Action of External Divalent Ion Reduction on Sodium Movement in the Squid Giant Axon

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ABSTRACT Voltage clamp measurements of the sodium potential have been made on the resting squid giant axon to study the effect of variations in external divalent ion concentration upon net sodium flux. From these measurements the intracellular sodium concentration and the net sodium inflow were calculated using the Nernst relation and constant activity coefficients. While an axon bathed in artificial sea water shows a slow increase in internal sodium concentration, the rate of sodium accumulation is increased about two times by reducing external calcium and magnesium concentrations to 0.1 times their normal values. The mean inward net sodium flux increases from a mean control value of 97 pmole/cm^2 sec. to 186 pmole/cm^2 sec. in low divalent solution. Associated with these effects of external divalent ion reduction are a marked decrease in action potential amplitude, little or no change in resting potential, and a shift along the voltage axis of the curve relating peak sodium conductance to membrane potential similar to that obtained by Frankenhaeuser and Hodgkin (1957). These results implicate divalent ions in long term (minutes to hours) sodium permeability.

INTRODUCTION

It is generally held that calcium and magnesium exert a stabilizing influence on excitable membranes (Lillie, 1923) in that calcium is supposed to have a direct effect on the permeability to monovalent ions (Shanes, 1958). Recent experiments on lobster giant axons have shown that the resting potential declines whenever the external calcium ion concentration is reduced below normal levels (Dalton, 1958, Dalton and Adelman, 1960). This decline of resting

potential in low external calcium was shown to be continuous (with no leveling off to a steady value) for the period of exposure (up to 30 min.). These experiments suggested that removal of external divalent ions introduces a severe continuous impairment in whatever system is maintaining the resting potential, such as might result from a slow continuous decline in the internal potassium concentration and a similar increase in the internal sodium concentration (Adelman and Dalton, 1960). Further, Gossweiler *et al.* (1954) have demonstrated that upon increasing the external calcium concentration four times, the resting potassium efflux of rat diaphragm decreases to less than 0.05 times its normal value. There was a 10 mv increase in the resting potential associated with this dramatic change in potassium flux. Therefore, this experiment represents a demonstration of the influence of extracellular calcium concentration both on ion movement through an excitable membrane and on membrane resting potential.

In a less direct manner, voltage clamp experiments (Frankenhaeuser and Hodgkin, 1957, Shanes *et al.*, 1959) on the squid giant axon indicated that the sodium and potassium permeability changes, which are associated with polarizations, depend on the external calcium concentration. Measurements of the "sodium potential" in low external calcium were suggestive of an enhanced sodium entry in the squid axon. Stämpfli and Nishie (1956) have shown, using the sucrose gap technique, that the membrane of myelinated nerve fibers of the Brazilian frog, *Leptodactylus ocellatus*, is slowly depolarized by external calcium-free solutions. In contrast to observations on invertebrate nerve, there was some tendency for the depolarization to cease upon prolonged exposure to calcium-free solution; *i.e.*, there was a transient depolarization. No adequate interpretation of this phenomenon was presented. However, the depolarization induced by calcium lack was shown to be practically prevented in sodium-free solution. They suggested that the depolarization in calcium-free solution was a result of an increased sodium permeability.

In order to test these suggestions as to the influence of divalent ions on sodium permeability a method was sought which would be sensitive enough to detect changes in the intracellular concentration and net flux of sodium in a rapid almost continuous manner. Such a method is outlined in the preceding paper (Moore and Adelman, 1961), in which evidence is presented that the sodium potential (Hodgkin and Huxley, 1952 *a*), as measured in a voltage clamp, can be used to estimate the intracellular sodium concentration of the squid giant axon. This method was developed to study the effect of an alteration in the external divalent ion concentration on the internal sodium ion concentration and the resting net sodium flux of the isolated squid giant axon. The peak sodium conductance change, associated with voltage clamp pulses, was also measured in our studies.

