cannulas which was also incorporated into this exchange. Measuring the current density, it was found that less than 1 mm beyond the tip of the cannula the current density abruptly decreased. We estimated an area with an absolute error no greater than 12 %.

Computation of the ionic flux during the early current. Most sodium flux determinations were carried out while applying rectangular pulses of membrane depolarization of short duration (*ca.* 3 msec). In order to subtract the actual later currents from the total current, it was necessary to carefully determine their kinetics. For this reason, various experiments were performed applying longer pulses of membrane depolarization (*ca.* 25 msec). Then, early transient currents were blocked with 10^{-8} M tetrodotoxin (TTX) added to the external artificial sea water (ASW). Next, the following expression was used to fit the later currents obtained (Dodge, 1963)

$$I(t) = I_{ss}\,[1-\exp(-t/\tau)]^4, \tag{2}$$

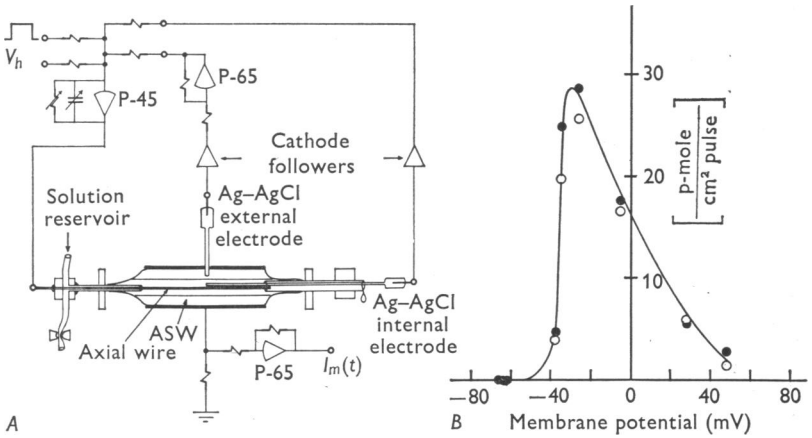

Fig. 1. *A*. Diagram of the electrode arrangement and control system. The internal electrode was a glass capillary tube (100 μm in diameter) filled with 600 mM-KCl and with a floating 50 μm platinum wire inside. All operational amplifiers used in the control system were from Philbrick Researches Inc. (Dedham, Massachusetts, U.S.A.) V_h is holding potential; $I_m(t)$ is membrane current.

B. Comparison between the measured tracer sodium flux (○) and electrically measured ionic flux during the early transient current (●). Curve drawn through filled circles. Experiment TN-38.

where I_{ss} and τ are constants of dimensions current and time, respectively. For those experiments in which only short duration pulses of membrane depolarization were used, an expression derived from (2) was used instead

$$I(t) = I(t_1)\left\{\frac{1-\exp(-t/\tau)}{1-\exp(-t_1/\tau)}\right\}^4 \quad (0 < t < t_1), \tag{3}$$

where t_1 is the duration of the membrane potential step. Finally, digital data of the total ionic current, $I_m(t)$, and of the later ionic current, $I(t)$, were fed to an IBM 1620 computer from which the net ionic flux during early transient current was obtained, i.e.

$$\int_0^{t_1}[I_m(t)-I(t)]\,dt. \tag{4}$$

Measurement of the sodium influx. To ensure that sodium efflux would be zero, the perfusing solution (550 mM-KF, 5 mM-Tris (Cl), pH = $7{\cdot}3 \pm 0{\cdot}1$) used during the present work did not contain sodium. The external ASW (430 mM-NaCl, 50 mM-$MgCl_2$, 10 mM-$CaCl_2$, 5 mM-Tris (Cl), pH = 7·5) was exposed to the air during each experiment, and as a result of slight evaporation its concentration changed slightly. For this reason osmotic pressure

(Mechrolab Osmometer, Hewlett Packard, Palo Alto, Calif.), as well as sodium content (Atomic Absorption Fotometer, Perkin, Elmer, Japan) were carefully checked in a sampling of experiments. The specific activity of the external ASW was also precisely determined. Its radioactivity was measured with a 3 in. crystal scintillation counter (Nuclear Chicago, Des Plaines, Illinois). The specific activity ranged from 0·19 to 0·12 counts per minute (cpm) per p-mole sodium.

