THE pH DEPENDENCY OF RELATIVE ION PERMEABILITIES IN THE CRAYFISH GIANT AXON

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ABSTRACT The dependence of the membrane potential on potassium, chloride, and sodium ions, was determined at the pH's of 6.0, 7.5, and 9.0 for the resting and depolarized crayfish ventral nerve cord giant axon. In normal saline (external potassium = 5.4 mM), the dependence of the membrane potential on the external potassium ions decreased with lowered pH while that for chloride increased. In contrast, in the potassium depolarized axon (external potassium = 25 mM), the dependence of the membrane potential on external potassium was minimum around pH 7.5 and increased in either more acidic or basic pH. In addition, the dependence of the membrane potential on external chloride in the depolarized axon was maximum at pH 7.5 and decreased in either more acidic or basic pH. The sodium dependency of the membrane potential was small and relatively unaffected by pH or depolarization. The data are interpreted as indicating a reversible surface membrane protein-phospholipid conformation change which occurs in the transition from the resting to the depolarized axon.

INTRODUCTION

One common characteristic of cell surface membranes is that of selective ion permeability. There is much evidence indicating that specific membrane ion permeabilities are involved in controlling membrane potential and that changes in ion permeabilities give rise to membrane excitability. However, the physical and chemical events which are involved in permeability regulation are currently not well understood. Thus, for example, attempts to discern structural membrane changes by examining the membrane capacity during an action potential have given negative results (Cole and Curtis, 1939; Strickholm, 1962). Although light scattering changes in nerve have been found to occur following one or several action potentials (Hill, 1950; Bryant and Tobias, 1952; Cohen et al., 1968; Tasaki et al., 1968), these observations have been interpreted in part as possibly due to water movement and volume changes (Tobias and Nelson, 1959). Ungar and Romano (1962) found an

absorption spectra change in protein following membrane excitation but these changes have been interpreted by Luxoro et al. (1963) as resulting from calcium ions being transported and interacting with cell proteins. Evidence for a physical-chemical change during the action potential comes from the work of Kornakova et al. (1952, 1957) and Kayushin and Lyudkovskaya (1954, 1955) where changes in nerve stiffness and diameter were found to occur with the action potential. Recently, Cohen, Keynes, and Hille (1968) reported changes in membrane birefringence during the actional potential which were voltage dependent and possibly could be due to molecular reorientation in the membrane. There are also the innumerable observations of impedance changes during the action potential which are ordinarily interpreted as a conductance change (Cole and Curtis, 1939), but which may have additional physical interpretations (Teorell, 1951, 1953; and Strickholm, 1962).

This paper describes experiments designed to investigate the possibility that fixed membrane charges might be involved in ionic permeability regulation and excitation (Teorell, 1953). If the membrane fixed charges are primarily dissociable amino, carboxyl, and phosphate groups, varying the external saline pH should change the membrane fixed charge distribution and thus possibly exert some effect on specific membrane ion permeabilities. In these experiments, the membrane potential dependence on various ions with pH was measured for resting and depolarized nerve. Since membrane excitation transiently occurs in the change from resting to depolarized nerve, any change observed between resting and depolarized nerve might possibly reflect the end points of a membrane reorganization (charge distribution, protein-phospholipid conformation, etc.) which might occur in excitation.

METHODS

Giant axons, 100–200 μ diameter, from the ventral nerve cord of the West Coast crayfish (*Procambarus clarkii*) were used in these experiments. The dependence of the membrane potential on chloride, potassium, and sodium was determined by the rapid external ion shift method which has been described in detail (Strickholm and Wallin, 1967). This technique consists of measuring the early change in membrane potential ΔV_m as a result of changing the concentration of one external ion by substitution with an impermeable ion. In these substitutions, no significant change in activity coefficients or osmotic pressure were detected. From this operation is determined the specific ionic membrane potential dependency for the ith ion as: $T_i = \Delta V_m/\Delta V_i$, where $\Delta V_i = (RT/F)\ln(C_{2i}/C_{1i})$, with C_{1i} and C_{2i} being the initial and final external ion concentrations. T_i is not necessarily a membrane transference number (Strickholm and Wallin, 1967) although for certain membrane models it may have this definition (Hodgkin and Horowicz, 1959). In the experiments of Strickholm and Wallin (1967), attempts to consider T_i as a transference number on the basis of the equivalent electrical circuit model (Finkelstein and Mauro, 1963; and Hodgkin and Horowicz, 1959) were not successful.

