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DIPOLE MOMENT CHANGES AND VOLTAGE DEPENDENT MEMBRANE CAPACITY OF SQUID AXON

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Summary

Changes in the membrane capacity of squid axons during hyper- and depolarizations are measured between —160 and +40 mV. After corrections for the series resistance and fringe effect, we found that the membrane capacity increased from 0.68 to 1.2 $\mu F/cm^2$ with depolarization. It was further observed that tetrodotoxin in the external medium eliminated the change in membrane capacity without affecting the conductivity. The voltage-dependent membrane conductivity is, in turn, greatly reduced by the internal cesium ion. These observations clearly indicate that the voltage-dependent membrane capacity and conductivity are closely related to ionic channels. Particularly, the increase in membrane capacity with depolarizations may be due to sodium channels. The change in the dipole moment associated with sodium sites was determined using values of α_m and β_m at various depolarizations. We found, based on voltage clamp measurements, that the increase in the dipole moment of the sodium site between —40 and —5 mV is 1230 Debye units (D.U.) and 930 D.U. between —5 and +60 mV, indicating that the depolarization of sodium channels may consist of two different steps.

Introduction

For the investigation of motions of large molecules such as proteins, dielectric constant measurements at radio frequencies have been one of the useful techniques for some time [1—3]. The principle of this technique is based on the orientation of polar molecules in response to the applied electrical field. By analyzing the frequency characteristics of permittivity, we can determine the dipole moment, relaxation time and approximate volume and shape of the macromolecule. Although no information is available as to the size of active elements in excitable membranes, the strong voltage dependence of ionic currents suggests that some polar molecules are involved in the activation and

inactivation of sodium and potassium sites. Recent measurements of gating currents [4—6] and the calculation of dipole moment changes due to ionic currents [7] support the notion by Hodgkin and Huxley [8] that the elementary process of the gate opening and closing may be the orientation of dipole moments (see also Schwan [9]).

The technique of capacitance and conductance measurements (or permittivity and conductivity) has been applied to many passive and excitable membranes [10—14]. Early work by Cole and Curtis and by Cole and Baker [15,16] demonstrate various unique features of nerve membranes. Of particular importance to us is their finding that membrane capacity of nerve axons remained unchanged during the action potential while membrane conductance underwent a tremendous increase. However, recently, Adrian and Almers [17] reported that membrane capacity of sartorius muscle is voltage dependent and that the muscle membrane capacity increased by 50% between —90 and +50 mV. This result indicates that the constancy of membrane capacity does not necessarily apply to all excitable membranes. On the other hand, in view of the complexity of impedance characteristics of muscle membrane [18], the notion obtained with muscle membranes does not necessarily hold for nerve membranes. However, Takashima [19] carried out a similar investigation with squid axons and studied the membrane capacity during depolarization as well as hyperpolarization. He found an increase in membrane capacity with depolarizing pulses when measurements were extended to sufficiently low frequencies and then corrections for the series resistance were properly applied. The nature or the origin of the voltage dependent capacity is still unknown. However, various analyses indicate that the increase in membrane capacity is not due to electrode polarization and that the increase arises either from some structural changes in the membrane or from, as discussed by Chandler et al. [20] using the linearized Hodgkin-Huxley equation, the reactive component of time-dependent ionic conductance.

The experiments done by Takashima were, however, limited to small hyper- and depolarizations within ±40 mV and in addition, the lowest frequency used was 1 kHz. Because of these reasons, he was unable to establish the full extent of capacity changes during hyper- and depolarizations. In the present study, a different electrode arrangement was used and this enabled us to use much larger hyper- and depolarizing pulses without causing deterioration of axon membranes. In addition, we extended the frequency to 0.5 kHz instead of 1 kHz, which was the lower limit in the previous experiments. With these modifications, we were able to measure the full amplitude of the capacity change with sufficiently large external pulses. In addition to these basic experiments, the effects of external tetrodotoxin as well as internal cesium ions on the capacity change were investigated.

Methods

All experiments were performed at the Marine Biological Laboratory, Woods Hole, Mass. using giant axons from squid *Loligo pealei*. Moderately cleaned axons were mounted in an axon chamber with circulating sea water at about 10°C. For the measurement of membrane admittance, a Pt-Ir wire with a

diameter of 75 μm was used as the internal electrode with a careful coating of Platinum Black. The use of a long electrode was preferred in order to minimize the effect of tip impedance as discussed later. Movable ground electrodes were placed on both sides of the axon as close to the axon wall as possible. For the measurement of membrane potential at the resting state and during hyper- and depolarizations, a glass microelectrode filled with 0.6 M KCl solution was used.

(A) Admittance bridge and application of pulses

For the measurement of membrane admittance, a Wayne-Kerr wide band bridge B-221 was used. The bridge has a frequency range between 100 Hz and 20 kHz with seven ranges of capacitance and conductance readings. As the oscillator and detector, a lock-in amplifier PAR model 124 having a frequency range from 2 Hz to 210 kHz was used. The advantage of using this amplifier is, in addition to its high sensitivity and stability, the availability of variable Q values. Therefore, we can optimize the value of Q of the amplifier between 1 and 100 so that the interference due to applied square pulses can be minimized with the minimum loss of sensitivity. Normally, the Q value was adjusted between 5 and 10 for transient capacitance and conductance measurements.

