IONIC PERMEABILITY OF K, Na, AND Cl IN POTASSIUM-DEPOLARIZED NERVE

DEPENDENCY ON pH, COOPERATIVE EFFECTS, AND ACTION OF TETRODOTOXIN

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ABSTRACT   The passive ionic membrane conductances (g_j) and permeabilities (P_j) of K, Na, and Cl of crayfish (*Procambarus clarkii*) medial giant axons were determined in the potassium-depolarized axon and compared with that of the resting axon. Passive ionic conductances and permeabilities were found to be potassium dependent with a major conductance transition occurring around an external K concentration of 12–15 mM ($V_m = -60$ to -65 mV). The results showed that K, Na, and Cl conductances increased by 6.2, 6.9, and 27-fold, respectively, when external K was elevated from 5.4 to 40 mM. Permeability measurements indicated that K changed minimally with K depolarization while Na and Cl underwent an order increase in permeability. In the resting axon ($K_0 = 5.4$ mM, pH $= 7.0$) $P_K = 1.33 \times 10^{-5}$, $P_{Cl} = 1.99 \times 10^{-6}$, $P_{Na} = 1.92 \times 10^{-8}$ while in elevated potassium ($K_0 = 40$ mM, pH 7.0), $P_K = 1.9 \times 10^{-5}$, $P_{Cl} = 1.2 \times 10^{-5}$, and $P_{Na} = 2.7 \times 10^{-7}$ cm/s. When membrane potential is reduced to 40 mV by changes in internal ions, the conductance changes are initially small. This suggests that resting channel conductances depend also on ion environments seen by each membrane surface in addition to membrane potential. In elevated potassium, K, Na, and Cl conductances and permeabilities were measured from pH 3.8 to 11 in 0.2 pH increments. Here a cooperative transition in membrane conductance or permeability occurs when pH is altered through the imidazole pK (~pH 6.3) region. This cooperative conductance transition involves changes in Na and Cl but not K permeabilities. A Hill coefficient n of near 4 was found for the cooperative conductance transition of both the Na and Cl ionic channel which could be interpreted as resulting from 4 protein molecules forming each of the Na and Cl ionic channels. Tetrodotoxin reduces the Hill coefficient n to near 2 for the Na channel but does not affect the Cl channel. In the resting or depolarized axon, crosslinking membrane amino groups with DIDS reduces Cl and Na permeability. Following potassium depolarization, buried amino groups appear to be uncovered. The data here suggest that potassium depolarization produces a membrane conformation change in these ionic permeability regulatory components. A model is proposed where membrane protein, which forms the membrane ionic channels, is oriented with an accessible amino terminal group on the axon exterior. In this model the ionizable groups on protein and phospholipid have varied associations with the different ionic channel access sites for K, Na, and Cl, and these groups exert considerable control over ion permeation through their surface potentials.

INTRODUCTION

The surface membranes of excitable cells are often considered as having voltage-dependent and nonvoltage-dependent ionic channels. Factors in addition to voltage appear involved, however, since the observed ionic permeability changes depend on the method of membrane

depolarization. Some of the methods used to alter membrane potential include (*a*) a forced voltage displacement from the stationary state, such as in a voltage clamp; (*b*) alteration of a specific ionic channel permeability by chemical reagents, etc.; and (*c*) a change in extra or intracellular ion concentrations that alters electrochemical gradients.

The last method, by which membrane potential is altered by changing external ions, most commonly potassium, is often used to determine the effect of membrane potential on ionic permeability. A depolarization produced by raising external potassium, however, does not necessarily produce the same membrane permeability changes as an equivalent depolarization produced by other means. Thus, in crayfish axons depolarization by external potassium produces a marked functional change in membrane-conductance regulating components (Clark and Strickholm, 1971). In contrast, when the same nerve is equally depolarized by changes in internal ions, minimal changes occur in ionic permeabilities (Strickholm, 1977). Similarly, the ionic conductances observed in the voltage clamp in steady state (Shrager, 1974) do not compare with those conductances produced by potassium depolarization as shown by this paper.

The reasons for these differences are not clear although there are physico-chemical reasons to believe that not only membrane potential but also local ion atmospheres may be contributory in determining the conformational state of membrane protein which might form permeability channels. Studies by Von Hippel and co-workers (Hamabata and Von Hippel, 1973; Von Hippel and Schleich, 1969; Haschemeyer and Haschemeyer, 1973) showed that protein structure in solution follows the Hoffmeister series with the "salting out" ions (i.e., K) preserving more the native helical form than the "salting in" or coil denaturing ions such as calcium. Extensive work by Tasaki and his co-workers (Tasaki, 1968) has also shown that excitability depends on the particular ions present at the inner membrane surface and also follows the Hoffmeister or lyotropic series. That specific ions affect the conformational structure of membrane protein was further corroborated in studies on isolated rat brain membranes with intrinsic protein (Papakostides et al., 1972), where it was found that in the presence of K membrane protein existed primarily in an antiparallel beta form. In contrast, upon the addition of Tetrodotoxin or in a medium of Na or Ca, the membrane proteins occur primarily in the disordered conformation, or as an alpha helix.
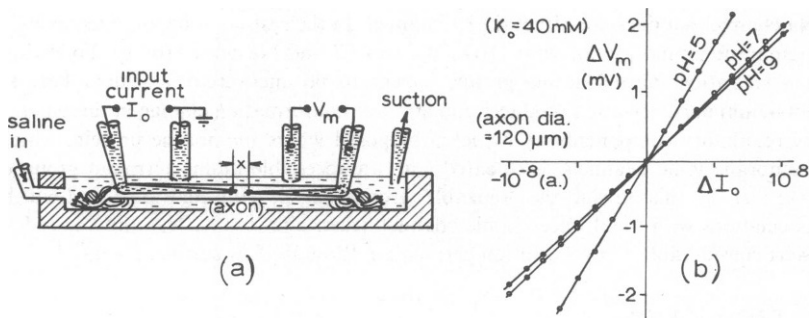

FIGURE 1 (*a*) Cannulation method for determining passive ionic conductances and permeability. (*b*) The relationship of membrane potential to input current across the axon cable input impedance in $K_o = 40$ mM. No membrane rectification is observed.

Previous research on crayfish nerve axons has shown that membrane fixed charges near ion permeation sites exert considerable control over resting K, Na, and Cl ionic conductances (Strickholm and Clark, 1977). It has also been reported that alteration of external potassium resulted in changes in the membrane ionic permeabilities (Strickholm and Wallin, 1967). Other studies have also suggested that ions (i.e., Ca) which enter neurons during excitation are able to mediate changes in ionic permeabilities (Eckert and Lux, 1977; Aldrich et al., 1979). These observations are important because they suggest that solution ion interactions with membrane protein and their charged groups have an important role in ion permeability regulation. Thus this paper examines in detail the action of potassium depolarization on passive K, Na, and Cl ion permeation in crayfish axons. In the depolarized axon the dependency of ionic permeability on extracellular pH was compared with that of the resting axon. The actions of Tetrodotoxin, local anesthetics, and certain group reactive reagents were also examined.

METHODS

Crayfish (*Procambarus clarkii*) medial giant axons from the ventral nerve code were used in these studies. Specific resting ionic conductances and permeabilities for K, Na, and Cl were determined by previously described methods (Hodgkin and Horowicz, 1959; Strickholm and Clark, 1977; Strickholm and Wallin, 1967) (Fig. 1 *a*). The method involves determining membrane resistance $R_m = 1/G_m$ from axon cable properties with cannulating microelectrodes to avoid errors observed in permeability when microelectrodes penetrate the surface membrane. The ionic transference number or dependency of membrane potential (T_j) on a specific *j*th ion is measured by a small step change in the concentration of the *j*th ion keeping other ion activities constant (Hodgkin and Horowicz, 1959). T_j is thus defined as $\Delta V_m / \Delta E_j$, where ΔV_m is the change in membrane potential when a specific ion is changed, and ΔE_j is the Nernst potential change for that ion and equals: $\Delta E_j = (RT/F)\ln(C_{jf}/C_{ji})$, where C_{jf} and C_{ji} represent the final and initial external concentrations for the *j*th ion.