METHODS

Following isolation and cleaning, a single squid giant axon was placed in the voltage clamp cell (Cole and Moore, 1960), and, as soon as feasibly possible, measurements of the sodium potential, E_{Na}, were begun. A detailed description of this procedure is given in the previous paper (Moore and Adelman, 1961). The initial measurements of E_{Na} were usually made every 2 min. in artificial sea water (Taylor, 1959) for a total period of 20 min., occasionally longer. The composition of the artificial sea water is given in Table I. Toward the end of this initial control period a series of step potential changes was applied while the membrane current was measured (see Cole, 1949, Hodgkin, Huxley and Katz, 1952, and Moore, 1959). Following the initial control period, the perfusing solution was exchanged for one in which both calcium

TABLE I

ARTIFICIAL SEA WATER COMPOSITION

Salt	Millimoles/liter
NaCl	430.00
KCl	9.18
$CaCl_2$	9.46
$MgCl_2$	23.40
$MgSO_4$	26.00
$NaHCO_3$	2.19
pH	7.9
Salinity	32 per cent

and magnesium were reduced in their concentrations to 0.1 times the control values (from 9.5 and 50 mM to 0.95 and 5 mM, respectively).

The osmolarity of the test solution was maintained identical with the control artificial sea water by replacing the calcium and magnesium with choline chloride. The choline chloride used was purified and recrystallized by our colleague, Dr. Robert E. Taylor, to whom we are indebted. Replacement with sucrose produced similar results and we were assured of the non-toxic nature of our choline chloride solutions.

Measurements of E_{Na} were continued for a test period of 20 to 30 min. While a measurement of E_{Na} required that the axon be clamped for 15 to 30 sec., usually the axon was not voltage-clamped during the intervals between E_{Na} measurements. Occasionally it was necessary to maintain the holding potential (*i.e.*, keep the axon voltage clamped at a value close to the expected resting potential value) during the entire test period inasmuch as spontaneous firing of the axon initiated by divalent ion reduction would occur at the resting potential. Whenever possible, continuous records of the resting potential, E_{RP}, and the voltage of the peak of the action potential, E_{AP}, were taken between E_{Na} measurements. Toward the end of the test period, a series of membrane current measurements was also made for step changes in mem-

brane potential. In this case the holding potential was increased so as to hyperpolarize the membrane prior to the steps in potential (Frankenhaeuser and Hodgkin, 1957). The perfusing solution was then exchanged for the control artificial sea water and the recovery of the axon was followed in terms of E_{Na}, E_{RP}, and E_{AP}.

RESULTS

Fig. 1 shows the result of a typical experiment in which peak voltage of the membrane action potential, E_{AP}, resting potential, E_{RP}, and sodium potential, E_{Na}, were measured. These all show a slow decrease in their values during the initial control period in artificial sea water. The significance of this decline of E_{Na} is discussed in the previous paper (Moore and Adelman, 1961). During exposure to a solution in which the concentrations of calcium and magnesium were reduced to 0.1 times their normal values, the action potential amplitude rapidly decreased. This effect is similar to that observed in lobster axons (Dalton, 1958, and Adelman and Dalton, 1960). In this experiment little or no difference in the rate of change of the resting potential could be detected except for the transients appearing just after solution change. The solution

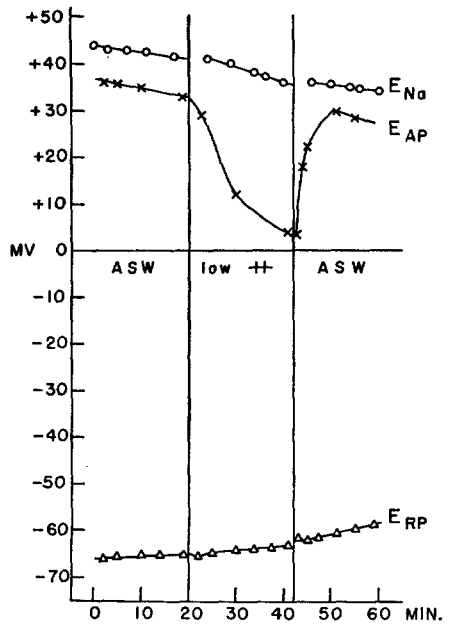

FIGURE 1. Values of the sodium potential (circles), the peak voltage of the action potential (*Xs*), and the resting potential (triangles) of a typical axon plotted as a function of exposure time to artificial sea water and to artificial sea water containing 0.1 times the normal divalent ion concentration.

change required as much as 4 min., and no measurements were obtained during this time. Consequently, in Fig. 1, the curve of E_{Na} has discontinuities following the solution changes. While the sodium potential changed 2.7 mv during the 20 min. control period in artificial sea water, this rate increased to 6.3 mv during the next 20 min. in the low divalent ion solution.

