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Control of Ionic Permeability by Membrane Charged Groups: Dependency on pH, Depolarization, Tetrodotoxin and Procaine

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Dedicated to Torsten Teorell

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ABSTRACT

The membrane permeabilities of K, Na, and Cl were determined in crayfish giant axons from pH 3.8 to 11.4. In general, cation permeability increases with pH while anion permeability decreases. In normal saline (K_o = 5.4 mM, pH = 7), $P_K = 1.33 \times 10^{-5}$, $P_{Cl} = 1.49 \times 10^{-6}$, and $P_{Na} = 1.92 \times 10^{-8}$ cm/s. Increasing external potassium results in a dramatic membrane conductance change around K_o = 12 mM (V_m = -60 mV) which results primarily from changes in P_{Na} and P_{Cl}. In elevated potassium (K_o = 40 mM, pH = 7), P_K, P_{Cl}, and P_{Na} increase by 1.45, 8.1, and 14.2. In the potassium depolarized axon, P_{Na} and P_{Cl} show a cooperative change when pH is altered through the imidazole pK region (pK = 6.3). These changes are not seen in normal saline, or with P_K. A Hill coefficient n = 4 was found for the cooperative change of P_{Na} and P_{Cl}. An interpretation here is that four protein molecules interact to form the Na and Cl ionic channels. Tetrodotoxin has minimal effects on passive permeabilities but reduces the Hill coefficient n for P_{Na} but not P_{Cl}, while procaine reduces n for both P_{Na} and P_{Cl}. The results show that membrane fixed charged groups have varied associations and control over the different ion permeabilities. In addition, membrane conformational changes are also involved in permeability control.

INTRODUCTION

An important role of the cell surface is the regulation of ion permeability which involves the free energy of transfer of an ion from its water hydration shell to a membrane association or selectivity site, the transfer process through the membrane, and then transfer to the opposite aqueous phase (1, 2, 7). The transfer process of an ion from solution to the membrane surface also involves passage through surface membrane potentials which result from ionized membrane groups. In artificial membranes, the fixed charges have been shown

to exert considerable control over both ion permeability and the cation/anion selectivity ratio (9, 20). Since the Debye length in physiological saline is estimated around 10 Å (10), the surface potentials which exert permeability control must be local and thus depend on charged groups at or near the ion permeability access sites. The research described here is a continuation of studies (17) which demonstrate that membrane fixed charged groups have varied distributions on the K, Na, and Cl permeability pathways through nerve membrane and can exert considerable control over ion permeation.

METHODS AND MATERIAL

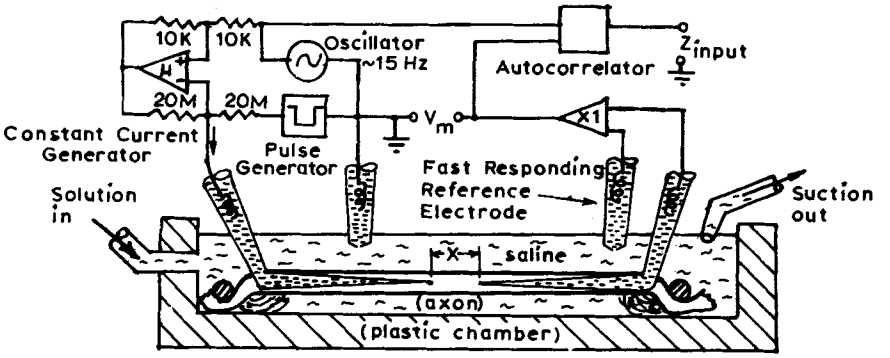

Fig. 1. Method for measuring passive ionic permeabilities.

