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TIME COURSE OF THE SODIUM PERMEABILITY CHANGE DURING A SINGLE MEMBRANE ACTION POTENTIAL

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SUMMARY

1. A method for measuring the time course of ionic fluxes during a non-propagated membrane action potential is described; the technique combines intracellular perfusion of the squid giant axon with radioactive tracer methods and with a method for controlling the membrane potential during small time intervals.

2. The method is used to determine the temporal course of the sodium extra influx during an action potential.

3. The results agree with the time course of the permeability change to sodium ions calculated with the Hodgkin & Huxley equations.

4. An average extra sodium influx of 7·13 p-mole/cm^2 per action potential was determined at 12° C and of 5·23 p-mole/cm^2 per action potential at 15° C.

INTRODUCTION

An important consequence of the sodium-potassium hypothesis in the Hodgkin & Huxley formulation (1952) is the prediction of the time course of the sodium and potassium ionic currents during a propagated action potential. It is possible, for example, to integrate the expression that gives the sodium current as a function of time and calculate the total extra sodium influx during a propagated action potential. At 18·5° C a net entry of sodium of 4·33 p-mole/cm^2 impulse was calculated (Hodgkin & Huxley, 1952). Keynes & Lewis (1951) measured a net sodium entry of 3·8 p-mole/cm^2 impulse in intact axon from *Loligo* while Shaw (1966) measured a net entry of 5·7 p-mole/cm^2 impulse in internally perfused axons of the same species. Although the agreement between predicted and measured sodium fluxes is quite remarkable (Rojas & Canessa-Fischer, 1968) it has not been

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possible yet to determine and compare the time course of these sodium and potassium fluxes. In part, this has been due to the inherent difficulties of such an experiment in which the ionic transfer across the axon membrane changes not only with time but also with distance along the nerve fibre. Non-propagated membrane action potentials obtained under space clamped conditions should in principle facilitate the experiment.

The aim of the present paper is to report a general method for measuring the temporal course of ionic fluxes during a non-propagated membrane action potential together with some of the results obtained with radioactive sodium. The measured temporal course of the sodium permeability change is compared with that calculated with the Hodgkin & Huxley equations and is found to be in good agreement.

METHODS

Giant-axons from the squid *Dosidicus gigas*, available at the Laboratorio de Fisiología Celular, Montemar, Chile, were used. A detailed description of the arrangements for intracellular perfusion has been presented in earlier publications (Rojas & Canessa-Fischer, 1968; Rojas, Taylor, Atwater & Bezanilla, 1969). We have shown that these axons can be used to determine sodium permeability changes under various experimental conditions (Rojas & Canessa-Fischer, 1968; Atwater, Bezanilla & Rojas, 1969; Bezanilla, Rojas & Taylor, 1970*a*). One can measure the extra sodium influx during a membrane action potential. This measurement provides the total permeability change during a non-propagated action potential. We have also shown that it is possible to interrupt the free course of the membrane action potential at different times (Bezanilla, Rojas & Taylor, 1970*b*). It is also possible to measure accurately enough the area under the current record just after the free course of the action potential has been interrupted, i.e. during the voltage clamp period. This is illustrated in Fig. 1. Upper trace represents the recorded membrane current. Lower trace represents the recorded membrane action potential. At time $t = t_1$ the membrane potential control system is turned on and the potential is maintained at -75 mV (near the reversal potential for the potassium currents) until $t = t_2$. Here the potential is suddenly returned to the resting potential value of -60 mV and kept there until the end of the cycle. The free course of a membrane action potential is shown as a dashed line. The extra sodium influx represented by the tail of inward current is given by

$$1/F \int_{t_1}^{t_2} i(t)\,\mathrm{d}t$$

(Atwater *et al.* 1969; Bezanilla *et al.* 1970). This calculated net influx during the tail can be subtracted from the measured tracer extra sodium influx. This difference as a function of time t_1 represents the temporal course of the integrated extra sodium influx between the initiation of the action potential until t_1. (For a more complete account of the results obtained with this system see Bezanilla *et al.* 1970*b*.) Some discussion of the experimental errors involved in these measurements have been presented elsewhere (Bezanilla *et al.* 1970*a*).

