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A STUDY ON THE POTENTIAL-DEPENDENCE OF PROTON BLOCK OF SODIUM CHANNELS

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Ionic currents through sodium channels in nodal membranes were measured under voltage clamp conditions both at normal and at low (4.8–4.9) external solution pH. The measurements of so-called 'instantaneous' currents were used to distinguish between the proton blockage in open channels and the influence of low pH on channel gating processes. It is shown that the amount of the proton blockage in open channels decreases as membrane potential becomes more positive. This result suggests that at least one of the acid groups accessible from the outside is located within the conducting pore. The influence of the other group(s) on the degree of potential-dependence of proton blockage is discussed.

Introduction

Inhibition of the sodium conductance of excitable membranes at low pH of external solution is consistent with the idea that there is one or more acid groups in the sodium channels [1–8]. An important approach in elucidating the mechanisms of channel selectivity and conductance is the determination of pK values of these groups and of their localization in the channel. Some recent works necessitate additional investigation of the proton block of sodium channels. Thus, in one of them [9], at variance with previous cases [2,7,8], no potential-dependence of proton blockage of sodium channels in muscle fibres was observed. Hence, the conclusion was drawn that all acid groups accessible from the outside are situated near the external end of the pore. In a previous paper [10], the inhibition of sodium conductance at low pH is accounted for by changes in negative surface potential near the channels.

In the present work, the effect of H^+ on sodium conductance (g_{Na}) was studied, the main attention being paid to the potential-dependence of g_{Na} inhibition.

Methods

The experiments were performed on myelinated nerve fibres from the frog *Rana riribunda* using a voltage-clamp technique described in detail earlier [12]. Membrane potential (E) was referred to the outside. Membrane currents were calibrated on assumption that the resistance of the current-feeding internode was equal to 20 mΩ. Sodium current measurements were accomplished with P/2 protocol, according to which, currents in response to positive steps were added with a digital averager to currents in response to two negative steps of half the amplitude. Thus, the linear components of membrane currents (leakage and linear capacity currents) were subtracted more precisely than with an electronic analogue device [7,8]. When compensating for resistance series to the membrane (R_s), its value was taken to be 300 kΩ. The experimental procedure was essentially the same

as described earlier [7,8].

For experiments, the fibres were chosen for which the setting time of the membrane potential was no longer than 20 μs. The setting time of the clamp was controlled both at the output of the clamp amplifier (point E in Dodge and Frankenhaeuser's nomenclature [13]) and at the output of the follower, which is connected to point C and consequently senses transient deviation of potential from the zero value in point D [12].

The control solution contained (in mM): 110 NaCl/2 $CaCl_2$/8 tetraethylammonium chloride/ 10 Tris-HCl (pH 7.5–7.6). The pH of the acid solutions was adjusted by adding bipthalate buffer (pH 4.8–4.9). Fibres were cut in solutions containing 100 mM KF plus 20 mM CsF or 120 mM CsF alone.

Experiments were performed at 9 °C.

Results and Discussion

To estimate separately the effect of pH on the conducting properties of open sodium channels and on the potential-dependence of the number of open channels, the 'instantaneous' currents were measured according to two programmes. The first was as follows: the instantaneous currents corresponding to a constant number of open channels were measured in response to varying post-pulse (E_p), following constant test pulse (E_t) (Fig. 1a). According to the second programme, instantaneous 'tail' currents were measured in response to constant E_p following varying E_t. Normalized tail currents give the dependence of fractional number of open channels on potential (Fig. 3).

Fig. 1 shows instantaneous currents and instantaneous current-voltage relations at normal and at low external solution pH obtained in one of the typical experiments. Currents measured at invervals of 50 μs or less between the step and post-pulse were usually used to plot instantaneous current-voltage relations. As a rule, the duration of E_t exceeded the time to reach peak values; therefore, to make instantaneous current-voltage relations correspond to the number of open channels at the current peak, the amplitude of the instantaneous currents was multiplied by the ratio I_p/I_t, where I_p and I_t are the peak current and the current at the end of E_t, respectively. Control

