

Proton Block of Rat Brain Sodium Channels

Evidence for Two Proton Binding Sites and Multiple Occupancy

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ABSTRACT The acid titration function of bilayer-incorporated batrachotoxin (BTX)-modified sodium channels was examined in experiments in which the pH was decreased symmetrically, on both sides of the membrane, or asymmetrically, on only one side. In an attempt to minimize interpretational ambiguities, the experiments were done in 1.0 M NaCl (buffered to the appropriate pH) with channels incorporated into net neutral bilayers. When the pH was decreased symmetrically (from 7.4 to 4.5), the small-signal conductance (g) decreased in accordance with the predictions of a simple (single-site) titration function with a pK of ~ 4.9 . As the pH was decreased below 6.5, the single-channel current-voltage (i - V) relation became increasingly rectifying, with the inward current being decreased more than the outward current. When the pH was decreased asymmetrically (with the pH of the other solution being held constant at 7.4), the titration behavior was different for extra- and intracellular acidification. With extracellular acidification, the reduction in g could still be approximated by a simple titration function with a pK of ~ 4.6 , and there was a pronounced rectification at $pHs \leq 6$ (cf. Woodhull, A. M. 1973. *Journal of General Physiology*. 61:687-708). The voltage dependence of the block could be described by assuming that protons enter the pore and bind to a site with a pK of ~ 4.6 at an apparent electrical distance of ~ 0.1 from the extracellular entrance. With intracellular acidification there was only a slight reduction in g , and the g -pH relation could not be approximated by a simple titration curve, suggesting that protons can bind to several sites. The i - V relations were still rectifying, and the voltage-dependent block could be approximated by assuming that protons enter the pore and bind to a site with a pK of ~ 4.1 at an apparent electrical distance of ~ 0.2 from the intracellular entrance. Based on the difference between the three g -pH relations, we conclude that there are at least two proton binding sites in the pore and that they can be occupied simultaneously.

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INTRODUCTION

Ion movement through voltage-dependent sodium channels is inhibited by a large variety of compounds, including H^+ (Hille, 1968; Drouin and The, 1969). Based on the voltage dependence of the H^+ -induced current block (Woodhull, 1973), it is generally accepted that H^+ can block Na^+ movement through sodium channels by binding to one or more sites within the pore. But it is not clear whether the Na^+ permeability is reduced by the titration of only one (Woodhull, 1973; Zhang and Siegelbaum, 1991) or several (Wanke, Carbone, and Testa, 1980; Mozhayeva, Naumov, and Negulyaev, 1981, 1982; Begenisich and Danko, 1983) H^+ binding sites, or how many sites are in the pore. Nor is it clear whether more than one H^+ can bind in the pore. These uncertainties arise, at least in part, because voltage-dependent sodium channels are asymmetric (e.g., Wanke et al., 1980) and H^+ permeable (Begenisich and Danko, 1983; Mozhayeva and Naumov, 1983). In addition, there is a net negative charge at the channel entrances (Drouin and Neumcke, 1974; Ohmori and Yoshii, 1977; Mozhayeva, Naumov, and Nosyreva, 1984; Green, Weiss, and Andersen, 1987; Smith-Maxwell and Begenisich, 1987), which means that H^+ not only can block Na^+ movement through the pore but can also inhibit Na^+ permeation through the titration of fixed charges at the channel entrance. Any such titration will alter the local charge density and electrostatic potential at the channel entrance (Gilbert and Ehrenstein, 1970; Nelson, Colonos, and McQuarrie, 1975) and thus the local $[Na^+]$ and electrostatic potential profile along the pore (Frankenhaeuser, 1960; Green et al., 1987), which in turn will alter the single-channel conductance (e.g., Green and Andersen, 1991).

We have examined the titration behavior of single bilayer-incorporated, batrachotoxin (BTX)-modified sodium channels from rat forebrain in the presence of symmetrical or asymmetrical pH changes. To minimize the interpretational complexity, the experiments were done at high salt concentrations (1.0 M NaCl), where the direct blocking action of H^+ is maximized relative to conductance changes that result from the titration of fixed charges in the vicinity of the channel entrances. The channel's titration behavior is asymmetric and is expressed as a strong dependence on the extracellular pH (pH_e), while intracellular pH (pH_i) changes are of minor importance. The results of the symmetrical or the extracellular titrations would be consistent with models in which at most one H^+ could bind within the pore and block Na^+ permeation. Taken together, however, the symmetrical and asymmetrical titrations cannot be described by such a scheme. At least two H^+ can bind to the channel, probably in a single-filing pore, and inhibit Na^+ permeation.

Some of this material has appeared in preliminary form (Daumas and Andersen, 1990, 1991).

MATERIALS AND METHODS

Single-channel experiments were done at room temperature (22–26°C) in buffered 1.0 M NaCl solutions containing 200 nM BTX. The membranes were formed from a 4:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC) (Avanti Polar Lipids, Inc., Birmingham, AL) in *n*-decane (Wiley Organics, Coshocton, OH) at 2–3% (wt/vol). To improve membrane stability, the lipids were further purified by DEAE-cellulose ion-exchange chromatography before use (Rouser, Kritchevsky, and Yamamoto, 1967).

The electrolyte solutions were buffered as follows: at $\text{pH} \geq 7.4$, with 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES; United States Biochemical Corp., Cleveland, OH); and at $\text{pH} \leq 6.5$, with 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES; Sigma Chemical Co., St. Louis, MO). In experiments with asymmetrical pHs, the electrolyte solutions were buffered with a mixture of 10 mM HEPES plus 10 mM MES. The solutions were nominally free of Ca^{2+} . (The free $[\text{Ca}^{2+}]$ is $< 1 \mu\text{M}$; e.g., Green et al., 1987.)

Rat forebrain synaptosomes were prepared following a modification of the procedure of Cohen, Blomberg, Berzins, and Siekevitz (1977) (see O'Connell, 1992), and the sodium channels were incorporated into planar bilayers. Once incorporated, the channels' orientations were determined from the gating behavior (Green et al., 1987). The single-channel current measurements were done with an Axopatch 1B patch clamp (Axon Instruments, Inc., Foster City, CA) using Ag/AgCl electrodes to make contact to the electrolyte solutions. For the analysis and presentation we use the standard membrane potential sign convention; the aqueous solution facing the channels' extracellular surfaces is the electrical reference.