The bridge B-221 is equipped with a neutral line output of the isolation transformer in addition to the outlet for unknown samples. Block and Haynes [21] postulated that large external DC fields can be applied to the sample using the neutral leads of the transformer which isolates the static field from the detector. Thus, the detector is isolated from the disturbance due to the external field and the bridge balancing can be achieved with a very small interference. In our measurements, two neutral leads are connected with a large capacitor of 10 μF and external pulses are applied across this blocking capacitor. By measuring the actual voltage across the membrane using a microelectrode, it was confirmed that the potential applied across the capacitor is indeed reproduced across the membrane. The capacitor is usually inserted to prevent the possible DC shunt of axon membranes. Elimination of this capacitor often resulted in deterioration of axons. Combination of this electrode arrangement and the use of the lock-in amplifier minimized undesirable interference of external pulses on the admittance measurement, which is normally performed with an exceedingly small AC signal. The procedure of transient measurements of membrane admittance with external pulses was already described in the previous publication [19]. It has been shown that the time required for the admittance to reach the quasi steady state is of the order of a few milliseconds although the rise time of applied pulses is 1—2 microseconds. The long transient is mainly due to the presence of the blocking condensor and also due to the time constants of the admittance bridge and amplifier. Because of the delay time in the change in the admittance due to these it is not possible, with the present measuring system, to investigate the time course or kinetics of the change in membrane capacitance and conductance. All the measurements of admittances of polarized membranes were, therefore, performed after the steady state was reached.

The range of hyper- and depolarizing pulses was ±100 mV, i.e., the membrane potential was varied from —160 to +40 mV. As seen later, the change in the membrane conductivity and capacity are nonlinear with the magnitude of

external pulses. However, for the measurement of membrane admittance, an AC field with a small amplitude of 2—3 mV peak-to-peak was used in order to minimize the shift of membrane potential due to the applied AC fields. With these small AC fields, the measured membrane capacity and conductivity were independent of the amplitude of AC fields indicating that the shift of the membrane potential due to AC fields is quite negligible.

(B) Correction for series resistance

In the previous publication, Takashima [19] demonstrated that the correction for the series resistance is a crucial step for the determination of membrane capacity during depolarizing pulses (see also Kishimoto [22]). As shown by Takashima and Schwan [23], the membrane capacity in the presence of a series resistance is given by the following equation.

$$C_P = \frac{CR^2/(R_s + R)^2}{1 + \omega^2 \left\{\frac{CR_sR}{R + R_s}\right\}^2} \tag{1}$$

At sufficiently low frequencies, the second term in the denominator becomes much smaller than 1 and Eqn. 1 reduces to a simple form, i.e.,

$$C = C_P \left\{1 + \frac{R_s}{R}\right\}^2 \tag{2}$$

where C and R are membrane capacitance (C_m) and resistance (R_m), R_s is the series resistance and C_P is the measured capacitance across the membrane including the electrolyte solution between the membrane and electrodes. At the resting state, the membrane resistance is much larger than the series resistance and measured capacitances are virtually equal to C_m. However, the resistance of depolarized membranes decreases considerably and, for a large depolarization, R_m becomes almost comparable to R_s. Under these circumstances, the measured capacitance is usually smaller than the true membrane capacitance. Thus, the correction for R_s is critically important in order to uncover the change in membrane capacitance during depolarizing pulses. In the present work, the series resistance was determined by use of impedance loci as described in detail by Takashima and Schwan [23]. The value of R_s with our present electrode arrangement was found to be between 6 and 10 Ω/cm^2.

(C) Correction for the fringe effect

It was observed by Takashima, Yantorno and Schwan (unpublished data) that the effect of tip impedance of the internal electrode causes a certain error in the value of membrane capacity and conductivity. The capacitance due to the fringe effect amounts to 0.06—0.07 μF (0.17—0.2 $\mu F/cm^2$) and is by no means negligible. As shown later, after these corrections, the membrane capacity of axons decreases to approximately 0.75 $\mu F/cm^2$ from the widely accepted value of 1 $\mu F/cm^2$. Also the value of membrane conductivity becomes much smaller than 1 $m\Omega^{-1}/cm^2$, a value which is ordinarily accepted for squid axon membranes. Thus, the consequence of the correction for fringe effects is far reaching.

Generally, there are two possible methods for the correction of fringe effects.

The one is the use of a guarded internal electrode and the other is the use of electrodes with various lengths. The latter may be, in principle, more straight foward than the former. However, the procedure is very cumbersome and requires repeated measurements of admittance with, ideally, one axon using different electrodes. On the other hand, the fabrication of guarded internal electrodes is very difficult and not practical at present. Therefore, no easy method is available for the correction of fringe effects currently.