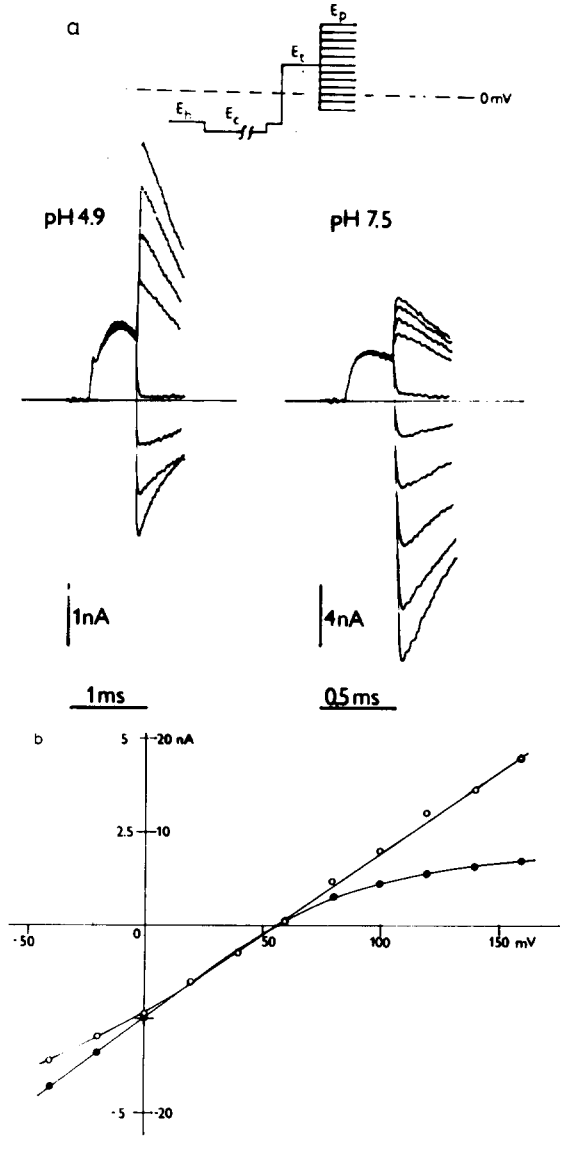

Fig. 1. The inhibition of sodium currents at low pH. (a) 'Instantaneous' currents after $E_t = +80$ mV at pH 4.9 and pH 7.5. The pulse programme is shown on the top of the figure. The post-pulse (E_p) is varied from −40 to +160 mV in 20-mV steps (current records in response to −20 and −40 mV at pH 4.9 are not shown). $E_h = -80$, $E_c = -120$ mV (50 ms). (b) ●, Current values at pH 7.5 (numbers on the right of ordinate axis); ○, current values at pH 4.9 (numbers on the left of ordinate axis). Current values are corrected for fast inactivation (see text). Node 249-83.

experiments showed that the result of such a calculation did not depend on the duration of E_t. In a number of experiments, currents were measured both with a hyperpolarizing prepulse to −120 mV

and with a depolarizing prepulse from −70 to −60 mV in order to check the influence of current size itself on the results. Current-voltage relations from both sets of measurements proved to differ from each other only by a constant factor, so the results obtained were not distorted by any kind of current-dependent artefacts.

It can be seen from the Fig. 1b that instantaneous current-voltage relations are nearly linear within the voltage range from −40 to +40 mV, both at normal and at low pH. At more positive potentials, the curve becomes concave downward at normal pH. At low pH, the curvature of this trace is much less pronounced, if present at all. The lowering of pH causes a decrease in current over the whole range of potentials. However, at more positive potentials, it is less pronounced. Control experiments with tetrodotoxin showed that within the range of potentials studied there are no nonlinear currents other than sodium currents. Thus, all features of currents registered must be attributed to the properties of sodium channels.

The ratio of chord conductance at low pH to that at normal pH (g_{pH}/g_{75}) was taken as a measure of H^+ block. As reversal potentials are equal both at normal and at low pH, this value is simply equal to the corresponding ratio of the currents. Fig. 2 shows the $g_{pH}/g_{7.5}(E)$-dependence calculated from instantaneous currents presented in Fig. 1b. Because the holding potential (E_h) was usually set at either −90 or −80 mV, a