For experiments with symmetrical pH changes, the procedures were similar to those previously described (Green et al., 1987). For the experiments with asymmetrical pH changes, the following modifications were made. The pH was kept constant (at 7.4) in either the intracellular or extracellular compartment, while the pH in the other compartment was decreased by addition of HCl. A predetermined aliquot (10–30 μl) of 5 or 2.5 N HCl was added to that aqueous compartment; the amount to be added was determined from a titration curve. After HCl addition, the chamber was stirred for 20–30 s. At the end of the experiment, the solutions in each compartment were removed and their pHs measured. The actual pH (measured after each experiment) varied from that expected by ± 0.3 pH units. Results from experiments in which the pH varied by < 0.1 pH unit of pH were averaged together.

The single-channel current records were stored on a VCR-based digital tape recorder (DAS 900; Unitrade/Dagan Corp., Minneapolis, MN) for later analysis. At playback on a stripchart recorder (Gould 220; Gould Inc., Cleveland, OH) the current signal was filtered at 30–125 Hz. The current transition amplitudes were determined manually from the stripchart record using a digitizing pad (Summagraphics Corp., Seymour, CT). Closures (or openings) lasting longer than 16 ms (125 Hz) to 40 ms (30 Hz) were used to determine the single-channel current transition amplitudes (i). (At pH 7.4, and potentials positive to -60 mV, BTX-modified sodium channels are open most of the time except for short closures [Krueger, Worley, and French, 1983; Moczydlowski, Garber, and Miller, 1984; Chabala, Urban, Weiss, Green, and Andersen, 1991]. As the pH is lowered, the channels' voltage-activation curves are shifted in the depolarizing direction [e.g., Fig. 1] and, at potentials negative to -40 mV or so, the open durations become very short. Moreover, the current traces usually became increasingly noisy as the pH was lowered, such that automated analysis procedures were less useful. All current transition amplitudes were corrected for temperature variations to a standard temperature of 24°C , using a Q_{10} of 1.53 (Correa, Latorre, and Bezanilla, 1991].)

The small-signal conductance (g) was estimated from the slope of the single-channel current-voltage (i - V) relations: using results between -20 and $+20$ mV when the i - V relations were rectifying, and between -40 and $+40$ mV when there was no apparent rectification. Except where noted, the results for each experimental condition are based on at least three channels from three independent experiments.

RESULTS

Experiments with Symmetrical pH Changes

Fig. 1 illustrates the effects of symmetrical pH variations on BTX-modified sodium channels. Three different alterations in channel function are seen as the pH is decreased from 7.4 to 4.5. First, the current is reduced at both positive and negative

potentials. There is little change between pH 7.4 and pH 6.5. But when the pH is decreased further to 5.0 or 4.5 there is a pronounced current decrease, consistent with the notion that H^+ can block the channel. Associated with this reduction in current there is an increased open-channel current noise (as compared with the closed state). Second, the current traces at low pHs show rectification (cf. the traces at +40 and -40 mV, pH 4.5), while there is no apparent rectification at pH 7.4. Third, the gating is altered. At pH 7.4 (1.0 M NaCl) the midpoint potential for the activation of rat brain sodium channels is about -80 mV (O'Connell, 1992). But the current traces to the left in Fig. 1 already show considerable gating activity at -40 mV at pH 4.5. These changes in channel gating were not examined further.

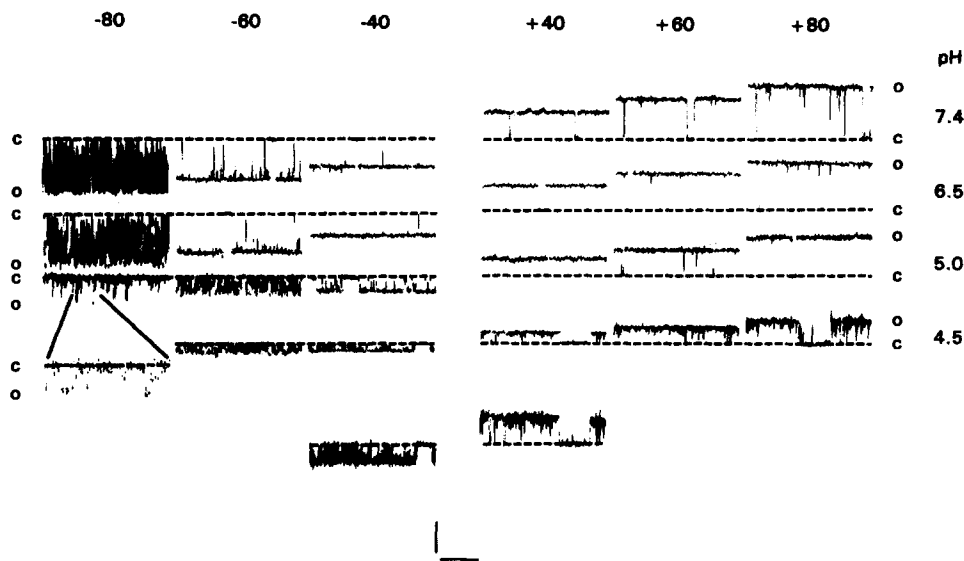


FIGURE 1. Current traces of a rat brain sodium channel at different $[H^+]$. The applied potentials are denoted at the top of the figure and the pHs at the right. The dotted line denotes the closed state. At negative potentials channels open toward the bottom of the figure; at positive potentials they open toward the top of the figure. Calibration bars: vertical, 2 pA; horizontal, 2 s, except where indicated. The frequencies used range from 35 to 70 Hz. For the partial enlargement of the trace at -80 mV for pH 5.0, the calibration bars are: vertical, 2 pA; horizontal, 0.2 s. Frequency, 125 Hz). The two bottom traces represent an enlargement of the pH 4.5 traces at -40 and +40 mV (calibration bars for these traces: vertical, 1 pA; horizontal, 2 s).

The i - V relations at different pHs are shown in Fig. 2. There is some scatter, which results from channel-to-channel conductance variation (Andersen, Green, and Urban, 1986). This variability seems to increase as the pH is decreased, possibly because the signal-to-noise ratio is decreased due to the increased current noise in the open state. Symmetrical acidification reduces the single-channel current amplitude at both positive and negative potentials, and produces a rectification that becomes more pronounced as the pH is decreased. At the lowest pH we could attain in this study, 4.5, the i - V relation shows a monotonic conductance decrease (from 12.4 to 7.3 pS)

when going from +100 to -50 mV. The conductance decrease is consistent with the notion that H^+ binds to a site within the pore, and thereby inhibits Na^+ permeation. No results could be obtained at potentials more negative than -60 mV (at pH 4.5) because of the titration-induced gating shift. (At pH 4.5 and 5.0, there is a hint of a conductance increase at the most hyperpolarized potentials. This question will be addressed more fully below.) In any case, these results demonstrate a pronounced asymmetry in the effect of H^+ on Na^+ permeation.