Two membrane models, the parallel branched (Hodgkin and Huxley, 1952), and homogenous domain (Teorell, 1953), were both utilized to analyze the data. The parallel branched model where the ionic channels are considered separate, gives:

$$I_m = G_m V_m - \Sigma g_j E_j, \text{ where } \Sigma g_j = G_m. \tag{1}$$

The membrane conductance G_m is obtained here from measurements on axon cable properties. In these experiments on resting nerve, $I_m = 0$. Therefore Eq. 1 gives:

$$V_m = \Sigma(g_j/G_m)E_j = \Sigma T_j E_j, \text{ where: } T_j = g_j/G_m \text{ and } \Sigma T_j = 1.0. \tag{2}$$

For a step change in a *j*th ion that alters E_j, Eq. 2 gives $T_j = \partial V_m / \partial E_j$. The specific resting ionic conductances are thus obtainable as:

$$g_j = T_j G_m = (\Delta V_m / \Delta E_j)G_m. \tag{3}$$

It is emphasized that g_j is defined here as the slope conductance at zero membrane current and not the chord conductance of the Hodgkin-Huxley formulation that is obtained with membrane current flow.

In the homogenous domain membrane model, where different ion species share common channels, the specific resting ionic permeabilities P_j were obtained from combining the Teorell or Hodgkin-Katz membrane potential and conductance equations, and the definition for T_j. This gives (Teorell, 1953; Hodgkin and Katz, 1949; Strickholm and Clark, 1977):

$$P_j \text{ (cation)} = (RT/F^2)(1/C_{jo}) [(\S - 1)/\S \ln \S] (T_j G_m), \tag{4}$$

and

$$P_j \text{ (anion)} = (RT/F^2)(1/A_{jo}) \, [(\S - 1)/\ln \S] \, (T_j G_m), \tag{5}$$

where C_{jo} and A_{jo} represent the external cation and anion concentrations, respectively, for the jth ion and $\S = \exp(-FV_m/RT)$, where V_m is the membrane potential with the outer surface referenced as zero, and $RT/F = 0.025$V at 20°C.

In a comparison of the above formulations it is seen that Eqs. 4 and 5 of the homogenous domain model are related to g_j of the parallel branched model (Eq. 3) as:

$$P_j = f(1/\text{conc.}) \times f(V_m) \times g_j. \tag{6}$$

Thus P_j and g_j would have the same dependency on pH in these experiments if membrane potential varies little over the range of measurement. This is the situation here (Fig. 4), which indicates that conductances or permeabilities will only differ by a constant multiplier in these studies (compare Figs. 8 and 9).

The normal saline utilized contained (mM): NaCl 205, KCl 5.4, CaCl$_2$ 13.5, and MgCl$_2$ 2.6. For the elevated potassium saline, Na was replaced by K one for one to provide $K_o = 40$ mM, and Na$_o = 170.4$ mM. Other ion substitutions are described in the paper. Early experiments utilized Tris or Tris Maleate buffer of 5 to 10 mM. These proved unsatisfactory for acidic pH studies. The "Good's buffers" (Good et al., 1966), Pipes (pK 6.8), Hepes (pK 7.55), and Mes (pK 6.15) were used in various combinations in most experiments at concentrations from 1 to 10 mM, most commonly at 2 mM. Buffer concentration effects were ordinarily not observed except for Mes which seemed to enhance chloride permeability in acidic pH. Its use was discontinued. Buffering was found not ordinarily necessary for the extremes of pH studied here since the solutions were continually being changed. Each solution change (pH or ion) replaced the existing bath volume typically 10-fold by adding fluid to one end and removal by suction from the other over a period of several minutes (Fig. 1 a). Measurements with dyes showed that much less than 1% of the initial solution remains with each exchange. Tetrodotoxin, procaine, and tetranitromethane (TNM) were from Sigma Chemical Co. (St. Louis, Mo.), 4,4'-diisothiocyanostilbenen-2,2 disulfonic acid (DIDS) was from Pierce Chemical Co. (Rockford, Ill.) and pentobarbital was from Robinson Laboratory Inc. (San Francisco, Calif.). Laboratory temperature was kept near 20°C. These studies used over 100 crayfish obtained from California and the Gulf of Mexico.

RESULTS

Procedures and Effects of Potassium Depolarization

Previous work had shown that the passive K, Na, and Cl permeabilities of crayfish axons varied with potassium depolarization (Strickholm and Wallin, 1967). Similarly, Wallin (1967) found that in crayfish axons intracellular ion concentrations were not in electrochemical equilibrium and that they changed with potassium depolarization until K_o neared 10 to 15 mM, where K and Cl ions approached electrochemical equilibrium. Fig. 2 shows the data of Wallin (1967) compared with the relative Cl/K ionic permeability as a function of potassium depolarization as determined by Strickholm and Wallin (1967). Observed are major changes in permeabilities around a potassium concentration of 15 mM, which corresponds to a membrane potential around -60 to -65 mV. To further delineate the effects of potassium on membrane properties, membrane conductance was determined vs. external potassium (Fig. 3). A transition in conductance occurs around $K_o = 12.5$ mM and is seen at all pH (3.8 to 11), but is most marked around pH 5.5 to 6.0, as seen here. Suggested here is that two different membrane conformation states possibly exist, one for the resting membrane and the other when potassium depolarized. Of interest is that at potassium concentrations above the
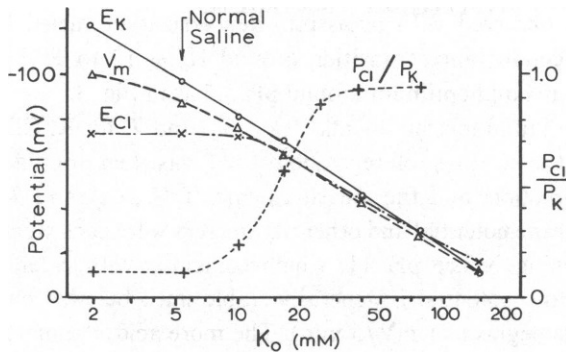
FIGURE 2 Effect of external potassium (K_o) on membrane potential (V_m), Nernst equilibrium potentials of potassium (E_K) and chloride (E_{Cl}), and the permeability ratio of chloride to potassium (P_{Cl}/P_K), pH 7.4.

transition point (K_o = 12.5 mM, Fig. 3), the axon is inexcitable. Figs. 2 and 3 show that at external potassium equal to 40 mM, K and Cl ions are near electrochemical equilibrium and the permeability and conductance changes with respect to increasing external potassium appear near completion. Moreover, no membrane rectification appears in K_o = 40 mM (Fig. 1 b). The membrane potential and permeabilities are generally reversible at this potassium concentration but are less so at higher potassium levels. Thus, in order to compare the two functional membrane states suggested by Figs. 2 and 3, K_o = 40 mM was chosen for the studies here as representative of the potassium depolarized membrane. The specific ionic conductances and permeabilities were measured here at K_o = 40 mM and then compared with previous results obtained on the resting axon at K_o = 5.4 mM (Strickholm and Clark, 1977).

The data described here were ordinarily obtained by initial dissection in normal crayfish saline (K_o = 5.4 mM) at pH 7.0 to prevent hysteresis shifts of the data (see Fig. 6 here and Fig. 6 of Strickholm and Clark, 1977). The external saline was brought to K_o = 40 mM (pH 7) from K_o = 5.4 mM by a step change or by incremental solution replacements. Although
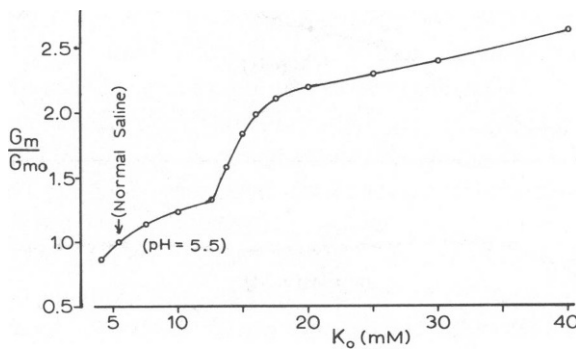

FIGURE 3 The dependence of resting membrane conductance (G_m) on external potassium (K_o). Potassium was substituted one-for-one for sodium. G_{mo} equals the membrane conductance in normal saline here at pH 5.5 The abrupt conductance transition observed here at K_o = 12.5 mM is less marked at other pH.

absolute conductances observed with potassium depolarization varied from axon to axon, every axon showed a conductance transition around $K_o = 12$ to 15 mM, which was less pronounced in alkaline pH and optimum around pH 5.5 as in Fig. 3.