For the present experiments, the correction for the fringe effect was performed using internal electrodes having different lengths as mentioned above. Since it is exceedingly difficult to measure membrane admittance repeatedly using one axon, nerve axons having a similar diameter were chosen and measurements of membrane capacitance and conductance were carried out with electrodes having lengths 1, 5, 10, 17 and 23 mm. The capacitances thus obtained are plotted against electrode lengths and the fringe capacitance was estimated from the intersection of the plot with the ordinate. Usually, the fringe capacitance was found to be approx. 0.07 μF (0.2 μF/cm^2). Therefore, we can correct the measured capacitance by subtracting this value. Similar plots were established at frequencies between 100 Hz and 50 kHz and these values were used for the corrections at these frequencies. A simplified procedure is to measure membrane capacitance and conductance at rest and during external pulses using a 23 mm electrode with one axon. Afterwards, we selected another axon with a similar diameter and repeated the same experiment using a 1 mm electrode. The values obtained with the 1 mm electrode were used for the correction of the fringe effect. This method is likely to cause an overcorrection of membrane capacitance by about 0.013 μF (0.04 μF/cm^2) and is, perhaps, acceptable.

(D) Analysis of data and electrode polarization

Analysis of data are based on the equivalent circuit shown in Fig. 1. This is a diagram having the Hodgkin-Huxley sodium activation branch added in parallel to the membrane admittance where the potassium activation and sodium inactivation are lumped together (see Cole [24]).

In this diagram, C_0 is the passive membrane capacitance, R_k and R_2 represent the potassium and leakage conductances. C_1 and R_1 are the capacity and resistivity of sodium channels. This circuit can be simplified by further lumping R_k

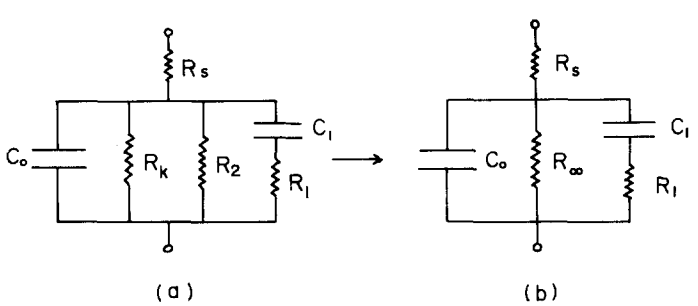

Fig. 1. Equivalent circuit of active squid axon membrane. C_0 is capacitance of passive membrane, R_k and R_2 are potassium and leakage resistances. C_1 and R_1 are capacitance and resistance of active sodium channel and R_s is series resistance. Diagram (b) is obtained by lumping R_k and R_2.

and R_2 and renaming it as R_∞. Analysis of the circuit 1b shows that measured capacitance and conductance are mutually related by Eqn. 3.

$$C = (C_0 + C_1) + \tau(G_0 - G) \tag{3}$$

where $\tau = C_1 R_1$. Eqn. 3 indicates that the plot of measured membrane capacitance against $(G_0 - G)$ will be a straight line if the diagram 1a and/or 1b are the correct presentation of active membranes. It must be noted that the active membrane is characterized by one time constant of the sodium site. The slope of the plot gives τ, and the intersection of the plot with the ordinate gives the sum of passive membrane capacity and that of active sodium channels, C_1. As shown later, the plot of C vs. $(G_0 - G)$ is a straight line for resting, hyperpolarized and moderately depolarized membranes. Deviation from the linearity indicates the presence of other processes which cause the distribution of time constants or the presence of electrode polarization. The plot deviates markedly from the linearity with large depolarizations. As well know, electrode polarization becomes significant when the conductance of the sample is high. Since membrane conductance becomes very high with large depolarizing pulses, the deviation of the plot from the linearity may be due to the presence of electrode polarization. However, it is also possible that the deviation is due to the increasing contribution of another process such as potassium activation.

(E) Voltage clamp experiments

For all voltage clamp experiments, a potential —60 mV was selected as the holding potential without a prepulse. We used a series resistance compensation up to the 2/3 of the total value. Pt-Ir wires with diameters 75 and 25 μm were used as the current and voltage electrodes. They are coated with Platinum Black and the offset of electrode potential was within ±3 mV. Sodium rate constants α_m and β_m are calculated using the Hodgkin-Huxley equation. Assuming the Q_{10} value to be 3, all kinetic parameters α_j and β_j ($j = m,n,h$) are scaled to the experimental temperature, i.e., 10°C. Further fine adjustments were carried out by fitting the calculated curves with those measured at each depolarization between —40 and +60 mV. The curve fitting was performed using a computer PDP9 combined with a X-Y recorder using a plotting subroutine. The sodium equilibrium constants at each depolarization were calculated by $K_m = \alpha_m/\beta_m$ and plotted against the field strength in the semilogarithmic scale.

