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Calcium ion as a cofactor in Na channel gating

(Ca block/surface charge/patch clamp/pituitary cells)

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ABSTRACT **Calcium ions in the external medium stabilize the resting state of voltage-dependent channels, including Na channels. This effect of calcium on channel gating is usually explained in terms of the surface charge hypothesis, which proposes that local adsorption of calcium ion to the outside of the membrane alters the intramembranous electric field, thus influencing channel behavior indirectly. Calcium ion has also been shown to block Na channels, most strongly at negative voltage. We have examined these two apparently separate effects of calcium, the gating effect and Ca block, and find the two are closely correlated. We propose that calcium (or a suitable substitute) is an essential cofactor in normal gating and that it produces gating and blocking effects by binding within the channel.**

Recent years have brought major advances in the understanding of the gating mechanism of voltage-dependent ionic channels. The Na channel peptide has four repeats of a positively charged region (called S4) with arginine or lysine at every third position (1). This region is widely thought to form an α-helix that lies within the membrane and moves outward in response to a positive change of the membrane voltage, thus driving the channel from closed to open conformation (2–5).

The presence of highly charged and presumably mobile regions in the Na channel raises the expectation that the channel should interact strongly with ions in solution on both sides, particularly multivalent ions. Such interactions are indeed observed. External Ca stabilizes the resting state of the channel, making it necessary to depolarize the membrane more strongly to open Na channels (6). We will refer to this as the *gate-shifting* action of Ca. This action has often been explained by saying that Ca screens negative surface charges on the membrane surface, changing the local voltage profile within the membrane and thus influencing the channels indirectly (7).

Ca has another prominent action on Na channels: it tends to block them when membrane voltage is negative (8–12). Channel blocking by Ca is a much faster process than channel gating and results from occupation of the channel lumen by Ca. Gate-shifting and blocking have for some years been regarded as two separate phenomena, mediated by Ca acting at two separate sites.

Recent experiments have shown that Ca is necessary to maintain the functional integrity of K channels. The experiments also suggest that Ca binds within the lumen of closed K channels and has a direct role in gating (13–15). The K channel experiments led us to reexamine the influence of Ca on Na channels and specifically to ask if blocking and gate-shifting are indeed separate phenomena or if they are in some way related. Here we report evidence showing that the two effects are very closely related.

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METHODS

Whole-cell patch-clamp experiments were carried out in the pituitary cell line GH3. Cells were replated every 10 days (16) with a subcultivation ratio of 1:15. We recorded from cells cultured for 2–7 days after replating.

The composition of the internal solution was (concentrations in mM) 100 NaF, 30 NaCl, 1 $CaCl_2$, 10 EGTA/CsOH, and 10 Hepes/CsOH (osmolarity = 292 mosmol). External solutions with Ca concentrations ranging from 0.2 mM to 50 mM were prepared by mixing the following two solutions. The "50 Ca" solution contained 50 $CaCl_2$, 80 NaCl, and 10 Hepes/NaOH. The "0 Ca" solution contained 80 NaCl, 10 Hepes/NaOH, and sucrose to raise the osmolarity close to 300 mosmol. pH was adjusted to 7.3.

The external recording solution in the experimental chamber was continually exchanged by a gravity-driven flow/suction arrangement. The chamber had a relatively small volume (about 0.3 ml), making possible a nearly complete exchange within 30 s. The temperature of the solution in the chamber was kept precisely at 15°C using a controller device connected to a Peltier cooler and with a thermistor as temperature sensor.

Under these recording conditions currents through K and Ca channels were eliminated or greatly reduced. With 2 mM external Ca, and after papain action (see below), channel activity resistant to 1 μM external tetrodotoxin was practically absent.

All solution changes were bracketed by averaging control measurements taken before changing to the test solution and after return to control medium.

Papain (1 mg/ml; type IV, Sigma) was included in the pipette solution. The enzyme removes the inactivation gating of Na channels within 10 min after break-in with the electrode (16).