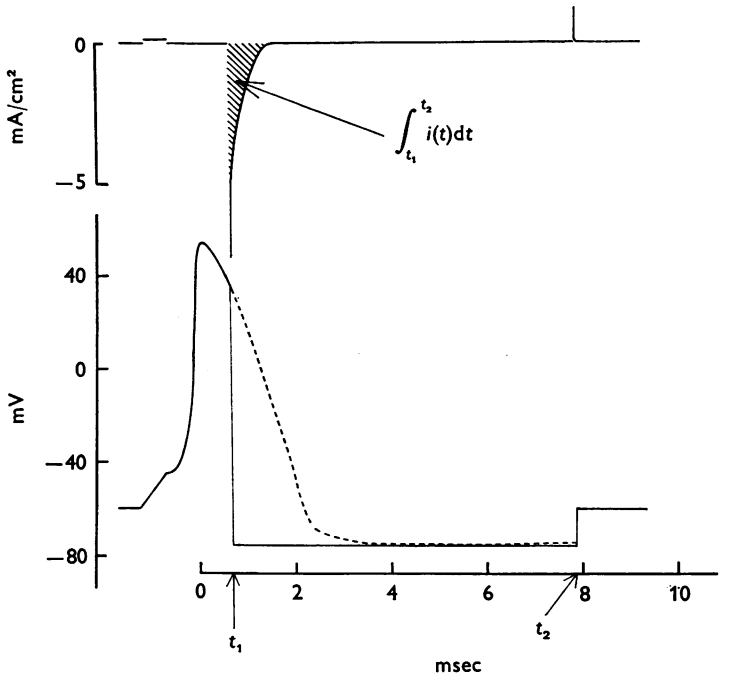

Fig. 1. Membrane current and membrane potential during one cycle: for explanations see text.

RESULTS

Sodium extra influxes as a function of duration of the free course of the membrane action potential

Fig. 2 illustrates an experimental protocol. This is a plot of the sodium influx in p-mole/cm² sec as a function of time in sec. Each column represents the average influx determined during an interval of approximately 200 sec. After the resting influx reached steady level of about 6 p-mole/cm² sec action potentials were elicited at a frequency of 10 sec^{-1} by application of a depolarizing rectangular pulse of current through the axial wire. During each cycle the control system was turned on 2·5 msec after the application of the stimulus and the membrane potential was controlled during 15 msec at −80 mV; after this the membrane potential was controlled at −60 mV for 5 msec. It can be seen that the net effect of this repetitive stimulation is to increase the rate at which sodium enters the fibre. That this increase in the rate of sodium entry is due to the action potential and not to either the brief stimulus or the 20 msec period under voltage clamp is shown by the results obtained during the fourth run

(period of repetitive stimulation). The action potential was interrupted at the beginning of the rising phase. The influx was not altered (during this run the membrane potential was clamped during 15 msec at -80 mV and then for 5 msec at -60 mV). This experiment demonstrates that it is the development of an action potential that causes the increase in the rate at which sodium enters the fibre. Each period under stimulation is shown between two arrows. The increase in sodium influx during the stimulation period is expressed in terms of the area under the columns above the

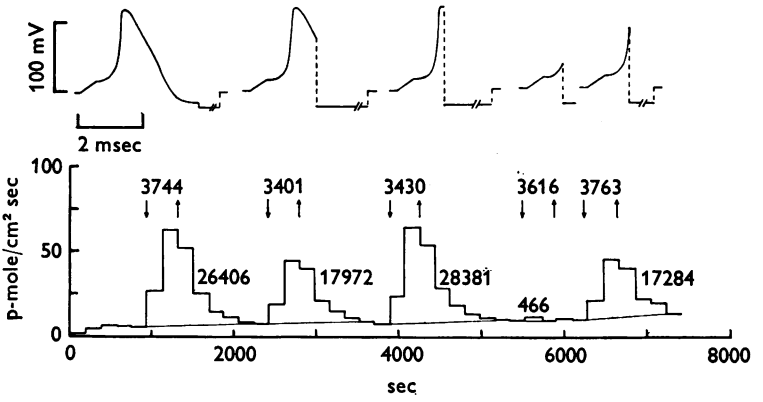

Fig. 2. Protocol of one experiment: recorded membrane potential and measured sodium influx during rest and during stimulation. Each column represents the average influx determined during intervals of approximately 200 sec. Every 30 sec, pictures of the ionic currents, measured during the period under membrane potential control during each cycle, were taken. Frequency of application of pulses was 10 sec^{-1}. The fibre was bathing in potassium-free artificial sea-water (K-free ASW) with 2 p-mole/c.p.m. ^{22}Na and internally perfused with potassium fluoride, 550 mM.