Experiments with Asymmetrical pH Changes

The asymmetric effect of H^+ on Na^+ permeation was further examined in experiments with asymmetrical pH changes. In these experiments, the pH in one solution was kept constant (at 7.4), while the other solution was acidified. Fig. 3 shows current traces obtained at different extracellular and intracellular pHs. There is a pro-

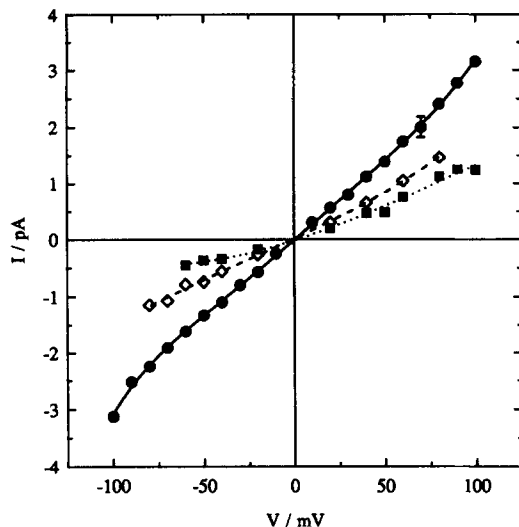


FIGURE 2. Single-channel i - V relations for rat brain sodium channels at three different symmetrical pHs: ●, pH 7.4; ◇, pH 5.0; ■, pH 4.5. Each point is the average of at least three independent measurements. The curves have no theoretical significance. Standard error bars are included, but are usually smaller than symbols.

nounced difference between the effect of extracellular and intracellular acidification of equal magnitude on the channels' Na^+ permeability. A comparison of the upper and lower set of current traces in Fig. 3 shows that an extracellular acidification is far more effective in decreasing the single-channel current than a comparable intracellular acidification. Compared with the situation at symmetrical pH 7.4 (Fig. 1), an extracellular acidification to pH 5.0 (upper set of traces) causes a strong reduction in the current amplitude, rectification, and gating shift. Much smaller changes are seen when the intracellular solution is acidified to pH 5.0 (lower set of traces).

The i - V relations for either condition are illustrated in Fig. 4 A. At low extracellular pHs, the i - V relations display a strong rectification: a pronounced block of the inward current and a lesser decrease of the outward current. By comparison, at low intracellular pHs one observes only a slight rectification: a small current reduction at positive potentials (outward current), and no significant current decrease at negative

potentials. The experiments with asymmetrical pHs also suggest a partial relief of the current block by H^+ transfer through the pore. Fig. 4B shows the results of experiments obtained with a pH_e of 5.2 and a pH_i of 7.4. There is a decrease in conductance (from 26.5 to 18.9 pS) when going from +100 to -60 mV, and an increase (from 18.9 to 20.5 pS) when going from -60 to -80 mV. As in the symmetrical experiments, the shift in gating precluded experiments at more negative potentials. (The conductance increase at hyperpolarized potentials is consistent with the notion that H^+ is permeant [cf. Begenisich and Danko, 1983 and Mozhayeva and Naumov, 1983]; but there is likewise a conductance increase at hyperpolarized potentials at pH_e 7.4, where any H^+ -induced channel should be minimal, which precludes a straightforward interpretation.)

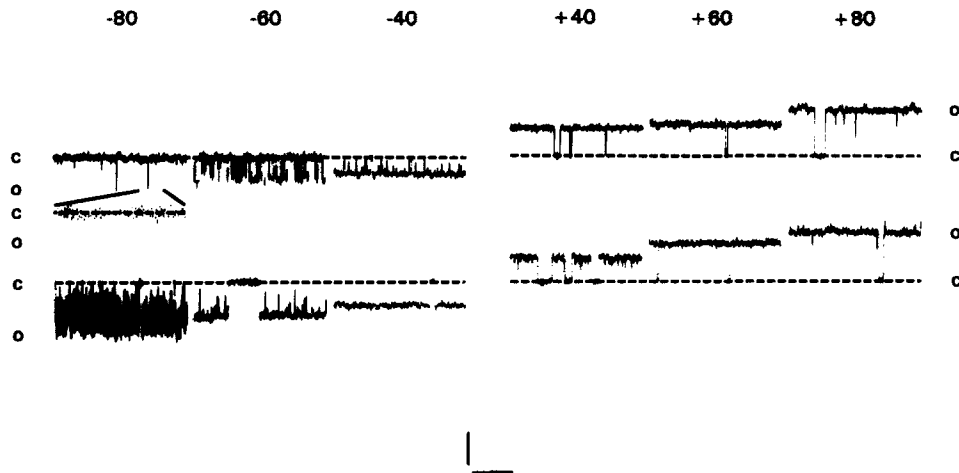


FIGURE 3. Single-channel current traces for rat brain sodium channels at asymmetrical pHs. (Top) pH_e 5.0; pH_i 7.4. (Bottom) pH_e 7.4; pH_i 5.0. Applied potentials are in the figure. Frequency, 50 Hz. The dashed line has the same meaning as in Fig. 1. Calibration bars: vertical, 2 pA; horizontal, 2 s.

Fig. 5 illustrates the small-signal conductance ($g([H^+])$) for the three experimental conditions used here: symmetrical acidification, extracellular acidification, and intracellular acidification. The three interrupted lines in the figure denote predictions based on a simple (single-site) titration curve:

$$g([H^+]) = g_{\max} \cdot [H^+] / (K_H^{ap} + [H^+]) \quad (1a)$$

$$= g_{\max} \cdot 10^{-pH} / (10^{-pK_{ap}} + 10^{-pH}) \quad (1b)$$

where g_{\max} denotes the maximal conductance and K_H^{ap} the apparent H^+ dissociation constant. K_H^{ap} is an apparent dissociation constant because of competitive Na^+/H^+ interactions; see Eqs. 5 and 7 in the Discussion. (The solid curves are drawn based on simulations using a two-site, double-occupancy model; see Discussion.)