Calculations of the intracellular sodium ion concentration were made assuming that the measured E_{Na} represents that potential at which Na^+ is at thermodynamic equilibrium and using the Nernst relation with activity coefficients assumed constant throughout the experiment. These calculations

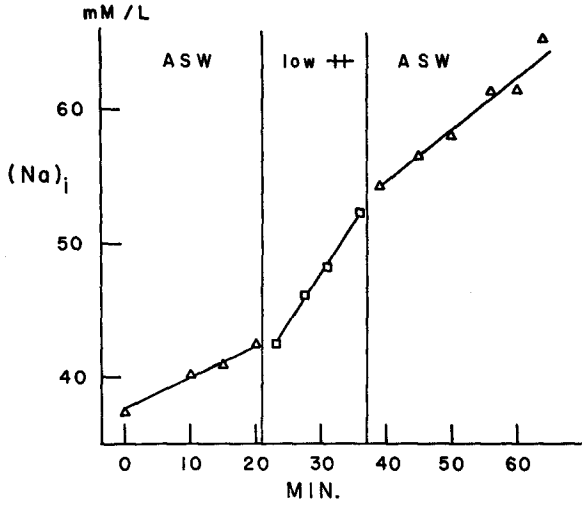

FIGURE 2. The internal sodium concentration, $(Na)_i$, of a typical axon plotted as a function of exposure time to artificial sea water and to artificial sea water containing 0.1 times the normal divalent ion concentration.

indicate a marked increase in the rate of accumulation of sodium ions inside an axon in low external divalent ion solution as compared to the rate of sodium ion accumulation inside an axon in normal sea water. In Fig. 2 the internal sodium concentration, $[Na]_i$, is plotted as a function of time for another typical axon. The slope of the $[Na]_i$ curve is constant over a 20 min. period. By expressing the change in internal sodium in terms of net flux as calculated from the slope of the $[Na]_i$ curve and the axon diameter, it can be shown that this inward net flux increases 2.9 times from a normal value of 49 pmole/cm² sec. in artificial sea water to a value of 140 pmole/cm² sec. in the low divalent ion solution. Similar alterations were seen in all axons so tested. Table II presents calculated flux data from five axons. The mean rate of sodium accumulation by these axons increased to 2 times the average of the initial and final control values upon reducing external calcium and magnesium concentrations to 0.1

times their normal values. These results substantiate the notion of Frankenhaeuser and Hodgkin (1957) that reduced external divalent ion concentration promotes sodium entry. In one experiment (axon 59-28), where the initial flux of sodium was quite low, the flux became 4.4 times normal in the low divalent solution. The effect of low external divalent ion treatment on the sodium flux was not completely reversible. In Table II it can be seen that the control inward net sodium flux has about twice the initial control value upon return to artificial sea water.

It should be apparent that the severe change in the membrane action potential amplitude (see Fig. 1) occurring in low external divalent ion solution cannot be related to the change in E_{Na} as effect and cause. Notice in Fig. 1 that the action potential amplitude changed about 30 mv while E_{Na} changed only 5 mv, during the test period, and that the values of E_{Na} are always lower

TABLE II

SODIUM INFLUX IN NORMAL AND LOW DIVALENT ION SEA WATER

Axon	Net inward Na^+ flux in pmole/cm² sec.			Ratio of test to control flux
	Initial in artificial sea water	Test in 0.1 times divalent	Recovery in artificial sea water	Test $\frac{\text{Initial} + \text{recovery}}{2}$
59-11	98	200	170	1.5
59-15	49	140	80	2.2
59-25	71	140	120	1.5
59-28	39	170	90	2.6
59-36	81	280	170	2.2
Means	68	186	126	2.0

during the recovery period than during the test period. Despite this the action potential will recover to almost normal amplitude upon return to normal divalent ion concentration. Either increased sodium inactivation or increased potassium conductance at the resting potential would qualitatively account for the reduction of the action potential seen here.

