

Interaction of H⁺ Ions with Acid Groups in Normal Sodium Channels

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Abstract. The ionic currents through sodium channels of nodal membrane were measured under voltage clamp conditions at normal and low pH external solutions. Inhibition of the currents at low pH was described on the assumption that there are two acid groups at the sodium channel: one (inner) within the pore, the second (surface) just near external end of the pore. The protonation of the inner group is potential-dependent and causes an almost complete blockage of the channel; the protonation of the surface group is potential-independent and results in a partial decrease in channel conductance. Calculations according to this model allowed an estimation of effective pK values for each of the groups, the electric distance from the outer to the inner group and the degree of Coulombic interaction between both groups. From reversal potential measurements, relative permeability of the sodium channels to H⁺ ions (P_H/P_{Na}) was estimated to be 274 ± 156 (\pm S.D.).

Key words: Nodal membrane — Voltage clamp — Sodium channels — Acid groups — Permeability

Introduction

A decrease in the pH of an external solution has been shown to inhibit the flow of current through sodium channels in nerve and muscle cell-membranes (Hille 1968; Woodhull 1973; Drouin and Neumcke 1974; Ulbricht and Wagner 1975; Shauf and Davis 1976; Campbell and Hille 1976). These data have been interpreted as evidence for existence of an acid group within the sodium channel. This group has been assumed to be a part of selectivity filter (Hille 1975a, b). Until recently, the same acid group had also been assumed to be part of a receptor for tetrodotoxin (TTX) (Hille 1975b, c). However, studies of sodium channels modified by alkaloid aconitine showed that channel selectivity and sensitivity to H⁺ ion blockage can be markedly altered without changes in sensitivity to blockage by TTX (Mozhayeva et al. 1976; Mozhayeva et al. 1977; Naumov et al. 1979). This suggests that the structure of the selectivity filter and TTX receptor are not equivalent. However, TTX blockage may be antagonized by a low pH external solution (Ulbricht and

Wagner 1975). Thus, it was inferred (Naumov et al. 1979) that there is at least one more acid group which seems to be part of a TTX receptor. Recent studies of sodium channels modified by carboxylic group reagent trimethyloxonium ions (TMO) demonstrated that TMO abolishes the sensitivity of sodium channels to TTX blockage without marked changes in selectivity (Spalding 1978, 1980). Thus, two types of experiments show that there are at least two acid groups incorporated in the structure of the sodium channel, which are accessible for protonation from an external solution.

In view of these studies it is important to determine how many acid groups are directly involved in the control of channel conductance and to estimate the parameters of these groups. In addition it would be of interest to estimate the permeability of the sodium channels to hydrogen ions. For this purpose, the effect of H^+ ions on sodium channels was studied over wide range of H^+ ion concentrations and membrane potentials.

Methods

The work was done on myelinated fibres from frog, *Rana ridibunda*, using the voltage clamp method (Mozhayeva et al. 1977). Holding potential (V_{ii}) was set at -100 mV. Each depolarizing test pulse was preceded by a hyperpolarizing pulse of -140 mV (100 ms) in order to remove fast inactivation. Membrane potential (V_m) is referred to the outside. When estimating the sodium currents (I_{Na}), the resistance of the axoplasm was assumed to be equal to 20 m Ω . Leakage and slow capacitative currents were subtracted electronically.

External solutions contained either 20 mmol.l $^{-1}$ biphthalate (pH from 4.0 to 6.3) or Tris-buffer (pH 7.65–7.70). In order to keep a constant content of permeable ions in all external solutions, Na^+ ion concentration was decreased to 80 mmol.l $^{-1}$ and K^+ ion concentration was increased to 30 mmol.l $^{-1}$. All external solutions contained 2 mmol.l $^{-1}$ Ca^{2+} ions and 10 mmol.l $^{-1}$ tetraethylammonium ions (TEA^+). The ends of the fibres were cut in a solution containing 115 mmol.l $^{-1}$ KF, 5 mmol.l $^{-1}$ TEA-Cl and 5 mmol.l $^{-1}$ Tris-Cl. TEA^+ ions were added to all solutions in order to block the potassium channels (Hille 1967; Koppenhöfer and Vogel 1969).

The experiments were carried out at 9 – $10^\circ C$.