For the symmetrical experiments (solid circles, long-dash curve), $g_{\max} = 28.1$ pS and $K_H^{ap} = 1.3 \cdot 10^{-5}$ ($pK_{ap} = 4.9$). The curve describes the experiments, except for the

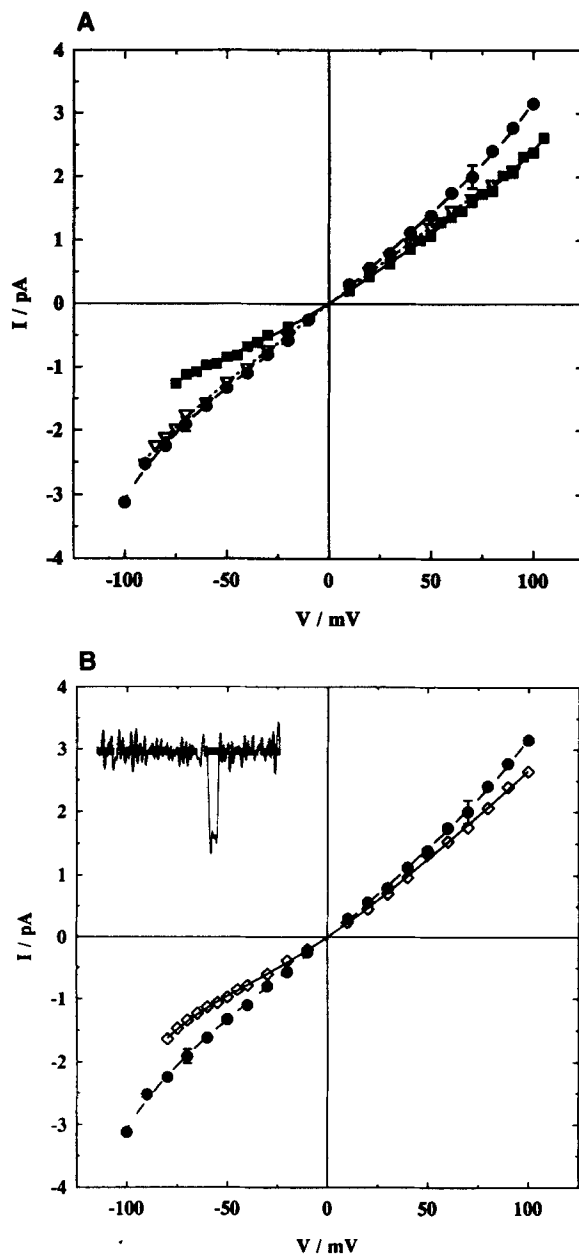


FIGURE 4. Single-channel i - V relations for rat brain sodium channel at asymmetrical pHs. (A) ●, Symmetrical pH 7.4. ■, pH_e 5.0; pH_i 7.4. ▽, pH_e 7.4, pH_i = 5.0. For each experimental condition, the points represent the average of results for five channels. The curves have no theoretical significance. (B) i - V relation based on two channels: ●, symmetrical pH 7.4. ◇, pH_e 5.2, pH_i 7.4. The superlinearity at hyperpolarized potentials could suggest a relief of the H^+ -induced block; i.e., that H^+ is permeant. The curves have no theoretical significance. The inset illustrates an event used to measure the current at -80 mV. Frequency, 125 Hz. The event lasts ~ 20 ms.

point at pH 4.5, where H^+ permeation through the channel could contribute to the measured current. At the high ionic strength used in these experiments, one can thus describe the H^+ -induced conductance decrease as if there were only a single H^+ binding site that could be titrated over the pH range used.

The conductance decreases are less when the pH is decreased asymmetrically. Importantly, the shape of titration function observed with extracellular acidification

(solid triangles, stippled curve) is similar to that of the symmetrical titration function, the major difference being that the apparent pK is shifted ~ 0.3 pH units in the acid direction. Intracellular acidification, in contrast, has almost no effect on the conductance (open triangles, short-dash curve) even at the lowest pHs, and the points do not fall along a simple titration curve. The latter result indicates that the conductance decrease results from the titration of several groups in the pore and at the channel entrance.

To obtain further information about the H^+ binding sites, the voltage dependence of the H^+ -induced current decrease was estimated following Woodhull (1973), by plotting $i_H(V)/[i(V) - i_H(V)]$ vs. V :

$$i_H(V)/[i(V) - i_H(V)] = (K_H(0)/[H^+]) \cdot \exp\{-\delta \cdot V \cdot e/kT\} \quad (2)$$

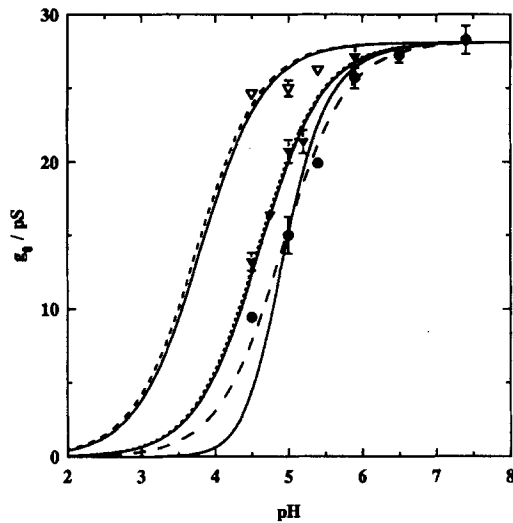


FIGURE 5. Variation of the small signal conductance in a function of pH for symmetrical acidification (\bullet), extracellular acidification (\blacktriangledown), and intracellular acidification (∇). The three discontinuous curves denote the least-squares fit of Eq. 1b to the results. For all the fits $g_{\max} = 28.1$ pS. (—, symmetrical acidification, pK_{ap} 4.9; ---, extracellular acidification, pK_{ap} 4.6; \cdots , intracellular acidification, pK_{ap} 3.8). The small-signal conductance (g) was estimated from the slope of the single-channel i - V relations: between -20 and $+20$ mV when the i - V relations were rec-

tifying, and between -40 and $+40$ mV when there was no rectification. The three solid curves denote simulated titration functions for all three conditions (see Discussion). The simulations were based on the following parameter choice (see also Fig. 8 B): $k_{NaO}^{OO} = 4 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; $K_{NaO} = 0.5$ M; $k_{ONa}^{OO} = 4 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; $K_{ONa} = 5$ M; $k_{ONa}^{NaO} = 4 \cdot 10^7$ s^{-1} ; $k_{NaNa}^{ONa} = 1 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; $K_{NaNa} = 5$ M; $k_{NaNa}^{NaO} = 1 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; $K_{NaNa} = 50$ M; $k_{HO}^{OO} = 1.25 \cdot 10^6$ $M^{-1} \cdot s^{-1}$; $K_{HO} = 1 \cdot 10^{-5}$ M; $k_{OH}^{OO} = 2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$; $K_{OH} = 2 \cdot 10^{-4}$ M; $k_{OH}^{HO} = 2.4 \cdot 10^{-4}$ s^{-1} ; $k_{HH}^{OH} = 4 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$; $K_{HH} = 4 \cdot 10^{-7}$ M; $k_{HH}^{HO} = 2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; $K_{HH} = 8 \cdot 10^{-6}$ M.