In an experiment the initial measurements of V_m, G_m, and T_j for K, Cl, and Na were made at pH 7.0, which was the starting reference point. pH was then altered by 0.2-pH units, in either acid or basic direction, and the measurements of V_m, G_m, and T_j were repeated. In alkaline pH, the membrane potential and other parameters were consistently stable, with axon failure typically beginning before pH 11, which coincided with calcium precipitation. In acidic pH, the membrane potential is generally stable until below around pH 5.5, when a gradual irreversible loss begins (~1 mV/5 min). The more acid, the more rapid the potential loss. Below pH 3.6–3.7, a very rapid irreversible potential loss begins (~1 mV/s) and data below this pH are not included. Variability was found in absolute magnitude and ratios of the specific K, Na, and Cl permeabilities. The greatest variability occurred near crayfish molting time, when, surprisingly, the crayfish saline utilized here could not maintain a viable axon, and resting potentials typically went to zero. This was correlated with (unpublished) measurements showing that blood-ion concentrations change dramatically at molting. Despite the variability in the relative and absolute ionic conductances or permeabilities, there was no apparent alteration in the pH location of these changes. Thus, the cooperative conductance transition around pH 6.3, and other changes with pH, etc. (see Figs. 5, 8, 9), appeared invarient with the seasons. This of course was only true when hysteresis errors in conductance with pH shifts were prevented by initiating all experiments at pH 7.0 (see Figs. 5, 6). The following data represent averages from more than 100 axons measured at different times over several years.

Membrane Potential

Fig. 4 shows the dependency of the steady-state membrane potential (V_m) on pH in $K_o = 40$ mM, compared with resting nerve ($K_o = 5.4$ mM). In the resting axon, a 17-mV change in membrane potential occurs from pH 4 to 11, which is consistent with changes in P_K, P_{Na}, and
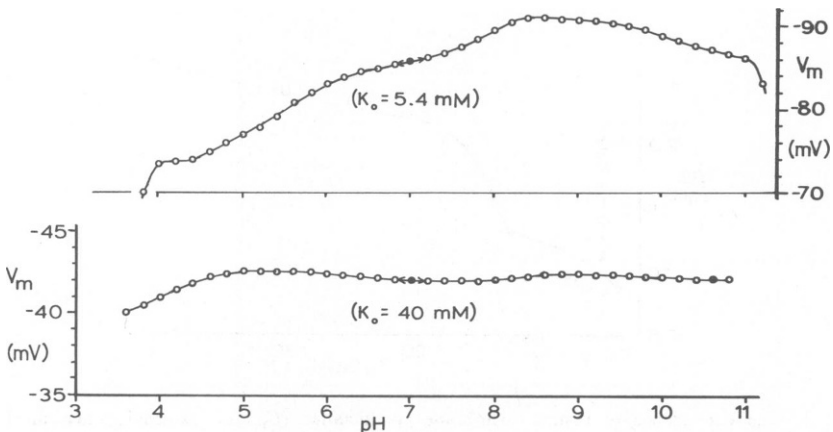

Figure 4 The dependence of steady-state membrane potential on pH in the potassium depolarized axon ($K_o = 40$ mM) compared with that of the resting axon ($K_o = 5.4$ mM). Potential measurements were initiated at pH 7.0 and obtained going either alkaline or acid in 0.1-pH U.

P_{Cl} with pH (Strickholm and Clark, 1977). This contrasts with the small potential dependency on pH in $K_o = 40$ mM, which is consistent with the view that both K and Cl are near electrochemical equilibrium in elevated potassium as demonstrated by Wallin (1967) (Fig. 2). Thus although K, Na, and Cl conductances or permeabilities vary considerably with pH (Figs. 8, 9), the effect of these permeability changes on V_m is minimized. In the homogenous domain model, this follows from either the constant field equation or that of Teorell (1953, Eq. 28), where if chloride adjusted to stay in electrochemical equilibrium ($V_m = E_{Cl}$), the membrane potential, although dependent on chloride, may be written without the chloride terms as: $E_{Cl} = V_m = (RT/F)\ln[(P_K K_o + P_{Na} Na_o)/(P_K K_i + P_{Na} Na_i)]$. In these experiments the ratio of P_{Na}/P_K does not exceed 0.015 over the pH range 3.6 to 10.8. The P_{Na}/P_K changes thus minimally affect potential.

For the parallel branched model, if $E_{Cl} = V_m$, the membrane potential may be written as: $V_m = [T_K/(1 - T_{Cl})]E_K + [T_{Na}/(1 - T_{Cl})]E_{Na}$, but since $\Sigma T_j = 1$, $V_m = [T_K/(T_K + T_{Na})]E_K + [T_{Na}/(T_K + T_{Na})]E_{Na}$. At its minimum (Fig. 7) $T_K/(T_K + T_{Na}) = 0.95$, while at its maximum $T_{Na}/(T_K + T_{Na}) = 0.05$ over the measured pH range. The effects of these T_j variations on V_m are small. Thus the small potential changes with pH observed here in $K_o = 40$ mM are consistent with the small Na contribution to ionic permeability and with the observation that Cl and K are near electrochemical equilibrium (Figs. 2, 8, 9).

Membrane Conductance and Resistance

When crayfish axon is depolarized by potassium to $K_o = 40$ mM from its normal value ($K_o = 5.4$ mM), the resting membrane conductance $G_m = 1/R_m$ (pH 7.0) increases from its resting value by 6.77-fold from 1.66×10^{-3} ($K_o = 5.4$ mM) to $1.12 \times 10^{-2} \pm 0.27$ (SD, $n = 44$) (ohm^{-1}cm^{-2}) ($K_o = 40$ mM). Fig. 5 shows the dependency of G_m on pH for the potassium depolarized axon compared with the resting axon. The data show that complex changes occur in membrane conductance when the axon is potassium depolarized, in particular around pH 6.3. In the depolarized axon, the steep conductance change with pH over the imidazole pK region (pH 6.0 to 6.6) suggests that the pH-titrated membrane groups that regulate ion
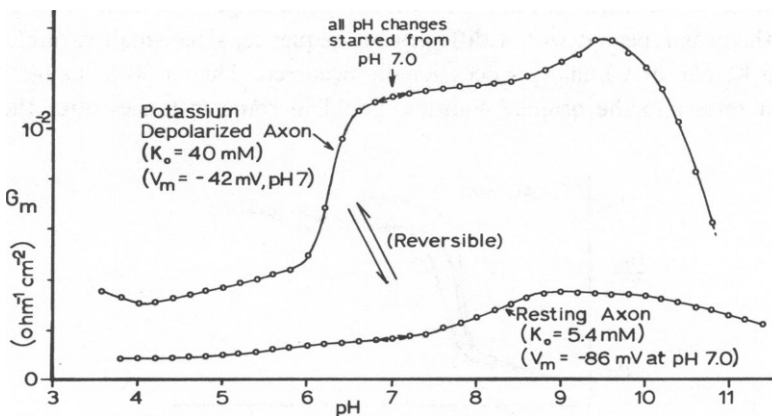

FIGURE 5   The dependence of membrane conductance (G_m) on pH for crayfish axon in normal saline ($K_o = 5.4$ mM) and for the potassium depolarized axon ($K_o = 40$ mM). All measurements were initiated from pH 7.0.

permeation interact. A cooperative interaction is implied, since ordinarily a similar titration of noncooperative elements, such as a free amino acid, would require a change of 2 pH U for equivalent effects. Also observed but quite variable and smaller in magnitude is a similar cooperative conductance change with pH variation around pH 8 that appears to involve potassium permeability (weakly indicated in Figs. 6, 8, 9).

Fig. 6 shows the hysteresis effects observed in membrane conductance when pH is reversibly changed. Such hysteresis effects depend on the pH excursion and are indicative of conformational changes and are commonly seen in protein molecules during pH titration, especially when cooperativity is involved.