assumed resting influx (a thin line drawn as a continuation of the resting influx before the stimulation period). These areas are given in p-mole/cm^2 by the numbers next to the columns. The numbers between the arrows give the total number of action potentials during the run. For the first run the ratio

$$26{,}406/3744 = 7{\cdot}05 \text{ p-mole/cm}^2$$

gives the average sodium entry per cycle. The recorded membrane action potential during each run is shown in the upper part of this figure above the corresponding sodium influx.

The interval between the application of the stimulus and the initiation of the voltage clamp period was predetermined and fixed during each run. However, the position of the action potential in this interval showed slight fluctuations from cycle to cycle during a run. As a consequence of these fluctuations the free course of the action potential was interrupted

at different points during each cycle in a run. When the membrane potential control system was turned on during the rising phase of the action potential the recorded currents change considerably. This made it necessary to take photographs of the oscilloscope traces every 30 sec. To obtain the corresponding sodium flux (see Methods) an average area under the current record was considered. A considerable error might have been introduced in the evaluation of the integrated extra influx in those runs in which very large current transients were recorded.

Some of these difficulties were avoided by eliciting the action potentials as follows. Each cycle was initiated with a short period under voltage clamp. The membrane potential was hyperpolarized from -60 to -89 mV during 2·8 msec and then the voltage clamp was turned off. An action potential was obtained. The voltage clamp was again turned on at various times after the initiation of the action potential.

In order to compare the results obtained with different fibres the origin of the time axis was placed at the time at which the membrane potential reaches its peak value. Table 1 summarizes the results obtained with six different fibres. Columns (*a*) an (*b*) give the resting potential and the resting influx before the run. Column (*c*) gives the time at which the free course of the action potential was interrupted relative to the initiation of the stimulating current pulse. Column (*d*) gives the time relative to the time at which the peak of the action potential takes place. Negative numbers in this column were obtained for membrane action potentials interrupted before they reached their peak value. Column (*e*), gives the measured extra influx per cycle (i.e. area under the flux curve divided by the total number of cycles with recorded membrane action potentials). Column (*f*) gives the computed net inward movement during the voltage clamp period corresponding to the tail current. Column (*g*) gives the difference between the measured and the calculated fluxes (i.e. column (*e*) – column (*f*)).

One way to examine the values in columns (*e*), (*f*) and (*g*) is to plot the extra sodium influx caused by one incomplete action potential as a function of time. This is shown for three experiments in Fig. 3. The recording of one action potential is shown for each experiment. During the first experiment shown in this Figure no measurable fluctuations of the point at which the free course of the action potential was interrupted were apparent. For the second and third fibres there were fluctuations which covered a range indicated in the figure by arrows. Open circles in this Figure represent the values in column (*e*) in Table 1 (that is, the extra sodium influx obtained during one cycle). Filled circles represent the values in column (*g*) i.e. the measured extra sodium influx, column (*e*), minus the flux calculated from the current record, column (*f*).