Results

(a) Membrane capacitance and conductance of resting and hyperpolarized membranes

The capacitances and conductances of resting and hyperpolarized membranes at various frequencies are shown in Fig. 2. The consequence of correcting for the fringe effect is clearly demonstrated in this figure. First of all, the membrane capacity of resting axons is found to be about 0.75—0.8 μF/cm^2 as an average. Before the correction, the membrane capacity of squid axons at the resting state is approximately 1.0 μF/cm^2 which is in agreement with the value obtained by Cole and Curtis [10]. Therefore, the correction for

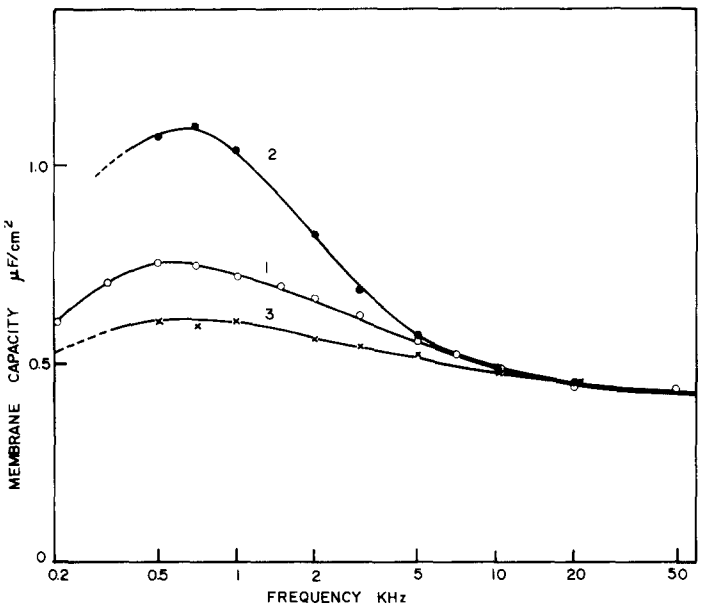

Fig. 2. Capacities of resting (—60 mV), hyperpolarized (—160 mV) and depolarized (+20 mV) membranes. Curves 1, 2 and 3 are for resting, depolarized and hyperpolarized membranes respectively.

the fringe effect results in a decrease of the membrane capacity by about 20—25%. Hyperpolarization further decreases the membrane capacity and it reduces to about 0.65—0.70 $\mu F/cm^2$ at —180 mV. At this membrane potential, all the Hodgkin-Huxley parameters are either 1 or 0 and the membrane is perhaps in the passive state. Curve 3 in Fig. 2, which represents the frequency characteristics of passive membrane capacitance, is still slightly frequency dependent. This means either that there is still some uncorrected errors or that membrane capacity of passive axons is truly frequency dependent.

The correction for the fringe effect has a substantial effect on the membrane conductivity. Conductivities of resting and hyperpolarized membranes at low frequencies become far smaller than 1 $m\Omega^{-1}/cm^2$, the value commonly used as the conductivity of resting axon membranes. For example, the conductivity of resting membrane is about 0.2—0.3 $m\Omega^{-1}/cm^2$ after the correction and that of hyperpolarized membranes is even smaller than 0.1 $m\Omega^{-1}/cm^2$. Therefore, the conductivity of a passive membrane is much smaller than the value ordinarily accepted. Under these circumstances, the leaky condensor model (a phase angle less than 90° C) may not be an accurate presentation of nerve membranes at rest.

(b) Membrane admittance of depolarized membranes

It is well known that depolarization causes a drastic increase in the membrane conductivity. Our results indeed indicate that the conductivity increases with a 80 mV depolarizing pulse is more than 100-fold if compared with that of the hyperpolarized membrane. The conductivity changes with various depolarizing pulses are shown in Fig. 3.

The capacities of depolarized membranes are shown in Fig. 2 (curve 2)

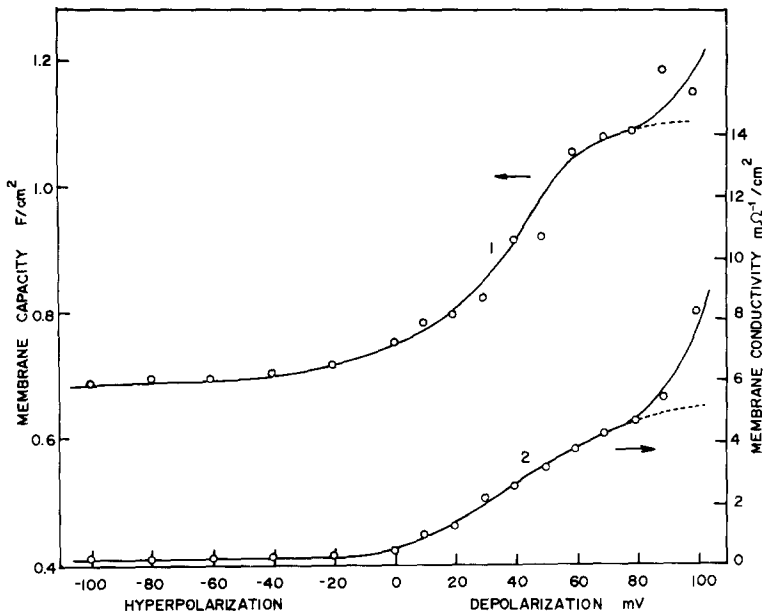

Fig. 3. Changes in capacity and conductivity at various membrane potentials. Curve 1, membrane capacity; curve 2, membrane conductivity. The zero potential indicates the resting state (—60 mV). The left ordinate is membrane capacity and the right ordinate, membrane conductivity. Frequency at 0.5 kHz. This figure represents one of the results without averaging.