Measurements were begun after a 25–30 minute delay to allow the temperature to stabilize. During this period Na^+ concentration in the internal solution increased, and therefore reversal potentials of I_{Na} (E_r) were generally rather low (20–30 mV).

In order to estimate the degree of the H^+ block, the ratio of chord conductance at low pH to conductance at high pH ($g_{pH}/g_{7.7}$) was used. The use of a conductance ratio instead of a current ratio was intended to reduce any error due to changes in reversal potential during the experiment. Changes in E_r were due both to its progressive drift to zero value and to lowering pH. In order to single out changes in E_r due to lowering pH, a difference was determined between the E_r value at low pH and the average of two E_r values (before and after) at high pH. In order to minimize the error due to a progressive rundown of sodium conductance, measurements at low pH were bracketed by measurements at high pH, and $g_{pH}/g_{7.7}$ was determined using the average of two (before and after) $g_{7.7}$ values. Over the course of the experiment sodium conductance was generally not decreased by more than 20–25 % of the initial value.

Results and Analysis

Fig. 1 shows peak current versus membrane potential at different pH values of the external solution. Lowering pH results in a shift of the voltage range of activation of I_{Na} towards a more positive V_m and leads to a decrease of I_{Na} .

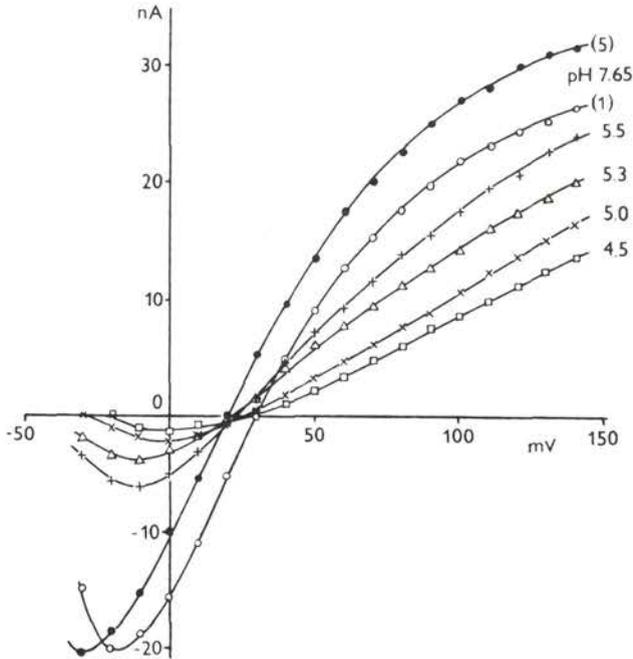


Fig. 1. Peak current-voltage relations for different pH of external solution. pH values are indicated by numbers near curves. The curves were visually estimated. For pH 7.65 only first (1) and last (5) curves are shown. See text for details. Node 42.

The shift of the voltage range of activation may be due to a neutralization of negative surface charge in the vicinity of sodium channels as a result of the protonation of "nonspecific" anionic groups (Hille 1968; Mozhayeva and Naumov 1970; Drouin and Neumcke 1974; Hille et al. 1975). These shifts on lowering pH from 7.65 to 4.5 amount to about 25 mV, which is in accordance with data of Hille et al. (1975).

The decrease of peak sodium currents at negative potentials is due both to this shift and to the blockage of open channels. Therefore the proton blockage of open sodium channels was determined from peak current measurements, only for positive membrane potentials, where the number of open channels at the peak of I_{Na} is approximately constant (Dodge and Frankenhaeuser 1959; Woodhull 1973),

Fig. 2a shows the family of experimental $g_{\text{pH}}/g_{7.7}$ -pH curves corresponding to four V_m levels during the test pulse. It can be seen that the increase of positive V_m values results in a shift of the $g_{\text{pH}}/g_{7.7}$ -pH curve along pH axis towards lower pH. This implies that the blockage decreases at more positive potentials. The effect is in qualitative accordance with Woodhull's data (Woodhull 1973). If only one acid group with potential-dependent proton binding were responsible for channel conductance, the $g_{\text{pH}}/g_{7.7}$ -pH curve should shift along the pH axis in response to V_m changes without altering its shape. However, this experiment demonstrates that the $g_{\text{pH}}/g_{7.7}$ -pH curve for $V_m = +140$ mV is much less steep than that for $V_m = +10$ mV. The theoretical titration curve of a single acid group fits rather well to experimental points for $V_m = +10$ mV, but it goes much steeper than the experimental curve for $V_m = +140$ mV.