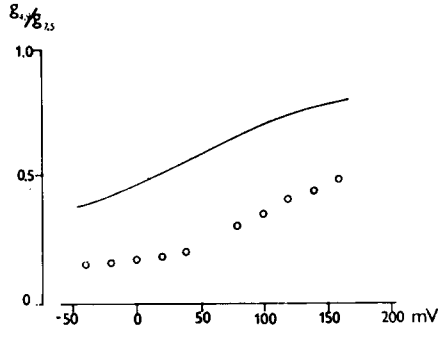

Fig. 2. The dependence of relative sodium conductance on potential for pH 4.9. O, Experimental values of $g_{4.9}/g_{7.5}$ obtained from the experiment presented in Fig. 1. When calculating $g_{7.5}$, values were corrected for slow inactivation. The continuous curve was calculated according to Eqn. 4 from Ref. 10; surface potentials on both sides of the membrane were taken to be equal to −75 mV. See text for details.

part of the channels at normal pH was inactivated by a slow inactivation mechanism [14]. Correction for this process was made using mean parameters of slow inactivation from work described earlier [15]. For the experiment presented in Fig. 2, the $g_{7.5}$ values were scaled up by 1.3. The ratio $g_{4.9}/g_{7.5}$ increases about 2.9-fold as the voltage rises from 0 to +160 mV. In seven experiments, where pH was lowered to 4.8–4.9, this figure was equal to 3.7 ± 0.2 (S.E.).

Since the instantaneous current-voltage relationship corresponds to a constant number of channels, the potential-dependence of the $g_{pH}/g_{7.5}$ ratio is determined by open sodium channel properties, not by the effect of low pH on gating processes. True, lowering the pH is known to affect the kinetics of activation and inactivation [16,17], so it is possible that the number of open channels at the current peak at normal and at low pH may differ to some extent. But, even if such a difference exists, it remains constant for instantaneous current-voltage relations over the complete potential range tested and therefore cannot account for the observed potential-dependence of $g_{pH}/g_{7.5}$. Thus, the results presented add further support to the previous data concerning the strong potential-dependence of g_{Na} inhibition with decreasing pH and are inconsistent with Campbell's data [9].

According to Campbell [9], the H^+ block potential-dependence shown in many works on the basis of current peak measurements occurs due to increase in the number of open channels at $E > 50$ mV, this increase being more pronounced at low pH. We checked this point. Fig. 3a shows tail currents in response to $E_p = 0$ mV. Such an E_p level was chosen because current kinetics at this potential are rather slow and, therefore, tail amplitude is measured more accurately. In this and in some other experiments, tail currents were measured with a depolarizing prepulse (50 ms in duration) in order to decrease current size and thereby to exclude any misinterpretation due to some kind of current-dependent artefact. It can be seen that tail currents after test pulses of the same duration become less as E_t increases from +80 to +120 and to +160 mV. This results from faster inactivation at more positive potentials. The correction for inactivation during the test pulse, quite analogous

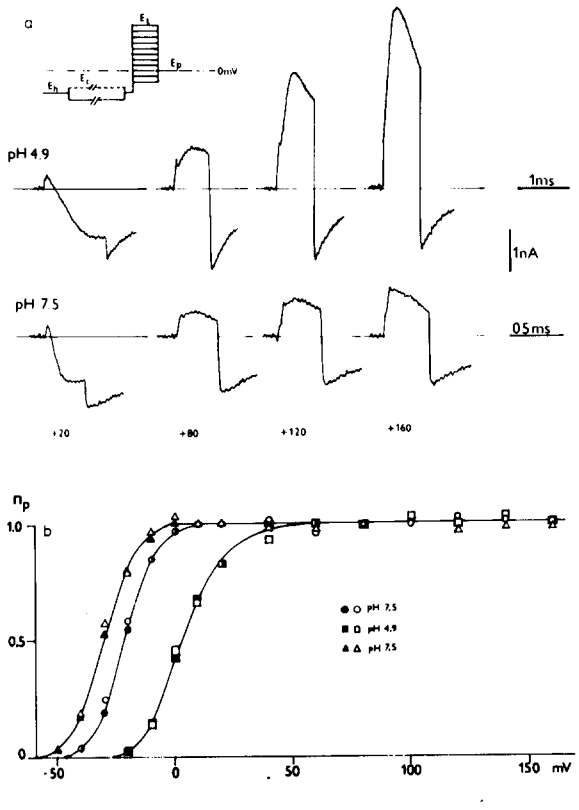

Fig. 3. The dependence of the number of open channels on membrane potential at low (4.9) and normal (7.5) pH. (a) 'Tail' currents in response to post-pulse E_p = zero after varying E_t. The pulse programme is shown on the top of the figure. Not all currents are shown. At pH 7.5, currents were measured with a prepulse to −70 mV, at pH 4.9, $E_c = -120$ mV. Numbers under the records indicate potential during E_t, in mV. (b) Normalized tail currents amplitude (○, □, △) and normalized peak chord conductance (●, ■, ▲) as function of E. Tail currents are corrected for inactivation during test pulse (see text). Solutions were applied in succession: pH 7.5 (○, ●), pH 4.9 (□, ■) and pH 7.5 (△, ▲) again. The same experiment as in Figs. 1 and 2.