Analogous to the previous measurement by Takashima [19], the membrane capacity increases with depolarizations if corrections for series resistances are applied and if measurements are extended to sufficiently low frequencies. The capacity increase with various depolarizing pulses is shown in Fig. 3 at 0.5 kHz. The curve indicates a capacity increase from 0.68 to 1.2 μF/cm^2 between membrane potentials —160 and +40 mV and reaches a saturation with 80—100 mV depolarizations (+20—+40 mV). This result is considerably different from the observation by Cole and Baker [16] who concluded that membrane capacity is independent of membrane potential. However, it must be emphasized that the difference arises only from our corrections for the series resistance and fringe effects. Without these corrections, the capacitance increases would not have been observed even with large depolarizations. A similar remark was already made by Kishimoto [22] for excitable Chara cell membranes and he pointed out that the correction for series resistance revealed a substantial increase in membrane capacitance of Chara cells during action potentials.

The plots of measured capacities (after corrections for series resistances and fringe effects) against $G_0 - G$ are shown in Fig. 4 at various membrane potentials. It is worthwhile to point out that the plots are remarkably linear with the resting and moderately depolarized membranes. The indication is that the equivalent circuit 1a or 1b are correct presentations of depolarized axon membranes under these conditions. The time constants determined from the slopes of these plots are tabulated in Table I. The time constant τ for the sodium activation calculated with the Hodgkin-Huxley equation are also shown in the same table. Clearly, observed time constants are one order of magnitude

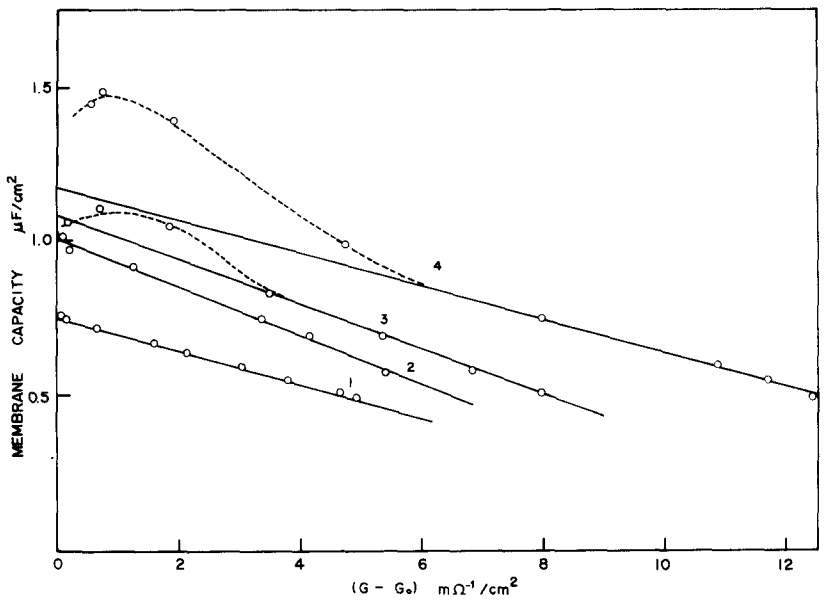

Fig. 4. The plot of membrane capacities against ($G - G_0$) at various depolarizations. Curve 1, resting membrane; curves 2, 3 and 4 with 40, 80 and 100 mV depolarizing pulses. Dotted lines connecting points indicate the observed values. Time constants and membrane capacities for curves 3 and 4 are obtained using the linear extrapolation.

smaller than those calculated except for large depolarizations. The plots begin to deviate from linearity with large depolarizations, and the non-linearity becomes pronounced above 80 mV as shown by dotted curves in Fig. 4. The deviation from the linearity indicates either the presence of electrode polarization or the contribution of additional processes such as the onset of potassium currents. For these cases, straight lines are drawn using points in the linear region. Membrane capacity and time constant are determined using the straight line by extrapolation.

(c) Membrane admittance change in the presence of tetrodotoxin

In these experiments, natural sea water was replaced by artificial seawater containing $3 \cdot 10^{-7}$ mol tetrodotoxin in order to block the sodium current. Other experimental procedures are exactly the same as before. The capacity and conductivity of axon membranes were measured at the resting state and with hyper- and depolarizing pulses. A few examples of measured values are shown in Table II. As shown, considerable decreases in the measured capacitance and increase in conductance are obvious when the membrane is

TABLE I

TIME CONSTANTS OF SODIUM ACTIVATION MEASURED BY MEMBRANE CAPACITY AND CALCULATED BY HODGKIN-HUXLEY EQUATIONS

Depolarization (mV)	0	20	40	60	80	100
Time constant observed (ms)	0.052	0.054	0.073	0.076	0.074	0.051
Time constant calculated (ms)	—	0.479	0.357	0.111	0.075	0.057