To optimize time resolution, we used a whole-cell patch clamp with "supercharging," and low-resistance (0.4–0.7 MΩ) patch pipettes (16). The estimated access resistance error in most experiments was <5 mV. Membrane current signals were sampled at 10- or 20-μs intervals, and their linear components were subtracted out using the current response to 50-mV hyperpolarizing steps as a control. The holding potential was −80 mV in all experiments. Na conductance, g_{Na}, was computed by dividing Na current (I_{Na}) by the driving force on Na ions, $V_m - V_{Na}$.

RESULTS

Ca Stabilizes the Resting Conformation of Na Channels. The activation of Na channels by depolarization is illustrated in Fig. 1. The traces show Na conductance (sodium current divided by driving force; see *Methods*), which is a direct measure of the number of channels in the conducting state. Shortly after the beginning of each sweep, V_m was stepped from −80 mV to the values given next to each trace. This caused an activation of Na conductance that, in 2 mM Ca, was slow and small at −50 mV and large and relatively fast

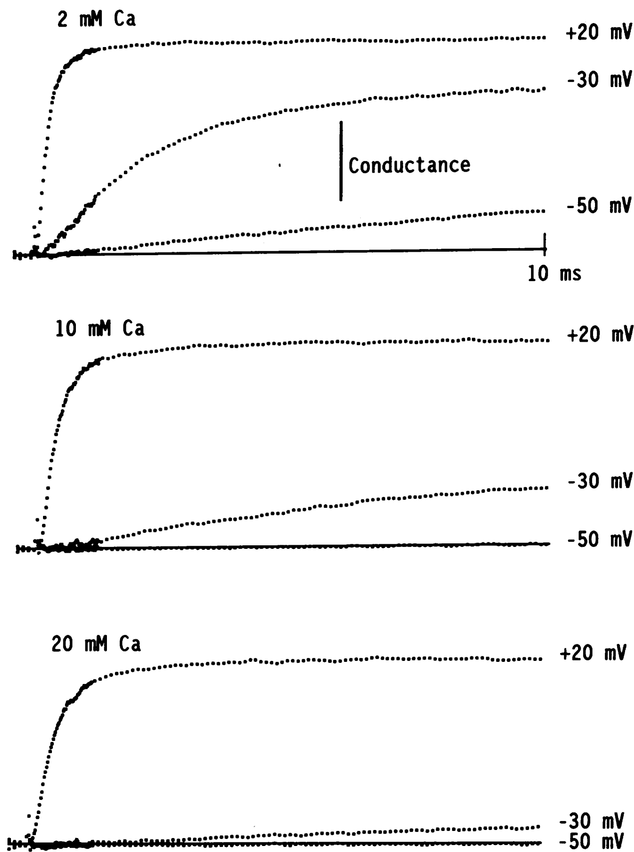

FIG. 1. Ca stabilizes the resting state of Na channels. Na currents were activated by steps to the voltages indicated in the presence of 2, 10, or 50 mM external Ca. Conductance was obtained by dividing current by the corresponding driving force for Na ions. Higher Ca slows and depresses conductance at low voltages, without much affecting the value attained at +20 mV.

at +20 mV. The dependence of conductance and kinetics on V_m is a normal property of Na channels.

A well-known effect of Ca is its tendency to stabilize Na channels in their closed conformation. This tendency can be seen by comparing the conductance at −50 and −30 mV in the three Ca concentrations illustrated. At −50 mV in 2 mM Ca, conductance rises slowly to about 18% of its maximum value (the value at +20 mV), whereas with 10 and 20 mM Ca there is no measurable conductance at −50 mV: essentially none of the channels has opened in higher Ca. A step to −30 mV activates most of the conductance in 2 mM Ca, about 25% of it in 10 mM Ca (still rising at the end of the step), and only about 10% of it in 20 mM Ca. The maximum conductance (recorded at +20 mV) is nearly the same in all three cases. Overall, Ca does not much affect the maximum conductance, but a larger depolarization is required to open a given fraction of the channels if Ca is high. This is the well-known "gate-shifting" action of Ca.

