Analysis of the Effects of Calcium or Magnesium on Voltage-Clamp Currents in Perfused Squid Axons Bathed in Solutions of High Potassium

EDUARDO ROJAS, ROBERT E. TAYLOR, ILLANI ATWATER, and FRANCISCO BEZANILLA

From the Laboratorio de Fisiología Celular, Facultad de Ciencias, Universidad de Chile, Clasificador 198, Correo Central, Santiago, Chile, and the Laboratory of Biophysics, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT Isolated axons from the squid, *Dosidicus gigas*, were internally perfused with potassium fluoride solutions. Membrane currents were measured following step changes of membrane potential in a voltage-clamp arrangement with external isosmotic solution changes in the order: potassium-free artificial seawater; potassium chloride; potassium chloride containing 10, 25, 40 or 50, mM calcium or magnesium; and potassium-free artificial seawater. The following results suggest that the currents measured under voltage clamp with potassium outside and inside can be separated into two components and that one of them, the predominant one, is carried through the potassium system. (*a*) Outward currents in isosmotic potassium were strongly and reversibly reduced by tetraethylammonium chloride. (*b*) Without calcium or magnesium a progressive increase in the nontime-dependent component of the currents (leakage) occurred. (*c*) The restoration of calcium or magnesium within 15–30 min decreases this leakage. (*d*) With 50 mM divalent ions the steady-state current-voltage curve was nonlinear with negative resistance as observed in intact axons in isosmotic potassium. (*e*) The time-dependent components of the membrane currents were not clearly affected by calcium or magnesium. These results show a strong dependence of the leakage currents on external calcium or magnesium concentration but provide no support for the involvement of calcium or magnesium in the kinetics of the potassium system.

INTRODUCTION

Squid axons bathed in a solution containing potassium as the only cation exhibit rapid and slow changes in membrane conductance (Segal, 1958; Moore, 1959; Ehrenstein and Gilbert, 1966). Although little is known about the mechanism of these changes in conductance, there are some observations which were used as background data for the experiments we describe and discuss in this paper.

Experiments using nonperfused giant axons of the squid, *Loligo pealei*, demonstrated that the steady-state current-voltage relation in high potassium was nonlinear and that there was a negative conductance region for hyperpolarizing pulses (Moore, 1959; Ehrenstein and Gilbert, 1966). This negative conductance would be predicted if the Hodgkin and Huxley parameters were not changed by high potassium except for the change in V_K (Stämpfli, 1958). These previous experiments led other investigators to study further the dynamic properties of this change in conductance in nonperfused giant axons of the same species. The existence of the negative conductance region in isosmotic potassium solution was confirmed. Further, it was found that combined amounts of magnesium and calcium added to this external potassium solution induced a displacement of the negative conductance region along the voltage axis (Ehrenstein and Gilbert, 1966; Gilbert and Ehrenstein, 1965), as was shown for axons from *Loligo forbesii* in seawater (Frankenhaeuser and Hodgkin, 1957). Later, it was reported that with intracellular perfusion of giant axons from the squid, *Dosidicus gigas*, no negative conductance was observed (Rojas and Ehrenstein, 1965; Rojas and Atwater, 1968).

We considered three possible reasons for the difference in steady-state current-voltage curves: (*a*) as no data were available for intact *Dosidicus* axons in isosmotic potassium, the difference in species appeared as the first possible cause; (*b*) if this were not the case, intracellular perfusion might have been removing intracellular substances responsible for these nonlinearities; among the possible substances, calcium and magnesium appeared as likely candidates (Keynes and Lewis, 1956); (*c*) it was also thought to be possible that a large increase in the time-independent, almost ohmic component of the membrane conductance might mask nonlinearities.

The first explanation was discarded when it was found that the steady-state current-voltage curve was almost linear in *Loligo* axons externally and internally perfused with solutions having potassium as the only cation (Lecar, Ehrenstein, Binstock, and Taylor, 1967). As reported in this paper we have investigated and discarded the second alternative stated above and have studied the effects of the addition of calcium and/or magnesium to the external potassium solution upon the membrane conductance in perfused fibers. It will be seen that our results strongly favor the third possibility and do not support the notion that calcium and magnesium interact directly with the time- and voltage-dependent potassium permeability.

A preliminary report of this investigation has been published elsewhere (Taylor, Rojas, and Atwater, 1967).

METHODS

Giant axons from the squid, *Dosidicus gigas*, were used. Because their large size made transport difficult, the squid were killed immediately after capture and only the part of the mantle containing the nerve bundles was kept in iced seawater during the trip

to shore. Within 2 hr after capture of the animal, the mantle nerves were removed, and the giant axons dissected from these bundles and prepared for experiment.

Perfusion

An axon was suspended in a temperature-controlled bath of potassium-free artificial seawater (K-free ASW) as illustrated in Fig. 1. A glass outlet cannula was introduced into one end, and while the axoplasm was gently sucked into the cannula, this cannula was slowly advanced. A glass inlet cannula, connected to a perfusion solution reservoir, was introduced into the other end of the axon until the tip of this cannula was

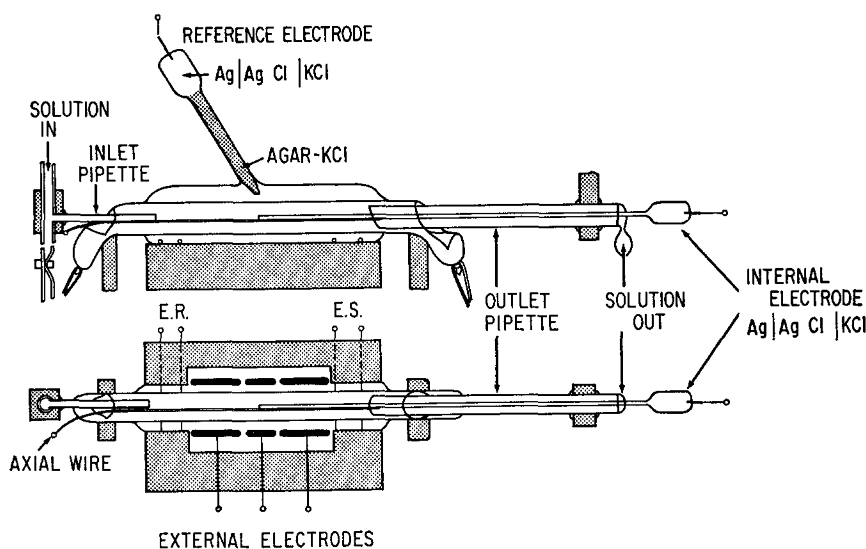

FIGURE 1. Simplified diagram of the combined suction-perfusion apparatus and point control voltage clamp. *E.R.*, external recording. *E.S.*, external stimulation. The platinum cell electrode used to measure the currents is in the center. The outer cell electrodes are grounded. Length of the fiber under internal perfusion about 25 mm.

inside the outlet cannula. A platinum wire, insulated except for 25 mm extending beyond the tip of the infusion cannula, was attached to the infusion (inlet) cannula and introduced at the same time. The axoplasm was then washed out of the outlet cannula with the perfusion solution and the outlet cannula was drawn back until 25 mm of the axon was under perfusion. The 25 mm of the platinum wire, plated with platinum black and extending 25 mm beyond the tip of the infusion cannula along the axis of the axon, rested just within the opening of the outlet cannula.