TABLE 1. Determination of sodium influxes during stimulation

Expt.	(a) Resting potential (mV)	(b) Resting influx (p-mole/cm² sec)	(c) (msec)	(d) (msec)	(e) $\int_0^t m^i_{Na}\,dt$ (p-mole/cm²)	(f) $\frac{1}{F}\int_{t_1}^{t_2} i\,dt$ (p-mole/cm²)	(g) (e)−(f) (p-mole/cm²)	(h)	Temp. (° C)
APINAF-11									
A	−57·0	41·5	4·20	2·90	4·07	0·02	4·05	1·00	—
B	−58·0	38·0	2·15	0·80	3·74	0·04	3·70	0·91	—
C	−59·0	43·8	1·45	0·05	3·90	2·90	1·00	0·25	—
D	−57·0	50·9	1·30	0·15	3·75	1·90	1·85	0·45	15·0
APINAF-14									
A	−57·7	12·3	2·10	0·65	8·80	0·90	7·90	0·75	—
B	−58·7	14·2	1·60	0·05	6·20	3·80	2·40	0·23	—
C	−57·0	17·1	1·30	−0·25	0·80	0·80	0·10	0·01	—
D	−57·0	26·0	1·10	−0·50	0·40	0·30	0·10	0·01	12·0
APINAF-15									
A	−68·0	5·8	3·75	2·25	7·05	0·14	6·91	1·00	—
B	−68·8	7·8	1·40	0·65	8·27	5·30	2·97	0·43	—
C	−66·2	9·0	1·05	0·00	0·13	0·48	−0·35	−0·05	—
D	−64·8	9·4	1·25	−0·20	4·59	5·40	−0·81	−0·11	12·0
APINAF-5									
A	−58·0	18·9	10·00	8·70	4·85	0·05	4·80	0·71	—
B	−62·7	14·0	1·65	0·25	4·33	0·80	3·53	0·52	—
C	−62·0	18·4	1·15	−0·13	1·31	1·75	−0·44	−0·06	—
D	−60·5	18·2	1·30	−0·02	6·04	3·30	2·74	0·40	—
E	−60·0	21·1	1·05	−0·16	0·90	0·32	0·58	0·09	—
F	−60·0	20·8	10·00	8·70	6·79	0·00	6·74	1·00	10·5
G	−58·0	25·9	2·95	1·50	4·93	0·10	4·83	0·71	10·5

TABLE 1. (*cont.*)

Expt.	(*a*) Resting potential (mV)	(*b*) Resting influx (p-mole/ cm² sec)	(*c*) (msec)	(*d*) (msec)	(*e*) $\int_0^t m_{Na}dt$ (p-mole/cm²)	(*f*) $\frac{1}{F}\int_{t_1}^{t_2} i\,dt$ (p-mole/cm²)	(*g*) (*e*) − (*f*) (p-mole/cm²)	(*h*)	Temp. (° C)
APINAF-6									
A	−62·5	16·8	0·75	−0·55	0·10	0·08	0·02	0·00	—
B	−63·5	15·6	1·05	−0·26	2·37	2·29	0·08	0·01	—
C	−60·7	22·2	1·12	0·00	11·92	9·38	2·54	0·34	—
D	−59·5	29·6	1·15	−0·25	8·31	8·29	0·02	0·00	—
E	−60·0	36·9	1·60	0·40	6·58	2·51	4·07	0·55	—
F	−57·0	45·4	4·10	2·80	6·62	0·02	6·60	0·90	—
G	−56·5	49·0	7·05	5·90	7·37	0·07	7·35	1·00	12·0
APINAF-12									
A	−66·7	21·3	0·75	−0·10	7·07	5·20	1·87	0·29	—
B	−65·0	39·0	0·70	−0·15	7·90	5·60	2·30	0·36	—
C	−64·0	44·4	0·90	0·02	9·78	7·40	2·38	0·37	—
D	−63·5	44·4	1·20	0·40	5·64	1·10	4·54	0·70	—
E	−61·0	51·1	2·20	1·40	6·62	0·20	6·42	1·00	—
F	−60·0	59·2	4·90	4·25	5·68	0·01	5·67	0·88	15·0

All experiments were performed in K-free ASW of the following composition: 430 mM-NaCl, 50 mM-$MgCl_2$, 10 mM-$CaCl_2$, 5 mM-Tris-Cl at pH 7·5. The fibres were internally perfused with 550 mM-KF, 5 mM-Tris-Cl at pH 7·3. For experiments APINAF-5, APINAF-6 and APINAF-12, 5 mM-TEA was added to the internal perfusing solution. Numbers in column (*e*) represent the average of the integrated extra sodium influx over many pulses. The limits of the integral are the initiation of the sweep, 0, and the end of sweep, *t*. These integrals represent average values.