To measure the inflow of radioactivity, artificial sea water with ^{22}Na was placed outside the axon and thereafter, every 2 or 3 min, samples of the perfusing solution were collected. The rate at which ^{22}Na appeared in the perfusate reached a steady value after no more than 5 min. Once this resting level of sodium inflow was reached, the measured resting potential was clamped, and then pulses of membrane depolarization were applied for about 10 min at a frequency of 10 pulses per second. As a result there was a change in sodium inflow which reached a steady value again after no more than 5 min. This last steady rate of ^{22}Na inflow was used to compute the tracer influx as follows

$$(\text{cpm/cm}^2\ \text{sec})_{\text{during pulse}} - (\text{cpm/cm}^2\ \text{sec})_{\text{during rest}},$$

where $(\text{cpm/cm}^2\ \text{sec})_{\text{during rest}}$ is the average between the values read before the voltage clamp run and after. This difference was divided by 10 to obtain the extra-influx during each pulse. The average resting influx before the experimental run was 28·6 p-mole/cm^2 sec and increased an average of 5·0 p-mole/cm^2 sec after voltage clamping. In most experiments the fibres were used for several determinations, with the membrane potential being clamped at a different level of depolarization during the pulses.

RESULTS

Sodium influxes during voltage clamp were determined in seven experiments. Results of six of these experiments are given in Table 1.

We mentioned in Methods that the holding potential during each voltage clamp run was equal to the measured resting potential. This is shown in column (*a*) of Table 1. In most experiments the resting potential changed between runs. For example, in experiment TN-38, in which six voltage clamp runs were performed, the greatest variation was 2 mV between consecutive runs. One has to recall, however, that the membrane was under voltage clamp for at least 7 min during each run. Although we were extremely careful to avoid differences between the measured resting potential and the holding potential, some unavoidable electrode polarization took place. We considered that if the resting level of sodium influx did not change considerably before and after each run, this was a good indication that the holding current was small. Column (*c*) of Table 1 represents the computed total ionic flux measured with the recorded membrane currents. Negative numbers in this column were obtained for membrane potential steps approaching the sodium equilibrium potential. The sign reflects which current is predominant during the integration; the inward will generate positive values and the outward will generate negative values.

The ionic flux computed using the recorded later currents are given in

column (*d*) of Table 1. Computed ionic influxes during the early currents for different membrane potential steps are given in column (*e*).

One instructive way of examining these values (computed ionic influxes) is to plot the increment in ionic influx during the early currents against the absolute membrane potential. This has been done in Fig. 1*B* (experiment TN-38 in which six values were collected). Column (*f*) of Table 1 gives the measured sodium influxes during each voltage clamp run

TABLE 1. Determination of sodium influxes during voltage clamp

Experiment	(*a*) V_h mV	(*b*) V_p mV	(*c*) $\frac{1}{F}\int_0^{t_1} I_m(t)\,dt$ p-moles/cm² pulse	(*d*) $\frac{1}{F}\int_0^{t_1} I(t)\,dt$ p-moles/cm² pulse	(*e*) (*c*) − (*d*) p-moles/cm² pulse	(*f*) m^i_{Na} p-moles/cm² pulse	(*g*) (*f*)/(*e*)
TN-36	−50·5	30	6·75	−0·15	6·90	6·7	0·97
	−52·5	50	6·92	−2·64	9·56	10·0	1·03
	−52·5	90	−7·43	−10·11	2·68	2·8	1·04
TN-38	−62·5	25	4·70	0	4·70	3·7	0·78
	−64·5	30	24·72	0	24·72	19·2	0·78
	−66·5	40	27·16	−1·27	28·43	25·5	0·90
	−64·5	60	13·18	−4·38	17·56	16·3	0·93
	−62·0	90	−2·71	−8·37	5·66	5·7	1·00
	−62·0	110	−9·04	−11·68	2·64	1·4	0·53
TN-40	−51·0	30	5·12	0	5·12	4·5	0·88
	−49·0	50	3·75	−1·79	5·54	5·6	1·01
TN-42	−66·5	30	10·36	0	10·36	13·0	1·25
	−65·5	40	10·22	−0·94	11·15	10·5	0·95
TN-43	−58·0	30	7·61	0	7·61	6·8	0·89
	−59·0	70	1·92	−5·96	7·88	7·2	0·91
	−60·5	90	−5·35	−9·88	4·53	4·3	0·96
TN-44	−62·0	100	−12·15	−15·85	3·70	3·3	0·89
					Average ± S.D.		0·92 ± 0·15

V_h is the holding potential (equal to the measured resting potential), V_p the membrane potential step, m^i_{Na} the sodium influx. Experiments were carried out at room temperature of 17 ± 1° C. Axons were internally perfused with 550 mM-KF, 5 mM-Tris (Cl) at pH 7·3 ± 0·1. External solution was K-free ASW except for experiment TN-36 for which 10 mM-KCl ASW was used.

and for a given membrane potential step. Here again data for experiment TN-38 were plotted in Fig. 1*B*. There is a remarkable agreement between both sets of values plotted in Fig. 1. A similar observation may be drawn from the fact that the ratios in column (*g*) of Table 1 are near unity.

Figure 1B shows that for an absolute membrane potential of about +50 mV, the net sodium movement is close to zero. This 'equilibrium potential' agrees remarkably well with the equilibrium potential measured from the reversal of the early currents in a plot of peak of early current against membrane potential.