Voltage-Clamp

The voltage-clamp technique used in these experiments has been described by Moore and Cole (1963). The voltage drop across the axonal membrane was maintained by an electronic feedback circuit which compares the measured membrane voltage,

V_m, to a command voltage, V_c; the difference, $V_c - V_m$, is fed to a P-45 operational amplifier (Philbrick Researches, Inc., Dedham, Mass.), used to supply current through the axial wire and radially along the length of the perfused region of 25 mm. The currents needed to maintain a given step potential were recorded on film as a function of time.

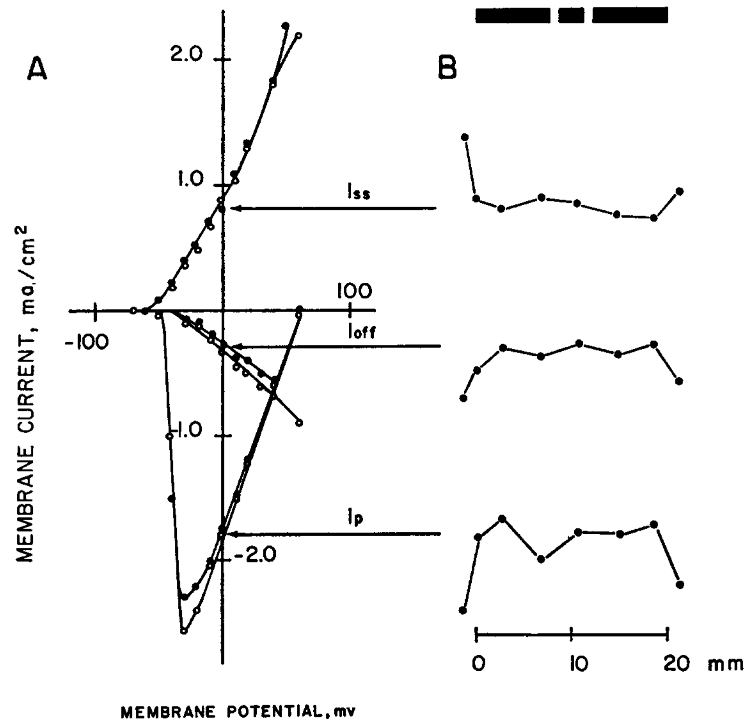

FIGURE 2. A comparison between membrane currents recorded with virtual ground and differential electrode systems. A, open circles correspond to membrane currents recorded with the virtual ground system. Solid circles correspond to membrane currents recorded with the differential system. B, test of current density along the nerve fiber. The three black rectangles at the top of the figure give the position of the electrodes of the virtual ground system relative to the differential system. The points show the variations of I_{ss}, I_{off}, and I_p when moving the differential electrode system along the fiber axis. The voltage-clamp pulse was kept constant at 60 mv (an absolute membrane potential of 0 mv). The ordinate for part B is the same as for part A.

Measurement of Membrane Current

Membrane currents were routinely measured through a central electrode with lateral guards as indicated in Fig. 1 (virtual ground system). Spatial uniformity was tested by measuring the longitudinal distribution of membrane current with external differential electrodes (Taylor, Moore, and Cole, 1960). (*a*) Virtual ground system: Fig. 1 shows the electrode arrangement with respect to the perfused fiber. In this figure, the electrodes used for measuring the currents during a voltage-clamp run are

shown as three bars. The platinum cell electrode used to measure the currents is in the center and is kept at virtual ground potential by means of a P-45 operational amplifier. The outer cell electrodes are grounded and serve to minimize fringe effects in the measuring region. (*b*) Differential electrodes: two C-shaped silver-silver chloride electrodes were made and used as differential electrodes. The radius of the internal C-shaped electrode was 1.2 mm and the radius of the external one was 4.0 mm. The two wires were connected directly to the input of a differential amplifier (3A3 plug-in amplifier, Tektronix, Inc., Beaverton, Oregon). From currents obtained while bathing the axons in ASW the following numerical values were extracted: peak early transient inward current, abbreviated I_p; steady-state outward current, or I_{ss}; and instantaneous current for the "off" of the voltage-clamp pulse, or I_{off}. To determine uniformity of the membrane currents along the fiber axis during voltage clamp, both systems for recording the currents were used simultaneously.

TABLE I

	Ionic composition							
Solution	K^+	Na^+	Cl^-	F^-	Ca^{2+}	Mg^{2+}	TEA	Osmotic pressure
mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*milliosmols*
97-B	500	—	50	500	—	—	50	—
97	550	—	—	550	—	—		960
21	—	430	550	—	10	50		940
123	—	500	520	—	10	—		950
125	—	450	550	—	50	—		950
49	550	—	550	—	—	—		975
96	535	—	555	—	10	—		1000
120	512.5	—	562.5	—	25	—		975
124	490	—	570	—	40	—		1030
117	475	—	575	—	50	—		990
118	535	—	555	—	—	10		1025
119	512.5	—	562.5	—	—	25		1000
121	490	—	570	—	—	40		975
109	475	—	570	—	—	50		975

pH was adjusted to 7.5 ± 0.3 with 5 mM Tris-Cl. Osmotic pressure was measured with a Mechrolab osmometer.

Fig. 2 represents the current-voltage relations (I_p, I_{ss}, and I_{off}) as measured with both systems. Fig. 2 B shows the variations in membrane currents obtained when moving the differential electrodes along the fiber axis. It is clear from this figure that the currents measured with these two systems are not significantly different (Fig. 2 A) and that the density of membrane currents is uniform around the central virtual ground electrode (Fig. 2 B). Thus, the lateral electrodes minimize the fringe effects. Beyond these lateral electrodes, in either direction along the fiber axis, there is an increase in membrane current; the extension of the axial wire beyond the end of the lateral electrodes explains why the current density is greater at the ends (see Fig. 1).

Measurements of Membrane Potential

The voltage drop across the axonal membrane was measured with two silver-silver chloride electrodes in contact with the intra- or extraaxonal solutions through appropriate KCl bridges. The internal electrode bridge was the usual glass capillary, about 200 mm in length and 125 μ in diameter, filled with 550 mM KCl and containing a floating 50 μ bright platinum wire. The external electrode bridge consisted of a glass capillary filled with 1 % agar 3-M KCl.