Passive ionic permeabilities for K, Na, and Cl were determined in crayfish giant axons by methods previously described (8, 17, 18) (fig. 1). Required to calculate specific ionic permeabilities is a measurement of membrane potential (V_m), conductance (G_m), and the dependency of the membrane potential on a particular ion (T_j). The technique involves cannulating an axon with microelectrodes from both ends to avoid damage to the surface membrane which would introduce leakage errors into the permeability measurements. One electrode injects constant current while the other records membrane potential and determines axon cable properties from which the membrane resistance R_m or conductance $G_m = 1/R_m$ is determined. The specific ionic dependency T_j of the membrane potential on a jth ion is determined by introducing a partial step change in one ion by equal substitution with an equivalent but much less permeable ion in order to keep other ion activities and osmotic pressure constant. From this operation, T_j is defined as: $T_j = \partial V_m/\partial E_j \simeq \Delta V_m/\Delta E_j$, where ΔV_m is the observed change in membrane potential following the ion change, and ΔE_j is the Nernst potential change for the jth ion and equals: $\Delta E_j = (RT/zF)\ln(C_{jf}/C_{ji})$, where C_{jf} and C_{ji} are the final and initial external

ion concentrations for the jth ion, z the charge, and (RT/F) = 0.025 mV at 20°C. An example of such step changes is shown in fig. 2. Here, $T_K = \Delta V_m/\Delta E_K$ = 10/15.5 = 0.65, and $T_{Cl} = \Delta V_m/\Delta E_{Cl}$ = 1.0/15.5 = 0.07. Note that the application of fluorodinitrobenzene (FDNB) reduces T_{Cl} to zero with minimal effect on T_K.

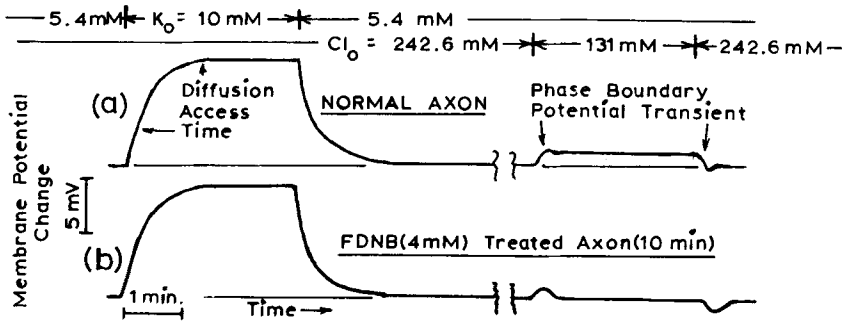

Fig. 2. a) The change in membrane potential following a step change in either external potassium or chloride. Sodium was substituted for potassium and glucuronate for chloride, one for one. b) The effects of treating the axon surface with fluorodinitrobenzene (FDNB).

The specific resting ionic permeabilities is obtained from combining the operational definition of T_j and the membrane conductance. From Teorell (20, eq. 28), the membrane potential for a fixed charged membrane may be written (with the external side referenced as zero) as:

$$\exp(-FV_m/RT) = \S = (rU_i + V_o/r)/(rU_o + V_i/r) \quad (1)$$

where

$$U_i = \Sigma u_i C_{ji},\ U_o = \Sigma u_j C_{jo},\ V_i = \Sigma u_j A_{ji},\ V_o = \Sigma u_j A_{jo} \quad (2)$$

and u_j is the ion mobility, C_{ji} and C_{jo} the inside and outside cation concentrations, and A_{ji} and A_{jo} the inside and outside anion concentrations for the jth ion. Summation is over the jth ion. The term r is the Donnan ion distribution ratio (concentration in the membrane with respect to that in solution) and is based on the assumption that equilibrium exists between the ion in the solution phase and at the membrane surface. Equation 1 was derived by Teorell (20) with no constraints on the form of the membrane potential gradient and is thus quite general, although it has been shown that with equal concentrations on both sides, a constant potential field is implied (5). With zero membrane fixed charges, r = 1, and equation 1 becomes the constant field equation.