TABLE II

MEMBRANE CAPACITIES AND CONDUCTIVITIES AT REST AND DURING A 60 mV DEPOLARIZATION IN THE PRESENCE OF $3 \cdot 10^{-7}$ MOL TETRODOTOXIN IN ARTIFICIAL SEAWATER

	Uncorrected		Corrected *	
	Conductivity ($m\Omega^{-1}/cm^2$)	Capacity ($\mu F/cm^2$)	Conductivity ($m\Omega^{-1}/cm^2$)	Capacity ($\mu F/cm^2$)
Resting	1.41	0.951	1.43	0.955
Depolarization	7.99	0.751	8.66	0.914

* No correction for fringe effects.

depolarized. The values of capacitances and conductances after the correction for the series resistance are also shown in this table. Undoubtedly, the apparent capacitance decrease is merely due to the presence of series resistances and the correction for R_s brings measured capacitances back to the level of the resting membrane. Fig. 5 illustrates the constancy of membrane capacity over a wide range of membrane potential in the presence of tetrodotoxin. In spite of the constancy in membrane capacity during depolarizations, membrane conductivity indicates a considerable increase as shown in Fig. 5. In any event, this experiment demonstrates that tetrodotoxin eliminates capacity changes of polarized membranes and suggests that the voltage-dependent membrane capacity may arise from the sodium channel.

(d) Membrane admittance with external tetrodotoxin and internal cesium ion

In these experiments, axons were internally perfused with the method

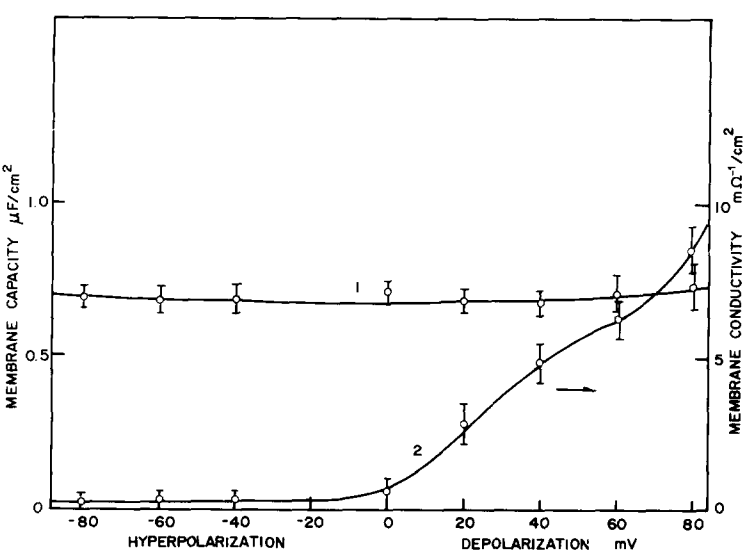

Fig. 5. Capacities and conductivities of squid axons at various membrane potentials in the presence of $3 \cdot 10^{-7}$ mol tetrodotoxin in artificial seawater. Curve 1, membrane capacity (left); curve 2, membrane conductivity (right). Frequency at 0.5 kHz. The zero potential indicates the resting state (—60 mV). The standard deviation is based on nine measurements.

described by Gilbert [25]. The internal potassium ion was replaced with Cs ion which is essentially impermeable to the axon membrane. After the internal perfusion, natural seawater was replaced by artificial seawater containing $3 \cdot 10^{-7}$ mol tetrodotoxin. Therefore, under these conditions, both sodium and potassium channels are blocked nearly completely. As before, membrane capacity and conductivity were measured at the resting state as well as with hyper- and depolarizing pulses. It was found that in the absence of sodium and potassium currents, changes in membrane capacity and conductivity are very small even with large depolarizations. In Fig. 6, membrane capacity and conducitivity are plotted against the membrane potential. This figure shows that changes in membrane capacity and conductivity are minimal for a wide range of the membrane potential in the absence of ionic currents.

This observations as well as the one mentioned in (c) above indicate that changes in membrane capacity and conductivity are closely related to the ionic channels. To summarize, we observed that (1) tetrodotoxin-sensitive capacity change and (2) tetrodotoxin-insensitive or cesium-sensitive conductivity changes are apparently two separate processes. The question why we did not observe tetrodotoxin-insensitive or cesium-sensitive capacity change may be answered by the fact that the cesium-sensitive capacity change is perhaps due to the potassium channels and is likely to be located at very low frequencies. Our technique is simply incapable of observing it because of the frequency limitation. Another question why we did not observe a tetrodotoxin-sensitive conductivity change is difficult to answer. It should be noted in Figs. 3 and 5 that the conductivity change in the presence of tetrodotoxin is as much as or