to that made above for instantaneous currents, showed that at pH 7.5, tail amplitudes corresponding to peak currents at E_t +20, +80, +120 and +160 mV are equal. At pH 4.9, corrected tail currents are equal after +40, +80, +120 and +160 mV, but after $E_t = +20$ mV the current is somewhat less. Fig. 3b shows normalized tail currents as a function of E_t. Because tail amplitude is determined by the number of open channels at the current peak, this quantity normalized to its maximal value gives the fractional number of open channels at the peak current (n_p). It can be seen that the number of the open channels at the peak is essentially constant over the voltage ranges from +10 to +160 mV at pH 7.5 and from +40 to +160 mV at pH 4.9. The same results were obtained in all analogous experiments. Thus, relative tail amplitudes (corrected for the inactivation) were 1.0, 0.98 ± 0.02, 0.94 ± 0.03, 0.94 ± 0.05 ($n = 8$) at pH 7.5 and 1.0, 1.02 ± 0.02, 0.99 ± 0.04, 0.98 ± 0.05 ($n = 6$) at pH 4.8–4.9 for E_t +40, +80, +120 and +160 mV, respectively. Thus, at least in frog myelinated fibre, the number of open sodium channels at the peak of the current reaches its limiting value at potentials from +10 to +50 mV (depending on pH and possibly on other factors influencing the position of the $n_p(E)$ curve relative to the voltage axis) and stays at this level as E becomes more positive.

Since Woodhull's work [2], the potential-dependent g_{Na} inhibition at decreasing pH has been interpreted as the result of sodium-channel blockage by protons following their binding with the acid group situated within the pore. According to an alternative model [10], inhibition of channel currents at low pH is due exclusively to a decrease in concentration of permeant cations near the mouth of the pore which is, in turn, due to a decrease in negative surface potential ($\Delta\psi$). $\Delta\psi$ near the channel mouth is, further, assumed to be equal to that $\Delta\psi$ which is sensed by gating mechanism and manifests itself in a shift of voltage dependence of the number of open channels along the voltage axis (see for example, Ref. 18). On these assumptions, a version of the constant field equation [19] has been derived which takes into account surface potentials on both sides of the membrane (Eqn. 4 from Ref. 10). By this equation, the pH-induced inhibition of the current at any given potential can be related to the shift of the $n_p(E)$ curve. Fig. 3 shows $n_p(E)$ curves for two pH values (7.5 and 4.9). These were determined either from tail current measurements or from peak current measurements. In the latter case, n_p values were calculated as normalized chord conductance. Because instantaneous current-voltage relations over the voltage range −40 to +40 mV are nearly linear (see above), both calculations gave practically the same results. It can be seen that lowering the pH to 4.9 results in the shift of

$n_p(E)$ curve to the right by 24 mV, and after returning to normal pH, the curve shifts backwards by 33 mV. This latter figure was inserted into Eqn. 4 from Ref. 10. It can be seen from Fig. 2 that this calculation predicts both the decreased inhibition of currents over all potential ranges studied and the decrease in potential-dependence of the inhibition compared to experimental values. Indeed, as E varies from 0 to +160 mV, the equation predicts a 1.64-fold decrease in current inhibition, whereas the observed inhibition decreases 2.9-times. It should be noted that in the rest of our experiments, the amount of H^+ block was larger and the shift of $n_p(E)$ curve was less. For pH 4.8–4.9, the g_{pH}/g_{Na} value at E = zero averaged 0.11 ± 0.06 and the shift of the $n_p(E)$ curve was 20 ± 2 mV ($n = 7$). Thus, the discrepancy between the surface charge equation and experimental data was on average even greater. Varying the initial ψ values of both internal and external surfaces within reasonable limits does not improve the interpretation of the experimental data by this equation.

