

VOLTAGE-DEPENDENT CALCIUM BLOCK OF NORMAL AND TETRAMETHRIN-MODIFIED SINGLE SODIUM CHANNELS

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ABSTRACT The mechanisms by which external Ca ions block sodium channels were studied by a gigaohm seal patch clamp method using membranes excised from N1E-115 neuroblastoma cells. Tetramethrin was used to prolong the open time of single channels so that the current-voltage relationship could be readily determined over a wide range of membrane potentials. Comparable experiments were performed in the absence of tetramethrin. Increasing external Ca ions from 0.18 to 9.0 mM reduced the single channel conductance without causing flickering. From the dose-response relation the dissociation constant for Ca block at 0 mV was estimated to be 32.4 ± 1.05 mM. The block was intensified by hyperpolarization. The voltage dependence indicates that Ca ions bind to sodium channels at a site located $37 \pm 2\%$ of the electrical distance from the outside. The current increased with increasing external Na concentrations but showed a saturation; the concentration for half-maximal saturation was estimated to be 185 mM at -50 mV and 204 mM at 0 mV. A model consisting of a one-ion pore with four barriers and three wells can account for the observations that deviate from the independence principle, namely, the saturation of current, block by Ca ions, and rectification in current-voltage relationship. The results suggest that the Ca-induced decrease of the macroscopic sodium current results from a reduced single sodium channel conductance.

INTRODUCTION

The classical description of ionic fluxes in excitable membranes uses forms based on Ohm's law with a Nernst-potential battery (Hodgkin and Huxley, 1952 *b*) or forms based on integrated versions of the Nernst-Planck electro-diffusion equations (Goldman, 1943; Hodgkin and Katz, 1949). This approach assumes that the ion permeation through membrane channels obeys the "independence principle" of Hodgkin and Huxley (1952 *a*), and that ions diffuse from one side to the other through a homogeneous membrane with a constant diffusion coefficient and with an identical ionic partition coefficient on the two sides of the membrane. However, there is a growing body of evidence that certain phenomena cannot be explained simply by free diffusion. This includes ionic flux coupling, ionic flux saturation, voltage-dependent block by permeant and impermeant ions, and concentration-dependent ionic selectivity (Begenisich and Busath, 1981; Begenisich and Cahalan, 1980 *a, b*; Cahalan and Begenisich, 1976; Chandler and Meves, 1965; Ebert and Goldman, 1976; Hille, 1975). To explain such a variety of membrane phenomena, ionic channels can be represented by a sequence of energy valleys (wells) separated by activation energy barriers. In the potential energy profile, ion binding

occurs at energy minima and an ion moves by hopping over the activation energy barriers (Cecchi et al., 1981; Hille, 1975; Hille and Schwarz, 1978). The ion flux can be calculated on the basis of absolute rate theory (Glasstone et al., 1941; Woodbury, 1971).

In the present paper we report evidence, at the single channel level, that external calcium ions interfere with sodium ion permeation through sodium channels by binding to a site within the channel, resulting in a reduction of the single channel conductance at large negative potentials. Single channel recording with an excised membrane patch has a great advantage in that the measured current is uncontaminated by gating processes and local ionic accumulation. We also show that a four-barrier, three-site permeation model, which has been used to describe "macroscopic" sodium currents (Hille, 1975), can account for nonindependent "microscopic" ion permeation through single sodium channels. A preliminary account of this study has appeared (Yamamoto et al., 1983 *b*).

METHODS

All experiments were carried out with N1E-115 neuroblastoma cells. These cells were maintained in tissue culture and grown in Dulbecco's modified Eagle's medium, supplemented with 10% newborn calf serum at 37°C in humidified air containing 10% CO₂. Three days to two weeks before use, cells were grown on coverslips in media to which 2% dimethylsulfoxide (DMSO) had been added in order to enhance the expression of neuronal characteristics (Kimhi et al., 1976).