Channel Blocking by Ca Ion. The second phenomenon of interest, channel blocking by Ca ion, is illustrated in the curves of Fig. 2. Blocking was examined by activating the Na channels with a short but strong depolarization that opened most of the channels, stepping the voltage to a new level, and measuring the current immediately after the step (Fig. 2 *Inset*). Ideally the current measurement is made before any channel gates have a chance to close. From many such measurements the *I–V* relation for open channels can be constructed, with effects due to gating eliminated.

Open Na channels are known to behave almost ohmically in many circumstances, which would give a straight line in a current–voltage plot. From −20 to +20 mV the channels

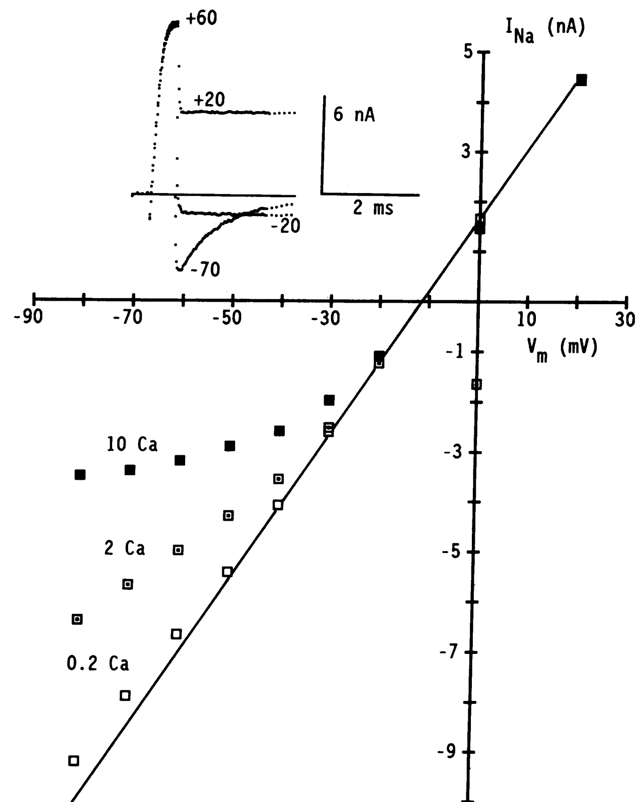

FIG. 2. Ca blocks Na channels at negative voltage. Na channels were activated by a brief step to +60 mV (see *Inset*), and the membrane was then repolarized to a new voltage, as given on the abscissa. Na current was measured immediately after the repolarizing step. Where appropriate (e.g., −70 mV), a single exponential was fit to the current tail and extrapolated to the origin of the pulse. The measured or extrapolated current is plotted as a function of the repolarizing voltage at three Ca concentrations. The deviation of the current from the ohmic straight line is more pronounced at negative voltage and at higher Ca concentration.

indeed behave ohmically regardless of Ca concentration. A straight line has been drawn through these points in Fig. 2. From −20 to −80 mV, the data points fall above the ohmic line, slightly above in 0.2 mM Ca, and far above in 10 mM Ca. Current at −50 mV in 2 mM Ca, for example, is 79% of the value expected for ohmic behavior and only 53% in 10 mM Ca.

The reduction of current magnitude below the ohmic value cannot be the result of channel gating, for open gates have no time to close under the conditions of the measurement. Instead, the reduction is the result of a very rapid block of the channels by Ca ion, which enters the channels readily but tends to stick. Despite calcium's stickiness, the flux of Ca ions into and out of the channel is so rapid that the block is developed, for practical purposes, instantaneously, and the fractional block depends on the relative values of entry and exit rates for Ca.

The plot in Fig. 3*A* shows the fraction of the channels that are blocked as a function of voltage, at several Ca concentrations. The fraction blocked in each case was estimated by comparing the observed current to the ohmic current, given by the straight line. If the current is 53% as large as the ohmic value, we estimate that 47% of the channels are blocked. The assumption is that behavior would be perfectly ohmic in 0 Ca, as suggested by the curves in Fig. 2.