The possibility that unspecific surface potential can influence the concentration of permeate or blocking ions near the channel mouth has been discussed in a number of studies [3,18,20]. In one of them [20] a calculation similar to that used in previous work [10] gave a reasonable agreement between shifts in voltage-dependence of activation of K channels and blocking-potency of tetraethylammonium cations when pH, divalent and monovalent cation concentrations in the solution were varied. However, with sodium channels, such an unequivocal relation between the shift of voltage-dependent functions does not hold. Thus, many divalent cations can induce a shift of voltage-dependent gating functions as large as that achieved by H^+, but with much less inhibition of the current [18]. Further, it should be noted that some recent data [11,17,21,22] suggest that the shift of the activation curve with increasing divalent cation or H^+ concentrations seems to reflect direct interaction of H^+ with gating machinery rather than neutralization of some unspecific fixed surface charges.

Experiments varying pH from 4.0 to 6.2 [7,8] suggest that there are at least two acid groups accessible from the outside, one of them (inner) within the pore, the second (surface) just near the external end of the pore. The protonation of the inner group is voltage-dependent and causes an almost complete blockage of the channel; protonation of the surface group is potential-independent and results in a partial decrease of channel conductance (by the factor α). Though this model simplifies considerably the real situation, in particular, it does not take into account protons passing through the channel [8,23,24]; however, it does give a reasonable description of the experimental data, at least for positive potentials. According to this model, the discrepancy between Campbell's data and ours' can be partially explained by the following assumptions. In muscle fibre, unlike nerve fibre the pK of the surface group is considerably higher than that of the inner group. Additionally (or alternatively), protonation of the surface group in muscle fibre results in a stronger inhibition of channel current than in nerve fibre, that is, α value is lower. In this case, channel current inhibition will be to a great extent determined by the protonation of the surface group and therefore the apparent potential-dependence of proton block will be weak. Further, the model predicts and experiments confirm [7,8] that, because of the presence of the surface acid group, the apparent potential-dependence of the blockage decreases as pH increases. It should be noted in this connection that in Campbell's experiments, the pH of the acid solutions was rather high, so that currents were inhibited by no more than 40%. Therefore, in order to judge accurately the presence of the acid group within the sodium channel, experiments with a pH low enough to suppress 80–90% of the sodium currents should be performed on the muscle fibres.

Recently, additional evidence for strong potential-dependent proton blockage was obtained on squid giant axon [24].

In order to explain potential-dependent blockage it is not strictly necessary in principle to assume that the acid group is situated within the pore. Indeed, one can imagine that there is some cation in the inside which can bind within the pore in a potential-dependent manner. Then, the rise of positive potentials will increase the binding of this internal cation (it can be penetrating or not) and, due to Coulombic repulsion between cations, will

decrease protonation of the acid group by externally applied protons. Thus, proton blockage from the outside can be potential-dependent without direct action of electric field on the blocking proton. It is possible that such a mechanism operates in some cases. For example, proton blockage in aconitine-modified sodium channels is significantly less potential-dependent than in untreated channels [25,26]. But this is true only when the ends of the fibre are cut in solution without Cs^+; with Cs^+ in artificial axoplasm, the proton blockage becomes nearly as potential-dependent as in normal channels (Mozhayeva, G.N. and Naumov, A.P., unpublished observations). As explained above, it may be a result of potential-dependent binding of Cs^+ within the aconitine-modified channel. In normal sodium channel, proton blockage from the outside is equally potential-dependent, irrespective of whether Cs^+ is present in the internal soltuion (this work) or not [7,8].

It should be noted that any explanation of the H^+ block over wide ranges of potentials and pH must incorporate the assumption about the existence of at least two acid groups in the channel which differ from each other both in inherent pK and in the extent of potential-dependence of the protonation. Then, according to the above-mentioned mechanism of indirect potential-dependency of the blockage, one should suppose that both groups are situated at the external end of the pore. In this case, the explanation of the reasons for which the potential-dependence of protonation of two groups will differ from each other, in our opinion, becomes too speculative.

Thus, for the present, the most reasonable explanation for the potential-dependent blockage of the sodium channel by externally applied cations involves the assumption that there is at least one acid group situated deeply in the pore, as was originally proposed by Woodhull [2].

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