Junction-potentials were measured as usual by using a 3-M KCl bridge in place of the membrane. A calomel cell was used to connect the external solution to ground potential.

Solutions

All solutions were prepared with double glass-distilled water. Fluoride solutions were kept in plastic containers and stored at 4°C for no more than 4 days. All salts were analytical grade. Osmotic pressures were measured with a Mechrolab osmometer (Hewlett-Packard Co., Palo Alto, Calif.). Composition of the solutions used is given in Table I.

RESULTS

A. *Controls in K-Free Artificial Seawater*

As a general procedure, the fibers were first bathed in K-free ASW for 5–25 min and internally perfused with potassium fluoride solution (see Table I). We have included in Table II various quantities measured at this time with a standard voltage-clamp run on perfused axons bathed in K^+-free artificial seawater as these are not readily available for *Dosidicus gigas* in the literature. (Potentials are referred to the external solution as ground; positive going membrane potential changes are referred to as depolarizations and negative going ones as hyperpolarizations.)

It is known that internal and external perfusion with solutions having potassium as the only cation results in a membrane with nearly linear steady-state current-voltage characteristics. Since complete removal of divalent cations from both sides of the axolemma eventually results in irreversible deterioration of the axon membrane, we will consider only those experiments in which most of the observed kinetic properties of the axon membrane were restored in seawater after the experimental runs in solutions with potassium as the only cation.

Table II shows control values for axons perfused with KF and bathed in K-free ASW for resting and action potentials (a, b) and for some quantities measured under voltage clamp, before (B) and after (A) (c, d, e, f, g) the external K-free ASW was replaced by potassium solutions. In two cases (experiments 3T and 14T) there was essentially no recovery, but this re-

TABLE II

CONTROLS IN K-FREE ARTIFICIAL SEAWATER

Experiment		3T	5T	7T	8T	9T	10T	11T	12T	14T	15T
(a) Corrected resting potential, *mv*	B	−67	−56	−58	−61	−56	−60	−60.5	−58	−60	−61
	A	−34	−50	−49	−50	−46	−54	−54	−56	−36	0
(b) Action potential height, *mv*	B	105	95	100	100	95	100	110	110	110	110
	A	0	48	72	70	70	80	110	110	0	28
(c) Maximum peak inward current, *ma/cm²*	B	2.8	1.5	1.75	2.5	2.5	1.25	2.5	2.05	2.1	2.0
	A	0.3	0.9	1.5	1.3	1.7	0.82	2.6	2.5	0.96	0.55
(d) Maximum transient conductance, *mmho/cm²*	B	47	19	19	33.5	36	16	28.5	24	25	29
	A	—	14	21.5	22	22.5	14.5	32	28	17	14.8
(e) Maximum steady-state conductance, *mmho/cm²*	B	14	16	15	26.5	20	8.5	16	21	5.2	7
	A	—	15	12.2	14	15	7.5	18	20	5.2	7
(f) Reversal potential, *mv*	B	—	—	+82	—	—	—	+84.5	+97	+60	+79
	A	—	—	+71	—	—	—	+69.5	—	—	—
(g) P_{Na}/P_K	B	—	—	35.8	—	—	—	36.6	67.4	14.7	31.8
	A	—	—	22.9	—	—	—	21.8	—	—	—
(h) Leakage conductance, *mmho/cm²*				2.35				4.0	2.82		2.9
(i) Temperature, °*C*	B	14	8	9	9	11.5	—	9	9	8.5	10
	A	—	—	10.5	—	9	—	8	9	11	10

The 550 mM KF used for internal perfusion had a junction potential with reference to K-free ASW of +6.5-9 mv.

sulted from known accidents before which these axons appeared to be in good condition.

The average corrected resting potential before the experimental run was −60 mv (see Table II) and after the experimental runs in isosmotic potassium solutions this average was −47 mv. The average membrane action potential height was 105 mv before and 73 mv after.

The peak early transient inward currents and late steady-state outward currents are plotted for a typical experiment in Fig. 2. From such plots we get for Table II the maximum peak inward current (c), the maximum transient conductance defined arbitrarily as the slope of the current voltage curve corresponding to the maximum inward current for large depolarizations (d), and the maximum steady-state slope conductance defined arbitrarily as the slope of the current voltage curve corresponding to the maximum outward current for large depolarizations (e).

Although incidental to this investigation, measurements of the potential were made on some axons during an applied pulse at which the initial transient current changed from inward to outward. This potential is termed "reversal potential" in Table II (f) and represents clearly that potential for which the rate at which potassium ions from the inside are flowing out is the same as that at which sodium ions from the outside are flowing in. If we interpret these results as Chandler and Meves did (1965), we arrive at permeability ratios for the fast channels of sodium to potassium shown in Table II as P_{Na}/P_K (g) which are larger than the average of 12 given by Chandler and Meves for *Loligo forbesii* (1965).

The data summarized in Table II demonstrate that the axons of the squid, *Dosidicus gigas*, can be used to perform experiments in high potassium and maintained in a medium deprived of divalent cations without any serious deterioration of the dynamic properties of the fiber as previously determined in seawater.

B. *Data in Solutions of High External Potassium*

EFFECTS OF INTERNAL TEA ON STEADY-STATE I-V CURVES WITH POTASSIUM AS THE ONLY INTERNAL AND EXTERNAL CATION

Before describing the effects of calcium or magnesium on voltage-clamp currents in perfused axons bathed in solutions of high potassium, we present indirect evidence which shows that about 70% of the outward currents measured in perfused fibers bathed in isosmotic potassium cross the axon membrane through the same channels through which the potassium currents seen in axons bathed in ASW pass. The evidence was obtained from four experiments with potassium inside and outside using the specific blocking agent for out-

ward currents through the potassium channels, tetraethylammonium chloride (TEA).