where $i_H(V)$ and $i(V)$ denote the current at acid pH and at pH 7.4, respectively, $K_H(0)$ is the apparent H^+ dissociation constant at $V = 0$ mV, δ is the equivalent valence (which denotes how the voltage affects the block), e is the elementary charge, k is Boltzmann's constant, and T is the temperature in Kelvin. Fig. 6 shows the results of this analysis. The straight lines were determined by linear regression. For extracellular acidification, $K_H(0) = 2.2 \cdot 10^{-5}$ M ($pK_e = 4.7$) and $\delta_e = 0.14$. For intracellular acidification, $K_H(0) = 7.9 \cdot 10^{-4}$ M ($pK_i = 4.1$) and $\delta_i = -0.21$. (That δ_i is negative means that a hyperpolarization will tend to reduce the magnitude of the block

because H^+ enters the pore from the intracellular solution.) The $K_H(0)$ estimates are similar to the K_H^{ap} estimates based on the results in Fig. 5 and Eq. 1.

DISCUSSION

We have examined the H^+ titration behavior of single voltage-dependent sodium channels by determining the titration function under three different conditions: symmetrical pH changes, extracellular pH changes only, and intracellular pH changes only. The results show a pronounced asymmetry of the titration functions, with a more pronounced sensitivity to extracellular pH changes. We first compare the three titration curves and show that the results are incompatible with "simple" titration models (in which there can be at most one Na^+ or H^+ in the pore). We then briefly discuss the voltage dependence of the H^+ -induced block. Finally, we examine

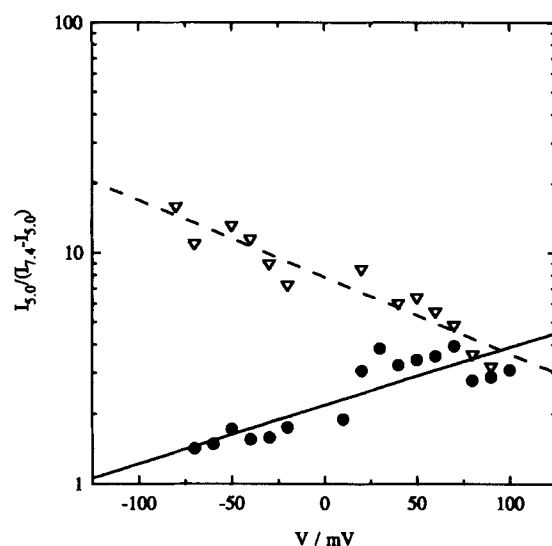


FIGURE 6. Voltage-dependent H^+ block of sodium channels by extracellular (\bullet) or intracellular (∇) H^+ . Results from Fig. 4A are plotted according to Eq. 2. The straight lines denote the best fits: for extracellular acidification (*solid line*), $K_H(0) = 2.2 \cdot 10^{-5}$ M, $pK_e = 4.70$, $\delta_e = 0.14$; for intracellular acidification (*dashed line*), $K_H(0) = 7.9 \cdot 10^{-4}$ M, $pK_i = 4.1$, $\delta_i = 0.21$.

two classes of double-occupancy models and conclude that the results favor a single-filing model in which there are two H^+ binding sites that can be simultaneously occupied.

The g-pH Titration Functions

H^+ interactions with voltage-dependent sodium channels are complex, as H^+ binding can alter both channel gating and Na^+ permeation (Hille, 1968; Ohmori and Yoshii, 1977; Sigworth, 1980; Zhang and Siegelbaum, 1991). Both kinds of alterations of channel function were observed here (Figs. 1 and 3). We will here only consider the alterations of Na^+ permeation, where the three major effects are (Fig. 7): (1) H^+ can bind within the pore and thereby block Na^+ permeation (Woodhull, 1973; Begenisich and Danko, 1983; Mozhayeva et al., 1984); (2) H^+ can permeate the pore (Mozhayeva et al., 1982; Begenisich and Danko, 1983; Mozhayeva and Naumov,

1983), such that a one-sided pH change may affect all titratable sites in the pore;¹ and (3) H⁺ can bind to sites just outside the pore in the vicinity of the extracellular entrance (Drouin and Neumcke, 1974; Sigworth and Spalding, 1980; Spalding, 1980; Mozhayeva et al., 1981) and the intracellular entrance, which will alter the local fixed (surface) charge densities (σ) and surface potentials (ψ_e and ψ_i for the extracellular and intracellular pore entrances, respectively), and thereby alter Na⁺ permeation by altering the local [Na⁺] at the entrance(s) as well as the electrostatic profile along the pore (Frankenhaeuser, 1960; Sigworth and Spalding, 1980; Green et al., 1987).² We tried to minimize these complications by working at high ionic strengths (1.0 M NaCl), such that the surface potentials at the channel entrances relative to the adjacent bulk solutions would be small in magnitude and vary little as a function of pH. Nevertheless, ψ_e (and ψ_i) are not zero; assuming a symmetrical charge distribution, the apparent charge density at the pore entrances of rat brain sodium channels

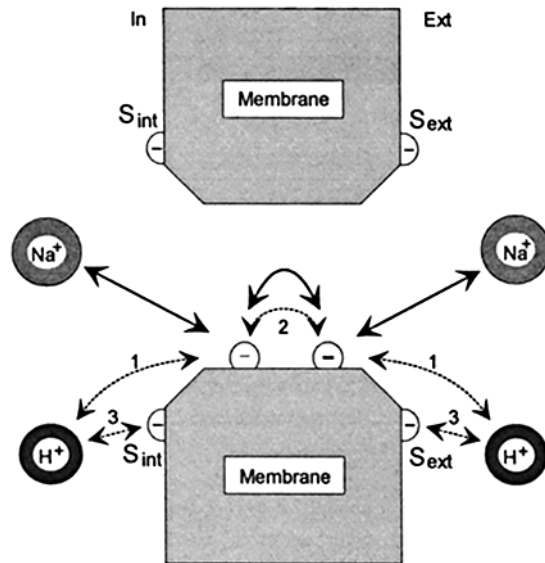


FIGURE 7. H⁺ interactions that alter the conductance of voltage-dependent sodium channels. (1) H⁺ can enter the pore and bind to titratable sites; (2) H⁺ can move through the pore and therefore bind to multiple sites; (3) H⁺ can bind to titratable sites outside the pore and alter Na⁺ permeation either through the ensuing changes in the local surface charge (surface potential) or through conformational changes that close the pore.