It seems necessary to differentiate between the effects of external divalent ion reduction on the changes of resting sodium permeability as indicated by the net sodium flux and the alterations brought about in the rapid sodium conductance system associated with step changes in potential in the voltage clamp (Hodgkin and Huxley, 1952 *b*). Inasmuch as hyperpolarization can activate the sodium fast conductance and also reduce the potassium conductance to a low value (for rationale see Taylor, 1959, p. 1073) one can determine the effects of low external divalent ions on the transient peak sodium conductance, $\acute{g}_{Na}$, in response to step changes in membrane potential without the complicating factors of resting sodium inactivation and potassium con-

ductance if the membrane is hyperpolarized prior to such step potentials (Frankenhaeuser and Hodgkin, 1957). An approximation (*cf.* Frankenhaeuser and Hodgkin, 1957, and Taylor, 1959) to the sodium conductance at the time of the transient peak is given by

$$\acute{g}_{Na} = \frac{\acute{I}_{Na} - I_l}{E_M - E_{Na}}$$

where $\acute{I}_{Na}$ is the total current at the peak, I_l is the leakage current, E_M is the membrane potential during the pulse, and E_{Na} is the sodium potential deter-

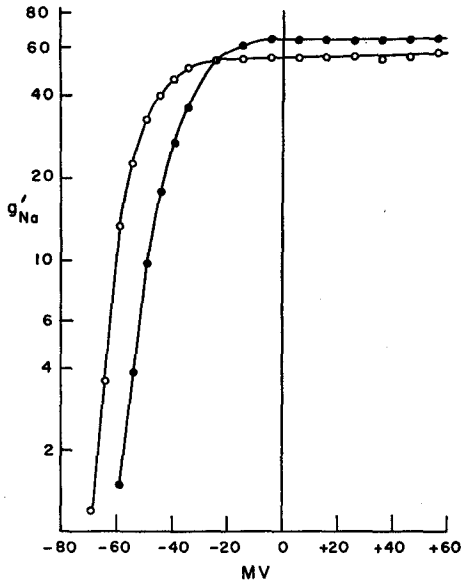

FIGURE 3. Approximate value (mmho/cm²) of the sodium conductance at the time of the peak transient current in response to step changes in membrane potential applied to a previously hyperpolarized axon. Closed circles, initially in artificial sea water; open circles, after 15 min. in artificial sea water containing 0.1 times the normal divalent ion concentration.

mined by the method of Moore and Adelman (1961). The leakage current was estimated by assuming a constant leakage conductance and zero leakage current at the resting potential. The value of the leakage current at the sodium potential was taken equal to the total membrane current 0.4 msec. from the onset of a step polarization to the sodium potential. This form was used to calculate $\acute{g}_{Na}$ and these values were plotted as in Fig. 3 and are in substantial agreement with those obtained by Frankenhaeuser and Hodgkin (1957). In Fig. 3 a tenfold reduction in external divalent ions produces a shift along the voltage axis of about 15 mv. While this shift is less than that obtained for re-

duction of external calcium by Frankenhaeuser and Hodgkin (1957), it is in the same direction and of the proper order of magnitude, and is closer to the value which was obtained previously in this laboratory (unpublished). This difference in results may have occurred as a result of the difference in the reference calcium concentration. The tenfold reduction in calcium in Frankenhaeuser and Hodgkin's work (1957) was made from a reference calcium solution having twice the normal divalent ion concentration.

DISCUSSION

From the results presented in this work it is clear that a tenfold reduction in the normal external divalent ion concentration of the isolated squid giant axon increases the inward resting net sodium flux to about 2 times normal. This result tells us nothing of itself about the unidirectional fluxes of sodium through the axon membrane. However, an accumulation in the internal sodium may be considered as resulting either from an inability of the sodium pump to keep up with an increased sodium influx, or from a decline in the activity of the sodium pump with no change in the sodium influx. Moore and Adelman (1961) have estimated from data of Hodgkin and Keynes (1955) the approximate sodium efflux to be 5 pmoles/cm^2 sec. for an axon bathed in sea water at 7.5°C. If the sole action of external divalent ion reduction were to decrease this efflux to zero, *i.e.* turn off the sodium pump, then one would expect the resting sodium net influx to increase to only 1.1 times normal rather than the measured 2 times normal. It is therefore suggested that a tenfold reduction in external divalent ion concentration primarily increases the resting inward leakage of sodium through the squid axon membrane. This is equivalent to saying that the resting sodium conductance of the membrane is increased upon reduction of external divalent ion concentration.