Fig. 3 shows several *I-V* curves obtained in one of the TEA experiments. For this axon a control voltage-clamp run in seawater was obtained before and after exposure to 550 mM KCl. Only the control run after exposure to high external potassium is shown in this figure (curves drawn through filled circles). 12 min after replacement of the external K-free ASW by 550 mM KCl, the I_{ss}-V curve drawn through the open squares was obtained. The curve

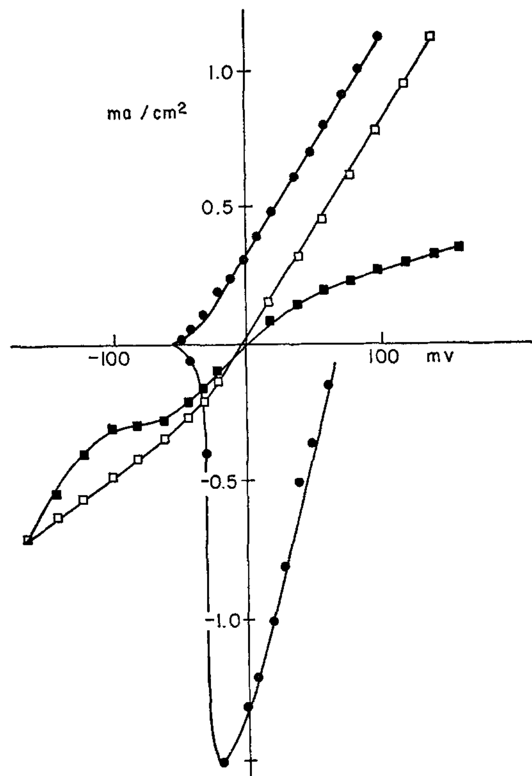

FIGURE 3. Effects of internal TEA on steady-state I–V curves with potassium as the only internal and external cation. 12 min after replacement of external K-free ASW by 550 mM KCl the I_{ss}–V curve drawn through the open squares was obtained. 5 min after addition of 50 mM TEA to the perfusing solution the I_{ss}–V curve drawn through the solid squares was obtained. I_p–V and I_{ss}–V curves for the last control run in K-free ASW are also shown (solid circles).

drawn through the filled squares was obtained 5 min after addition of 50 mM TEA to the internal solution. If one compares the outward steady-state currents (represented by the I_{ss}-V curves shown in Fig. 3) obtained with potassium solutions inside and outside one can see that although TEA strongly reduces the outward currents, about 30% of these currents are not affected by TEA. Depolarizing pulses of membrane potential presumably drive the TEA molecules into the membrane (Armstrong and Binstock, 1965; Armstrong, 1966) producing a reduction of the outward potassium current.

Thus, we conclude that with potassium as the only cation there are at least

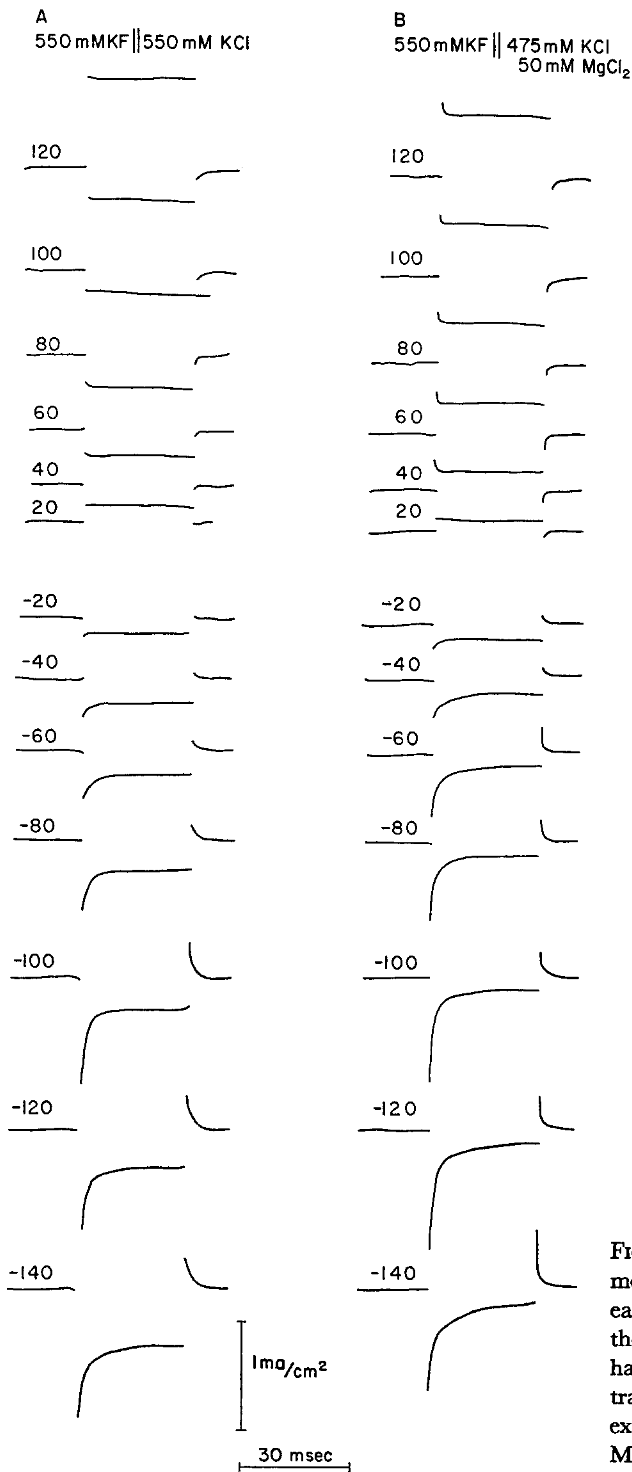

FIGURE 4. Effect of 50 mM Mg^{2+} upon membrane currents. Numbers attached to each record correspond to the magnitude of the membrane potential step. These records have not been corrected for capacitative transients or leakage. A, before addition of external Mg^{2+}. B, after addition of 50 mM $MgCl_2$.

two components of the outward currents. The first, about 70% of the total outward current, is reversibly inhibited by TEA and the second, about 30% of the total outward current, is not affected by TEA. The analysis of inward currents in the presence of TEA is more complicated than for outward currents. As shown by Armstrong and Binstock (1965, Fig. 4) and Armstrong (1966, Fig. 3), the results (Fig. 3) are in essential agreement with those for