From the plot it is clear that the fraction of blocked channels increases as the Ca concentration is raised. It also increases as voltage is made more negative, because the membrane field draws external Ca ions into the channels.

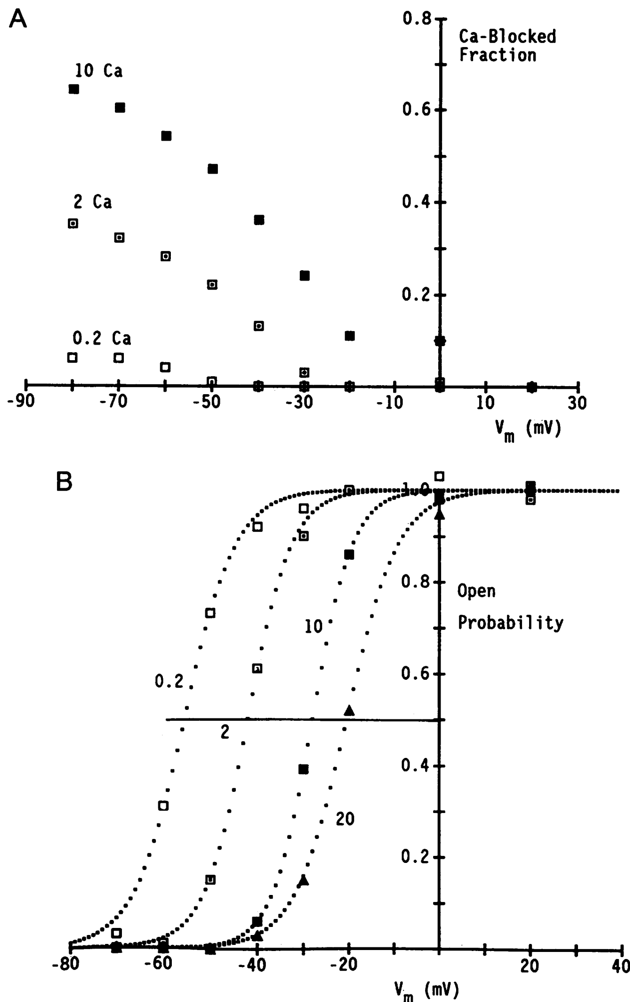

FIG. 3. (*A*) The fraction of Na channels that are blocked by Ca is plotted as a function of voltage at three calcium concentrations. The fraction blocked was derived from instantaneous current–voltage curves like those in Fig. 2. (*B*) The fraction of Na channels that have open activation gates was estimated from the Na conductance measured at −70 mV at the end of 10-ms voltage steps to the activating voltage given on the abscissa. Channels with open activation gates can be either Ca-blocked or conducting. The plotted points give all of the channels that have open activation gates, whether conducting or blocked. The data in *A* were used to calculate the fraction open from the conductance. Raised Ca increases the blocked fraction (*A*) and favors the closed conformation of the channel (*B*).

Open Probability. The interpretation of conductance traces like the ones in Fig. 1 is complicated by the fact that a channel can be nonconducting for two reasons: its voltage-sensitive gate can be closed or, as shown in Fig. 2, it can be blocked by Ca. We would like to know the open probability (p_{open})—that is, the fraction of channels that have open activation gates—regardless of whether they are conducting or Ca blocked. The p_{open} can be obtained from the conductance traces by adjusting for the channels that are Ca blocked, using the data in Fig. 3 and the formula: p_{open} = fraction conducting/(1 − fraction blocked). We used this adjustment to obtain p_{open} at the end of the 10-ms pulses like the ones shown in Fig. 1.

The results are plotted in Fig. 3*B* for several Ca concentrations. The p_{open} is a steep sigmoid function of voltage, with a saturation value reached near 0 mV. The gate-shifting action of Ca is evident from the displacement of the curves along the voltage axis. In 0.2 mM Ca, p_{open} begins to rise near −70 mV and reaches its half-maximal value near −55 mV. Raising the Ca concentration shifts the p_{open} curve to the right with little or no change in shape: the gating of the channel is shifted to a different voltage range. An empirical measure of the shift can be obtained by measuring the displacement of the curves measured at half maximum, along the continuous horizontal line in Fig. 3*B*. Changing from 0.2 to 50 mM Ca, for example, shifts the midpoint of the p_{open} curve to the right by 47 mV.