Dependence of the Membrane Potential on K, Na, and Cl: (T_j)

The dependence of the membrane potential on external potassium (K_o), sodium (Na_o), and chloride (Cl_o) was examined by small step concentration changes in one particular ion by solution changes that replaced >99% of the initial solution. Ion substitutions were one-for-one to keep ion activities and osmotic pressure constant. Thus, a small change in K was replaced by an equivalent alteration in sodium, Na by choline, and Cl by isethionate, although glucuronate was used in some studies. From the definition of T_j, $\Delta V_m = T_j \Delta E_j = T_j$ $(RT/F)(\ln C_{jf} - \ln C_{ji})$, where C_{jf} and C_{ji} are final and initial external ion concentrations of the jth ion (Hodgkin and Horowicz 1959, Strickholm and Clark, 1977). For each ion and the substitutions (C_{jf}) used here, the membrane potential change ΔV_m appeared linear with respect to $\ln(C_{jf})$, up to about a 35% ion change. Thus in these studies, step changes in ion concentrations were normally kept below 35%. In the experiments here, the liquid junction potential changes at the reference electrode (fast responding in a nonionic gel to avoid errors with pH and solution changes; Strickholm, 1968), when ions were changed, were calculated from the Henderson (1907) equation as 0.0 mV for K (40–47 or 55 mM), 0.4 mV for Na (170.4–131 mM), and 0.4 mV for Cl (242.6–187 mM). The parenthesis here represent the initial (C_{ji}) and final (C_{jf}) ion concentration for each ion step change. Potential changes were measured to an accuracy of 0.05 mV on a strip chart recorder. The sequence of ion change was most often that for Na, followed by a return to initial saline (K_o = 40 mM), then Cl, with a return to initial saline, then K, followed by a 0.2 pH change, and then repeated. In some experiments this was repeated with a different ion sequence, since small variable hysteresis effects due to K, Na, or Cl changes occasionally occurred. Thus a 30% change in one ion, followed by a return to the original solution, could in some instances alter the potential
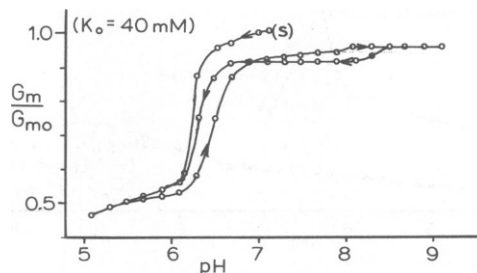

FIGURE 6 Hysteresis of membrane conductance (G_m) with pH changes. G_{mo} equals the membrane conductance at pH = 7.0 (K_o = 40 mM). (S) represents the starting point for the measurements.

dependence (T_j) of the next ion measured by 5–10%. This effect depended on the pH utilized and other treatments of the axon (group specific reagents, etc.). These observations additionally support the data and views of Von Hippel and Schleich (1969) and Papakostidis et al. (1972) that the simple ions K, Na, and Cl can affect protein conformation and introduce hysteresis, an affect which could be separate from that produced by potential.

Fig. 7 shows in K_o = 40 mM the effect of pH on the dependency of the membrane potential (T_j) on the external ions K, Na, and Cl. Observed is a steep change in T_j for all three ions over the imidazole pK region (~pH 6.3) that corresponds to the similar steep change observed for membrane conductance. Around pH 5.8 the dependency of the membrane potential on Na and Cl becomes zero, and T_K = 1.0, which is that predicted if the membrane were only K-permeable (Eq. 2) (Hodgkin and Horowicz, 1959; Strickholm and Clark, 1977). These effects on T_j at this pH are absent in normal saline (K_o = 5.4 mM). In alkaline pH, T_{Cl} goes to zero around pH 10.7. This corresponds to the situation in normal saline (K_o = 5.4 mM) where T_{Cl} also goes to zero around pH 10.6. At pH 7.0, T_K = 0.54 ± 0.13 (SD, n = 28), T_{Na} = 0.033 ± 0.012 (n = 23), T_{Cl} = 0.39 ± 0.07 (n = 25), ΣT_j = 0.96 ± 0.08 (n = 16). Fig. 7 indicates that the sum of T_j is near unity over the entire pH range. This is consistent with the parallel branched membrane model for permeability where the ionic channels are separate (Eq. 2). It is also consistent with the Teorell membrane model (Teorell, 1953) for diffusion through a homogenous domain which predicts that ΣT_j = 1 if chloride is in electrochemical equilibrium as is seen here (Wallin, 1967; Strickholm and Clark, 1977).

Specific Ionic Conductances and Permeabilities

Fig. 8 shows the passive specific ionic conductances $g_j = T_j G_m$ for K, Cl, and Na based on the data from Figs. 5 and 7 and G_{mo} (pH 7.0, K_o = 40 mM) = 1.12 × 10^{-2} (ohm^{-1} cm^{-2}). Quite striking is the finding that although T_K and G_m showed marked changes with pH over the imidazole pK region, their combined product (g_K) shows that potassium conductance changes
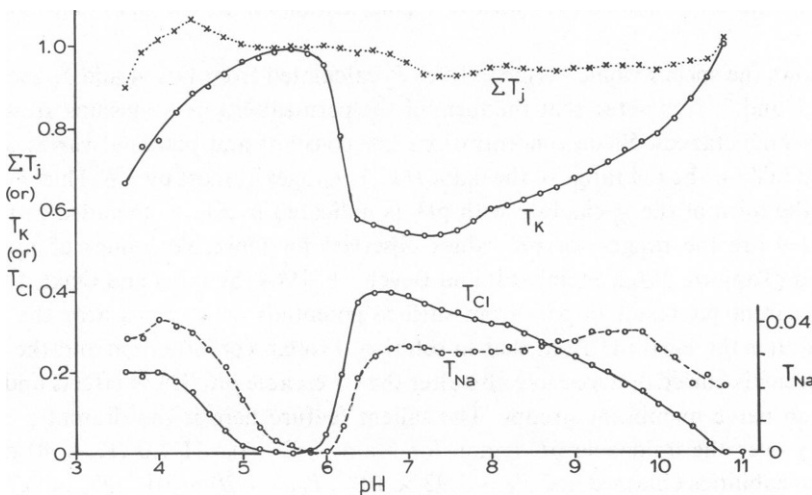

FIGURE 7 The specific ionic dependence of membrane potential on potassium (T_K), sodium (T_{Na}), and chloride (T_{Cl}) as a function on pH in the potassium-depolarized axon (K_o – 40 mM). ΣT_j – T_K + T_{Na} + T_{Cl}.
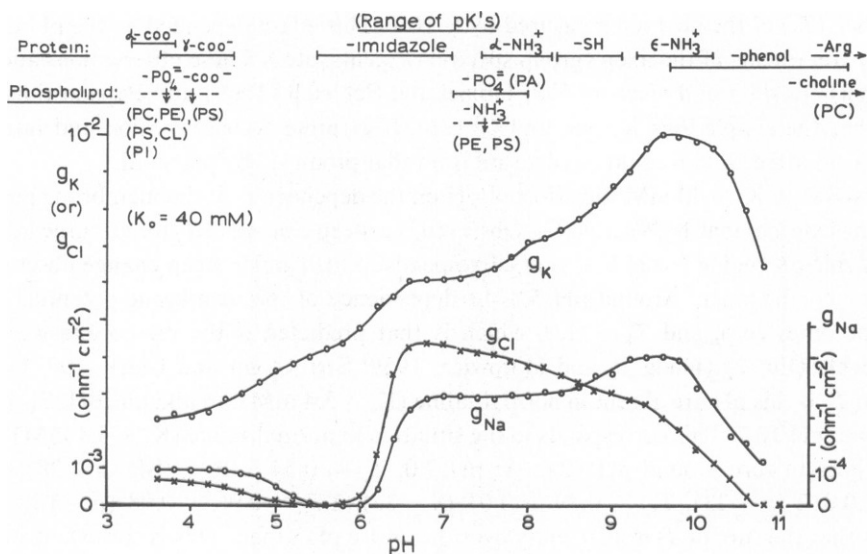
FIGURE 8 The dependence of specific ionic conductances (g_j) on pH for the potassium-depolarized axon ($K_o = 40$ mM). Indicated by horizontal bars at the top are the experimentally observed ranges of pK of ionizable groups on protein and phospholipid.

appear not cooperative over this region. Only sodium and chloride passive ionic channels show cooperative behavior over the imidazole pK region. The passive ionic conductances in $K_o = 40$ mM at pH 7.0 are: $g_K = 6.08 \times 10^{-3}$, $g_{Cl} = 4.34 \times 10^{-3}$, $g_{Na} = 0.366 \times 10^{-3}$ (ohm^{-1} cm^{-2}). The conductance ratios are: $g_K:g_{Cl}:g_{Na} = 16.6:11.8:1.00$. This compares with the values and ratios in normal saline ($K_o = 5.4$ mM): $g_K = 9.75 \times 10^{-4}$, $g_{Cl} = 1.62 \times 10^{-4}$, $g_{Na} = 0.533 \times 10^{-4}$ (ohm^{-1} cm^{-2}); and: $g_K:g_{Cl}:g_{Na} = 18.3:3.04:1.00$ (Strickholm and Clark, 1977). The increase in membrane conductances with K depolarization for K, Cl, and Na are 6.24, 26.8, and 6.87.