If channel conductance were controlled by one acid group, the apparent pK of

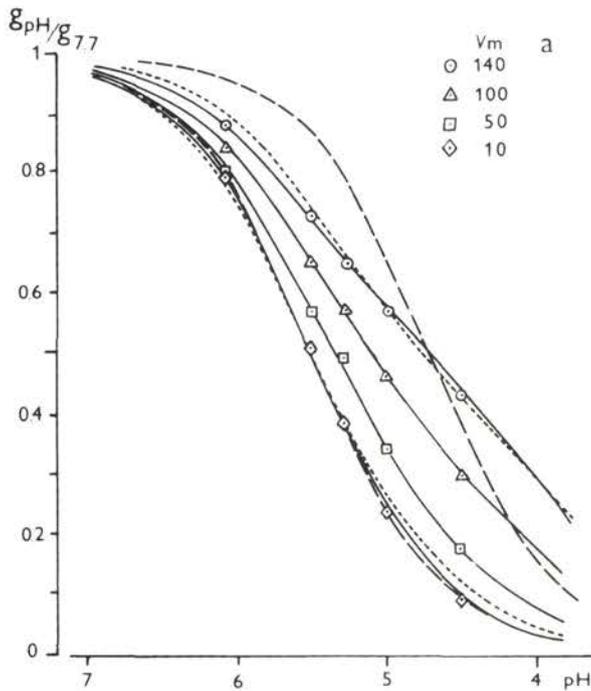


Fig. 2. Inhibition of the sodium conductance by hydrogen ions. **a)** The dependence of the sodium conductance on pH of external solution. Symbols are experimental $g_{\text{pH}}/g_{7.7}$ values. The numbers near to symbols indicate V_m during the test pulse. Dashed lines are theoretical titration curves of a single acid group. Solid lines are calculated by equation (2) with the following parameter values: $F^2 = 1$, $\delta = 0.40$, $\text{pK}_{1(0)} = 4.90$, $\text{pK}_2 = 5.60$. Dotted lines were calculated by the same equation with: $F^2 = 4$, $\delta = 0.40$, $\text{pK}_{1(0)} = 5.42$, $\text{pK}_2 = 5.42$.

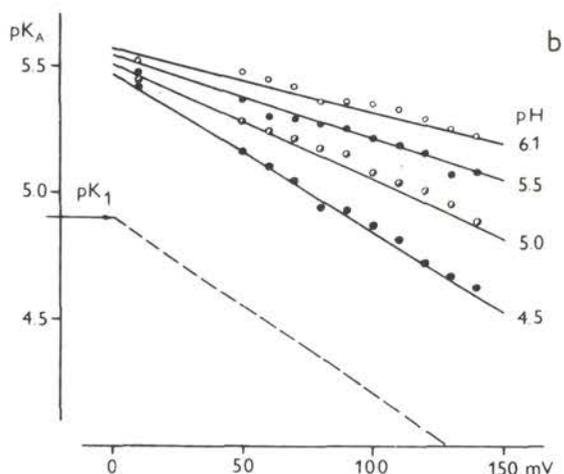


Fig. 2. Inhibition of the sodium conductance by hydrogen ions. **b)** The dependence of apparent pK (pK_A) on potential. Symbols denote pK_A values calculated by substitution of experimental $g_{pH}/g_{7.7}$ values into equation (1). Solid lines were calculated by substitution of theoretical $g_{pH}/g_{7.7}$ values from equation (2) into equation (1). The dashed line indicates the pK of the inner acid group. The same parameter values were used as in Fig. 2a with $F^2 = 1$. See text for details. Node 43.

this group (pK_A) should be given by following equation (Naumov et al. 1979):

$$pK_A = \lg [(g_{7.7}/g_{pH}) - 1] + pH \quad (1)$$

However, it can be seen from Fig. 2b that pK for every particular V_m level is not constant. It depends on the pK at which measurements were made. Experimental pK_A - V_m curves are different at different pH values: the lower pH is, the steeper the curve.