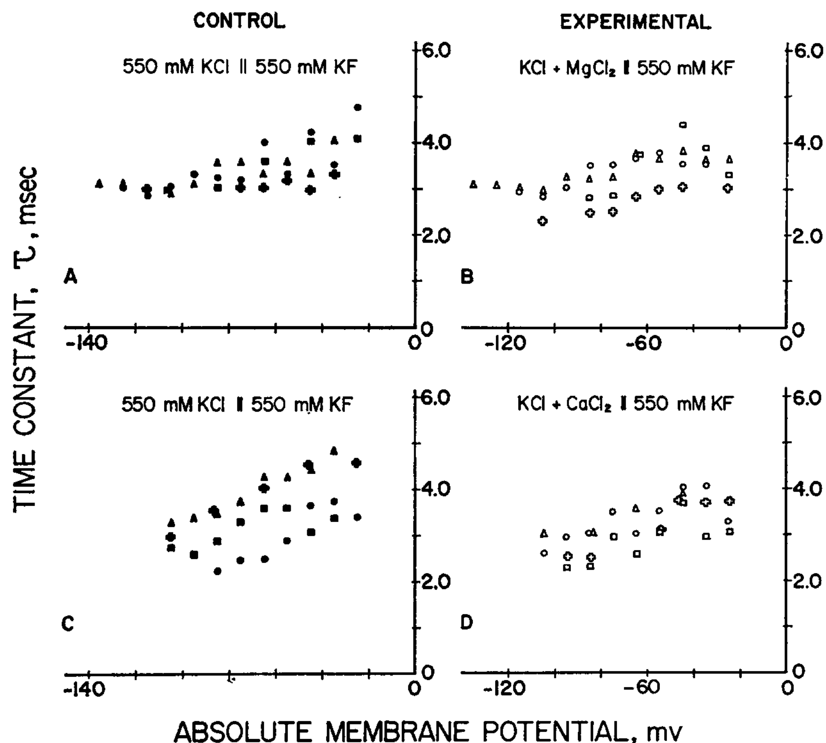

FIGURE 5. Effects of adding varying amounts of magnesium or calcium to the external solution upon the time constant of decay of the membrane currents for hyperpolarizing pulses. For Figs. 5 A and 5 B: open and solid circles, 10T; open and solid triangles, 11T; open and solid squares, 12T; open and solid stars, 7T. For Figs. 5 C and 5 D: open and solid circles, 5T; open and solid triangles, 14T; open and solid squares, 15T; open and solid stars, 9T.

unperfused axons bathed in high potassium solutions containing calcium and magnesium.

EFFECTS OF MAGNESIUM OR CALCIUM UPON MEMBRANE CURRENTS

With potassium as the only internal and external cation, membrane currents recorded under voltage-clamp conditions are not strongly time-dependent for depolarizing steps (positive inside during the membrane potential step). Currents measured for hyperpolarizing membrane potential steps (negative inside

during the step), however, are more time-dependent. Current records taken 50 min after replacing the external potassium-free ASW by isotonic potassium chloride are shown in Fig. 4 A. Numbers attached to each record correspond to the magnitude of the membrane potential step.

Fig. 4 B shows the membrane currents measured in the same experiments 20 min after the addition of 50 mM $MgCl_2$ to the external potassium solution. The membrane currents were not drastically changed. However, it is clear from these records that I_{ss} is reduced.

We have computed the time constant for the decrease in membrane currents observed during hyperpolarizing membrane potential steps of different magnitude by fitting the time-dependent current with the equation:

$$I_t - I_{ss} = (I_{inst} - I_{ss}) \exp(-4t/\tau),$$

where τ is a voltage-dependent time constant. This expression follows from the Hodgkin and Huxley equations provided that the final value of the time-

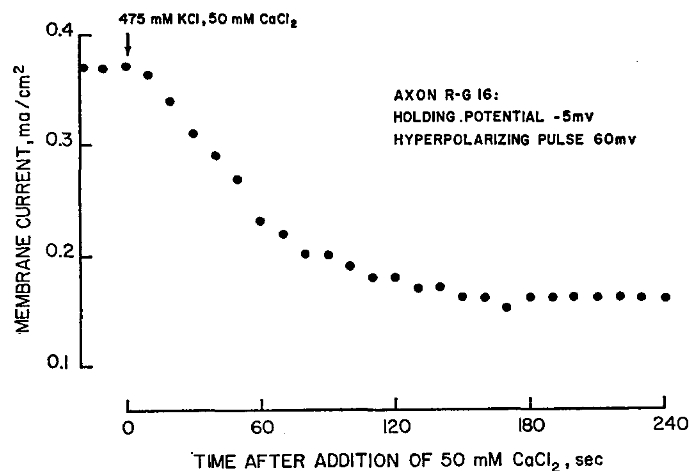

FIGURE 6. Effect of 50 mM $CaCl_2$ on I_{ss} as a function of time. For explanation see text.

dependent current is zero (Hodgkin and Huxley, 1952). Time constants plotted vs. absolute membrane potential appear in Fig. 5.

The effects upon τ of the addition of different amounts of magnesium or calcium to the external potassium solution are shown in Fig. 5 B and D. Although there is considerable scattering of the data, no systematic effects are apparent. In both cases τ seems to be inversely dependent on absolute membrane potential ranging from about 2.5 to 4.4 msec.

Although it was clear that I_{ss} was greatly reduced by the addition of calcium to the external solution, the magnitude of the time-dependent component was not affected. The effect of calcium upon I_{ss} was clearly determined in the experiment plotted in Fig. 6. Immediately after a linear *I-V* curve was recorded