Fig. 9 shows the specific ionic permeabilities P_j calculated from Eqs. 4 and 5, and the data of Figs. 4, 5, and 7. It is noted that the form of the permeability relationships are similar to that of the conductances. Since concentrations are constant and potential varies only from -40 to -43 mV for the pH range of the data, $f(V_m)$ changes at most by 7%. Thus P_j will vary little from the form of the g_j changes with pH as indicated by Eq. 6. Included at the top of Figs. 8 and 9 are the ranges for pK values observed for ionizable groups of protein and phospholipid (Tanford, 1962; Steinhardt and Beychock, 1964; Seimiya and Ohki, 1973). The range of apparent pK result in part from surface potentials on proteins near the ionizable groups that alter the local pH from that in solution. Protein conformation and the extent to which the group is buried or accessible also alter the observable pK. These effects undoubtedly also occur on nerve membrane groups. The salient feature here is the dramatic change in permeability over the imidazole pK region for Na and Cl. At pH 7.0 ($K_o = 40$ mM), the specific permeabilities obtained are: $P_K = 1.93 \times 10^{-5}$, $P_{Cl} = 1.20 \times 10^{-5}$, $P_{Na} = 2.73 \times 10^{-7}$ cm/s. The ratio $P_K:P_{Cl}:P_{Na} = 70.7:44.0:1.00$. For comparison with the resting nerve ($K_o = 5.4$ mM, pH 7.0), $P_K = 1.33 \times 10^{-5}$, $P_{Cl} = 1.99 \times 10^{-6}$, $P_{Na} = 1.92 \times 10^{-8}$ cm/s, and the ratio
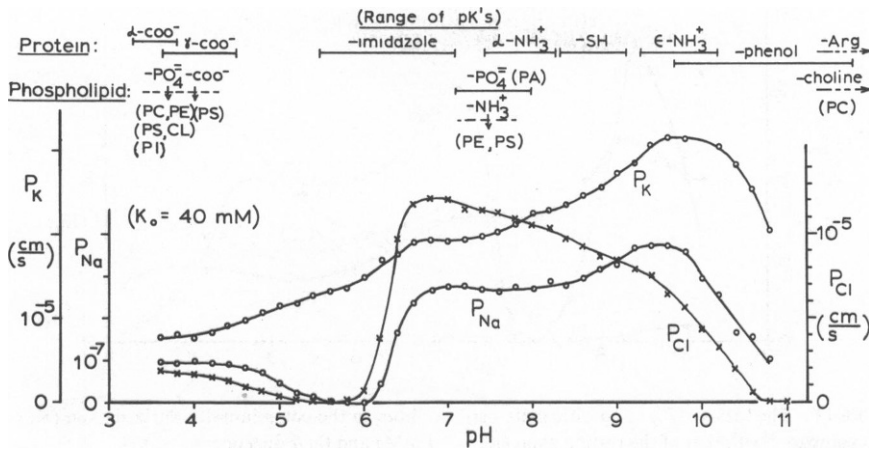
FIGURE 9 The dependence of specific ionic permeabilities (P_j) on pH for the potassium-depolarized axon. Indicated by horizontal bars at the top are the experimentally observed ranges of pK of ionizable groups on protein and phospholipid.

$P_K:P_{Cl}:P_{Na} - 693:78:1.00$ (Strickholm and Clark, 1977). Thus, potassium depolarization results in changes both in the absolute and relative ionic permeabilities.

To illustrate the permeability changes which occur with potassium depolarization, Figs. 10 and 11 show the specific ionic conductances and permeabilities of K, Na, and Cl in $K_o = 40$ mM compared with that obtained in the resting axon ($K_o = 5.4$ mM) (Strickholm and Clark, 1977). All the resting slope conductances g_j are seen to increase with K depolarization. In the parallel-branched model, the reciprocal of specific ionic conductance $1/g_j$ depends on the integral of $1/F^2 u_j C_j$ over the membrane, where F is the Faraday, and $u_j C_j$ the membrane-mobility concentration product (Schwartz, 1971). The K conductance increase is 6.2-fold (pH 7.0), when external K increases by 7.2 (from 5.4 to 40 mM). Thus the g_K changes could result in part from an elevated external K which would increase $u_K C_K$. For Na and Cl, however, whose external concentrations remain essentially constant, major changes in the ionic channels appear necessary to explain their conductance increases.
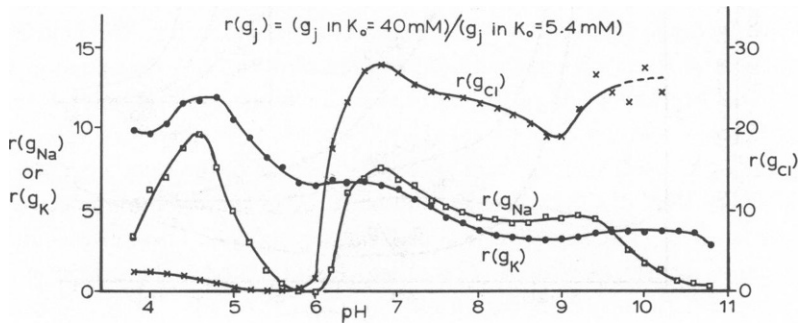

FIGURE 10 The ratio $r(g_j)$ of specific ionic conductances in the potassium-depolarized axon ($K_o - 40$ mM) compared with that of the resting axon ($K_o - 5.4$ mM) and their dependence on pH. In extreme alkaline pH, g_{Cl} approaches zero and the ratio $r(g_{Cl})$ becomes uncertain.
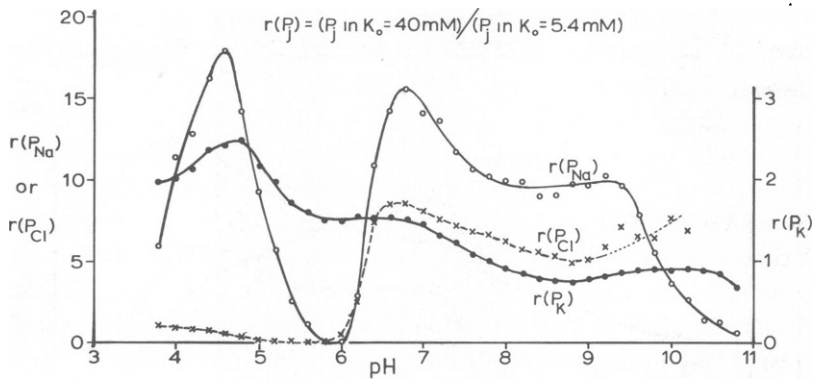
FIGURE 11 The ratios $r(P_j)$ of specific ionic permeabilities in the potassium-depolarized axon ($K_o = 40$ mM) compared with that of the resting axon ($K_o = 5.4$ mM) and their dependency on pH.