Single-channel currents were recorded from excised membrane patches

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using the gigaohm sealing patch-clamp technique (Hamill et al., 1981). The normal external solution contained 125 mM NaCl, 5.5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 25 mM dextrose, 20 mM sucrose, and 20 mM HEPES; the pH was titrated to 7.3 with NaOH. For low calcium solutions, part of the calcium was replaced by magnesium on an equimolar basis. Solutions with higher concentrations of calcium or sodium ions were made by reduction of sucrose and/or dextrose on an iso-osmotic basis. The internal solution for outside-out patch experiments contained 20 mM K glutamate, 5 mM HEPES, 20 mM K₄EGTA, and 525 mM sucrose. The internal solution for inside-out patch experiments contained 150 mM CsF, 2 mM Na HEPES, and 20 mM HEPES, and the pH was titrated to 7.2 with CsOH. All solutions were filtered immediately before use through a membrane filter, 0.45 μm pore size (Gelman Instrument Co., Ann Arbor, MI).

Inside-out membrane patches were obtained as follows: After the gigaohm seal was established, depolarizing steps of 60 mV in amplitude, superimposed on a hyperpolarizing holding potential of -30 to -40 mV (in addition to the cell resting membrane potential), were applied to the bath. After observing activity of single sodium channels in the membrane patch, we changed the bathing solution to the internal solution for inside-out patch experiments. Three minutes after switching the solution, the membrane patch was excised by sudden withdrawal of the electrode from the cell.

In many cases, the bathing solution was further switched to an internal solution containing tetramethrin, a sodium channel modulator. This compound has been found to prolong the open time of the single sodium channels without affecting the single channel conductance (Yamamoto et al., 1983 a). This allowed us easily to change the membrane potential to various levels while the channel was kept open. The single channel conductance could be measured accurately even at large negative potentials. (+)-*trans*-tetramethrin was first dissolved in DMSO to make a stock solution, and the latter was diluted by the internal solution. The amount of DMSO in the final solution was below 0.3% (vol/vol), and had no effect on the sodium current.

Outside-out membrane patches were obtained as follows: After a gigaohm seal was established, negative pressure was continuously applied to the interior of the pipette until the membrane within the pipette was disrupted. The disruption of the membrane was signaled by a sudden appearance of large capacitive and leakage currents. The pipette was then gently pulled from the cell. The tip of the pipette was sealed again, forming an outside-out membrane patch.

Experiments were performed at 8–12°C except when the effect of temperature was examined. The temperature of the chamber was controlled by a Peltier device and measured with a thermocouple probe (model BAT-12, Bailey Instruments Co., Inc., Saddle Brook, NJ).

RESULTS

Effects of External Calcium Concentration on the Current-Voltage Relationship

Fig. 1 *A* and *B* illustrates single sodium channel currents recorded from membrane patches exposed to 50 μM tetramethrin and bathed in external solutions containing two different concentrations of calcium. The membrane was depolarized from a holding potential of -100 mV to -40 mV for a period of 120 ms, and then repolarized to -100 mV. Some of the channels remained in an open state even after the membrane potential returned to -100 mV. In the membrane patch exposed to the normal external solution containing 1.8 mM Ca and 125 mM Na, the membrane repolarization, if applied while a channel was open, resulted in a decrease in single channel current (Fig. 1 *A*) in the face of an increased driving force for inward

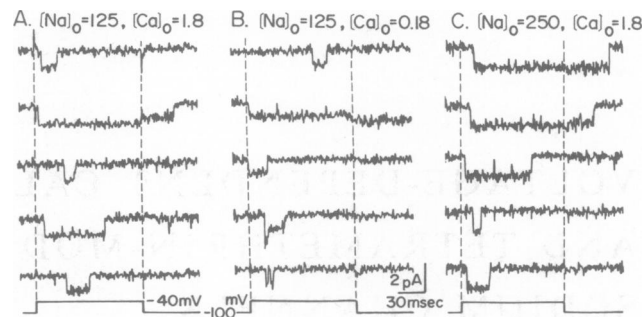


FIGURE 1 Single sodium channel currents recorded from inside-out membrane patches exposed to 50 μM tetramethrin. *A*, Currents in the external solution containing 1.8 mM Ca and 125 mM Na; *B*, 0.18 mM Ca and 125 mM Na; *C*, 1.8 mM Ca and 250 mM Na. The membranes were depolarized from a holding potential of -100 mV to -40 mV. *A*, *B* and *C* were obtained from three different membrane patches. Temperature 12°C.

sodium current. Interestingly, the situation was different when the external calcium concentration was lowered to 0.18 mM (Fig. 1 *B*). In this condition, the current amplitude did not decrease but increased upon repolarization, as expected from an increase in driving force. These observations indicate that the current-voltage (*I-V*) relationships of single channels are not linear but show rectification, and that the shape of the *I-V* curve can be modified by changing the external calcium concentration.