Correlation Between Gate-Shifting and Blocking. The central question of the paper is whether gate-shifting and the propensity of Ca to block the channels are related. The shift was measured as illustrated in Fig. 3*B*. As a measure of the general tendency of Ca to enter and block channels, we used the Ca-blocked fraction at −70 mV. Using the fraction blocked at −50 or −80 mV gave almost identical results. When the shift is plotted as a function of the fraction of blocked channels as in Fig. 4, it can be seen that there is a very strong, indeed almost perfect, correlation between the shift and the fraction blocked.

DISCUSSION

The results show a close relationship between calcium's tendency to "shift" gating along the voltage axis and its tendency to block channels (Fig. 4). This correlation at first seems puzzling but can be partially understood by plotting gate-shifting and blocking as a function of Ca, as shown in Fig. 5. The triangles are taken from the shift data of Hille *et al.* (17), whereas the filled squares are our shift data, and the open squares are our Ca-blocked-fraction data. The shift and the fraction of Ca-blocked channels saturate as a function of [Ca], but neither curve is a simple saturation isotherm of the type expected for a single binding site with fixed K_d. Fitting of shift data in terms of surface charge requires more than one type of binding site (8). The fraction-blocked curve, on the other hand, is not simple because the site is in the membrane field, and the apparent affinity of the site for Ca varies with membrane voltage. Nonetheless, though neither is simple, the saturating curves for the two effects are similar or

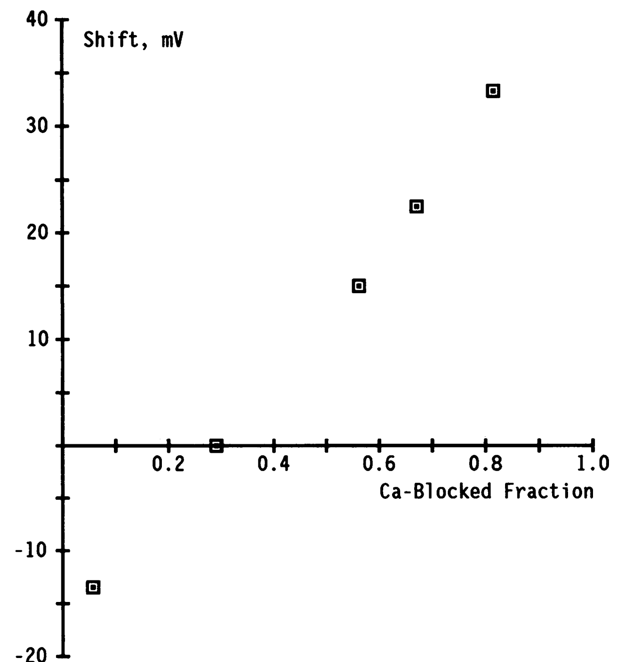

FIG. 4. The shift of the open-probability curve is plotted as a function of the fraction of the Ca channels that are Ca-blocked. The shift is the horizontal displacement of the midpoint of the open probability curves of Fig. 3*B*. The fraction blocked was measured from Fig. 3*A* at −70 mV.

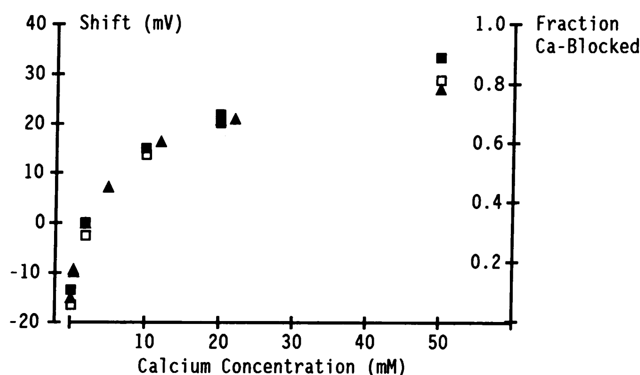

FIG. 5. Shift and Ca-blocked fraction as a function of Ca. The filled triangles give shift data replotted from Hille *et al.* (17). The filled squares are our shift data, and the open squares give our data for the Ca-blocked fraction. The shift and the fraction-blocked are very similar functions of Ca.

identical, and this underlies the close correlation observed in Fig. 3. Clearly this suggests that in some way, gate-shifting and blocking are closely related mechanistically.