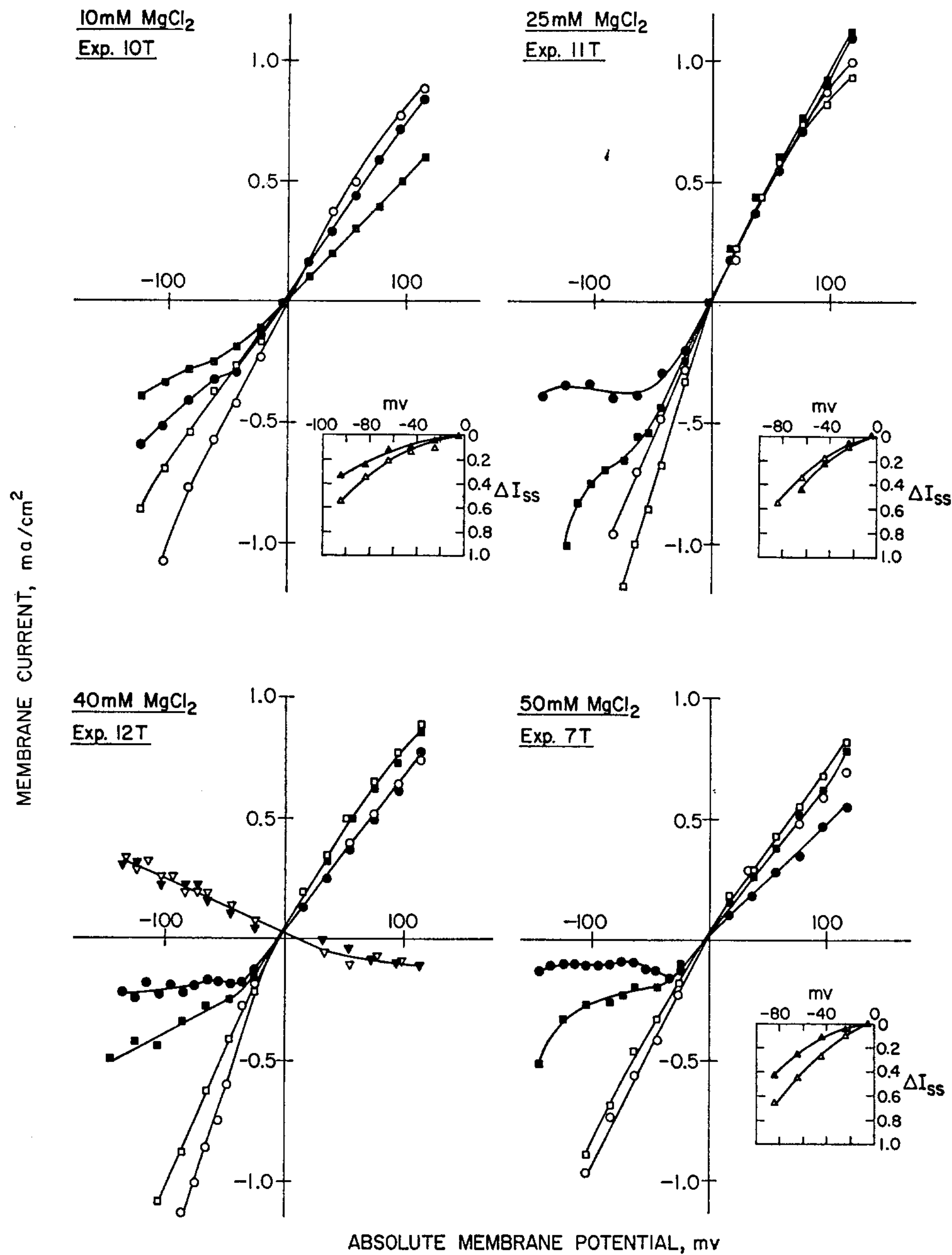

FIGURE 7. Effects of magnesium upon membrane conductance. See text for explanation.

with 550 mM KCl outside, the membrane potential was clamped at −5 mv; then, every 10 sec, hyperpolarizing pulses of 60 mv and 50 msec duration were applied. Next, the external solution was changed for 475 mM KCl plus 50 mM $CaCl_2$, and the changes in membrane currents were recorded at every pulse. Fig. 6 shows the variation in membrane currents, I_{ss}, with time after the addition of 50 mM $CaCl_2$ to the potassium solution. The steady-state currents were decreased from 0.36 ma/cm^2 to 0.16 ma/cm^2. A semilogarithmic graph of these currents showed a first-order kinetics with a rate constant of 65 sec. This effect might be related to an adsorption of calcium to the membrane.

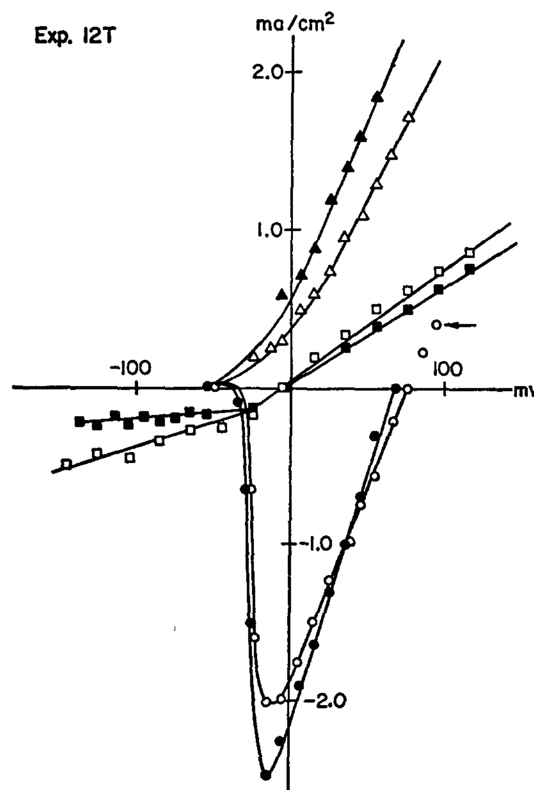

FIGURE 8. *I-V* curves in K-free artificial seawater and in isosmotic potassium. Open circles (I_p) and open triangles (I_{ss}), obtained in K-free ASW before the experimental runs in KCl. Open squares (I_{ss}) and solid squares (I_{ss}) obtained in 550 mM KCl and 475 mM KCl plus 50 mM $CaCl_2$, respectively. Solid circles (I_p) and solid triangles (I_{ss}) obtained in K-free ASW after the experimental runs in KCl. Arrow indicates the reversal potential where the leakage currents were measured.

EFFECTS OF MAGNESIUM OR CALCIUM UPON MEMBRANE CONDUCTANCE

In order to analyze current records such as those shown in Fig. 4 with more accuracy, we plotted the membrane currents as functions of absolute membrane potential, as can be seen in Fig. 7. In each set of *I-V* curves we have plotted the instantaneous current, I_{inst}, and the steady-state currents, I_{ss}, before and after the addition of a given concentration of magnesium to the external solution. It can be seen that in general, magnesium will reduce the I_{ss}

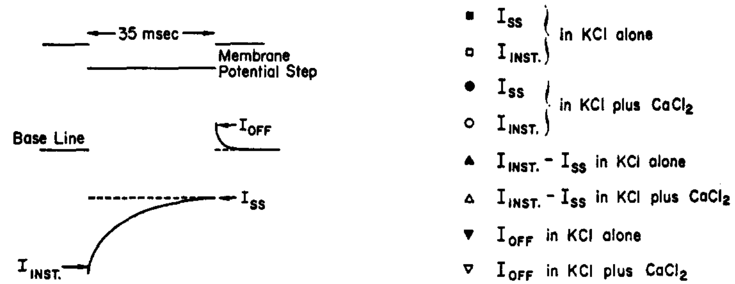

FIGURE 9. Effect of calcium upon membrane conductance. See text for explanations.

for hyperpolarizing pulses. Thus, it will transform the almost linear relationship measured with potassium as the only internal cation to a nonlinear *I-V* curve, but only upon the addition of 50 mM $MgCl_2$ did a negative conductance region become apparent (see graph corresponding to experiment 7T). The small inserts in this figure represent $I_{inst} - I_{ss}$ for hyperpolarizing pulses vs. absolute membrane potential. This value measures possible effects of magnesium upon the magnitude of the time-dependent component of the membrane currents. With the exception of those data for 25 mM $MgCl_2$ (axon 11T) in Fig. 7 there appears to be an increase in the component of membrane current with the addition of Mg ions. A quantitative study of this effect in terms of Mg^{++} concentration is difficult because the properties of the membrane, particularly the leakage, change with time in the absence of divalent ions.

The instantaneous currents for the "off" of the membrane potential step were not affected by the addition of magnesium as shown in the *I-V* curves for experiment 12T.

These off currents represent a transient shift of the apparent equilibrium potential (in the face of no gradient in concentration of potassium ions) and unless there are transient permeability changes for fluoride or chloride ions this probably represents a local accumulation or depletion of potassium ions in or near the membrane. Such an effect is well-known for axons in ASW and is presumably the result of changes in K^+ concentration in the Schwann cell spaces (Frankenhaeuser and Hodgkin, 1956).