Sodium extra influxes with internal tetraethylammonium

Tetraethylammonium (TEA) ions specifically block the outward potassium currents (Armstrong & Binstock, 1965; Armstrong, 1966; Hille, 1967). When internally applied to squid giant axons the action potential is prolonged but its rising phase is not affected. Three experiments similar to

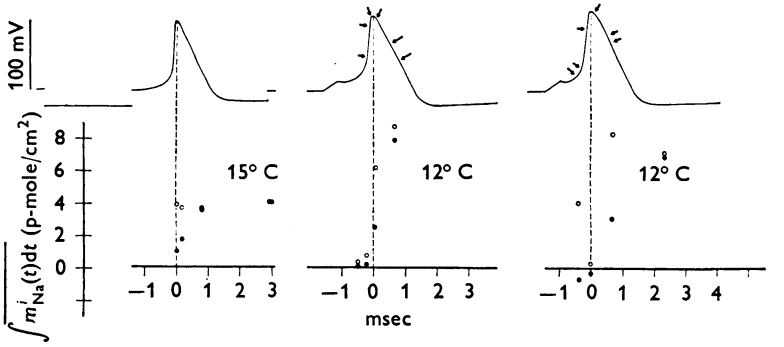

Fig. 3. Sodium extra influx as a function of time for three fibres internally perfused without TEA. Each insert in the upper part of this Figure represents one of the records of the membrane action potential during one of the consecutive runs. The points represent sodium influx; they are plotted on the same time scale as the action potentials, and thus correspond to the flux measured for an action potential interrupted by the voltage clamp at that point in time. The point at which this interruption took place fluctuated from cycle to cycle. The recorded potential changed its free course between the arrows shown for the second and third fibre. (For example, the second point obtained for the second action potential shown marks the extra influx of sodium for action potentials interrupted between the times indicated by the first two arrows, the next point for the next two arrows, etc.) Open circles represent the measured extra sodium influx given in Table 1 in column (*e*). Filled circles represent this measured extra influx minus the extra influx during the inward current calculated from the integration of the current record. The fibres were bathed in K-free ASW and internally perfused with 550 mM-KF. Experiments APINAF-11, APINAF-14 and APINAF-15.

those described in the previous section were performed using 5 mM-TEA in the perfusing solution. Fig. 4 illustrates the results obtained in one of these fibres. Action potentials recorded during consecutive runs are shown in the upper part of this Figure above the corresponding extra sodium influx. During each cycle the membrane potential was controlled during 15 msec at the measured resting potential. Before the initiation of a run the effect of the TEA upon the outward potassium currents was examined. In less than 300 sec these currents were effectively blocked. However, the development of a plateau action potential took a considerably longer time.

At the end of the last run the duration of the action potential was still increasing.

The difference 'measured extra sodium influx (column (*e*) in Table 1) minus calculated sodium influx from the tail current record (column (*f*))' as a function of time for three experiments is shown in Fig. 5.

To be able to compare the experimental results obtained at different temperatures and in different axons, the data summarized in Figs. 2 and 5 were standardized as follows. For each fibre the extra sodium influx

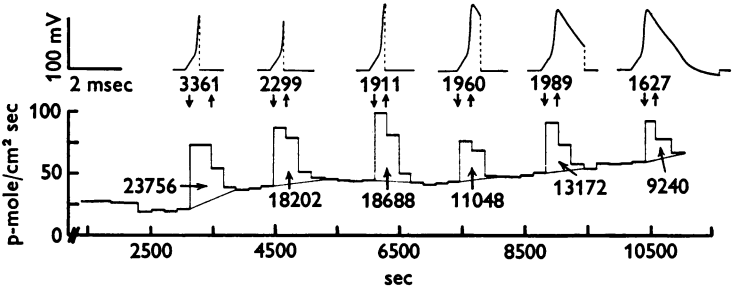

Fig. 4. Protocol of one experiment internally perfusing with 550 mM potassium fluoride plus 5 mM-TEA. Each insert in the upper part of this Figure represents one of the recorded membrane action potentials. Calibrations: 100 mV and 2 msec. Each column represents the average influx determined during the intervals between the arrows (approximately 200 sec). Frequency of application of the pulse 10 sec^{-1}. Total number of action potentials is given by the number between the arrows. The number under the columns gives the extra sodium influx in p-mole/cm^2 during the stimulation period.

obtained in a run with the longest free course for the action potential was considered to represent the maximum integrated extra sodium influx. All the other experimental points for the same fibre were divided by this value. The standardized values are given in column (*h*) in Table 1. This procedure permits a comparison of extra influxes obtained at different temperatures. The origin for the time axis was chosen as the time at which the action potential reached its peak value. Times relative to this origin are given in column (*d*) in Table 1. Finally, the time scale for each experiment was changed to allow for the temperature effect upon the time constants of the sodium and potassium processes (Hodgkin & Huxley, 1952). 12° C was the temperature chosen for the comparison.