The changes in K permeation may also be examined from the Teorell model, which provides the intrinsic membrane permeability P_j separate from changes in the membrane electrochemical gradients. Here at pH 7.0, the proportional change in the ionic permeabilities $r(P_j) = P_j(K_o = 40 \text{ mM})/P_j(K_o = 5.4 \text{ mM})$ is: $r(P_K) = 1.45$, $r(P_{Cl}) = 8.05$, $r(P_{Na}) = 14.2$. Fig. 11 shows that potassium permeability changes minimally from the resting axon with K depolarization in alkaline pH, and that it only approximately doubles in acidic pH. In contrast, sodium and chloride permeabilities show complex changes with pH after potassium depolarization. The data here on permeability thus better support the parallel-branched membrane model, since the passive ionic channels regulating K, Na, and Cl appear separate because they each behave differently with pH and depolarization. But the data could also
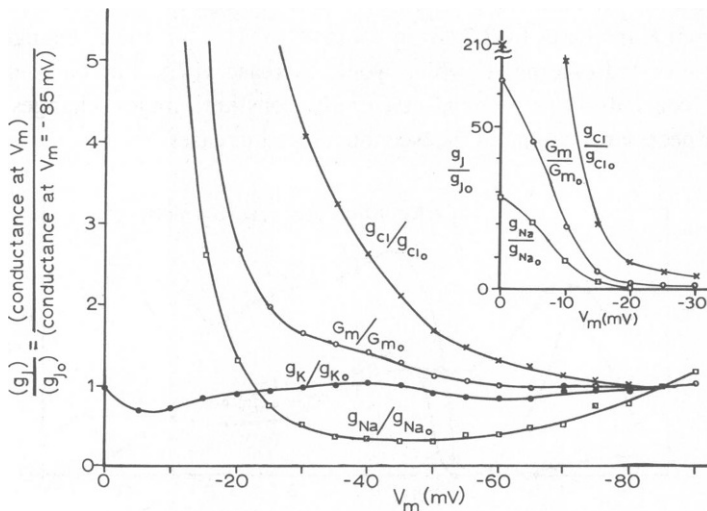

FIGURE 12 The changes in specific ionic conductances with depolarization resulting from intracellular K loss and Na gain as an axon deteriorates with time. g_{jo} represents the conductance of K, Na, or Cl for $V_m = -85$ mV, while g_j is that at the given membrane potential. G_m equals the total membrane conductance. pH 7.0.

result from changes in selectivity binding sites of a homogenous domain. Figs. 10 and 11 also clearly indicate that the conductance or permeability state of the potassium-depolarized axon is different from that of the resting axon.

It has been reported that membrane depolarization resulting from intracellular K depletion and Na gain showed minimal changes in ionic conductance or permeability (Strickholm, 1977). In these experiments the axon was allowed to run down and lose potential over many hours. Intracellular ion concentrations were not measured. Fig. 12 shows the changes in K, Na, and Cl conductances with membrane potential in this situation at pH 7.0. The external solution was normal saline (K_o = 5.4 mM). No major conductance changes are observed until membrane potential falls to < -30 mV. A similar situation exists for pH 5.0 and 9.0. The changes observed here for K, Na, and Cl conductances during gradual membrane potential failure evidently contrast with the results obtained by potential changes with external potassium alteration (Fig. 8). Particularly absent in Fig. 12 is the striking conductance change seen with external K depolarization (Fig. 3) when membrane potential is near -60 to -65 mV. Clearly, equivalent depolarizations produced by a change in either the intra- or extracellular solutions produce different ionic conductance changes.

The Cooperative Conductance or Permeability Change Over the Imidazole pK Region. Estimation of the Number (n) of Sites Involved

The narrow pH range over which conductance or permeability changes over the imidazole pK region (Figs. 8, 9) suggests that the membrane elements involved interact in a cooperative manner to control ion permeation. This cooperative interaction will be examined here with the simplest of assumptions. These are: (a) the observed ion permeability or conductance is a measure of the fraction of sites θ occupied by protons on the receptor sites of the ionizable membrane groups, and (b) the binding of the ligand strongly "activates" other sites so that they fill up more readily. With these assumptions, both the Adair equation, and the Monod-Wyman-Changeux models (Monod et al., 1965; Wyman, 1964; Whitehead, 1970; Van Holde, 1971; Triggle, 1978) become equal to the Hill equation in the limiting case of strong positive cooperativity. A Scatchard plot of the data here shows positive cooperativity. The Hill equation may be written as: $\theta/(1 - \theta) = K(A)^n$, where $\theta/(1 - \theta)$ represents the ratio of the fraction of ligand (A) binding sites occupied to those vacant on the protomer and (A) is the hydrogen ion concentration in this case. Wyman (1964) and Whitehead (1970) have indicated that in general, n represents in the ideal limit, the number of interacting elements.
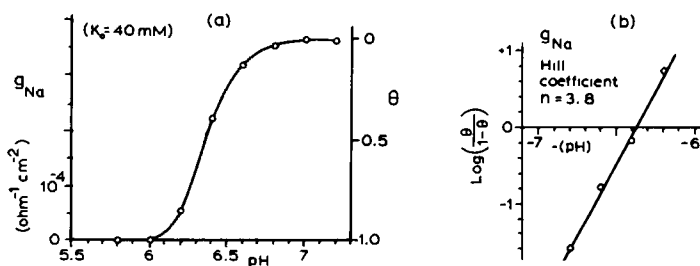

FIGURE 13   (a) The conductance of sodium with respect to pH over the imidazole pK region. θ equals the fraction of proton binding sites occupied on the sodium channel regulating component. (b) The Hill plot of the data of figure (a).

The measured Hill coefficient n may thus be less than the actual number of interacting components. The coefficient n could represent n sites on one single molecule, or equally could represent n separate but interacting protein molecules forming the ionic permeability channel. Figs. 13 a and 14 a show g_{Na} and g_{Cl} redrawn from Fig. 8. Here it is assumed that above pH 7 hydrogen ion binding to the cooperative group is minimal and the fraction of sites occupied (θ) equals zero, whereas below pH 5.8, where the ionic conductances (or permeabilities) of Na and Cl are maximally altered and zero, all the proton binding sites are occupied and $\theta = 1.0$. The Hill equation gives $\log [\theta/(1 - \theta)] = \log K + n \log (H) = \log K - n(\text{pH})$. Figures 13 b and 14 b show the Hill plot: $\log [\theta/(1 - \theta)]$ vs. pH for the sodium and chloride conductance. A least-square fit of the data gives a slope $n = 3.8$ for g_{Na} and $n = 4.3$ for g_{Cl}. Similar values for n are obtained for P_{Na} and P_{Cl} as expected for Eq. 6. The Hill coefficient here is quite sensitive to the value of g_j where θ is taken as zero. Fig. 8 shows a decline in g_{Cl} going alkaline from pH 7.0, which seems to reduce the asymptote of g_{Cl} for imidazole deprotonation. If deprotonation of imidazole increased g_{Cl} another 3% from pH 7.0 to 7.4, the observed Hill coefficient n for g_{Cl} would be 4.0. The observed Hill coefficient n equal to ~4 for g_{Na} and g_{Cl} thus strongly suggests that four protein molecules associate to form the ionic membrane channels of Na and of Cl, although other explanations are possible.

Action of Tetrodotoxin, Local Anesthetics, and Reagents

Tetrodotoxin (TTX) was added initially to the resting axon, maintained for several minutes after action potential failure, and then maintained during potassium depolarization. The TTX concentrations used ($\sim 10^{-7}$ M) were typically an order more than necessary to block the action potential at pH 7.0 to ensure binding site saturation over a wide range of pH. It was found that TTX reduced the cooperative conductance or permeability changes seen for Na when pH is altered through the imidazole pK region and changed the Hill coefficient n for sodium from near 4 to around 2 (Fig. 15 a). In contrast, potassium and chloride conductances appeared minimally affected over the imidazole pK region by TTX. It was also observed that the action of TTX is different and less effective if it is added after the membrane is potassium-depolarized than if TTX is added before, and maintained during, potassium depolarization (Fig. 15 b). These observations additionally support the view that the membrane components subserving ionic permeability have different conformational or TTX binding states in the resting and depolarized axon.