The membrane conductance (per unit area) $G_m = \Delta I_m/\Delta V_m$ at zero membrane current, is given by Teorell (20, eq. 34) and is rewritten here as:

$$G_m(I_m = 0) = (F^2/\delta)(r\,U_o + V_i/r)(\S \ln \S)/(\S - 1) \quad (3)$$

where δ is the membrane thickness.

The diffusion coefficient $D_j(cm^2/s) = u_jRT$. The membrane permeability may thus be defined as:

$$P_j(\text{cation}) = rD_j/\delta,\ P_j(\text{anion}) = D_j/r\delta \quad (4)$$

Utilizing the operation $T_j = \delta V_m/\delta E_j$ and equations 1 through 4, expressions for specific ionic permeabilities are obtained as (17):

$$P_j(\text{cation}) = (RT/F^2)(1/C_{jo})((\S-1)/\S\ln\S)(T_jG_m) \quad (5)$$

$$P_j(\text{anion}) = (RT/F^2)(1/A_{jo})((\S-1)/\ln\S)(T_jG_m) \quad (6)$$

Equations (5) and (6) thus allow the specific ionic permeabilities P_K, P_{Na}, and P_{Cl} to be determined from measurements of T_K, T_{Na}, T_{Cl}, V_m, and G_m.

The studies here utilized medial giant axons from the ventral nerve cord of crayfish (Procambarus clarkii) obtained from California or the Gulf of Mexico. The normal saline contained in mM; NaCl 205, KCl, 5.4, $CaCl_2$ 13.5, $MgCl_2$ 2.6. In studies where the membrane was potassium depolarized, K replaced Na one for one to provide K_o = 40 mM, and Na = 170.4 mM. The non penetrating "Goods' buffers" (6) were utilized to prevent alteration of intracellular pH. Buffer concentrations ranged from 1 to 10 mM, most commonly 2 mM. Tetrodotoxin (TTX), procaine, and tetranitromethane (TNM) were from Sigma Chemical (St. Louis, MO) and 4,4'Diisothiocyanostilbene-2,2 disulfonic acid (DIDS) was from Pierce Chemical (Rockford, IL). Laboratory temperature was kept near 20°C.

RESULTS

In normal crayfish saline, the membrane permeability of K, Na, and Cl was determined as a function of pH in 0.2 pH steps from pH 3.8 to 11.4 utilizing equations (5) and (6) (fig. 3). Below pH 3.8 axon failure rapidly occurs and in alkaline pH axon failure coincides with the precipitation of calcium in the saline.

Figure 3 shows the range of pK's of ionizable groups which have been experimentally observed on protein and lipid. The pK's of protein groups are rarely found more than 1 pH unit away from the native pK in free solution. Thus figure 3 indicates which groups most likely regulate the ionic permeabilities. Clarification of which membrane groups are probably associated with regulating each ion came from utilizing group reactive reagents (11). Figure 3 suggests that chloride permeability is almost entirely regulated by the protonation of side amino groups on protein. Evidence for this is that amino peptidase, which cleaves from amino terminal ends of protein had minimal effect. DIDS, which is quite specific for amino groups, reduced P_{Cl} to zero. FDNB, which reacts primarily with amino groups and also but slower with imidazole, sulfhydryl, and phenol (11), reduced P_{Cl} to zero (see fig. 2). PCMB, which