is about $-0.26 e/\text{nm}^2$ (Chabala and Andersen, 1989); using the Gouy-Chapman approximation (e.g., Aveyard and Haydon, 1973), ψ_e and ψ_i ($= \psi$) are thus predicted to be -18 mV in 1.0 M NaCl. Changes in pH may alter ψ (when σ is titrated), but that will not have a significant effect on g , which in this concentration range varies only weakly as a function of [Na⁺]. Nevertheless, variations in ψ will alter the local [H⁺] ($[\text{H}^+]_s = [\text{H}^+]_b \cdot \exp(-\psi \cdot F/RT)$, where $[\text{H}^+]_b$ denotes the bulk [H⁺]), which will alter the shape of the titration functions. Generally, the effect will be to "stretch out" the

¹ The H⁺ permeability of lipid bilayers and biological membranes is quite high (e.g., Andersen, 1989b), and the intracellular pH may have been affected as well. In fact, Wanke et al. (1980) found that the extracellular titration function differed between perfused and unperfused axons, and proposed that this was due to poor pH control of the unperfused axons.

² H⁺ could also bind to sites distant from the pore and inhibit Na⁺ permeation through a channel closure that is not related to gating per se.

titration function (e.g., Edsall and Wyman, 1958, Ch. 9), because protonating a fixed negative charge in the vicinity of the pore entrance will decrease the magnitude of σ (and thus of ψ and $\exp\{-\psi F/RT\}$). The ratio $[H^+]_s/[H^+]_b$ will therefore decrease as the pH is lowered, which will affect the shape of the titration function, which is reference to $[H^+]_b$, e.g., $g([H^+]_b)$. This effect will be modest in our case, however, because $\exp\{-\psi F/RT\}$ is only ~ 2 at pH 7.4. Moreover, the distortion imposed by titrating the charges adjacent to the pore entrance will depend on the pKs of the different titratable groups, being maximal when the charges within and outside the pore have similar pKs, which is unlikely to be the case because of their different environments. We therefore analyze the titration function under the assumption that changes in ψ_e (and ψ_i) can be disregarded.

When the pH is decreased symmetrically, the g -pH relation can be approximated by a simple titration function (Fig. 5; Eq. 1), with a pK_{ap} of 4.9 and a g_{max} of 28.1 pS. The shape of the curve is consistent with the notion that one H^+ binds in the pore and blocks Na^+ permeation. At the lowest pH, 4.5, the conductance is slightly larger than predicted. This could arise from H^+ permeation through the pore, as also may be suggested by the superlinear i - V relations at low pHs (Fig. 4 B). Other possibilities are that H^+ are imperfect blockers (i.e., that Na^+ can permeate through a pore in which H^+ has bound [because of single-file flux coupling or Na^+ "slippage" past a site to which H^+ has bound]), and that the shape of the $g([H^+]_b)$ relation is affected by the titration of fixed charges in the vicinity of the pore entrances. If that were the case, however, one would expect to see a similar deviation in the extracellular titration curve, which was not observed.

When only pH_e was decreased (and pH_i was held constant at 7.4), the g -pH relation again could be approximated by a simple titration function (Fig. 5), but the pK_{ap} was ~ 0.3 pH units less than for the symmetrical situation. The pK_{ap} is less than that previously found for sodium channels in frog node of Ranvier (Woodhull, 1973; Mozhayeva et al., 1981) and muscle (Campbell and Hille, 1976). This is probably because our experiments were done at a higher $[NaCl]$ (higher ionic strength), which has two consequences: first, any surface charge at the channel entrance would be screened more effectively and the ratio $[H^+]_s/[H^+]_b$ would be correspondingly reduced; and second, any direct competitive H^+/Na^+ interactions would be augmented.

In contrast, when only pH_i was decreased (and pH_e was held constant at 7.4), the conductance was only weakly dependent on pH_i . The g -pH relation could not be approximated by a simple titration function (Fig. 5), indicating that we could be titrating groups in the pore as well as negative charges at the intracellular channel entrance (Smith-Maxwell and Begenisich, 1987). The asymmetry in the titration behavior is opposite to that observed for macroscopic sodium currents in the squid giant axon (cf. Carbone, Fioravanti, Prestipino, and Wanke [1978] and Wanke et al. [1980]), where there was an increased sensitivity to intracellular acidification. This difference could, in part, result from the higher ionic strength (higher intracellular $[NaCl]$) used here; but the BTX modification, which is known to decrease sodium channels' sensitivity to a variety of intracellular channel blockers (Khodorov, 1985), is also likely to play a role.

This shift in the titration functions (symmetrical vs. extracellular or intracellular)

cannot be explained by simple titration models in which only a single H^+ can bind within the pore. We note first that any linear sequence of first-order reactions can be collapsed into a single equivalent step (Britton, 1966; Cleland, 1975). Thus, if the channel can be occupied by at most a single ion (Na^+ or H^+), the inhibition by H^+ can be described using the kinetic scheme in Fig. 8A, and the channel's Na^+ permeability at 0 mV (p_{Na}) is (cf. Andersen, 1989a):

$$p_{Na} = p_{Na}^0 / (1 + [Na^+]/K_{Na} + [H^+]_e/K_H^e + [H^+]_i/K_H^i) \quad (3)$$

where p_{Na}^0 denotes the limiting p_{Na} (when $[Na^+]$ ($=[Na^+]_e = [Na^+]_i$), $[H^+]_e$, and $[H^+]_i$ approach 0), K_{Na} the $[Na^+]$ for half-maximal conductance, and K_H^e and K_H^i the $[H^+]$