In order to describe the data presented let us assume that there are two acid groups at the channel: one of them within the pore and another just at the external end of the pore. Then, protonation of the first (inner) group would be potential-dependent (Woodhull 1973), while protonation of the second (surface) one would not. Let us further assume that the protonation of the inner group results in complete blockage of the channel, whereas the protonation of the surface group reduces channel conductance (current) to a fraction α of its normal value. Generally, Coulombic interactions between protons bound to both groups should be taken into account. The repulsive effect of one H^+ ion occupying one site on the rate of movement of another H^+ ion into or out of second site can be expressed by multiplying the respective rate constants by the factors F_{in} and F_{out} (Aityan et al. 1977; Hille and Schwarz 1978). Let us assume for simplicity that these factors are equal for both groups and that $F_{out} = F = 1/F_{in}$. Then relative sodium conductance is expressed by the following equation:

$$\frac{g_{pH}}{g_{7.7}} = \frac{F^2(1 + \alpha C_H K_2)}{F^2(1 + C_H K_2) + C_H K_1 (F^2 + C_H K_2)} = \frac{F^2(1 + \alpha C_H K_2)}{F^2(1 + C_H K_2) + C_H S K_2 (F^2 + C_H K_2)} \quad (2)$$

where C_H — activity of H^+ ions, K_1 and K_2 — binding constants of H^+ ions with inner and surface groups respectively, $S = K_1/K_2$.

Absolute values of K_2 only affect the position of the $g_{pH}/g_{7.7}$ -pH curve and can be determined by shifting calculated (with initially arbitrary K_2 values) curves along the pH axis until they fit the experimental curves. The shape of $g_{pH}/g_{7.7}$ -pH curves depends on α , S and F values, which are not known in advance.

Sigworth and Spalding (1980) and Sigworth (1977) have shown that both decrease of pH and the treatment of the membrane with TMO results in an approximately three-fold reduction in single channel conductance at $V_m = 0$ mV. It is natural to think that the surface groups assumed in the model and groups accessible for esterification by trimethyl- or triethyloxonium ions (Backer and Rubinson 1977) are the same groups. Therefore α at $V_m = 0$ mV can be assumed to be equal to 0.3. Fig. 3 shows peak current-voltage relations before and after the

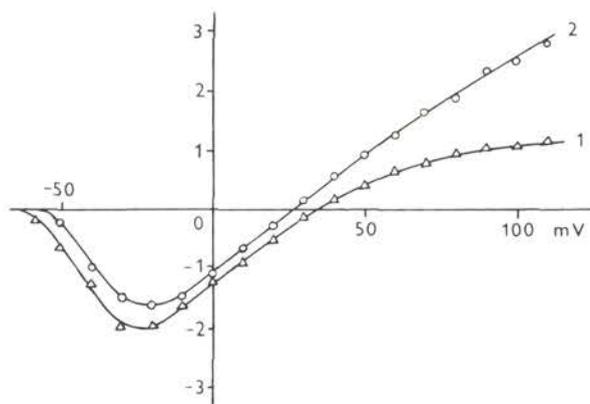


Fig. 3. Peak current-voltage relations for a node prior to (curve 1) and following treatment with triethyloxonium ions (curve 2). Curve 2 was measured in the presence of TTX (10^{-7} mol.l $^{-1}$). For curve 1 every division on ordinate is 10 nA, for curve 2 — 1 nA. The treatment with triethyloxonium was performed after the manner previously described for trimethyloxonium ions (Spalding 1980). Node 92.

treatment of the node by triethyloxonium ions. A part of the channels was modified in this and other analogous experiments. Therefore I_{Na} after triethyloxonium ion treatment was measured in the presence of TTX (10^{-7} mol.l $^{-1}$) in order to eliminate currents passing through unmodified channels. It can be seen that current-voltage relation for modified channels is almost linear, whereas that for

normal ones is convex at positive potentials (see also Fig. 1 and 5). Within the frame of our model this implies that α must increase with V_m . From three experiments analogous to that presented in Fig. 3 and from a study by Sigworth and Spalding (1980) it was concluded that α should increase approximately from 0.3 to 0.6 as V_m increases from 0 to +150 mV.