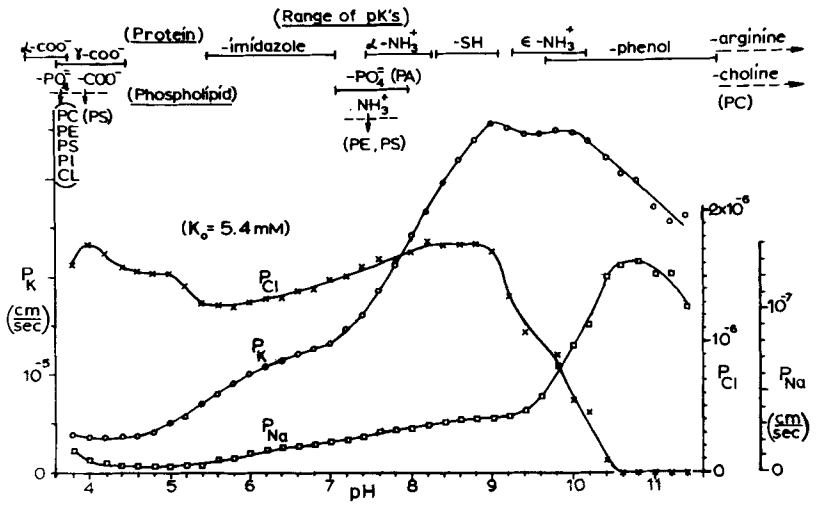

Fig. 3. The dependency of resting membrane permeabilities on pH.

reacts with sulhydryl groups and introduces a negatively charged benzoate, also reduced P_{Cl}. In resting nerve, the imidazole group appears to have no effect on P_{Cl}. Upon potassium depolarizing nerve, the protonation of imidazole quite dramatically reduces P_{Cl} to zero (fig. 7). This suggests a conformational change occurs with K depolarization, which either exposes a buried imidazole group, or moves the group to where it can affect the chloride channel.

Figure 3 suggests that P_K is initially activated by ionization of phospholipid phosphate and carboxyl (ca. pH 4), and secondarily by deprotonation of imidazole groups (pH 5 to 7). From pH 7 to 9, amino, SH, and phosphatidic acid (PA) groups could be involved in increasing P_K. The use of exopeptidases which cleave from either the carboxyl or amino terminal ends of protein showed no effects on P_K. Similarly, the amino reactive reagents DIDS and FDNB, and the SH reagent PCMB had minimal effects on P_K. Thus the secondary ionization of PA, and not amino or SH groups appear involved here in regulating P_K.

Sodium permeability appears to follow that of potassium with respect to pH except for the alkaline region (ca. pH 9.5 to 11) where resting P_{Na} increases. Figure 3 suggests that deprotonation of amino groups and/or ionization of tyrosine could increase P_{Na}. The application of DIDS (for amino groups) and TNM (for tyrosine) showed that both reagents reduced P_{Na}, which suggests that both tyrosine and amino groups contribute to regulating P_{Na} in resting nerve.

Membrane ionic channels are often considered as being voltage dependent or non voltage dependent. Among the methods used to change potential are: a) A forced potential change as in a voltage clamp. 2) Chemically altering membrane components regulating specific permeabilities. 3) Altering external or internal ion concentrations. The latter method, where membrane potential

is altered by changes in ion concentrations, was examined and found to give different results depending on whether internal or extracellular ions are changed. Figure 4 shows the changes in membrane permeability which occur