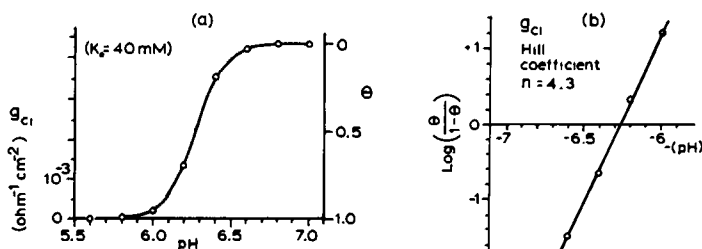FIGURE 14 (a) The conductance of chloride with respect to pH over the imidazole pK region. θ equals the fraction of proton binding sites occupied on the chloride channel regulating component. (b) The Hill plot of the data of figure (a).
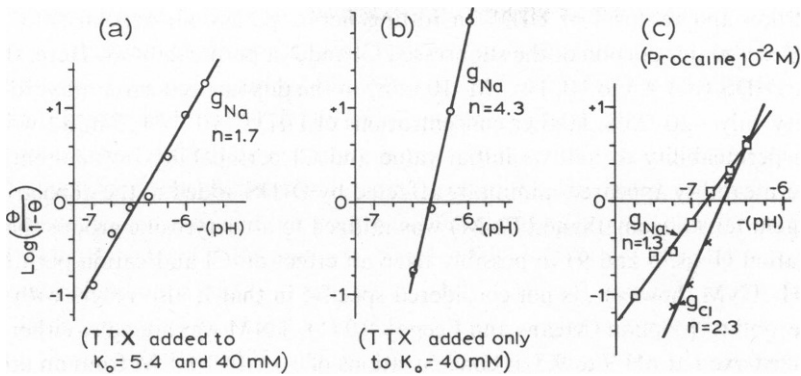
FIGURE 15 (a) The Hill plot of sodium conductance change with pH over the imidazole pK region in elevated K_o (40 mM). Tetrodotoxin (TTX) (10^{-7} M) was initially added in resting saline (K_o – 5.4 mM) and then maintained as the axon was depolarized to K_o = 40 mM. (b) Similar Hill plot of sodium conductance as in (a). The axon was initially depolarized (K_o – 40 mM) without TTX. TTX was added later to the K-depolarized axon. (c) Similar Hill plot of sodium and chloride conductance as in (a) but with only procaine (0.01 M) present at a concentration which just blocks the action potential.

The local anesthetics procaine and pentobarbital were examined at concentrations which just blocked the action potential (0.01 M and 0.04 M). The actions of procaine (pK = 8.95) were complex but it reduced the Hill coefficient n for g_{Na} and g_{Cl} (Fig. 15 c). Procaine also raises g_{Na} and suppressed g_K and g_{Cl} in normal saline (K_o = 5.4 mM) at pH 7.0, but suppresses all conductances in K_o = 40 mM (pH 7). Pentobarbital (pK = 8) had effects which changed with pH. It suppressed chloride permeability in alkaline pH (~>8), where it is predominantly in the negative form. At the concentrations here, g_{Cl} became zero at pH 9.5, where ordinarily it is not zero until pH 10.7 (Fig. 8). As suggested by McLaughlin (1977), both procaine and pentobarbital could function here as an amphipath with the charged polar group situated at the solution membrane interface. Their action here on the exterior membrane surface could in part be due to alteration of the local charge field at the entry site to the ion permeability channel. The action of procaine here, however, is not consistent with the expected effect of its positive charge. Similarly, although pentobarbital suppresses g_{Cl} in alkaline pH, it increases g_{Cl} at pH 7.0, where it is predominantly neutral, which suggests more complex interactions.

The reagent 4,4'-diisothiocyanostilbene-2,2 disulfonic acid (DIDS) was applied to the resting and depolarized axon and showed different results. The reagent DIDS is considered quite specific for cross-linking amino groups and has been used to reduce anion permeability in red cells (Cabantchik and Rothstein, 1972, 1974). The reagent was mixed immediately before use and added to the resting or K-depolarized axon in the dark for periods of 5–15 min and then washed out. Typically, the initial reaction appeared complete within the diffusion access time to the membrane surface (~1–2 min), as evidenced by the time-course of change in membrane resistance. Around neutral pH, where DIDS is not very reactive, and in both the resting and potassium-depolarized axon, Cl and Na permeabilities were reduced in the presence of DIDS (10^{-4} M), and the effect was reversible when DIDS was removed. In alkaline pH (~9.5–10), however, DIDS (10^{-5}–10^{-4} M) added to resting nerve reduced irreversibly both Cl and Na permeability to near zero while slightly raising that of K. After

the application and removal of DIDS in resting nerve, potassium depolarization ($K_O = 40$ mM) restored a major portion of the suppressed Cl and Na permeabilities. Here, the repeated addition of DIDS (pH 9.5 to 10, 10^{-5} M, 10 min) in the depolarized axon reduced Cl and Na permeability only ~20–25%. Higher concentrations of DIDS (10^{-4} M, 5 min), would further reduce Na permeability to half its initial value and Cl permeability to one-tenth its initial value. K permeability appeared minimally affected by DIDS added to the depolarized axon.

The reagent tetranitromethane (TNM) was utilized to alter tyrosine groups that appeared on pH titration (Figs. 8 and 9) to possibly have an effect on Cl and cation permeabilities in alkaline pH. TNM, however, is not considered specific in that it also reacts with sulfhydryl, methionine, and tryptophan (Means and Feeney, 1971). TNM was added to either the resting or depolarized axon at pH 9 to 9.5 at concentrations of 10^{-4} to 10^{-5} M from an ethanol stock solution so that the maximum ethanol concentration in the saline was <1%. The TNM solutions were added slowly to avoid ethanol osmotic shock and membrane alteration, which readily occurs here with 5% ethanol solutions. The results showed TNM to have minimal effects at pH 9 on Na and K permeabilities when added to depolarized nerve, although when added to resting nerve, P_{Na} was reduced. Cl permeability was reduced partially here (at pH 9) to three-fourths its normal value by TNM only in depolarized nerve. In addition, the permeability changes with pH through the imidazole pK region (~pH 6.3) were minimally affected by TNM and the Hill coefficient n for P_{Na} and P_{Cl} appeared unaltered here.

DISCUSSION

Previous work (Strickholm and Clark, 1977) on this axon in normal saline showed that membrane fixed charges exert considerable control over ion permeation through most likely their local potentials. In general, it was found that cation (Na and K) permeability decreased with protonation of membrane groups while that of chloride increased. This followed qualitatively the form predicted by Teorell (1953). Evidence was obtained that passive Na and K permeability appeared initially activated by ionization of carboxyl and phosphate, then by deprotonation of imidazole, and finally and maximally by what appeared to be phosphatidic acid. With respect to chloride, the protonation of side amino groups on protein had a major role in activating its permeability.

When the axon is K-depolarized a major change occurs in total membrane conductance, but minimal changes occur in K permeability after depolarization (Fig. 11). Thus, K permeability in elevated K behaves as in the resting axon and appears to depend initially on carboxyl and phosphate, then imidazole, and possible phosphatidic acid. The role of sulfhydryl and amino groups is uncertain here, although cross-linking of amino groups with DIDS had a very small effect on P_K. In extreme alkaline pH, P_K is reduced for both the resting and depolarized axon, which suggests involvement of tyrosine groups perhaps as a selectivity site since ionization should enhance P_K. The effects here of TNM on P_K, however, appear not major, which suggests that the tyrosine group may not be near the resting K channel.

In contrast to K, Na permeability appears to behave differently in the resting and depolarized axon with respect to pH and group specific reagents. TNM, which alters tyrosine, reduced P_{Na} in resting but had no effect in depolarized nerve. Similarly, DIDS which affects amino groups, appears more effective in resting than in depolarized nerve in reducing P_{Na}. P_{Na}

also shows a striking cooperative type response with pH changes over the imidazole pK region in depolarized but not resting nerve, an effect that is absent with P_K. These observations support the view that the Na and K passive ionic channels could be separate. They also suggest that a major conformational change occurs in the passive Na ionic channel with depolarization but not with K. Of interest is that the imidazole and tyrosine groups, which appear here to regulate passive Na permeability, are also implicated in regulating the active Na and K channels (Hille et al., 1975; Brodwick and Eaton, 1978; Shrager, 1974). Moreover, the carboxyl group here has been suggested to form part of the absorption site for the active sodium channel (Hille et al., 1975; Ulbricht, 1977; Neumke, 1977; Spalding, 1978).