One of the aims of the present investigation was to obtain experimental data from two independent types of measurements. We limited ourselves to those unavoidable hypotheses and have tried to provide clearer experi-

mental evidence supporting the sodium hypothesis, as formulated by Hodgkin & Huxley (1952*a–d*). There are several aspects, however, which need to be clarified. For example, if the membrane depolarization during a voltage clamp experiment is turned off when the early current reaches the peak value, a large 'tail' of inward current develops and the current record looks discontinuous. Hodgkin & Huxley (1952*b*) provided some evidence that this 'tail' of inward current was also carried by sodium. Again they replaced the external sodium by choline and described a complete disappearance of this 'tail' from the current records. Table 2 summarizes the data obtained

TABLE 2. Data from one experiment TN-50, in which sodium influxes were measured when depolarizing the axon membrane with short pulses

(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
V_p	$\frac{1}{F}\int_0^{t_1} I_m(t)\,dt$	$\frac{1}{F}\left(\int_0^{t_1} I_m\,dt+\int_{t_1}^{t_2} I_m\,dt\right)$	m^i_{Na}	(*d*)/(*b*)	(*d*)/(*c*)
(mV)	p-mole/cm² pulse				
20	2·3	2·4	2·4	1·0	1·00
30	5·6	8·3	7·5	1·3	0·91
40	3·9	7·4	6·5	1·7	0·88
50	2·4	6·0	5·6	2·4	0·94
70	1·0	5·1	4·1	4·1	0·80
100	0·2	5·4	3·3	16·7	0·61
				Average ± S.D.	0·86 ± 0·12

t_1 is the duration of the membrane potential step; the voltage clamp pulse was turned off when the early transient inward current reached its peak value. t_2-t_1 is the duration of the 'tail' of inward current. During each determination of sodium influx the holding potential was equal to the resting potential. Internal solution: 550 mM-KF, 5 mM-Tris (Cl) at pH 7·3; external solution: K-free ASW. Temperature of the bathing solution, 15° C.

in one experiment in which sodium influxes were measured when depolarizing the axon membrane with short pulses, i.e. of a duration no greater than the time needed for the early current to reach a peak value. We have prepared this table differently from Table 1. Column (*b*) is the ionic flux computed from the current just before the beginning of the 'tail'. Column (*c*) is the ionic flux computed also considering the 'tail'. Column (*d*) is the measured sodium influx. It is obvious that unless one considers the 'tail' in the computation of the ionic flux, the ratio, measured influx/calculated influx, becomes greater than one, which is difficult to accept. This ratio became close to unity again when including the 'tail'. This is shown in column (*f*) of Table 2.

DISCUSSION

One of the predictions of the sodium hypothesis is that the equilibrium potential for the early currents ought not to exceed the sodium equilibrium potential. This point was tested by Chandler & Meves (1965) in experi-

ments made by the voltage clamp method on *Loligo* axons perfused with chloride solutions. They showed that the equilibrium potential for the early currents conformed to the equation

$$V_{Na} = \frac{RT}{F} \ln \left\{ \frac{a^o_K + \alpha a^o_{Na}}{a^i_K + \alpha a^i_{Na}} \right\},$$

where R, T, F and a have their usual meanings and α is the permeability ratio, P_{Na}/P_K, for the active membrane which was found to be about 12. Considering this equation for an axon internally perfused with 550 mM-KF (a^i_K = 368 mM, a^i_{Na} = 0) and externally with K-free ASW (a^o_{Na} = 298 mM, a^o_K = 0) α can be computed measuring the reversal potential (Rojas & Atwater, 1967). For experiment TN-38 (see Table 1) a reversal potential close to 50 mV was obtained which according to the above equation gives a selectivity ratio equal to 10. Thus, the equivalent sodium activity inside the axon fibre was 368·5/10 or 36·8 mM for this experiment. If we assume that for the active membrane the sodium fluxes are proportional to the sodium activity in the corresponding solution, i.e. a^o_{Na} = 298 mM and a^i_{Na} = 36·8, the sodium influx near the reversal potential for this experiment (1·4 p-mole/cm^2 pulse) indicate that the extra efflux of potassium (representing sodium) equals $1{\cdot}4 \times 36{\cdot}8/298$ or 0·17 p-mole/cm^2 pulse. Therefore, 1·40–0·17 = 1·23 p-mole/cm^2 pulse represents a net influx of sodium. Thus, the net influx is 88 % of the measured influx. This net flux also becomes very small near the reversal potential suggesting that 50 mV is in fact the sodium equilibrium potential.

The results presented in this communication indicate that for an axon bathed in K-free ASW and internally perfused with 550 mM-KF

(i) the early inward current can be completely accounted for by the sodium influx;

(ii) the membrane potential at which the sodium influx becomes negligible is the potential at which the early current reverses; and

(iii) the 'tail' of inward current is also carried by sodium alone, as was suggested by Hodgkin & Huxley (1952*b*).

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