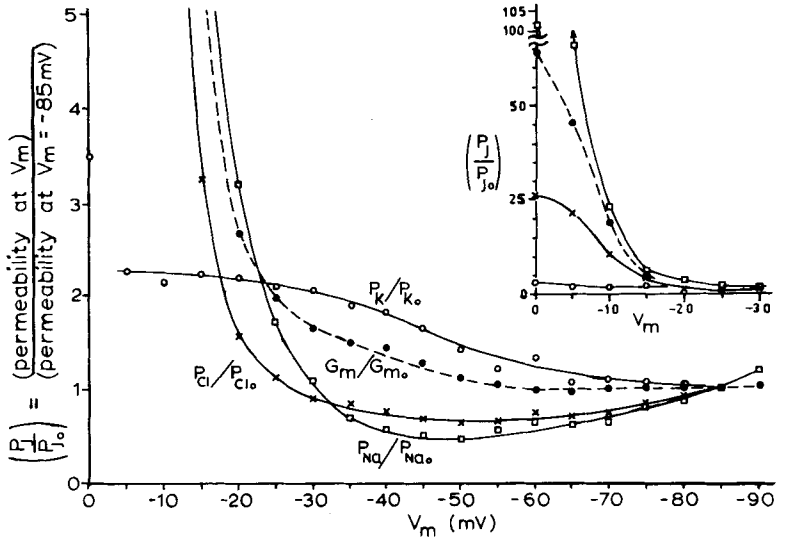

Fig. 4. The dependency of membrane permeability on membrane potential. Depolarization is produced here by intracellular potassium loss and sodium gain with time during gradual axon failure. Extracellular concentrations are kept constant. pH = 7.0.

(at pH 7) when intracellular ion concentrations change gradually due to K depletion and Na gain with time. Observed is that no major change occurs in ionic permeabilities and total membrane conductance until membrane potentials falls below -30mV. In contrast, when the axon is potassium depolarized by increasing external K, a major change in membrane conductance G_m occurs around K_o = 12-13 mM (V_m = -60 to -65 mV)(fig. 5). Suggested here

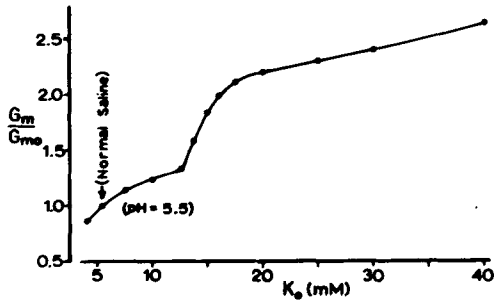

Fig. 5. The dependency of membrane conductance G_m on external potassium. Sodium replaced potassium one for one. G_{mo} equals the membrane conductance in normal saline.

is that a major change in the conformation of components has occurred. This change occurs at all pHs and is most dramatic at pH 5.5 as shown here. For K concentrations above 25 mM, the permeability transitions seem nearly complete. The dependency of membrane conductance G_m on pH in K_o = 40 mM was compared with that obtained in normal saline (fig. 6). The data shows that for pH = 7.0,

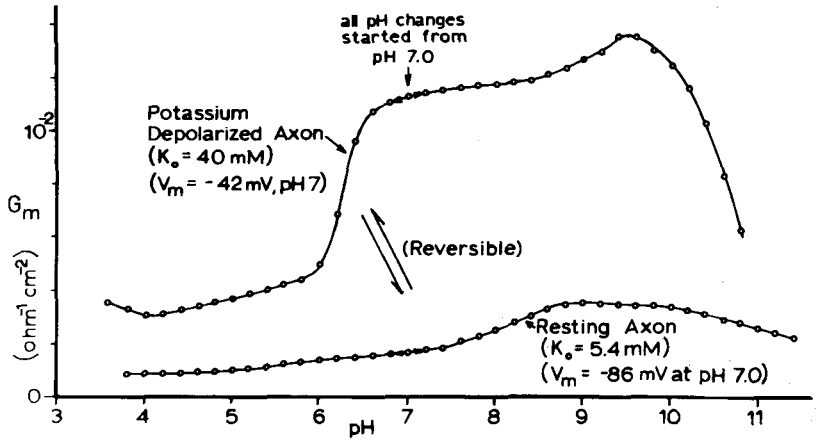

Fig. 6. The total membrane conductance G_m in normal saline (K_o = 5.4 mM) compared with the potassium depolarized membrane (K_o = 40 mM), and the dependency on pH.

total membrane conductance increases by 6.8, and a steep conductance transition occurs with pH over the imidazole pK region, an effect which is absent in normal saline. The specific ionic permeabilities was determined in elevated potassium (40 mM) as a function of pH (fig. 7).