If the above operations on the membrane potential are performed by varying separately the external concentrations of sodium, chloride, and potassium ions to obtain T_{Na}, T_{Cl}, and T_K, the relative chloride to potassium permeability ratio P_{Cl}/P_K can be calculated from the

constant field equation to give: $P_{Cl}/P_K = r_1 = T_{Cl}K_0\xi/T_KCl_0$, where K_0 and Cl_0 are the potassium and chloride external concentrations and $\xi = \exp(-V_mF/RT)$. Similarly, it is found that $P_{Na}/P_K = r_2 = T_{Na}K_0/T_KNa_0$. The relations obtained above appeared to satisfy the data for resting nerve. However, for depolarized nerve, the agreement between the data and the above equations, which were derived from the constant field equation, was unsatisfactory. Agreement with the equivalent electrical model was equally unsatisfactory. Therefore, in this paper, although r_1 and r_2 are also calculated for depolarized nerve, the only meaningful parameter for this situation is perhaps the operational definition: $T_i = \Delta V_m/\Delta V_i$ which defines the measurement made (i.e., the change in membrane potential corresponding to a change in external ion concentration).

The external crayfish salines of various pH's in which T_K, T_{Cl}, T_{Na} were measured are shown in Table I. Solution (*a*) is the normal crayfish saline which was adjusted to pH 7.5 by adding small amounts of HCl. These solutions (*a* to *e*) were duplicated at an elevated potassium concentration of 25 mM where potassium ions replaced sodium one for one. The pH values of 6.0, 7.5, and 9.0 were chosen since this represented the pH range which the axon could temporarily tolerate for a short period (10 min) without undergoing serious deterioration and irreversibility. Axon failure was more rapid below pH 6.0 than above 9.0. Solution pH was measured in every experiment.

These different pH buffer solutions were utilized to ascertain whether the buffer ions affected the results and also whether intracellular pH changes could possibly have an influence. Caldwell (1958) has provided evidence which indicates that internal pH will vary with external pH in a bicarbonate saline but probably not in phosphate or Tris buffer. Thus, solution (*a*), in which axon dissection was done, or solution (*d*), presumably standardized intracellular pH and the changes into solution (*b*), (*c*), or (*e*) should only have changed external pH. To check these points, intracellular pH was examined in some experiments by intracellular perfusion or injection with saline containing KF = 230 mM, NaCl = 15 mM, and a pH sensitive dye (Brom Thymol Blue, Cresol Red, or Phenol Red).

To obtain T_K in normal saline at each of the designated pH's, external potassium was changed from 5.4 to 10 mM by direct substitution of potassium ion for sodium. Similarly, T_{Cl} was obtained by changing external chloride from 242.6 to around 131 mM by direct replacement of chloride ion with glucuronate or isethionate. T_{Na} was measured only in solutions (*a*), (*d*), and (*e*) by reducing sodium to 115 mM by replacing sodium ion with choline or Tris (Hydroxymethyl)aminomethane.