Fig. 2 illustrates the *I-V* relationships of single sodium channels in various external calcium concentrations. In a solution containing 0.18 mM calcium, the *I-V* relation is linear or even superlinear (downward curvature) in the membrane potentials ranging between 0 and -80 mV (○). Hyperpolarizations to the level more negative than -80 mV reduce the current amplitude. In the normal solution

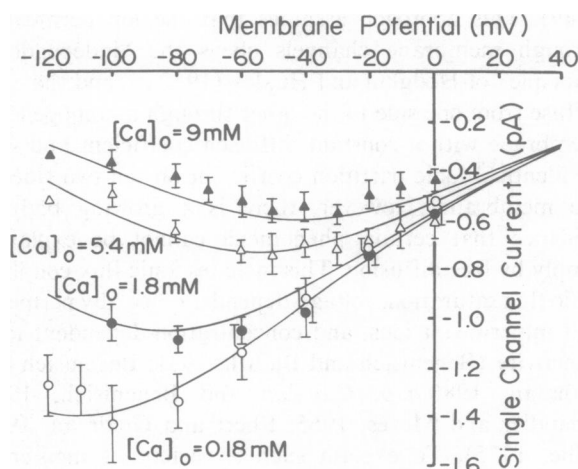


FIGURE 2 Current-voltage relationships for single sodium channels in various external calcium concentrations: ▲, 9 mM; △, 5.4 mM; ●, 1.8 mM; ○, 0.18 mM. Each point with a vertical bar represents the mean ± standard deviation. Lines are drawn by the four-barrier model using the parameters shown in Fig. 6 and the ionic concentrations indicated for each curve (see text). Each curve was obtained from different inside-out membrane patches treated with 50 μM tetramethrin. Temperature 12°C.

containing 1.8 mM Ca (Fig. 2, ●), the amplitude of single channel currents increases almost linearly by hyperpolarization at potentials between 0 and -40 mV, but levels off at more negative potentials, reaching a maximum at about -80 mV. The current decreases with further hyperpolarization to -100 mV. As the calcium concentration is raised to 5.4 mM, the I - V curve bends at less negative potentials, the current amplitude attaining a maximum at about -50 mV (Fig. 2, Δ). In a solution containing 9 mM Ca, the current amplitude attains a maximum at about -30 mV (Fig. 2, ▲). These results suggest that external calcium ions block sodium channels in a voltage-dependent manner. The rectification observed in 0.18 mM Ca solution seems to be due to a magnesium effect rather than a calcium ion block. This will be discussed later.

Calcium ions did not seem to permeate sodium channels, because no single channel current was observed when sodium ions were totally replaced by calcium ions. Therefore, the calcium block of sodium channels is due to a binding of calcium ions to a site in the channels. To estimate the apparent dissociation constant (K_d) for calcium block, dose-response curves were constructed at each membrane potential. Fig. 3 illustrates the curves for fitting data obtained at -20 and -100 mV. The current amplitudes measured in low Ca (0.18 mM) solution at membrane potentials between 0 and -60 mV (i.e., the linear portion of I - V curve) were assumed to represent those without Ca block. To estimate the current amplitude without block at potentials more negative than -60 mV, the linear portion of the I - V curve was extrapolated in the hyperpolarizing direction. The mean amplitude of single sodium channel currents obtained at each calcium concentration and at each membrane potential was normalized to the current without block. These normalized values are plotted in Fig. 3. Assuming one-to-one stoichiometric

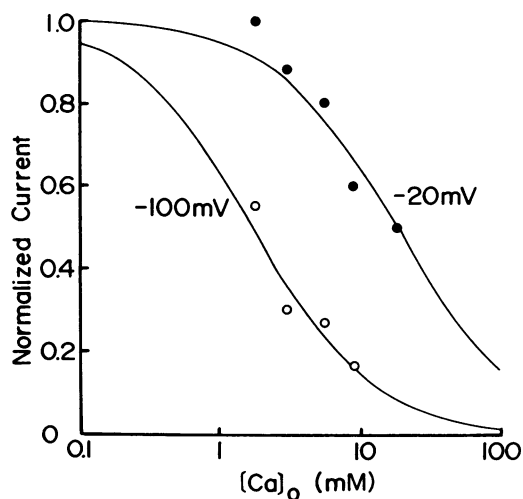


FIGURE 3 Concentration dependence of Ca block of sodium channels at -20 mV (●) and at -100 mV (○). Curves were drawn by Eq. 1 (see text). The K_d is estimated to be 18 mM at -20 mV and 1.7 mM at -100 mV. Temperature 12°C.