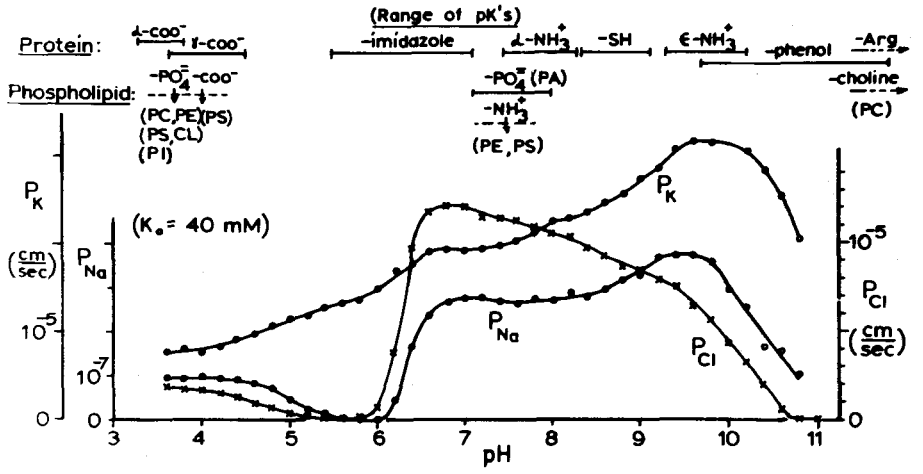

Fig. 7. Membrane permeabilities as a function of pH in the potassium depolarized axon.

The data of figure 7 shows that P_K minimally increases with K depolarization (compare with fig. 3), while P_{Na} and P_{Cl} both increase by 14 and 8 fold, respectively, at pH 7.0. Most striking is that the steep conductance change observed over the imidazole pK region (fig. 6) does not involve P_K but only P_{Na} and P_{Cl}.

The actions of the group reagents DIDS and TNM were compared for the resting and depolarized axon. DIDS is most reactive in alkaline pH and here (10^{-5}M, pH 10), it irreversibly reduced P_{Na} and P_{Cl} to near zero with minimal effects on P_K in resting nerve (K_o = 5.4 mM). With DIDS removal and potassium depolarization (K_o = 40 mM), a major portion of the suppressed P_{Na} and P_{Cl} was restored. The reapplication of DIDS here again reduced P_{Na} and P_{Cl} to low values.

TNM (10^{-4}M), was utilized in a 1% ethanol solution of saline. In resting nerve, TNM suppressed only P_{Na}. In potassium depolarized nerve however, TNM only suppressed P_{Cl}. Both of these studies with DIDS and TNM thus indicate the presence of buried groups in resting nerve which become accessible with potassium depolarization. Additionally, the results support the view that two different conformational states exist for the resting and depolarized nerve.

The steepness of the permeability changes of P_{Na} and P_{Cl} in depolarized nerve (fig. 7) suggest that the proton binding sites in each ion channel are interactively linked and cooperative. This cooperative type interaction will be examined with the assumption that the observed permeability is proportional to the fraction of sites θ occupied by protons on the ionizable membrane groups (figs. 8, 9). A Scatchard plot of figures 8a and

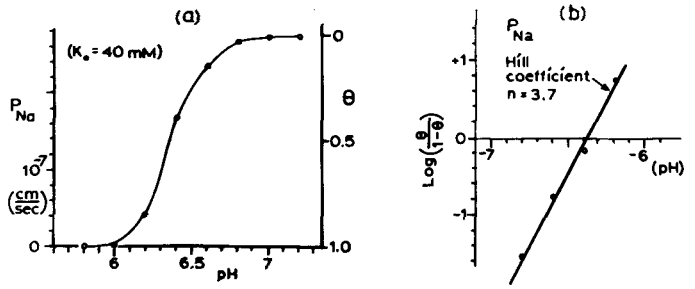

Fig. 8. a) Sodium permeability and pH. θ represents the fraction of titratable sites occupied by protons which regulate sodium permeability over the imidazole pK region. b) Hill plot for the sodium permeability data of (a).