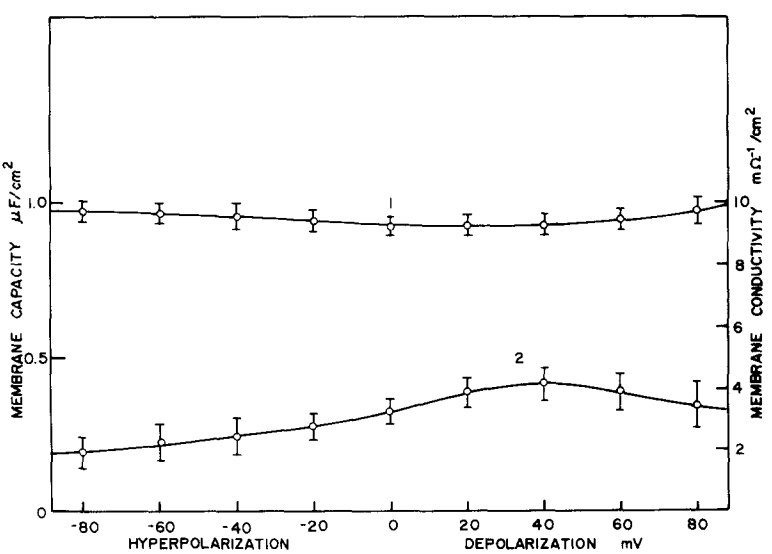

Fig. 6. Capacities and conductivities of squid axons at various membrane potentials. Axons are internally perfused with a cesium internal perfusion solution and external solution is artificial seawater containing $3 \cdot 10^{-7}$ mol tetrodotoxin. The zero potential indicates the resting state (—30 mV). Note that cesium mixture causes depolarization of the membrane. Action potential, 120 mV and 150 ms. The standard deviation is based on 12 measurements. Curve 1 is membrane capacity, curve 2 is conductivity.

even greater than the change without it. These observations call for further investigations of the problem.

The results reported in this paper indicate certain similarities between the voltage dependent membrane capacity and the gating current observed by Armstrong and Bezanilla [4] and by Keynes and Rojas [5]. However, these results also suggest subtle differences between these two processes. Above all, Armstrong and Bezanilla showed that the gating current is tetrodotoxin insensitive while our results indicate that the voltage dependent membrane capacity is tetrodotoxin sensitive. Moreover, in the presence of internal cesium and external tetrodotoxin, neither capacitance nor conductance change was observed. Fishman, Moore and Poussart [26] performed measurements of admittance changes at ultralow frequencies under similar conditions and observed a decrease in membrane capacitance with depolarizations. Our results also indicate a small decrease of membrane capacitance with some depolarizations and are in agreement with those by Fishman et al. These observations suggest either that the origin of gating current and membrane capacitance changes are different or that some inactivation process blocks the membrane capacitance change in the absence of ionic currents (Bezanilla, personal discussion).

(e) Calculation of rate constants and determination of dipole moment change

As stated before, the Hodgkin-Huxley parameters α_m and β_m are determined using the voltage clamp data. The procedure is to calculate α_j and β_j ($j = m,n,h$) using the H-H equation for 6.3° C. Assuming Q_{10} to be 3, all these parameters and sodium and potassium conductances are scaled to those at 10° C at which all experiments were carried out. Calculated ionic currents are compared, after summation, with experimental curves for each depolarization. Usually, fine adjustments were necessary by trial and error to match the calculated curve with those observed. The equilibrium constants calculated for each depolarization are plotted in a semi-logarithmic scale vs. the field E in cgs units and shown in Fig. 7. From the slope of this plot, we can calculate the dipole moment change as discussed below. If a reaction

$$A \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} B$$

is characterized by a change in Gibbs free energy dG, the change is a function of temperature (T) and pressure (P). The dependence of rate constants on the field strength can be interpreted as the indication of involving changes in dipole moment. Therefore,

$$dG = -SdT + VdP - MdE \tag{4}$$

where S is entropy, V is volume and M is dipole moment. Under isothermal and isobaric conditions, the dependence of equilibrium constant on field strength E is given by the well known equation (see Schwarz [27] and Levitan and Palti [7]).

$$\frac{\partial \ln K}{\partial |E|} = \frac{\Delta M}{RT} \tag{5}$$

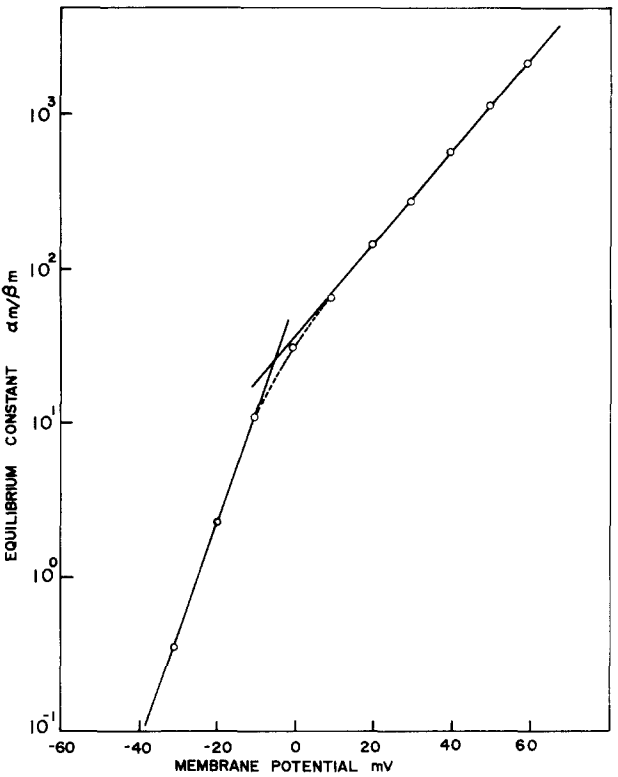

Fig. 7. Plot of equilibrium constants K against the membrane potential in mV. Equilibrium constants are calculated by $K = \alpha_m/\beta_m$. Field strength in statvolts are calculated by the following formula: $E = (V - V_r)/d \times 0.0033$ where V and V_r are membrane potentials of depolarized and resting axons in volts and d is the membrane thickness in centimeters, i.e. 80 Å.