Graphs of the standardized extra influx data obtained with three fibres internally perfused without TEA and the extra influx data obtained with three fibres internally perfused with TEA as a function of the standardized time showed no clear differences. For this reason the experimental points from both graphs were put together in Fig. 6.

Comparison of the experimental results with the predicted values from the Hodgkin & Huxley model nerve

Fig. 6 shows all the standardized experimental points available. This Figure also shows the calculated membrane action potential and the temporal course of the integrated sodium extra influx for the Hodgkin & Huxley model nerve. All calculations were done for a temperature of 12° C using the same equations and parameters published by Hodgkin & Huxley.

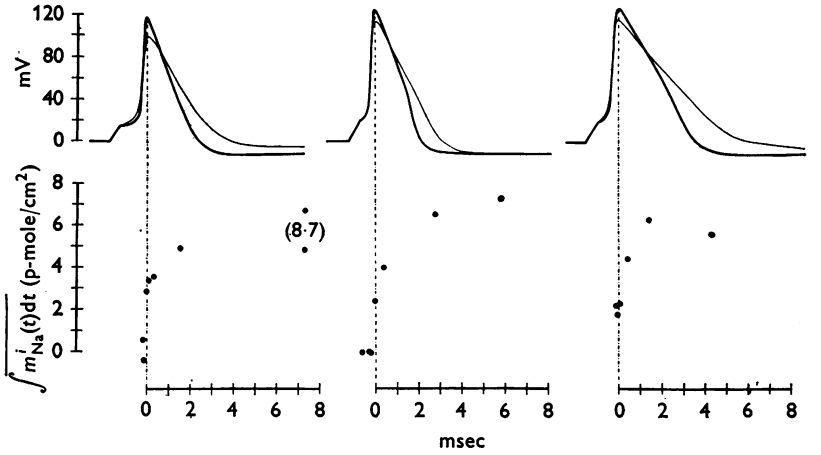

Fig. 5. Sodium extra influx as a function of time for three fibres internally perfused with 550 mM potassium fluoride plus 5 mM-TEA. Each insert in the upper part of this Figure represents two action potentials recorded during the first run (thick tracing) and during the last run (thin tracing). Filled circles represent the data given in column (*g*) of Table 1, i.e. measured extra influx minus extra influx calculated from the current record. For the first experiment shown in this Figure the number in parentheses represents the time for both experimental points.

DISCUSSION

The experiments presented here were designed to resolve the time course of the sodium permeability change during a membrane action potential. To achieve this objective it was necessary to interrupt the free course of the action potential by fixing the voltage and to integrate the area under the current record during the voltage clamp period. As results of previous experiments demonstrate that this 'tail' of inward current is carried by sodium ions (Bezanilla *et al.* 1970*a*) the corresponding sodium flux was subtracted from the measured extra sodium influx. This difference as a function of time immediately gave the temporal course of the change in permeability to sodium ions.

Fig. 6 shows that the standardized experimental points fall near the curve predicted by Hodgkin & Huxley for the integral of the permeability change during a membrane action potential. The scattering of the points is well within the range of the experimental errors particularly the errors introduced by the fluctuation of the point at which the free course of the action potential was interrupted.