9a shows a downward concavity and thus non interacting sites cannot be assumed. A Scatchard plot with downward concavity is also considered

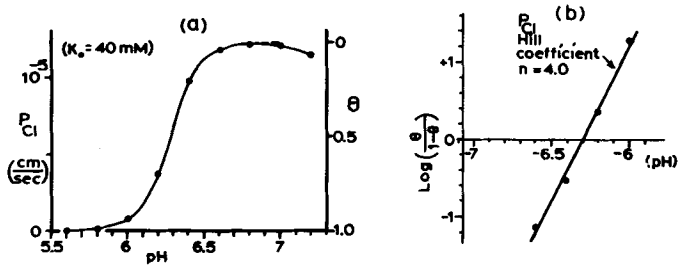

Fig. 9. a) Chloride permeability and pH. θ represents the fraction of titratable sites occupied by protons which regulate chloride permeability over the imidazole pK region. b) Hill plot for the chloride permeability data of (a).

definite evidence for interactive sites where the free energy change of ligand to site interactions decrease as more sites are occupied (3). Thus strong positive cooperativity appeared to occur here where the binding of protons strongly activates other sites so that they fill up more readily. For the cooperative model of Monod-Wyman-Changeux, and for the Adair equation, the Hill equation becomes the limiting case of strong positive cooperativity. The Hill equation can be written as: $\theta/(1-\theta) = K(H)^n$, where $\theta/(1-\theta)$ represents the ratio of the fraction of ligand (H) binding sites occupied to those vacant on the protomer. K is an equilibrium constant and n represents in the ideal limit, the number of interacting elements (3, 12, 21, 23). The coefficient n could thus represent n interacting protein molecules or n sites on one protein. Figures 8a and 9a show P_{Na} and P_{Cl} redrawn from figure 7. It is assumed here that above pH 7, hydrogen binding to the cooperative imidazole group is zero and the fraction of sites occupied (θ) equals zero, while at pH 5.6, θ = 1 where both P_{Na} and P_{Cl} are maximally altered and equal zero. The Hill equation gives: $\log((\theta/(1-\theta)) = \log K - npH$ and is plotted in figures 8b and 9b for P_{Na} and P_{Cl}. A Hill coefficient n of near 4 is obtained for both these permeabilities which suggests that four protein molecules could be associating to form the membrane ionic channels for Na and Cl.

It is not known whether the active sodium ionic channel is created de novo during excitation, or whether it is a modified passive ionic channel. It has been suggested that partial membrane fluctuations, which create active sodium channels could represent the resting sodium permeability (13). Tetrodotoxin (TTX) was added to resting and depolarized nerve at concentrations which blocked excitation. In resting nerve, TTX had minimal effects on the measured passive ionic permeabilities of Na, K, or Cl, although the action

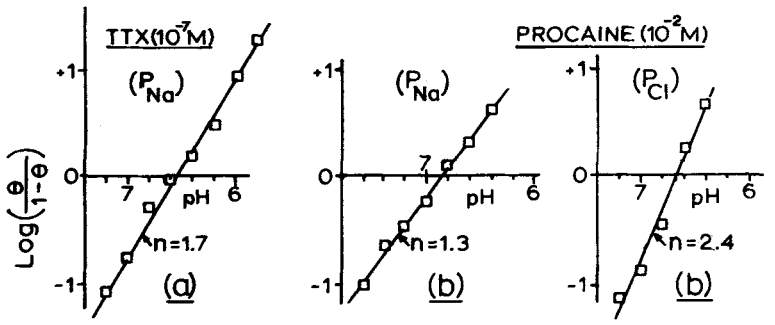

Fig. 10. a) The action of Tetrodotoxin on the Hill coefficient n of the cooperative permeability change of sodium when pH is altered over the imidazole pK region. b) The action of procaine on the Hill coefficient n for P_{Na} and P_{Cl}.