where R is the gas constant. Thus, the plot of ln$K$ at various field intensities E electrostatic units (cgs) will give the change in dipole moments. As shown in Fig. 7, the plot of ln$K$ vs. E consists of two linear portions. The one at small depolarizations yields a dipole moment change of 1230 D.U. (1 D.U. = 10^{-18} electrostatic units (cgs) and the slope with large depolarizations gives 930 D.U. These values are slightly larger than those given by Levitan and Palti [7] but by and large in good agreement qualitatively. The discontinuity of the ln$K$ vs. E plot is observed also with α_m and β_m values which are directly calculated with the Hodgkin and Huxley equations for 6.3° C. This observation, also reported by Levitan and Palti [7], indicates the presence of two steps in the activation of sodium channels. Unfortunately, we did not observe two steps in the increase of membrane capacitance with depolarizations. In order to correlate the dipole moment change and increase in membrane capacitance, we had to use the over-all change in dipole moment ignoring the discontinuity in the ln$K$ vs. E plot. The relationship between dipole moment and permittivity (consequently, capacitance) is given by the following equation [2].

$$\Delta M^2 = (9kTM_r/4Nhc)\Delta E \qquad (6)$$

where N is Avogadro's number, k is the Boltzman constant, h is an empirical

parameter having a value of 5.8 for many macromolecules. ΔE is the increment of permittivity and c is the weight fraction of the solute, i.e., particles in sodium channels. Since the change in dipole moment and that of membrane capacity (or permittivity) are determined experimentally, we can reduce Eqn. 6 to a simple form by substituting numerical values. Namely,

$$M_r/c = 3.9 \cdot 10^7 \tag{7}$$

for 10° C. If we know the value of c, i.e., the fraction of sodium sites per unit area or volume, we can calculate the molecular weight or size of active elements in sodium channels. Since the value of c is not known, we cannot solve Eqn. 7 uniquely.

Discussion

The present research confirmed the results reported previously by Takashima [19] that membrane capacity of squid axons is voltage dependent and increased with depolarizations. Using a different electrode arrangement from the previous one, we were able to use larger hyper- and depolarizing pulses and investigate the full extent of admittance changes during these pulses. Although the nature or the origin of the capacity increase is not well understood, the results reported in this and previous papers indicate the involvement of molecules having a large dipole moment or the formation of space charge due to the movement of ions. On the other hand, the strong voltage dependence of ionic currents was interpreted by Levitan and Palti [7] in terms of a dipole moment change during depolarizations. By inter-relating the dipole moment change determined by voltage clamp experiments and the capacity increase measured by an AC bridge method, we obtained a simple equation (7). This equation, unfortunately, contains two unknowns and cannot be solved uniquely unless the value of either one of them is available.

The change in membrane capacity observed by us may be related to the gating current observed by Armstrong and Bezanilla [4] and Keynes and Rojas [5]. However, according to Bezanilla and Armstrong [28], the gating current is unaffected by tetrodotoxin which blocks sodium currents selectively. Contrary to the gating current, the voltage-dependent membrane capacitance is sensitive to tetrodotoxin. Addition of tetrodotoxin in seawater blocks the change in membrane capacity nearly completely. These results indicate that the capacity change and gating current may have different origins or the presence of inactivation process which is effected by tetrodotoxin (see Fishman et al. [26] and Bezanilla, personal discussion).

The origin or the nature of the voltage-dependent membrane capacity is still unknown. We observed, as mentioned before, tetrodotoxin-sensitive capacity and cesium-sensitive conductivity changes. In addition, we suspect that the tetrodotoxin-sensitive capacity is coupled with a tetrodotoxin-sensitive conductivity. In view of these considerations, the voltage dependent membrane capacity is closely related to the activation of sodium channels. In general, we can think of two possible origins of the voltage dependent membrane capacity. (a) The reactance component of time-dependent ionic currents

[20] and (b) orientation of permanent dipoles in the membrane. The calculation by Chandler et al. is based on linearization of the Hodgkin-Huxley equation and holds only for small depolarizations. On the other hand, we have very little information as to the polar molecules in the membranes. The inherent difficulty of the study with biological membranes is that those membranes are not purified systems but a mixture of essential and non-essential components. Under these circumstances, we must answer the question to what extent are the observed capacitance change, observed by us and gating currents, observed by others, due to components which are truly essential to the gating mechanism?

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