If we assume that the sodium pump is virtually inoperative in our experiments and that its action may be neglected, then all the sodium flux values may be expressed as inward currents. Then it is possible to consider that the action of external divalent ions is on the resting sodium conductance of the axon membrane. The sodium current at the resting potential is given by the value of the sodium flux in mole/cm^2 sec. times the value of the Faraday constant, 96500 amp sec./mole, and the approximate resting sodium conductance is obtained by dividing this current by the difference between the resting potential and the sodium potential. These values are given in Table III. The values of the resting and sodium potentials used in the calculations were determined at the end of the 20 min. control and test periods.

It is now possible to compare the peak sodium conductance changes with the resting sodium conductance changes. The rapid peak sodium conductance response to step changes in membrane potential in the voltage clamp during

external divalent ion reduction seems to be in substantial agreement with the results reported by Frankenhaeuser and Hodgkin (1957). Inasmuch as there is a shift in the peak sodium conductance curve on the voltage axis upon reducing the external divalent ion concentration 10 times, one would expect that the resting sodium conductance *vs.* voltage curve would show a similar shift such that at the resting potential there is a higher than normal resting sodium conductance such as is indicated in Table III. Qualitatively this shift would be sufficient to account for the increased resting net sodium influx seen in our experiments. It is interesting to note that a reduction in external divalent concentration to 0.1 times the normal concentration would occasionally produce spontaneous firing of our axons at the resting potential. Recent calculations (Huxley, 1959) of the effects of changes in the external calcium con-

TABLE III

SODIUM CONDUCTANCE IN NORMAL AND LOW DIVALENT ION SEA WATER

	Approximate resting Na^+ Current in 10^{-6} amp/cm²			Approximate resting Na^+ Conductance in 10^{-3} mho/cm²			Conductance ratios
Axon	Initial in artificial sea water	Test in 0.1 × divalent	Recovery in artificial sea water	Initial in artificial sea water	Test in 0.1 × divalent	Recovery in artificial sea water	Test $\frac{\text{Initial} + \text{recovery}}{2}$
59-11	9.5	19.3	16.4	8.9	19.5	17.7	1.5
59-15	4.7	13.5	7.7	3.9	11.8	7.1	2.1
59-25	6.8	13.5	11.6	5.9	12.4	11.6	1.4
59-28	3.8	16.4	8.7	3.1	14.5	8.6	2.5
59-36	7.8	27.0	16.4	7.2	27.3	18.8	2.1
Means	6.5	18.1	12.2	5.8	17.1	12.8	1.9

centration upon the Hodgkin and Huxley equations (1952 *d*) predict this instability upon considering the Frankenhaeuser and Hodgkin (1957) data.

From the work of Frankenhaeuser and Hodgkin (1957) it is apparent that two of the main effects of low external divalent ion concentration on the squid giant axon are to increase the extent to which the sodium-carrying system is inactivated in the steady state (Hodgkin and Huxley, 1952 *c*) and to increase the resting potassium conductance. While inactivation studies were not carried out in this work, previous unpublished investigations in this laboratory on the effects of divalent ions on the squid giant axon, which were performed independently of Frankenhaeuser and Hodgkin's experiments, produced similar results.

The effects of lower than normal external divalent ion concentration on the resting membrane sodium permeability cannot at this time be ascribed to any specific mechanism such as a direct effect on the sodium pump. Rather, the

results presented here would suggest that the effect is somewhat non-specific and may be due to a "rotting" of the membrane induced by divalent ion withdrawal as proposed to us by Dr. B. Frankenhaeuser.

The authors wish to express their appreciation to Dr. K. S. Cole and Dr. R. E. Taylor and to other members of the Laboratory of Biophysics, National Institute of Neurological Diseases and Blindness, National Institutes of Health, for their cooperation and their helpful discussion of this work, and especially to the Marine Biological Laboratory, Woods Hole, Massachusetts, for the facility and services which made this work possible.

Received for publication, November 16, 1960.

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