relation for calcium binding to a site, one can calculate the dissociation constant (K_d) for calcium block using Eq. 1:

$$r = (1 + [Ca]_0/K_d)^{-1} \quad (1)$$

where r is the normalized current amplitude and $[Ca]_0$ is the external calcium concentration.

The K_d values were calculated for other membrane potentials as well. In Fig. 4, the K_d for calcium block is plotted semilogarithmically as a function of the membrane potential. The relationship is well-fitted by a straight line. The K_d decreased e -fold for a 32-mV hyperpolarization, suggesting that the binding site for calcium ions is located 37% of the way along the membrane field from the external side.

Effect of External Sodium Concentration on the Current-Voltage Relationship

Fig. 1 C illustrates examples of single channel currents in a high-Na medium. The external sodium concentration was doubled to 250 mM while the external calcium concentration was kept constant at 1.8 mM. The amplitude of single channel currents is larger in the high-Na medium than in normal solution as can be seen by comparison with the records in Fig. 1 A. The current amplitude does not increase appreciably upon hyperpolarization from -40 mV to -100 mV. Fig. 5 illustrates the I - V relationship in high-Na solution. The shape of the I - V curve in high-Na condition (squares) is not exactly the same as that in normal Na solution (circles). In spite of a two-fold increase in the external sodium concentration, the amplitude of

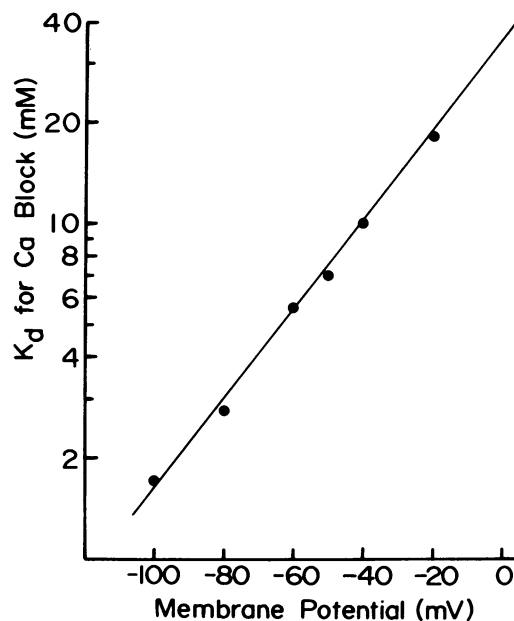


FIGURE 4 Dissociation constant (K_d) for Ca block as a function of the membrane potential. The straight line (—) is a least-squares fit using Eq. 2 (see text). Temperature 12°C.

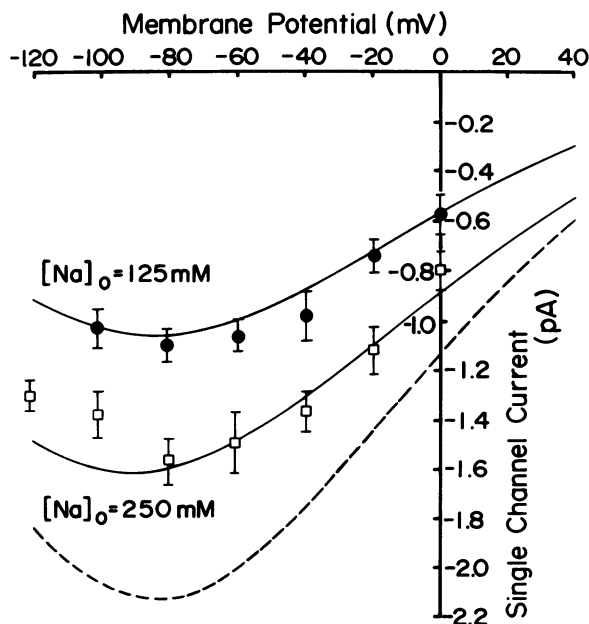


FIGURE 5 Current-voltage relationships of single sodium channels in external solutions containing two different concentrations of sodium: ●, 125 mM Na and 1.8 mM Ca; □, 250 mM Na and 1.8 mM Ca. The two sets of measurements were made with different membrane patches. Solid lines (—) are drawn by the four-barrier model (see text). The broken line (---) is obtained by scaling up the solid line for normal solution so as to double the current amplitude at each membrane potential. Temperature 12°C.