TABLE I

EXTERNAL CRAYFISH SALINE

	Phosphate Buffer	Tris-Base: Tris-Maleate Buffer	Tris-Base: Tris-HCl Buffer	$NaHCO_3$	KCl	NaCl	$CaCl_2$	$MgCl_2$	Approx. pH
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	
(a)	—	—	—	2.3	5.4	205	13.5	2.6	7.5
(b)	10	—	—	—	5.4	205	13.5	2.6	6.0
(c)	—	—	10	—	5.4	205	13.5	2.6	9.0
(d)	—	10	—	2.3	5.4	205	13.5	2.6	6, 7.5, 9.0
(e)	—	10	—	—	5.4	205	13.5	2.6	6, 7.5, 9.0

In an experiment, T_{Cl}, T_{Na}, and T_K were measured initially at normal potassium levels (5.4 mM) sequentially at the pH's of 7.5, 6.0, 7.5, 9.0, and 7.5. In some experiments the reverse order of pH was utilized to insure that hysteresis effects in the response of the surface membrane to pH did not affect the data. Normally, no obvious hysteresis was found. The pH solutions utilized in an experiment used one of the three following buffer combinations: (1) the solutions (*a*), (*b*), and (*c*) at pH 7.5, 6.0, and 9.0; (2) solution (*d*) at pH's 6.0, 7.5, 9.0; and (3) solution (*e*) at pH's 6.0, 7.5, 9.0.

If the axon appeared healthy after these ionic and pH changes, external potassium was elevated to 25 mM by one to one replacement of potassium for sodium. T_K was measured by changing potassium to 40 mM by equal replacement of potassium ion for sodium and T_{Na} and T_{Cl} were measured as described before at each pH. Some experiments were started initially at elevated external potassium to avoid possible artifacts which might have resulted from pH manipulations at the normal potassium level. The results in these various situations were the same indicating reversibility over the range of buffer type, pH and ions utilized.

An experiment was considered satisfactory if the resting potential did not deteriorate by more than a few millivolts over the course of the experiment and an action potential remained. This was of importance since both T_{Cl} and T_K are membrane potential dependent. T_{Na} is however, not much affected by membrane potential in a healthy axon.

RESULTS

Fig. 1 shows typical membrane potential changes observed when external pH is suddenly altered for nerve in resting and elevated external potassium. At resting potassium levels (K_0 = 5.4 mM) a lowering of pH decreases the dependence of the membrane potential on potassium (T_K), raises T_{Cl} and lowers the membrane potential (Figs. 1 and 2). No significant change in T_{Na} with pH has been observed. In resting nerve, the variation of T_K and T_{Cl} with membrane potential has been found to be -0.0028 and $+0.0046$ per millivolt change in membrane potential. The observed change in T_K and T_{Cl} with pH (Figs. 2 and 4) cannot, therefore, be accounted for on the basis of the change in membrane potential with pH observed in Fig. 1. In this crayfish axon, the Nernst equilibrium potential for potassium and chloride have been found to be respectively greater and less than the resting mem-

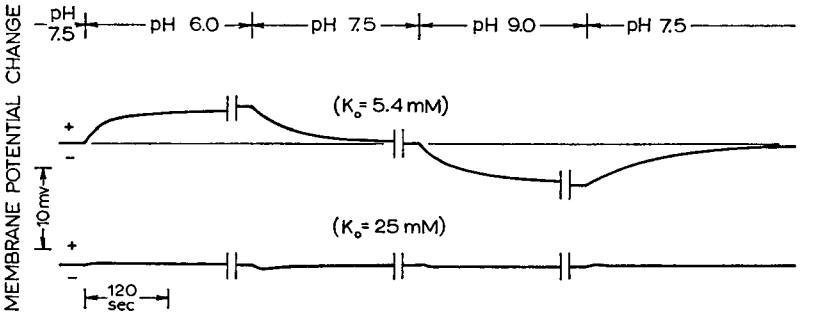

FIGURE 1 The dependency of membrane potential on external pH in the resting (K_0 = 5.4 mM) and depolarized (K_0 = 25 mM) crayfish axon.