Chloride permeability appears predominantly activated by the protonation of side amino groups on protein in both the resting and potassium-depolarized axon. Buried amino groups also appear to become accessible with K depolarization as indicated by application of DIDS. Altering the imidazole group by pH in the resting axon seems to have little effect on P_{Cl}, whereas in depolarized axon a buried imidazole group appears uncovered and protonation here reduces Cl permeability to zero. This clearly is in contrast to a simple fixed charge effect, where imidazole protonation would be expected to increase anion permeabilities. In the resting axon, protonation of carboxyl and phosphate had no major effect on P_{Cl}, whereas in the depolarized axon, protonation of these groups appears to restore some chloride permeability, which was near zero due to imidazole protonation.

The evidence here suggests that the ionic channels are generally specific for particular ions and sharing by dissimilar ions is minimal. This follows from findings that pH, depolarization, or group specific reagents can alter a specific ion permeability without affecting the other ions. It is difficult to conceive of a multi-ion channel having a selectivity filter being altered by reagents and affecting only one ion and not having effects on all the ions entering that channel. Thus the parallel branched membrane model seems more appropriate for passive permeabilities than that of the homogenous domain (Finkelstein and Mauro, 1963).

The results here thus show that both surface charges and conformational states of surface membrane protein and lipid appear involved in regulating ionic permeability. That surface charges can regulate ion permeation through effects on the membrane surface double layer potential has been demonstrated in innumerable studies (Gilbert, 1971; Haydon and Hladky, 1972; Mozhayeva and Naumov, 1972; McLaughlin, 1977a, b). In protein chemistry, it is ordinarily quite difficult to separate changes in ionization of molecular groups from corresponding changes in molecular structure. Thus in the titration of proteins, it is the typical rule that ionization changes introduce simultaneous conformational changes (Tanford, 1962, 1973). This is supported here by the hysteresis effects observed with pH changes. These conformational changes additionally often introduce alterations in the degree of buried and exposed groups. Also in protein chemistry is the finding that replacement of one ion for another, such as Na for K, can also introduce conformational changes (Von Hippel and Schleich, 1969). That the above could be involved in various amounts here is suggested by the altered conformation state that follows depolarization by external K, where buried protein groups appear to become accessible, and from the difference in action of reagents on the resting and depolarized axon permeabilities.

The role of membrane potential here is uncertain since equivalent depolarizations by the different methods here (Figs. 3, 5, 10, and 12) and by voltage clamp produce different results.

Voltage-clamp studies on similar crayfish axons (Shrager, 1974) show that steady state potassium slope conductance (g_K^*) has an initial dramatic increase at $V_m = -60$ mV (pH 7.5). At $V_m = -40$ mV, the slope conductance $g_K^* = 1.5 \times 10^{-2}$, and it increases further until it asymptotes at 2.2×10^{-2} S/cm^2 for positive membrane potentials. In contrast, at pH 5.8, g_K^* does not begin to increase until V_m is more positive than -40 mV. In comparison, the K-depolarized axon gives: $g_K = 6.3 \times 10^{-3}$ (pH 7.5), and 4.4×10^{-3} (pH 5.8) S/cm^2 for $V_m = -42$ mV. Also with K depolarization, the membrane conductance transition (Fig. 3) occurs around -60 to -65 mV for all pH. This thus contrasts with the voltage-clamp results where a strong pH dependency exists for the potential at which g_K changes appear initially. A major difference, however, is that g_K measured in this paper is at zero membrane current, whereas the voltage-clamp studies described above are at a short time and far removed from the zero current stationary state. In addition, voltage-clamp studies on the squid axon show a slow or long term inactivation process for the later g_K (Ehrenstein and Gilbert, 1966). A similar g_K inactivation has been found in puffer fish neurons when in either normal saline or isotonic KCl (Nakajima and Kusano, 1966). In addition, Frankenhauser (1963) described the K current in the frog node of Ranvier as having a slow inactivation process. The results here, which show a minimal increase in g_K after K depolarization, could thus be the result of a slow g_K inactivation with depolarization that would not be detected in these experiments.

Fig. 16 represents a proposed schematic of the external organization of the passive ionic controls based on these and previous results in the resting axon. The orientation of the intrinsic membrane protein proposed here is based on the finding that leucine amino peptidase, which cleaves from the N terminal end of protein, broadens the action potential, gradually reduces Cl permeability, and has pH dependent effects on P_K and P_{Na}, whereas carboxy peptidase C has no visible effect (unpublished observations). Moreover, the endopeptidases trypsin and chymotrypsin affect Na, K, and Cl permeabilities in complex time-dependent ways (unpublished observations) that suggest that with protein cleavage different charged groups at
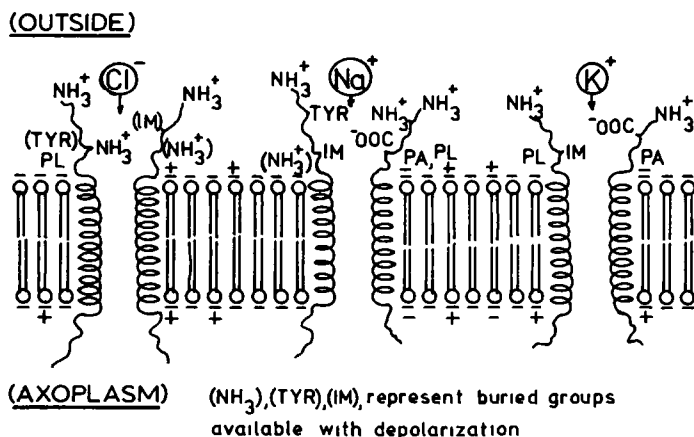

FIGURE 16 Schematic model indicating ionic channels and postulated ionizable groups involved in regulating passive ionic permeability on the crayfish axon external surface. The precise location of these groups on protein is uncertain. PA is phosphatidic acid, Im is imidazole, Tyr is tyrosine, and PL refers to other ionizable groups on phospholipid, in particular carboxyl and phosphate groups near the cationic channels. The location of the terminal carboxyl group on the regulating protein is uncertain.

different times affect access to the ion channels. The above results thus suggest that the amino terminal end of membrane protein that regulates ionic permeability is external and partially exposed, whereas the carboxyl terminal is possibly inaccessible, buried, or intracellular. In addition, the data on K depolarization and the action of DIDS suggest that some amino groups are perhaps buried or inaccessible in the resting axon, but become accessible during K depolarization. The orientation proposed here for membrane protein with the amino terminal end exposed to the cell exterior is opposite to that proposed for the integral band 3 protein of the red cell but similar to others (Marchesi et al., 1976).

The data here clearly indicate that the ionic channels in the resting and the K-depolarized axon have two different conformation states. This agrees with other evidence that changes occur in membrane binding and reagent reactive sites during membrane depolarization or excitation. Thus Marquis and Mautner (1974) found that the rate of reaction for sulfhydryl reagents is much faster and effective in blocking excitation when the axon is stimulated and depolarized than when it is at rest. Strichartz (1975) found that the rate of blockage of active Na current by local anesthetics occurs more rapidly when repetitive (conditioning) depolarizing voltages are applied. Similarly, Tasaki (1968) has suggested that a conformational change in the membrane surface occurs during depolarization and excitation.

The action observed here with TTX and the local anesthetics in altering the cooperative changes that occur with pH shifts over the imidazole pK region suggests that these reagents may act by preventing a normal conformational change from occurring with depolarization. This could occur either by immobilizing a conformational state or by altering normal structure. This view agrees with those proposed by Eyring et al. (1973), who suggest that local anesthetics act by causing conformational changes in membrane lipoproteins. Similarly, Seeman (1972, 1975) has suggested that anesthetics could function by producing membrane expansion of hydrophobic regions and thus block ionic channels by introducing extensive conformational changes in membrane protein.

In summary, the data suggest that both the conformational state of membrane protein and lipid and the local potential of charged groups close to or within ionic channels appear to be in a complex interrelation in regulating ionic permeability and are dependent on pH and external K. The precise role of membrane potential here is also evidently unclear. The interpretations presented here are tentative and clearly much further research is necessary to delineate the location and function of membrane fixed charges near ionic channels and their relation to conformational changes.

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