The physical picture that emerges is quite different from the surface-charge view, as shown by the cartoons in Fig. 6. According to the surface-charge theory, Ca ions are adsorbed to fixed negative charges on the outer surface of the membrane. The field in the membrane changes as a result of Ca adsorption and influences the voltage sensor of the channel in the same sense as would making the external voltage more positive—i.e., it tends to keep the channel gate closed. Since a component of the field within the membrane depends on the fraction of surface sites that are Ca occupied, the shift is a saturating function of the Ca concentration. Ca block of the channel is the result of Ca occupation of a separate site, located within the channel lumen.

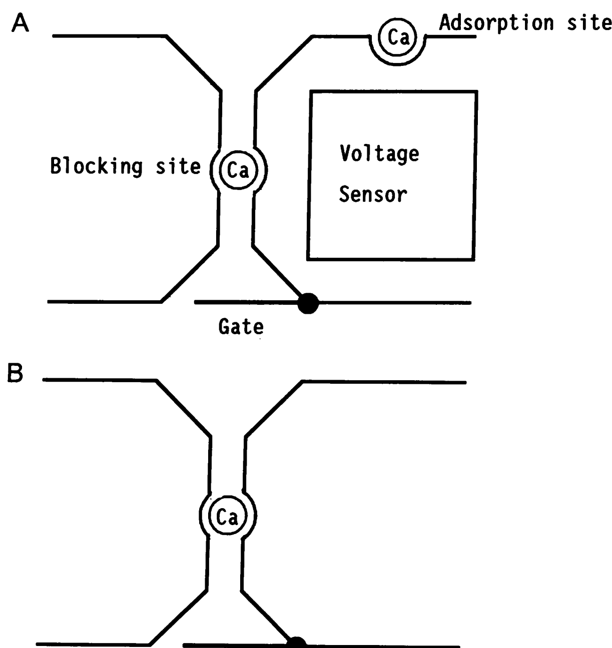

FIG. 6. Hypotheses for gate-shifting and block. (*A*) The surface charge hypothesis explains gate-shifting as a local alteration of the membrane field by Ca occupying the adsorption site. The voltage sensor cannot distinguish the local change from a change of the membrane voltage. Ca block is a separate phenomenon, produced by Ca occupation of a blocking site in the channel. (*B*) The correlation between gate-shifting and Ca block suggests that both effects are produced by Ca at a single site in the channel. Occupation of this site by Ca blocks the channel and, by an unknown mechanism, predisposes it to close.

Fig. 6*B* depicts channel block by Ca ions. The site for blocking is in the channel, and far enough into the membrane that voltage has a substantial influence on the equilibrium between external Ca and the site. The blocked fraction increases as Ca is drawn toward the site by internal negativity. Rough quantitation by standard methods (8) suggests that the site is about halfway into the channel. Binding of a Ca ion to the channel is transient, and an occupying ion may dissociate from the site either by passing on through the channel or by returning to the external medium. Raising the external Ca concentration increases the blocked fraction of channels, and the fraction of occupied sites would be expected to follow a saturation curve.

The correlation between gate-shifting and block can be explained in two ways. First, one could postulate that the properties of the blocking site and the surface site are the same. Or, more parsimoniously, one can postulate that Ca exerts its gate-shifting and blocking actions by occupying the lumen of the channel. We prefer the latter explanation but cannot rigorously exclude the former. In the latter view, occupation of a site in the channel by a Ca ion would aid in closing the channel, or Ca occupation might retard opening, or both. By either mechanism, lowering Ca would make the closed conformation of the protein less stable.

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