single channel currents increases less than twice at all membrane potentials. This observation is not compatible with the independence principle, which predicts approximately a two-fold increase in current amplitude with doubling of the external sodium concentration. The results indicate that sodium ions interact with a site within the sodium channel while passing through it.

Model

The constant field theory (Goldman, 1943; Hodgkin and Katz, 1949), which requires the independent ion permeation, cannot adequately describe the present results, including ionic saturation and Ca block. An alternative approach is to use a barrier model, which is based on Eyring's rate theory in combination with standard chemical kinetics (Woodbury, 1971). A barrier model consisting of two barriers and one site was used by Woodhull (1973) to describe the block of macroscopic Na currents in nodes of Ranvier by hydrogen or calcium ions. This two-barrier model described the block adequately but was not intended to fit the current-voltage relation. However, barrier models consisting of four barriers and three sites (Hille, 1975) or of three barriers and two sites (Begenisich and Cahalan, 1980 *a, b*) have been successfully used to describe the current-voltage relation and other phenomena related to macroscopic measurements of Na currents which deviate from the independence principle. In the present paper,

Hille's four-barrier model (Hille, 1975) has been adopted to simulate the I - V relationships of single sodium channels. This model pictures the ionic channel as a narrow pore with ions moving stepwise between binding sites separated by four barriers across the membrane. It assumes that no more than one ion can occupy the pore at any one time.

To solve for the steady-state ionic current in such a one-ion pore, a set of kinetic equations similar to that developed by Hille (1975) was used. In this model, the depth and location of the external primary energy well are constrained by the experimentally determined K_d for the ions in question and the fractional electrical distance, δ , derived from the slope of the curve relating K_d to the membrane potential. The K_d at 0 mV, $K_d(0)$, for calcium ions was estimated to be 32.4 ± 1.05 mM in the present study (Fig. 4). This value corresponds to a binding energy well of about -3.5 in RT units. Because no calcium ion current flows through sodium channels, the rates of calcium ions moving from the blocking site to the cytoplasmic side are assumed to be negligible. The voltage dependence of K_d is expressed by Eq. 2 (Woodhull, 1973):

$$K_d(E) = K_d(0) \exp(z\delta FE/RT). \quad (2)$$

In the present study, δ was estimated to be 0.37 ± 0.02 . As described below, magnesium ions seem to have some blocking action. Therefore, it is assumed that the energy profile for magnesium is exactly the same as that for calcium except that the primary well is shallower for magnesium. For simplicity, sodium, calcium, and magnesium ions are assumed to share the same binding site within the channel. The primary well for sodium ions has been estimated to be -1.59 RT units from the concentration-dependent saturation curves.¹ The heights of the energy barriers and other parameters were chosen by a method of trial and error to give the observed values of the single channel currents. All parameters of the model determined in this way are summarized in Fig. 6, and are used for the curve fitting in Figs. 2, 5, 7 and 8.

Smooth lines in Figs. 2 and 5 are drawn by the four-barrier model and show that the rectification of the I - V relation was well-fitted by the model. All I - V relationships in various ionic environments, except for those in low-Ca (0.18 mM) solution, could be fitted by the model without contributions of magnesium. However, the model predicts an increase in current amplitude by hyperpolarization from -80 to -120 mV in low Ca (0.18 mM) solution, whereas the observed current was decreased by hyperpolarization in this membrane potential range. This indicates the magnesium block of the sodium channel. In fact, magnesium ions have been shown to have some blocking action on the macroscopic sodium currents (Taylor et al., 1976). The model gave a value of 100 mM as an apparent K_d for magnesium ions at 0 mV.

¹Yamamoto, D., J. Z. Yeh, and T. Narahashi. Unpublished data.