Let us assume according to Woodhull (1973) that K_1 (and consequently S) is exponentially dependent on potential (at least at positive potentials) with the exponent factor equal to a fraction of the total potential drop from the outer to the inner group (δ). The substitution of equation (2) into equation (1) shows that the slope of calculated pK_A - V_m curves increases as pH decreases. The comparison of calculated and experimental pK_A - V_m curves for the lowest pH used in our experiments indicates that δ value should not be less than 0.4.

Table 1. Parameters of the first (inner) and the second (surface) acid groups determined in accord with the equation (2). See text for details.

Node	δ	$pK_{1(0)}$		pK_2	
		$F^2 = 1$	$F^2 = 4$	$F^2 = 1$	$F^2 = 4$
42	0.40	5.12	5.57	5.82	5.57
43	0.40	4.90	5.42	5.60	5.42
44	0.40	5.17	5.42	5.47	5.42
45	0.40	5.10	5.42	5.50	5.42
46	0.40	4.95	5.50	5.35	5.50
100	0.51	5.05	5.42	5.15	5.12
101	0.40	4.85	5.20	5.15	4.90
103	0.45	5.00	5.32	5.10	5.02
<i>Mean</i>	0.42	5.02	5.41	5.39	5.30
$\pm S.D.$	± 0.04	± 0.11	± 0.11	± 0.25	± 0.25

If one assumes that there is no interaction between H^+ ions bound to both groups, that is $F^2 = 1$, an optimal agreement between the model and experiment presented in Fig. 2 is obtained with following values of parameters: $\delta = 0.40$, $pK_{1(0)} = 4.90$, $pK_2 = 5.60$, $pK_{1(0)}$ is pK_1 at $V_m = 0$ mV. On average, these parameters for $F^2 = 1$ are: $\delta = 0.42$, $pK_{1(0)} = 5.02$, $pK_2 = 5.39$ (see Table 1).

No reasonable agreement between the model and the experimental data can be obtained with $F^2 > 4$, so it should be concluded that $1 \leq F^2 \leq 4$. If one assumes $F^2 = 4$, a rather good agreement between the model and experiment presented in

Fig. 2 can be reached with the following values of parameters: $\delta = 0.40$, $pK_{1(0)} = 5.42$, $pK_2 = 5.42$.

Here, as in Table 1, the pK of each group is relevant to the case when another group is not protonated. In this case the binding of protons on each of groups depends (at given pH) on both the inherent binding properties of given group and Coulombic effect of another group. In order to determine inherent binding constants for each group, K_1 and K_2 from equation (2) must be divided by F^2 . Naturally, with $F^2 = 1$ the binding constants from equation (2) are inherent ones irrespective of protonation of either group. Inherent $pK_{1(0)}$ and pK_2 for the experiment presented in Fig. 2 with $F^2 = 4$ are equal to 4.82. On average the inherent $pK_{1(0)}$ and pK_2 values with $F^2 = 4$ are equal to 4.81 and 4.70 respectively.

So, with Coulombic interaction between protons, the lower inherent affinities of both groups to H^+ ions are required to describe experimental data. In as much as 1 and 4 are permissible extreme values for F^2 , the inherent pK values of inner (5.02 and 4.81) and surface (5.39 and 4.70) groups obtained in the above calculations should be considered as maximal and minimal possible ones.

Blockage of sodium channels by H^+ ions at negative potentials can be studied by measuring so called "instantaneous" currents (Dodge and Frankenhaeuser 1959). For this purpose two successive test pulses were used (Fig. 4). V_m during the first pulse was constant (+50 or +60 mV) and its duration was set so that I_{Na}