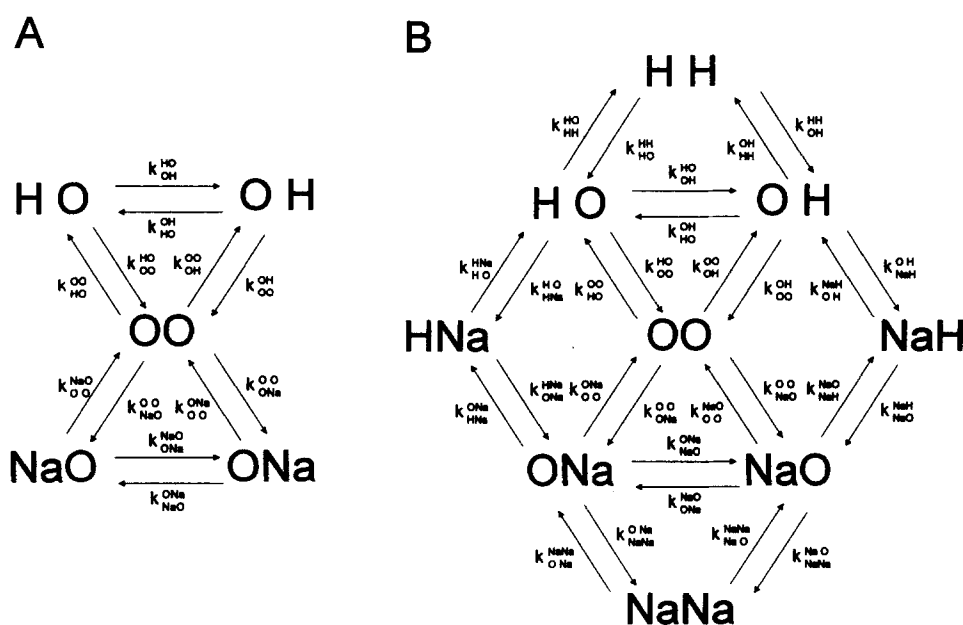


FIGURE 8. Kinetic models of Na^+/H^+ interactions in a pore. (A) The kinetic steps in a three-state (three-barrier, two-site, one-ion) pore in the presence of two permeant ions (H^+ and Na^+). The rate constants are denoted as $k_{\text{final state}}^{\text{initial state}}$ (k_{HO}^{OO} denotes, for example, the association rate constant for the $OO \rightarrow HO$ transition). (B) The kinetics steps in a four-state (three-barrier, two-site, two-ion) pore in the presence of two permeant ions (H^+ and Na^+). The rate constants have the same meaning as in A.

for half-maximal inhibition when the *trans* $[H^+]$ is zero and $[Na^+] \rightarrow 0$. When $p_{Na} \cdot [Na^+] \gg p_H \cdot [H^+]$, the single-channel conductance will be given by

$$g = (F^2/RT) \cdot [Na^+] \cdot p_{Na} \quad (4)$$

We will use this approximation below.

In the case of symmetrical pH changes, the $g([H^+])$ relation is given by Eq. 1, with an apparent inhibition constant $K_H^{\text{ap,sym}}$:

$$K_H^{\text{ap,sym}} = K_H \cdot (1 + [Na^+]/K_{Na}) \quad (5)$$

where

$$K_H = K_H^e \cdot K_H^i / (K_H^e + K_H^i) \quad (6)$$

In the case of asymmetrical pH changes (when the *trans* $[H^+] \approx 0$), the $g([H^+])$ relation is again given by Eq. 1 with apparent inhibition constants

$$K_H^{ap,e} = K_H^e \cdot (1 + [Na^+]/K_{Na}) \quad (7a)$$

or

$$K_H^{ap,i} = K_H^i \cdot (1 + [Na^+]/K_{Na}) \quad (7b)$$

in the case of extracellular and intracellular acidification, respectively. Combining Eqs. 5 and 7a and b we have a general relation for single-ion channels:

$$K_H^{ap,sym} = K_H^{ap,e} \cdot K_H^{ap,i} / (K_H^{ap,e} + K_H^{ap,i}) \quad (8)$$

From the shift in pK_{ap} for the symmetrical and extracellular titration functions ($pK_{ap}^{sym} - pK_{ap}^e$), ~ 0.3 pH units, one finds (cf. Eq. 8) that $K_H^i/K_H^e \approx 1$, which implies that the intracellular acidification should be more effective in terms of blocking Na^+ permeation than extracellular acidification. The predicted shift in pK_{ap} for the intracellular titration function $pK_{ap}^{sym} - pK_{ap}^i$ is only ~ 0.3 pH units, which is incompatible with the results in Fig. 5.

Thus, even though each of the three titration curves by itself might be consistent with the notion that Na^+ is blocked when only a single H^+ has bound in the pore, taken together they are incompatible with such a "simple" titration model. To our knowledge, this is the first demonstration that single-ion models cannot account for the H^+ -induced channel block. These results thus provide strong support for the notion that there are at least two H^+ binding sites (Wanke et al., 1980), and that they can be occupied simultaneously (Begenisich and Danko, 1983).³

Voltage Dependence of Channel Block

The inhibitory effect of H^+ is voltage dependent (Fig. 6), which is consistent with the notion that H^+ can bind to sites fairly deep within the pore (Woodhull, 1973). The voltage dependence is relatively shallow as compared with previous studies on macroscopic currents (Woodhull, 1973; Wanke et al., 1980; Mozhayeva et al., 1981, 1984; Begenisich and Danko, 1983), but is more pronounced than was observed in another single-channel study (Zhang and Siegelbaum, 1991). The modest voltage dependence we see may be due to our experimental conditions (high $[NaCl]$), because the voltage-dependent block of Na^+ permeation reflects the voltage dependence of $K_H^{ap,e}$ (or $K_H^{ap,i}$), not the voltage dependence of K_H^e (or K_H^i). This can be illustrated using the single-ion occupancy approximation: at low $[Na^+]$, when $[Na^+]/K_{Na} \ll 1$, $K_H^{ap,e} \approx K_H^e$ (cf. Eq. 7a), and the voltage dependence of $K_H^{ap,e}$ will be similar to that of K_H^e ; at high $[Na^+]$, when $[Na^+]/K_{Na} \gg 1$, $K_H^{ap,e} \approx K_H^e \cdot [Na^+]/K_{Na}$,

³ The second H^+ binding site is not the external site deduced by Drouin and Neumcke (1974) and Mozhayeva et al. (1981, 1984), because the high $[NaCl]$ used in the present experiments would minimize changes in ψ at the channel entrances and blunt the effect on g . (Titration of a site in the vicinity of the intracellular pore entrance, however, may account for the shape of the intracellular titration function.)

and $K_H^{ap,e}$ could be almost voltage independent even though K_H^c is voltage dependent. In addition, H^+ is permeant (Begenisich and Danko, 1983; Mozhayeva and Naumov, 1983), as is also indicated by the nonmonotonic g - V relations in Fig. 4, which again will cause δ to be underestimated (Woodhull, 1973).