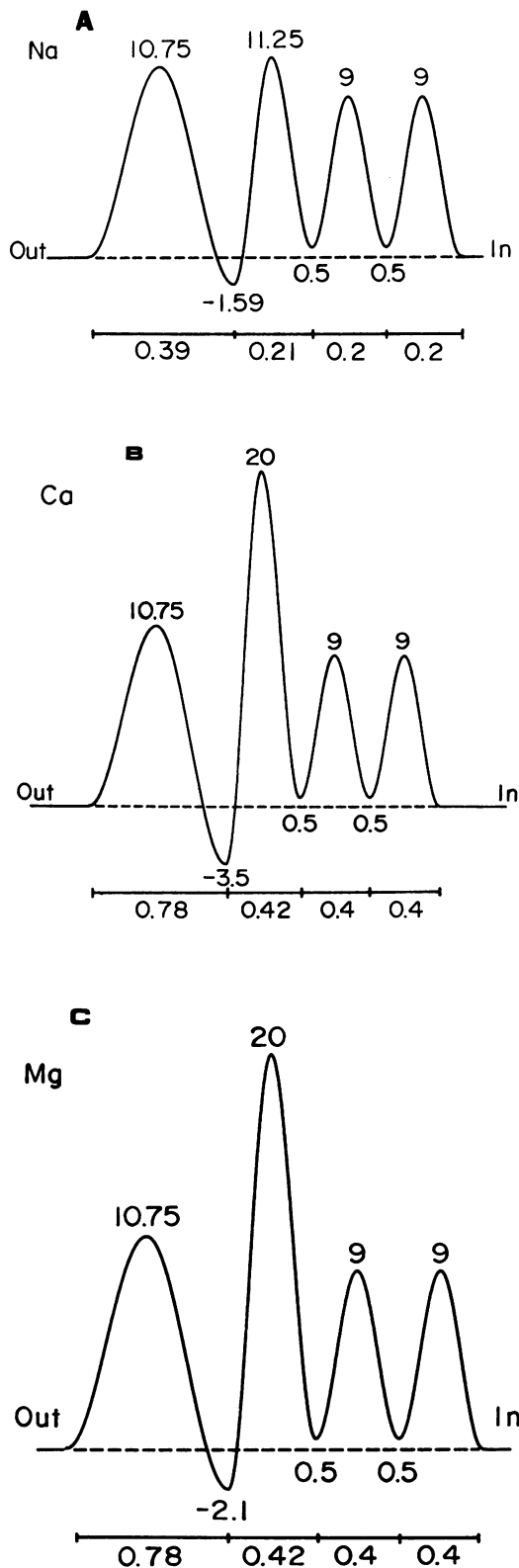


FIGURE 6 Parameters of the four-barrier model for Na (A), Ca (B), and Mg (C). The height of the energy barrier is shown above each peak, and the depth of the well is shown below each valley (RT units). The distance given on the line beneath the profile was chosen to fit the data. The valence of Na, Ca or Mg ions is included in the electrical distance to simplify the form of the electrical energy term in the total energy profile.

The four-barrier model predicts saturation properties of sodium channels. Fig. 7 shows the relationship between the single channel current amplitude and the external sodium concentration. The curves were calculated by the four-barrier model, and show a saturation of ionic currents at high sodium concentrations. The measurements fall on the calculated curves reasonably well.

Another prediction from the four-barrier model is the temperature dependence of single channel current. The net ion fluxes were calculated at various temperatures without changing any parameters. The curve (—) in Fig. 8