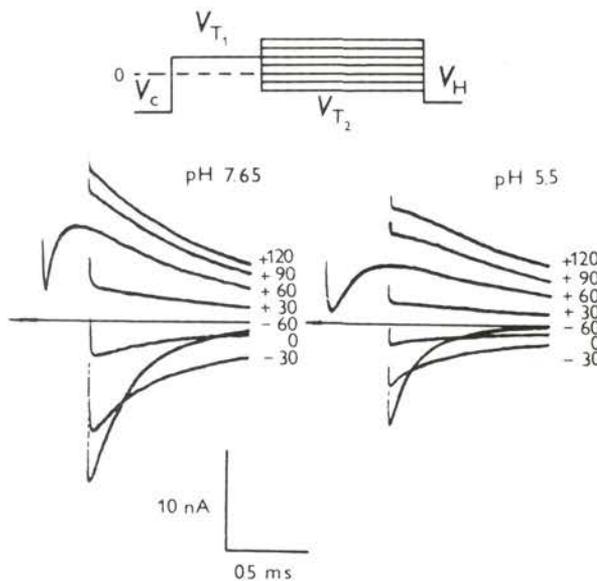


Fig. 4. "Instantaneous" currents at two pH values of external solution. At the top of the Figure the pulse programming is shown. Numbers near records indicate the potential during the second test pulse. Node 46.

reached a peak value. "Instantaneous" I_{Na} were measured at the beginning of the second test pulse, the voltage level of which was varied from -50 to $+150$ mV. Fig. 5 shows "instantaneous" and peak current-voltage relations for three pH values. It can be seen, that at positive V_m "instantaneous" currents are the same as

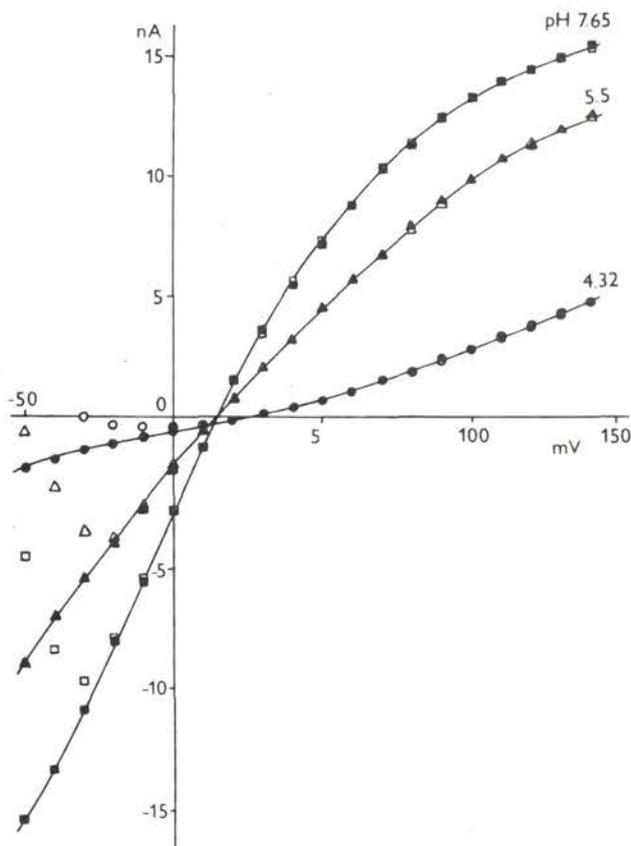


Fig. 5. "Instantaneous" current-voltage relations for three pH values. Solid symbols indicate "instantaneous" currents, open — peak currents. Node 46.

peak ones at all pH values tested. This implies that the number of open channels at the peak sodium current is approximately constant for positive potentials. If so, it is possible that the "instantaneous" current-voltage relations relate to a constant number of the open channels at all potentials. In other words, the "instantaneous" current-voltage relations reflect properties of open sodium channels.

It can be seen from Fig. 5 that the current-voltage relations for open channels tend to be symmetrical on both sides of the reversal potential. Thus, at normal pH they tend to be convex at negative potentials although to a lesser degree than at positive potentials. A similar "instantaneous" current-voltage relationship was

observed by Sigworth (1977). At low pH the current-voltage relations become approximately linear or even concave on both sides of the reversal potential. Fig. 6 shows $g_{pH}/g_{7.7}$ values versus V_m at low pH of solution. It can be seen that $g_{pH}/g_{7.7}$ increases both at positive and at negative potentials. In all experiments where such