potential was blocked. Thus the possibility, that the random creation of active sodium channels represents passive permeability does not seem probable since TTX presumably blocks active channel formation. However in potassium depolarized nerve, TTX reduces the Hill coefficient n for P_{Na} (fig. 10a). The Hill coefficient n for P_{Cl} was not affected. Suggested therefore is that TTX acts by altering or modifying the passive Na channel by reducing cooperative interactions. Procaine was also added at concentrations which blocked excitation and reduced the Hill coefficient n for both P_{Na} and P_{Cl} in the depolarized axon (fig. 10b). The action of procaine is quite complex since its pK is 8.95 and it changes ionization and membrane penetrability with pH. At pH 7, where procaine is predominantly positively charged, it raises P_{Na}, but suppresses P_{Cl} and P_K. In the depolarized axon, procaine suppresses all permeabilities at pH 7.0.

DISCUSSION

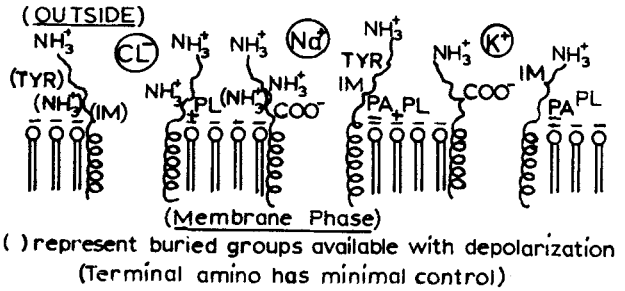

Fig. 11. Schematic of membrane charged groups believed to be involved in regulating K, Na, and Cl passive permeabilities.

The results here clearly indicate that various membrane fixed charged groups exert a major control over ion permeation and these are depicted in figure 11. In addition, a conformational change appears to occur in the permeability regulating components following external potassium depolarization. This is evidenced by the appearance of buried groups which appear inaccessible to reagents in resting nerve but become accessible with depolarization. These membrane conformational changes appear to primarily involve P_{Na} and P_{Cl} but not P_K. The role of membrane potential in inducing these changes is not certain since equivalent potential changes produced by an alteration of intracellular ions result in minimal changes for P_K, P_{Na}, and P_{Cl}. These constrasting results, which show different permeabilities depending on which surface sees changes in ions, suggests that local ion atmospheres contribute to membrane conformation and permeability. Support for this view are experiments which show that protein structure depends on the Hoffmeister series of ions with the "salting out" ions (i.e. K) preserving more the helical form than the "salting in" or coil denaturing ions such as calcium (22). Similarly, studies with isolated rat brain membranes containing intrinsic protein showed that in the presence of K, membrane protein was primarily in an antiparallel beta form. However, in a media of Ca or Na, the membrane protein structure shifted to either a disorded conformation or alpha helix (14). The results here in elevated external K, thus suggest that a Ca-K displacement interaction on the external surface may be involved in altering the membrane conformational state and not the change in membrane potential. A related similar view has also been proposed by Tasaki (19).

The analysis here of the linked cooperative interactions observed when pH is altered over the imidazole pK region suggests that 4 protein molecules may strongly interact to form the Na and Cl ionic channels. When procaine or TTX block excitation, they also reduce the Hill coefficient n for the sodium channel in depolarized nerve. TTX affects the coefficient n with minimal affects on passive permeabilities. This suggests that the blockage of excitation possibly involves the limiting of cooperative interactions of membrane groups on or near the ionic channel and that excitation involves changes in the passive sodium channel. This view corresponds with other views proposed (4) where local anesthetics are believed to act by causing conformational changes in membrane lipo-proteins, possibly by producing membrane lipo-proteins, possibly by producing membrane expansion of hydrophobic regions (15, 16).

This paper is dedicated in honor of Professor Torsten Teorell, in whose laboratory this investigator received encouragement for this work. The research was supported by grants from the American Heart Association, Indiana Affiliate, and from NIH.

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