Fig. 8 represents the control *I-V* curve and the experimental steady-state *I-V* curve corresponding to axon 12T (also used in Fig. 7). This figure compares the magnitude of potassium currents when externally perfusing with artificial seawater or high potassium with or without magnesium. Clearly, the magnesium is inducing a reduction in the steady-state hyperpolarized currents. In this particular experiment the potassium and sodium conductances in seawater were completely recovered, and therefore, it can be claimed that the observed effect induced by magnesium added to the external high potassium solution is not caused by deterioration of the fiber.

The same experimental analysis performed with magnesium and presented above, was done with calcium, and the essential data are summarized in Fig. 9. The results obtained with calcium were very similar to those obtained with magnesium with the exception of the following aspects: (*a*) calcium induced a reduction in the steady-state hyperpolarizing currents but the time-dependent component (ΔI_{ss}, Fig. 9) remained the same with or without calcium, (*b*) although calcium induced a nonlinear *I-V* behavior, there was no indication of a negative conductance region, unlike 50 mM magnesium which induced a negative conductance.

DISCUSSION

Effects of External Potassium with or without Internal Perfusion

Let us first consider the results obtained with unperfused and perfused axons. The steady-state current-voltage curves for unperfused axons exhibit a negative resistance region in solutions of high potassium (Moore, 1959; Ehrenstein and Gilbert, 1966) while perfused axons do not (Rojas and Ehrenstein, 1965; Rojas and Atwater, 1968).

One possible reason for this difference in steady-state *I-V* curves was that intracellular perfusion might have been removing some small axoplasmic components responsible for nonlinearities. We have determined the potassium and sodium composition of the axoplasm of *Dosidicus gigas* fibers. Our analysis yielded the following ranges: 400 to 480 mM for potassium and 30 to 80 mM for sodium. These concentrations are very similar to those reported for axons of other species (Steinbach and Spiegelman, 1943; Keynes and Lewis, 1951). Thus, internal perfusion with 550 mM KF does not significantly alter the internal potassium concentration.

There are, however, some other cationic constituents such as calcium and magnesium normally present in the axoplasm (Keynes and Lewis, 1951, 1956) which are not included in the fluoride perfusion solution. In an attempt to restore the ionic composition, we performed experiments in which we perfused with a solution which mimics the complete ionic composition of the axoplasm of intact axons (Deffner, 1961). Internal perfusion with 500 mM potassium aspartate, 50 mM sodium glutamate, 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ at pH 7.1, resulted again in a membrane *I-V* curve similar to that seen when perfusing with 550 mM KF.

If the differences in perfused and unperfused fibers in external isosmotic potassium are caused by the removal of some axoplasmic constituent, it does not appear that this is calcium, magnesium, sodium, aspartate, or glutamate.

One could interpret the linear steady-state *I-V* curve for perfused axons as an indication that nonlinearities are not an intrinsic property of the membrane, but a property determined and modulated by the external environment. Fig. 4 shows, however, that although the steady-state *I-V* curve of the axon membrane with potassium as the only internal and external cation is almost linear, the currents for depolarizing and hyperpolarizing pulses are quite different. While depolarizing currents are not time-dependent, hyperpolarizing currents are strongly time-dependent. These currents are different depending on the direction of the ionic flow (Fig. 4).

Effects of Magnesium or Calcium Added to the External High Potassium Solution

The fact that the kinetics of the time-dependent hyperpolarizing currents were not essentially altered by the addition of different amounts of calcium and

only slightly by magnesium (as shown in Figs. 7 and 9) led us to consider the effects of these ions on the ohmic component of the membrane currents, i.e. upon the "leakage." Control runs with potassium as the only internal and external cation gave slope conductances for depolarizing steps ranging from 5.0 to 10.2 mmho/cm² (average in 18 experiments ±SD was 7.2 mmho/cm² ± 1.2). We have normalized the data of the calcium and magnesium runs to this average slope conductance. In this way, the effects of adding increasing amounts of either calcium or magnesium could be adequately compared.

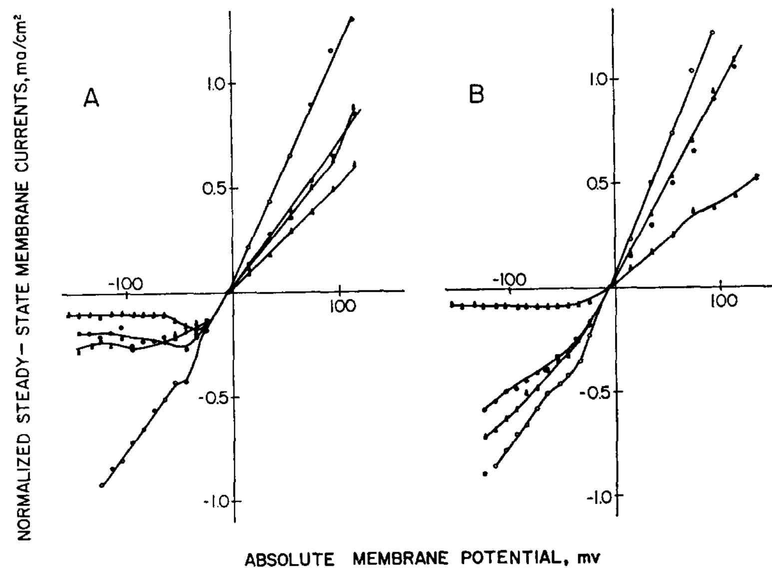

FIGURE 10. Normalized data on the effect upon I_{ss}-V curves of adding increasing amounts of calcium or magnesium to the external potassium solution. A, the effect of the addition of increasing amounts of magnesium upon I_{ss}-V curves (open circles, 10T; open triangles, 11T; solid circles, 12T; solid triangles, 7T). B, the effect of the addition of increasing amounts of calcium upon I_{ss}-V curves (open circles, 5T; open triangles, 14T; solid circles, 15T; solid triangles, 9T).

Normalized data on the effects of adding increasing amounts of either calcium or magnesium are shown in Fig. 10.

Clearly the net effect of either calcium or magnesium added to the external potassium solution is to gradually decrease both depolarizing and hyperpolarizing membrane currents. A possible interpretation of these effects could be that divalent cations are adsorbed by negatively charged groups in the leakage channels present in the axonal membrane (Rojas and Atwater, 1968), resulting in neutralization of these charges. As a consequence, the number of mobile counterions within the leakage channel could be reduced and the steady-state conductance decreased (Helfferich, 1962).

Leakage current is not an accurately defined quantity. An example is

shown by the arrow in Fig. 8, and others are included in Table II, when the leakage is estimated for an axon in K^+-free ASW as that current during a voltage step to the reversal potential for sodium channels. For *Dosidicus gigas* axons the leakage so taken averaged about 3 mmho/cm^2 in this series of experiments. This value is in the usual range (compare values of 1.3 to 2.5 mmho/cm^2 in Rojas and Atwater (1968, Table I). Evidence for some rectification is clear since leakages estimated from hyperpolarizing steps from the resting potential are smaller.