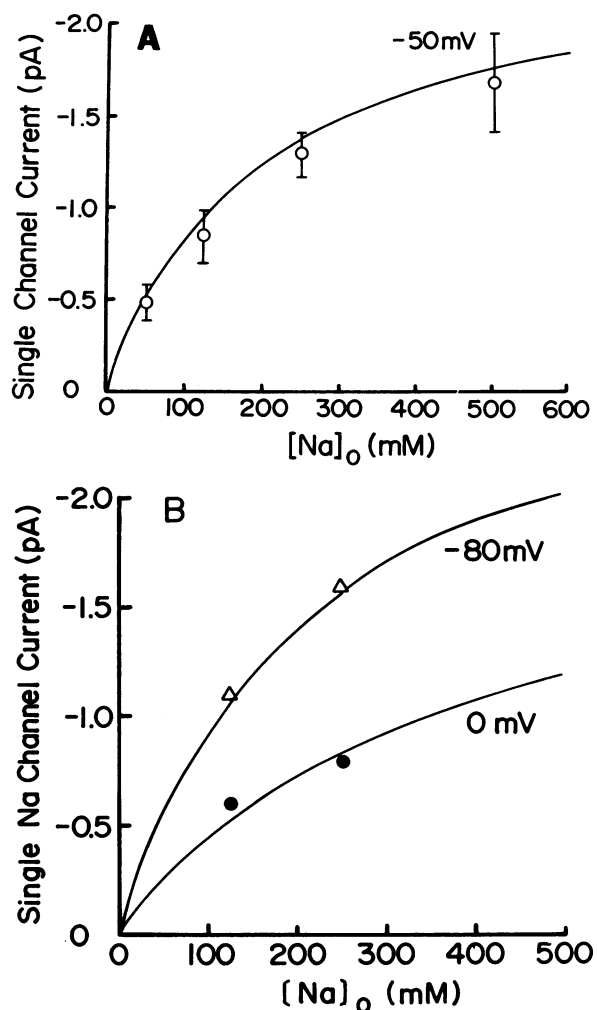


FIGURE 7 Saturation of inward current through the single sodium channels. Current amplitudes are plotted against external sodium concentrations. A, Data were obtained from a single outside-out membrane patch, not treated with tetramethrin, at -50 mV. External solutions with a desired sodium concentration were made by diluting a high-Na solution (500 mM NaCl, 0.18 mM CaCl₂, 2.42 mM MgCl₂, 5 mM HEPES) with an appropriate amount of Na-free solution (700 mM sucrose, 0.18 mM CaCl₂, 2.42 mM MgCl₂, 5 mM HEPES). Temperature 10°C . B, Data in 125 mM Na and 250 mM Na were obtained from two different inside-out membrane patches treated with tetramethrin ($50\ \mu\text{M}$). Points were replotted from Figs. 2 and 5. Δ , -80 mV; \bullet , 0 mV. The external solution contained 1.8 mM Ca. Temperature 12°C . All curve fittings were performed using the four-barrier, three-site model.

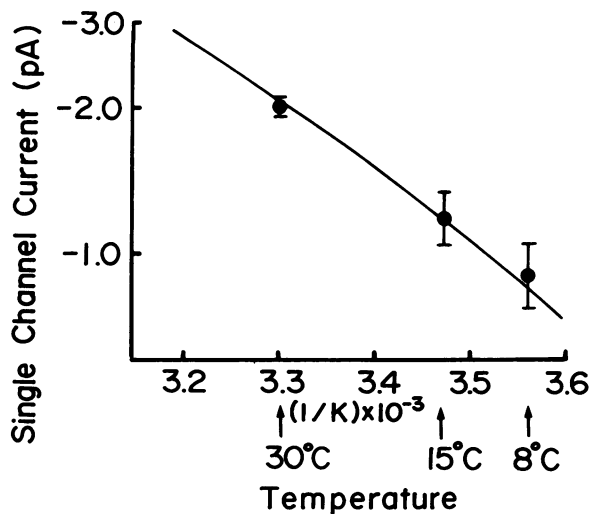


FIGURE 8 Temperature dependence of single channel currents of the sodium channel. The measurements (mean \pm SD) were obtained from an inside-out membrane patch not treated with tetramethrin. The external solution contained 0.18 mM Ca and 125 mM Na. The curve is drawn based on the four-barrier model.

represents the temperature dependence of the single channel current predicted by the four-barrier model. The value of Q_{10} expected from the model is 1.47 between 20° and 30°C. The Q_{10} value estimated from the experiment (Fig. 8, •) is 1.41. Thus there is a good agreement between the model and the experimental value.