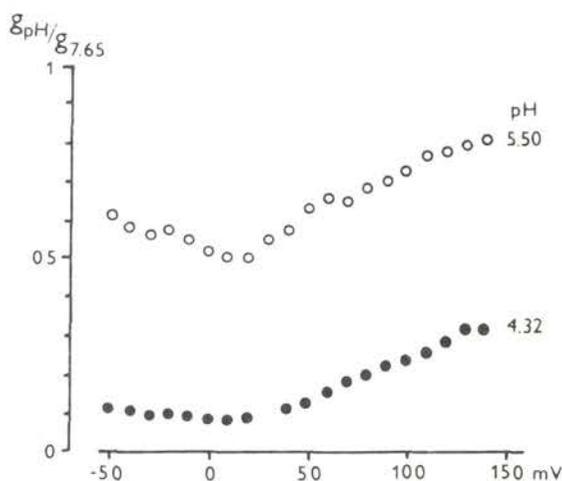


Fig. 6. Dependence of the relative sodium conductance on potential for two pH values. Symbols indicate $g_{pH}/g_{7.7}$ values. Node 46.

measurements were made this quantity either increased or remained unchanged as negative potentials increased. These results imply that the degree of protonation of the inner group (at a given pH) does not increase indefinitely with increasing negative potentials. This implies that the rate of H^+ ions passing from a binding site to the internal solution at zero potential is comparable to the rate of its returning from a binding site to the external solution. Occupancy of the binding site by H^+ ions at negative potentials can be reduced due to increased rate of H^+ ions moving from the site to the internal solution. Such a possibility follows from Woodhull's general model (Woodhull 1973).

If the rate of H^+ ions moving from the inner acid group to the internal solution is, in fact, comparable to the rate of H^+ ions returning to the external solution, the permeability of the sodium channels to H^+ ions would be expected to be high, and a decrease in pH may result in the shift of the reversal potential towards more positive potentials. Indeed, this appears to be the case. Shifts in ΔE_r are evident in Fig. 1 and 5. The relative permeability of sodium channels to H^+ ions can be calculated from the following equation (Hodgkin and Katz 1949):

$$\frac{P_H}{P_{Na}} = \left(\frac{P_K C_K}{P_{Na} C_H} + \frac{C_{Na}}{C_H} \right) [\exp(E_r/24.4) - 1] \quad (3)$$

where C_K , C_{Na} and C_H are activities of K^+ , Na^+ and H^+ ions in the external solution.

C_H for the control solution was neglected when deriving the equation. For all univalent cations the activity coefficient was assumed to be 0.78. ΔE_r is the shift in

Table 2. Relative permeability of sodium channels to H^+ ions. ΔE_r is the shift in reversal potential of I_{Na} associated with the substitution of the control solution (pH 7.65) by the acid solutions (pH 3.7–4.47). P_H/P_{Na} is calculated by equation (3).

Node	pH	ΔE_r (mV)	P_H/P_{Na}
38	4.47	2	157
40	4.47	2	157
41	4.47	3	240
45	4.47	2	157
45	4.32	2	111
46	4.47	6	512
46	4.32	9	580
95	4.29	9	545
96	4.00	6	174
97	4.00	4	111
97	3.71	8	128
100	4.25	6	310
101	4.25	6	310
101	4.00	10	316
102	4.24	7	357
103	4.00	7	215
Mean			274
\pm S.D.			\pm 156

reversal potential with lowering pH. P_K/P_{Na} is assumed to be 0.1.

Table 2 presents measured ΔE_r values and calculated P_H/P_{Na} values for 12 experiments. On average P_H/P_{Na} is equal to 274.

Discussion

The results presented show clearly that the inhibition of sodium conductance at low pH is due to protonation of at least two acid groups in the sodium channel. It is natural to assume that the inner one is a part of selectivity filter and the surface one is a part of TTX receptor.

The functional role of the external group is to increase channel conductance. Sigworth and Spalding (1980) assumed that the external group increases channel conductance (in comparison with the case when its negative charge is removed by protonation or esterification) due to an enhancement of local cation concentration near the mouth of the pore. In this case the increase in cation concentration near to an external negative charge is due to the deepening of an energetic well for cations.