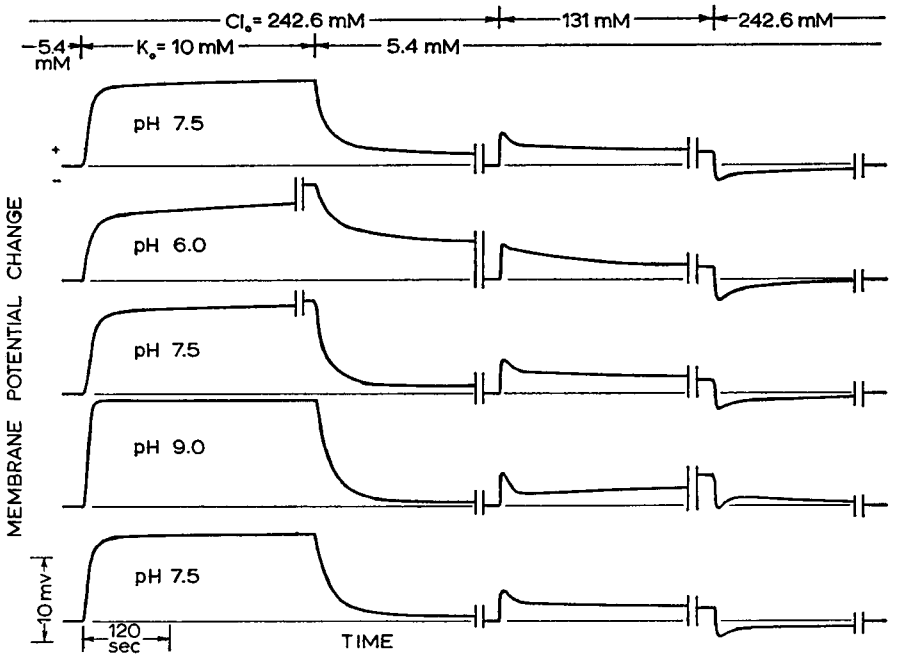

FIGURE 2 The dependency of the membrane potential on potassium and chloride as a function of pH in the resting crayfish axon (K_0 = 5.4 mM). The data was obtained sequentially at pH 7.5, 6.0, 7.5, 9.0, and 7.5 in order to check for reversibility. The early potential transients observed when chloride is changed represent transient liquid junction potentials at the axon surface and reference electrode.

brane potential, (Strickholm and Wallin, 1965, 1967; Wallin, 1966, 1967). The observed decrease in membrane potential with lowering pH is therefore consistant with the known ionic equilibrium potentials and the observed decrease in T_K and increase in T_{Cl} with lowered pH.

Fig. 3 shows the dependence of the membrane potential on potassium and chloride with pH in elevated potassium (K_0 = 25 mM). No significant change in T_{Na} with pH or from resting to depolarized nerve was observed. The concentration of 25 mM for external potassium was chosen because T_{Cl} and T_K are not very sensitive to changes in membrane potential at this and smaller membrane potentials (Strickholm and Wallin, 1967). Thus, at K_0 = 25 mM, any fluctuations in T_K and T_{Cl} due to small potential shifts with pH changes are minimized. Also, reversibility is more readily obtainable at K_0 = 25 mM than higher potassium values. In addition, the membrane potential change when going from 5.4 to 25 mM K_0 is 30 mv which if applied as a voltage depolarization is more than sufficient to elicit an action potential.

Fig. 4 depicts the variation of T_K, T_{Cl}, and T_{Na} with pH at K_0 = 5.4 mM and K_0 = 25 mM. The general form of the data did not depend on the various buffer