DISCUSSION

The conductance of single sodium channels modified by tetramethrin was not constant over the membrane potentials ranging between 0 and -120 mV; instead, it was reduced at large negative potentials. This reduction was not due to the action of tetramethrin, because similar I - V relations of single-sodium channels have been obtained under normal conditions not only in neuroblastoma cells¹ but also in tunicate egg cells (Fukushima, 1981) and bovine chromaffin cells (Fenwick et al., 1982). The results in the present paper unequivocally show that rectification of the I - V curve in single sodium channels is due to the voltage-dependent block of the channel by external calcium ions. This finding at the single-channel level accounts for nonlinear characteristics of the instantaneous I - V curves for macroscopic sodium currents observed in normal squid axons (Taylor et al., 1976) and in nodes of Ranvier treated with aconitine (Mozhayeva et al., 1977) or batrachotoxin (Mozhayeva et al., 1982).

The present study suggests that the channel block is due to rapid movement of a calcium ion into and out of the blocking site in the sodium channel. This is analogous to the proposed ionic block of single anomalous-rectifier K channels in tunicate eggs (Fukushima, 1982). In the anomalous-rectifier K channels, single channel currents are clearly interrupted by the movement of blocking ions

into the channel without affecting the single channel conductance, in much the same way as the local anesthetic block of single acetylcholine-activated channels (Neher and Steinbach, 1978; Ogden et al., 1981). Unlike the ionic block of the anomalous rectifier channel or the local anesthetic block of acetylcholine-activated channels, Ca block of single sodium channels is manifested as a simple reduction of channel conductance without any sign of flickering between open and blocked states. The time constant of the block by calcium ions may be so fast that we could not resolve it. In fact, the time constant for the Ca block of single sodium channels is calculated to be $0.3 \mu\text{s}$ or 3 MHz at 0 mV using the parameters of four-barrier model. The simple reduction of conductance due to ionic block has been reported in Ca block of the gramicidin channel (Bamberg and Lauser, 1977), Cs block of potassium channels in sarcoplasmic reticulum (Coronado and Miller, 1979), and the block of sodium channels by tetramethylammonium from the cytoplasmic surface (Horn et al., 1981). On the analogy of the local anesthetic block of acetylcholine-activated channels (Neher and Steinbach, 1978), it is possible that the apparent open-channel lifetime is increased by the Ca block. There are not enough data yet available to assess this possibility.

In the present study, we found that a one-ion pore model adequately described the blocking action of Ca ions on sodium channels. This does not preclude the possibility of a multi-ion pore as proposed by other investigators (Begenisich and Cahalan, 1980 *a, b*). It has been suggested that under physiological conditions, the binding sites are rarely occupied by ions simultaneously (Begenisich and Busath, 1981; Busath and Begenisich, 1982). Hence, a one-ion pore model can approximate this Ca-blocking behavior. Further studies of the interactions between permeant and impermeant (blocking) ions may reveal deviations from the one-ion pore model. The one-ion pore model predicts that as the concentration of permeant ions is increased, the potency of blocking ions should decrease (because they compete with each other for a site in the channel), and the voltage dependence of block should not change. Both of these criteria were satisfactorily met in the case of the isolated sarcoplasmic reticulum potassium channels (Miller, 1982).

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DISCUSSION

Session Chairman: Charles F. Stevens *Scribe:* Thomas A. Rando

HORN: It is known that Ca currents can be measured through sodium channels, so your model should be able to predict that at sufficiently hyperpolarizing voltages the current-voltage relationship will not just bend toward the abscissa but will turn around and increase again with voltage. Do you ever see that experimentally and is that what you would predict?

YAMAMOTO: It is very difficult to measure the single channel current at negative potentials, say -140 mV. When we tried we didn't see any second increase of the current. If I reduce the barrier height for Ca ions from 20 RT to 15 RT (see Fig. 6), I can predict a second increase of Na

channel current at very negative potentials. For 20 RT barriers, the Na current will be completely blocked at very negative potentials. Ca ions cannot pass through the channel.

JAKOBSSON: There is a result that I seem to remember from the dark ages when people looked at macroscopic currents. When one looked at Na current tails while stepping to hyperpolarizing voltages, the tails became larger with increasing hyperpolarization. That result would not seem to be predicted by your Fig. 5 if you imagine the macroscopic current to be made up of a number of single channels with that I-V curve. Have you looked at the macroscopic currents?

BEZANILLA: Perhaps I can answer that. Clay Armstrong, Bob Taylor, and I did similar experiments with squid axons in 1973-4. In low Ca the tail currents did indeed increase as you say, at more negative membrane potentials, but in high Ca the tail currents saturated. Using a simple