However, according to saturable pore models (Chizmadzhev et al. 1974; Hille 1975a) deepening the mouth well alone should cause a decrease in the channel conductance rather than an increase. The increase in the channel conductance due to the presence of the negatively charged surface group may be expected if the height of the main rate limiting barrier(s) is reduced. Because the inner acid group is assumed to be a part of the selectivity filter (Hille 1975a, b) the main barrier for alkali cations and the inner binding site for H^+ ions seem to fall close together in the channel. Therefore the energetic levels of the main barrier and the inner well should both change by the same value, as the surface group is protonated (or deprotonated). Thus, the parameter F^2 (determining the Coulombic effect of the surface group on the binding of H^+ ions to the inner group) and the parameter α (determining the effect of the same group on the height of the rate limiting barrier) are interrelated. A simple evaluation shows that $F^2 = 3$ can be enough to ensure the required approximately threefold reduction of the channel conductance as the surface group is protonated. This F^2 value is within the range of values (from 1 to 4) permissible for H^+ ion blockage. Thus, at the present stage of investigation, one may assume a value of F^2 equal to 3. If one assumes minimal and maximal possible values for the effective dielectric constant around the group to be 20 and 60, the maximal and minimal possible distances between surface and inner groups are 2.6 nm and 0.9 nm respectively. Because the assumed contribution of the surface group to the height of the rate limiting barrier(s) is pure electrostatic, removal of its charge (by protonation or esterification) should not alter a selective sequence for cations, which is in accordance with results of Spalding (1978, 1980).

The proposed model gives a more adequate description of the inhibition of the sodium conductance by H^+ ions that a single acid group model does. An important feature being that it seems to yield more realistic values of parameters for the inner group. Ignoring the effect of the surface group on channel conductance results in an overestimation of the $pK_{i(o)}$ value and an underestimation of the δ value (see e. g. Fig. 2b). The presence of the surface group, protonation of which is not potential-dependent makes the apparent voltage dependence of H^+ blockage less pronounced. Therefore the δ value which was estimated earlier (Woodhull 1973; Campbell and Hille 1976; Naumov et al. 1979) to be about 0.25, is according to the two acid group model not less than 0.40. Hence, it can be inferred that the inner acid group and consequently the whole selective filter is located deeper in the channel than was assumed earlier.

It should be noted, however, that because of H^+ ions passing through the channel the interaction of H^+ ions from the external solution with the inner acid group is not at equilibrium, and K_i determined from the model should be considered as some effective constant characterizing the degree of protonation of the inner acid group. The possibility of H^+ ions moving from the inner group to the internal solution is equivalent to an increase in the effective rate of deprotonation

of this group, i.e. to a decrease in the value of the binding constant. Therefore the equilibrium binding constant should be somewhat higher than that of K_1 determined according to the model. Further, an allowance for the nonequilibrium interaction of H^+ ions with the inner group should affect evaluation of the δ value. For example, according to the Woodhull's general model (Woodhull 1973) $1/K_1$ should be given by sum of two exponential terms — one of them with a factor δ in the exponent, as assumed in our calculations; and another with a factor $(\delta - 0.5)$. At not very high positive potentials K_1 should change with potential less steeply than a single exponential with factor δ in the exponent. Thus, for the description of experimental data it may be necessary to use a δ value which is even higher than that estimated from the calculations according to the model. The above consideration shows that the calculations presented in this work may need further refinement. However this will only be possible with additional data. Therefore the use of a more general model for description of the inhibition of the sodium conductance by H^+ ions would be premature at the present time.

In principle, there is a possibility that the shifts of reversal potential towards more positive voltage values when lowering pH are due to the increase in sodium to potassium or calcium to potassium permeability ratios. However this seems to be unlikely. The high permeability of the sodium channels to H^+ (or, may be, hydronium) ions is not surprising if one considers that the H^+ ion is the smallest and has extremely high mobility in water solutions (Robinson and Stokes 1959). Essentially the same P_H/P_{Na} values as presented in this study were obtained from direct measurements of the proton currents in the sodium channels (in preparation). From P_H/P_{Na} values a difference in the heights of rate limiting barrier(s) between H^+ and Na^+ ions has been estimated to be about 5.6 RT (Chizmadzhev et al. 1974; Mozhayeva et al. 1977).

The high permeability determined from reversal potential measurements does not by any means rule out the high blocking potency of the H^+ ion. Because of the strong binding of H^+ ions to the acid group, they move slowly through the sodium channels (Chizmadzhev et al. 1974; Hille 1975a) and that is why they block the movement of other ions.

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