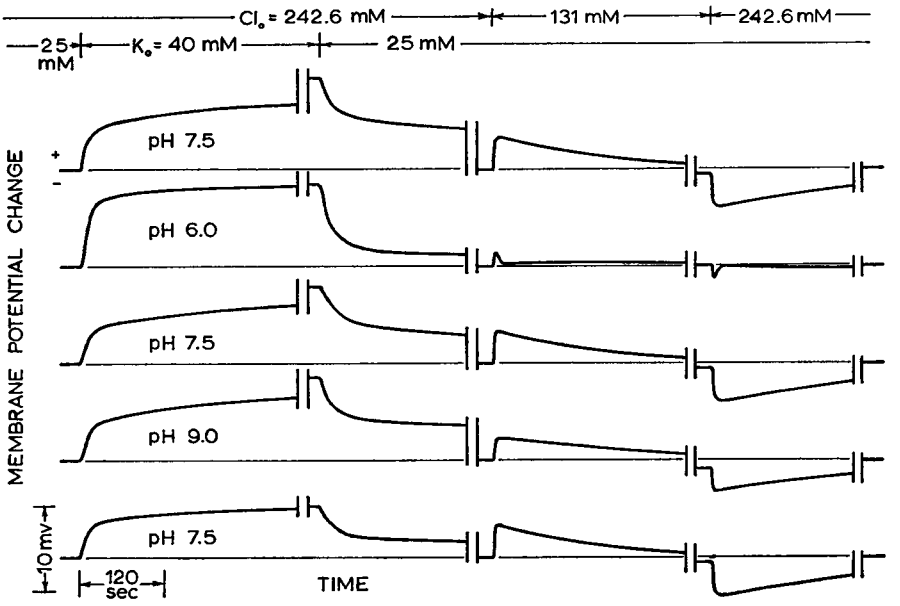

FIGURE 3 The dependency of the membrane potential on potassium and chloride as a function of pH in the depolarized crayfish axon. The data was obtained sequentially at pH 7.5, 6.0, 7.5, 9.0, and 7.5.

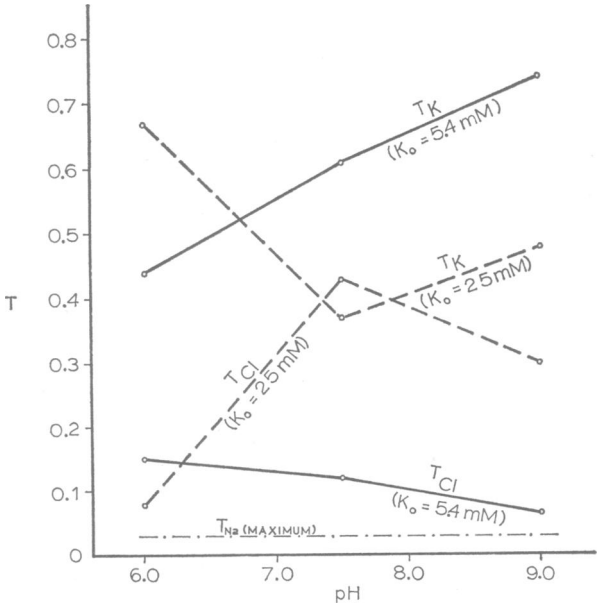

FIGURE 4 The variation of T_K, T_{Cl}, and T_{Na} with pH for resting (K_o = 5.4 mM) and depolarized (K_o = 25 mM) crayfish nerve.

TABLE II

COMPILED RESULTS OF EXTERNAL ION CHANGES

	pH	V_m	T_K	T_{Cl}	P_{Cl}/P_K
		mv			
K_0 = 5.4 mM (resting nerve)	6.0 (11)*	80.4	0.44	0.15	0.19
	7.5 (12)	84.9	0.61	0.12	0.12
	9.0 (9)	88.4	0.74	0.065	0.06
K_0 = 25 mM (depolarized nerve)	6.0 (8)	54.7	0.67	0.08	0.11
	7.5 (8)	55.0	0.37	0.43	1.05
	9.0 (7)	55.0	0.48	0.30	0.56

* Numbers enclosed in parentheses indicates number of measurements.

solutions utilized (*a* to *e*), and indicates that there is a difference in the ionic dependence of the membrane potential on pH between resting and depolarized nerve. It should be noted that if the *T*'s are interpreted as transference numbers in the equivalent electrical circuit model, the sum, $T_K + T_{Cl} + T_{Na}$ should equal unity. This is not observed here (Fig. 4).