Multi-Ion Models

The three g -pH relations in Fig. 5 are incompatible with single-ion models of channel block, and therefore suggestive of multiple H^+ occupancy. The voltage-dependent H^+ block of Na^+ permeation suggests that one of the H^+ binding sites, or possibly both, are in the pore. We examined two double-occupancy models: one in which there can be two ions in a single-filing pore (Begenisich and Danko, 1983; see also Fig. 8 B), and one in which there can be only one ion in the pore and an extracellular H^+ binding site that is not in the pore. Both models can produce titration functions that violate the constraint given by Eq. 8, but neither model was fully satisfactory.

The flux equations for the single-filing model were solved following Urban and Hladky (1979) (see also Andersen, 1989a). There are 22 independent rate constants in the final expression for p_{Na} , and there is not sufficient information to determine them. We therefore used simulations to see if the results would be compatible with such a model. (The simulations were done using a grid-search that minimized weighted sums of squared deviations between the experimental estimates of pK_2^{2p} and the theoretical pHs for half-maximal inhibition of the single-channel conductance.) One can approximate the experimental results using a variety of parameter sets; one such simulation is shown in Fig. 5. General features of the simulations we have done are (1) that the symmetrical titration function tends to be too steep (as compared with a simple titration curve); (2) that there is a finite H^+ flux through the pore; (3) that the intrinsic H^+ dissociation constants for binding only one H^+ ($K_{HO} = k_{OO}^{HO}/k_{HO}^{OO}$ and $K_{OH} = k_{OO}^{OH}/k_{OH}^{OO}$) are comparable (usually with $K_{OH} > K_{HO}$); and (4) that the corresponding dissociation constants for binding a second H^+ ($K_{HH|} = k_{OH}^{HH}/k_{HH}^{OH}$ and $K_{|HH} = k_{HO}^{HH}/k_{HH}^{HO}$) are less than those for binding the first ion ($K_{HO} > K_{HH|}$; $K_{OH} > K_{|HH}$), that is, the second H^+ binds with a higher affinity than the first.

The last conclusion appears counterintuitive, because one would expect that electrostatic repulsion should reduce the affinity for the second ion. Experimental determinations of the repulsion between fixed charges at the entrance to gramicidin channels show, however, that electrostatic interactions between charges close to the two channel entrances may be insignificant (Cifu, Koeppe, and Andersen, 1992), which also is why we did not impose (extrathermodynamic) constraints on the ion-ion interactions. In any case, H^+ binding could induce alterations in the channel protein itself, which could cause the increased H^+ affinity as an allosteric effect.

Given the very large number of free parameters, it is surprising that we were unable to achieve a better fit to the symmetrical titration function. That deficiency appears to be inherent to the model, in the sense that parameter sets that allow for significant deviations from the constraint imposed by Eq. 8 also allow for significant cross-product terms involving $[H^+]_e$ and $[H^+]_i$, and a higher affinity for the second, as compared with the first, H^+ that binds. The failure of this single-filing model to describe the symmetrical titration function could also result from neglecting the consequences of titrating the fixed charge in the vicinity of the (extracellular)

entrance. But in that case we should not have been able to describe the extracellular titration function. Interestingly, there was little if any constraint on the choice of Na^+ parameters, and there is not even a clear requirement for double occupancy by Na^+ .

In an attempt to improve the fit, we also examined an alternative class of models where there is a modulating site outside the pore. Pietrobon, Prod'homme, and Hess (1988) observed, for example, allosteric interactions between Ca^{2+} occupancy in the pore and H^+ binding to some site outside the pore. In our case we propose that Na^+ permeation can be inhibited by protonation of sites in the pore and by protonation of the modulating site(s). Assuming single-ion occupancy (Fig. 8A), if there is only a single (extracellular) modulating site, the $g([\text{H}^+])$ relation becomes

$$g([\text{H}^+]) = \{p_{\text{Na}}^0 \cdot [\text{Na}^+] / (1 + [\text{Na}^+] / K_{\text{Na}}) \cdot F([\text{H}^+]_e, [\text{H}^+]_i)\} \quad (9)$$

where

$$F([\text{H}^+]_e, [\text{H}^+]_i) = 1 / \{1 + [\text{H}^+]_e / K_{\text{H}}^{\text{ext}} + ([\text{H}^+]_e / K_{\text{H}}^{\text{ef,e}} + [\text{H}^+]_i / K_{\text{H}}^{\text{ef,i}}) \cdot (1 + [\text{H}^+]_e / (K_{\text{H}}^{\text{ext}} \cdot R_{\text{rep}}))\} \quad (10)$$

$K_{\text{H}}^{\text{ext}}$ denotes the dissociation constant for H^+ binding to the modulating site, $K_{\text{H}}^{\text{ef,e}}$ and $K_{\text{H}}^{\text{ef,i}}$ are equal to $K_{\text{H}}^{\text{ap,e}}$ and $K_{\text{H}}^{\text{ap,i}}$ as defined in Eqs. 7a and b, and R_{rep} is a factor that denotes how the modulatory sites interact with the H^+ binding sites in the pore: if $R_{\text{rep}} > 1$, there are repulsive interactions (negative cooperativity); if $R_{\text{rep}} < 1$, there are attractive interactions (positive cooperativity). Models of this kind do not obey the constraint in Eq. 8 unless $R_{\text{rep}} \gg 1$, in which case the symmetrical titration becomes much too steep when we simulate the pK_{ap} shifts.

Summary

Our main conclusion is negative: the H^+ titration of voltage-dependent sodium channels cannot be described by a single-ion model. The basic features of our results can be described using the single-file model originally proposed by Begenisich and Danko (1983). That we have not been able to obtain a quantitative simulation of the results seems to be intrinsic to the model insofar as the parameter choices that allow Eq. 8 to be violated cause the symmetrical titration function to be too steep. It is not clear that the incorporation of triple or higher H^+ occupancy will remedy the deficiency.

We would like to thank Dr. Anne M. O'Connell for helpful discussions and assistance with the synaptosome preparation.

This work was supported by the USPHS through NIH grant GM-40062.

Original version received 17 August 1992 and accepted version received 17 October 1992.

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