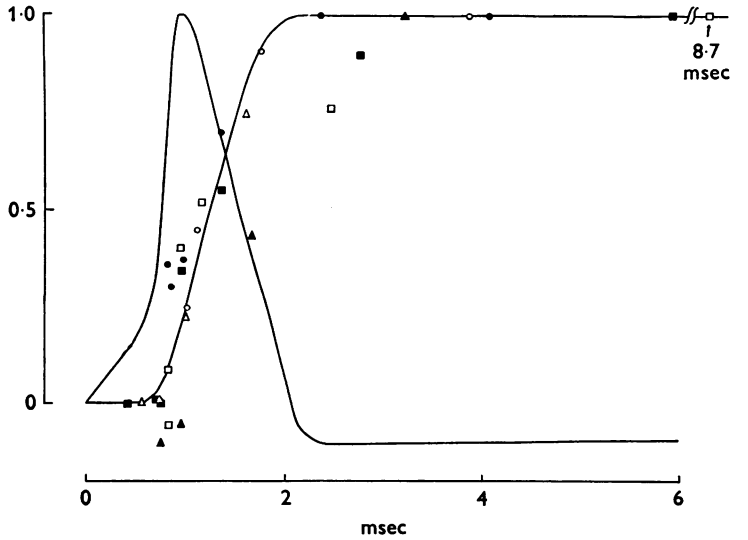

Fig. 6. Standardized experimental data and the Hodgkin & Huxley predictions for the sodium permeability change during a membrane action potential. □—□: experiment APINAF-5, ■—■: experiment APINAF-6; ○—○: experiment APINAF-11; ●—●: experiment APINAF-12; △—△: experiment APINAF-14; ▲—▲: experiment APINAF-15.

Vertical axis represents the standardized sodium extra influx; the horizontal axis represents the standardized time expanded or compressed for each experiment according to Hodgkin & Huxley (1952) by multiplying the time axis by $\phi = 3^{(0\cdot1t'-0\cdot63)}$ where t' is the temperature of the experiment. The membrane action potential drawn was calculated with the Hodgkin & Huxley unmodified equations at 12° C. The curve represents the standardized Hodgkin & Huxley prediction for the time course of the integrated sodium extra influx.

For a complete membrane action potential the measured extra sodium influxes can be directly compared with the calculated values. For example, we have measured an average extra sodium influx of 7·13 p-mole/cm² per action potential at 12° C. For the same temperature we calculated an extra influx of 10·39 p-mole/cm² and a net extra influx of 7·96 p-mole/cm². Our measurements at 15° C gave smaller values, the average being 5·23 p-mole/cm² per action potential. The equations predict an extra influx of 5·01 p-mole/cm² at 18·5 °C (see Table 5, Hodgkin & Huxley, 1952). A similar

comparison for an incomplete membrane action potential has to take into account the fact that the experiments reported here were done using uncompensated feed-back and for this reason the rate of fall off of the current transients changed considerably between runs and from fibre to fibre (Bezanilla *et al.* 1970*b*). Values of the extra sodium influx corresponding to

$$\frac{1}{F}\int_{t_1}^{t_2} i(t)\,dt$$

with $i(t)$ as predicted by the Hodgkin & Huxley equations and as measured in this work are given in Table 2. It can be seen that the calculated extra

TABLE 2. Sodium extra influxes corresponding to the inward current tails

(*a*)	(*b*)	(*c*)	(*d*)
		$\frac{1}{F}\int_{t_1}^{t_2} i_{exp}\,dt$	$\frac{1}{F}\int_{t_1}^{t_2} i_{H-H}\,dt$
No. of determinations	msec	(p-mole/cm²)	
2	−0·52	0·19	0·0
4	−0·24	4·19	0·9
3	0·02	4·41	2·9
1	0·40	2·51	3·0
2	0·65	3·10	—
1	2·25	0·14	0·0
1	2·80	0·02	0·0
1	5·90	0·07	0·0
1	8·70	0·05	0·0

Column (*b*) represents the time relative to the time at which the membrane action potential reaches its peak value. Only those experiments performed at 12° C were used for the comparison. To obtain an average the time interval accepted was 0·08 msec; for example, the numbers used to get the second average in column (*b*) fall within the interval 0·24 ± 0·08 msec. Hodgkin & Huxley equations solved for a fibre at 6·3° C.

sodium influx is greater for the experimental currents ($i(t)_{exp}$) than for the theoretical currents ($i(t)_{H-H}$). It is possible to correct for the effects of the lack of compensation for the series resistance (Bezanilla *et al.* 1970*b*). For example, one can solve the equations having the series resistance as a parameter and adjust its value until the rate of fall off of both the experimental and the theoretical currents is similar; then both fluxes can be compared.

It is clear that the method used to obtain the time course of the sodium permeability change is not affected by the above considerations. Thus, the time course of the sodium permeability change measured in this work is well described by the kinetic parameters of the Hodgkin & Huxley equations.

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