In the experiments with injected pH sensitive dyes, no change in intracellular pH was detected in solution (*e*) when pH was changed (from pH 5.5 to 9.2) for the resting and potassium depolarized nerve. Axon failure usually occured within a period of 10 min with the dyes utilized. When the axon failed and lost its membrane potential, intracellular pH changed with extracellular pH. The results therefore suggest that the observed pH effects result from changes on the axon external surface and support the view that the outer surface membrane may be a fixed charge membrane (Tasaki, 1968). However, this interpretation needs further examination with an intracellular pH electrode since the kinetics of pH change across the membrane is in part affected by the dye concentration which was high to allow for visible observations.

In Table II are compiled averages for the data presented here. T_{Na} in these experiments was usually near zero and within the range of experimental error (± 0.03) for the various pH solutions. An upper limit for P_{Na}/P_K is then 1.8×10^{-3} for resting nerve and 10^{-2} for depolarized nerve.

DISCUSSION

One interpretation of the data at normal external potassium level is that ionizable fixed charges on the membrane surface contribute to the regulation of membrane permeability. In the theory of fixed charge membranes (Teorell, 1951, 1953) a decrease in pH would be expected to increase the ratio of positive to negative bound charged groups and thus raise the permeability ratio of chloride to potassium. This behavior is observed in Fig. 4 for resting nerve. This data also suggests an acidic

isoelectric point for the membrane fixed charges. These results are consistent with data from frog nerves and crayfish muscle which suggest the presence of fixed charges which become protonated in acidic pH (Hille, 1968; Hagiwara et al., 1968), and with observations on red cells where the surface isoelectric point is estimated around pH 1.5 to 2.0 (Furchgott and Ponder, 1941; Cook et al., 1961; Seaman and Ullenbruch, 1963). In contrast to that above, the chloride conductance in frog skeletal muscle cells has been observed to decrease with decreasing pH which is opposite to the observations presented here (Hutter and Warner, 1967).

However, the data observed here at elevated potassium are not readily explainable by a simple fixed charge theory as outlined above. A specific explanation cannot be given at present but several points are worth mentioning. For one, the membrane characteristics at normal pH change when the axon is potassium depolarized. Thus, at normal resting potential and neutral pH, the permeability ratio for chloride to potassium is 0.13, but depolarized by a roughly fivefold increase in external potassium, it is 0.91 (Strickholm and Wallin, 1967). This effect is quite reversible and probably reflects the labile character of the surface membrane.

Secondly, it is probably an oversimplification to discuss the effect of pH on the cell membrane only in terms of the number of fixed charge groups, as can be done in a rigid porcelain membrane (Teorell, 1951, 1953). In a biological membrane, other changes in addition to local ionizations are likely to occur with pH variation. It is well known that proteins change their tertiary structure with pH (Tanford, 1961) and for cell membrane proteins this could have profound effects on membrane permeability (Pardee, 1968). Further support for such a view comes from studies on thin black artificial phospholipid membranes where ionic permeabilities are low when the membranes contain only phospholipid and a filler (tetradecane etc.). However, with the addition of certain proteins to these membranes, the ionic permeability increases considerably and excitation is occasionally possible (Mueller and Rudin, 1963, 1968). In natural membranes it has also been shown that proteases acting on the inner membrane surface eliminate excitability without major effects on the resting potential (Rojas and Luxoro, 1963; Tasaki and Takenaka, 1964; Rojas and Atwater, 1967). Although externally applied proteases have no effect on membrane excitability or potential (Tobias, 1955), unpublished experiments by the authors have indicated that externally applied proteases prevent the normal increase in relative chloride permeability which occurs with depolarization in crayfish axons. Additional experiments by the authors have indicated that almost any agent which affects protein structure also affects relative ionic permeabilities and the action potential. Thus for example, the reagent 2,4-fluorodinitrobenzene which combines with free amino groups reduces considerably the membrane potential dependence on chloride (Shrager et al, 1968). These experimental evidences all suggest that the membrane protein structure is involved in permeability and excitability control. The data at elevated potassium appears therefore suggestive of

the pH effects around the isoelectric point of a protein, where physical characteristics such as swelling, viscosity, osmotic pressure, conductivity, etc., are at a minimum. A possible explanation for the data observed here at $K_0 = 5.4$ and 25 mM may be that depolarization has shifted the membrane isoelectric point from an acidic value to one near neutrality.