The leakage, estimated in external isosmotic potassium calculated from steady-state currents at hyperpolarizing steps to 100–150 mv ranged in these experiments from 3 to 10 mmho/cm^2 (average of eight experiments shown in Figs. 9 and 11, 5.25 mmho/cm^2). The value with TEA (Fig. 3) was 1.7 mmho/cm^2. With high (40–50 mM) Ca^{++} or Mg^{++}, this leakage value was less (0.6 mmho/cm^2 for Fig. 10).

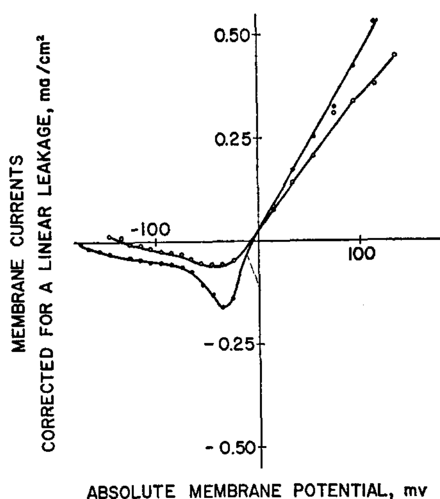

FIGURE 11. Steady-state I–V curves corrected for a linear leakage. Current-voltage curves for 50 mM Ca^{++} (open circles) and 50 mM Mg^{++} (solid circles) obtained from the curves of Fig. 10 by subtraction of a linear leakage of 0.6 mmho/cm^2 estimated for the 50 mM Mg^{++} case.

The simplest assumption for our purposes is that the leakage *I-V* curve is linear and the conductance is reduced by addition of either calcium or magnesium. In Fig. 11 we have subtracted the smallest leakage *I-V* curve from those obtained with the addition of 50 mM of either magnesium or calcium to the external potassium solution. It is clear from these curves that in both cases, the *I-V* curves resemble the *I-V* curves obtained from isosmotic potassium with unperfused axons. Accordingly, internal perfusion with 550 mM KF of axons bathed in 550 mM KCl will increase the leakage conductance and this in turn will appear as though the nonlinearity of the membrane had been decreased by the perfusion. Our data indicate that external calcium or magnesium reduces the ohmic conductance of perfused fibers. We should like to stress that

in isosmotic potassium without corrections of any sort, internally perfused axons seem to have a greater leakage than unperfused fibers. Thus, unperfused fibers behave, in the absence of externally supplied calcium, as if the external calcium concentration were not zero. Other results from the laboratory have shown that calcium continues to leak from intact axons for many hours (Rojas and Hidalgo, 1968).

This research was supported in part by a National Institutes of Health Grant (NB-06503-03) and by Universidad de Chile. It was carried out at Laboratorio de Fisiología Celular, Universidad de Chile, Montemar, Chile.

Received for publication 27 November 1968.

REFERENCES

ARMSTRONG, C. M. 1966. Time course of TEA^+-induced anomalous rectification in squid giant axons. *J. Gen. Physiol.* **50**:491.

ARMSTRONG, C. M., and L. BINSTOCK. 1965. Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride. *J. Gen. Physiol.* **48**:859.

CHANDLER, W. K., and H. MEVES. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol.* (*London*). **180**:788.

DEFFNER, G. G. 1961. The dialyzable free organic constituents of squid blood: a comparison with nerve axoplasm. *Biochim. Biophys. Acta.* **47**:378.

EHRENSTEIN, G., and D. L. GILBERT. 1966. Slow changes of potassium permeability in the squid axon. *Biophys. J.* **6**:553.

FRANKENHAEUSER, B., and A. HODGKIN. 1956. The after effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* (*London*). **131**:341.

FRANKENHAEUSER, B., AND A. HODGKIN. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol.* (*London*). **137**:218.

GILBERT, D. L., and G. EHRENSTEIN. 1965. Abstracts of the 23rd International Congress Physiological Society, Tokyo. No. 148. P. 86.

HELFFERICH, F. 1962. Ion Exchange. McGraw-Hill Book Company, Inc., New York.

HODGKIN, A. L., and A. F. HUXLEY. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* (*London*). **117**:500.

KEYNES, R. D., and P. R. LEWIS. 1951. The sodium and potassium content of cephalopod nerve fibres. *J. Physiol.* (*London*). **114**:151.

KEYNES, R. D., and P. R. LEWIS. 1956. The intracellular calcium contents of some invertebrate nerves. *J. Physiol.* (*London*). **134**:399.

LECAR, H., G. EHRENSTEIN, L. BINSTOCK, and R. E. TAYLOR. 1967. Removal of potassium negative resistance in perfused squid giant axons. *J. Gen. Physiol.* **50**(6, Pt. 1): 1499.

MOORE, J. W. 1959. Excitation of the squid axon membrane in isosmotic potassium chloride. *Nature* (*London*). **183**:265.

MOORE, J. W., and K. S. COLE. 1963. Voltage clamp techniques. *In* Physical Techniques in Biological Research. W. L. Nastuk, editor. Academic Press, Inc., New York. **5**:263.

ROJAS, E., and I. ATWATER. 1968. An experimental approach to determine membrane charges in squid giant axons. *J. Gen. Physiol.* **51**(5, Pt. 2):131s.

ROJAS, E., and G. EHRENSTEIN. 1965. Voltage clamp experiments on axons with potassium as the only internal and external cation. *J. Cell. Comp. Physiol.* **66**:71.

ROJAS, E., and M. C. HIDALGO. 1968. Effect of temperature and metabolic inhibitors on ^{45}Ca outflow from squid giant axons. *Biochim. Biophys. Acta.* **163**:539.

SEGAL, J. R. 1958. An anodal threshold phenomenon in the squid giant axon. *Nature* (*London*). **182**:1370.

STÄMPFLI, R. 1958. Die Strom-Spannungscharakteristik der erregbaren Membran eines einzelnen Schnürrings und ihre Abhängigkeit von der Ionenkonzentration. *Helv. Physiol. Pharmacol. Acta* **16**:127.

STEINBACH, H. B., and S. SPIEGELMAN. 1943. The sodium and potassium balance in squid nerve axoplasm. *J. Cell. Comp. Physiol.* **17**:187.

TAYLOR, R. E., J. W. MOORE, and K. S. COLE. 1960. Analysis of certain errors in squid axon voltage clamp measurements. *Biophys. J.* **1**:161.

TAYLOR, R. E., E. ROJAS, and I. ATWATER. 1967. Analysis of the effects of Ca^{++} or Mg^{++} on voltage clamp currents in perfused squid axons in internal and external solutions of high K^{+}. *J. Gen. Physiol.* **50**:2487.