One theoretically possible cause of the observed shift in pH-dependency of T_K and T_{Cl} with depolarization is that the membrane ionization constants have changed as a result of the Wien dissociation field effect (Wien, 1927; Onsager, 1934; Cole, 1965; Agin, 1967; Bass and Moore, 1967). For the dissociation field effect to be effective the ionizable groups must be in the voltage field, but even granted that this is the case, a quantitative calculation shows that the effect is not enough to account for the observed changes between $K_0 = 5.4$ and 25 mM. Following Onsager's theory (1934), with a membrane dielectric constant of 6 to 8 and a potential change of 30 mv at 25°C, the expected change in isoelectric point is calculated to be no more than 0.2 pH units for uni-univalent ions. A similar small change in pH is obtained for a di-univalent ion pair. Results similar to these have been obtained by Bass and Moore (1967) who calculated a membrane pH change of around 0.1 to 0.3 for a 1-1 and 1-2 electrolyte and 70 mv depolarization.

An alternative explanation could be that ionization constants change as a result of changing local dielectric constants. It has been pointed out by Fuoss (1934), Gurney (1953), Davies (1962), and Fuoss and Hsia (1967), that ion pair formation for all ions begins to be complete if the dielectric constant of the supporting medium falls below 40. With calcium ion, some ion pair formation occurs even in dielectric media near that of water (Davies, 1962). In going from the solution to the phospholipid membrane phase, the dielectric constant changes from 80 to a value below 10. At critical membrane regions where calcium, sodium, and potassium may be located, the dielectric constant may be at a metastable value where a small change in dielectric constant, by changes in water hydration or by other means, could markedly change relative ion dissociation and binding. For example, in a solution of dielectric constant = 37.8, $pK_{N \cdot Cl} = 0.818$ and $pK_{KCl} = 1.109$ where $pK = -\log K$; and $K = (A^-)(C^+)/(A^-C^+)$, the dissociation constant for the ion pair association (Davies, 1962). In this dielectric medium ($D = 37.8$) a small change in the average dielectric value will markedly change the ion pair association and relative binding for these ions. Evidence that water movement may be involved in these experiments comes from studies which show that cell surfaces become dense and opaque when hyperpolarized and translucent and transparent under depolarization (Lyudkovskaya, 1952). There is also the unexplained action of heavy water (D_2O) which affects the action potential without affecting the resting potential (Thies and Carlson, 1956). Although heavy water has almost the same dielectric constant as protonated water (H_2O), D_2O changes dissociation constants by a factor of around 3 (Gurney, 1953). Additional evidence suggesting that water

movement may occur with depolarization comes from the light scattering measurements of Bryant and Tobias (1952), by Hill (1950), and more recently by Cohen, Keynes, and Hille (1968).

The observed change in membrane potential in going from K_0 = 5.4 to 25 mM is around 30 mv which is more than sufficient to elicit an action potential in this axon. Therefore, the results at K_0 = 5.4 and 25 mM could reflect two end points of a membrane molecular change which might occur during excitation, with the action potential resulting from the transitional process. Admittedly however, the total steady-state currents here at K_0 = 25 mM (30 mv depolarization) are essentially zero and cannot be compared with the steady state currents obtained in a voltage clamp experiment with 30 mv depolarization.

In summary, the events where T_K, T_{Cl}, and the dependency of T_K and T_{Cl} on pH, change with depolarization, are best interpreted as resulting from a change in protein-phospholipid conformation with depolarization.

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