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Effects of the external pH on Ca channels: Experimental studies and theoretical considerations using a two-site, two-ion model

(surface potential)

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ABSTRACT **Some effects of the external pH on Ca channels were studied in a hybridoma cell line (mAb-7B), by using the whole-cell configuration of the patch-clamp technique. As the pH was lowered, both the activation and the inactivation curves shifted toward less negative membrane potentials, suggesting a pH-induced decrease of an external negative surface potential, sensed by the mechanism of gating. The potential for half-activation, $V_{1/2}$, and that for half-inactivation, V_h, were related by a straight line with a slope of one. The inward current varied exponentially with $V_{1/2}$, as would be expected if the field inside the channel and the Ca^{2+} concentration at the entrance were sensitive to the surface potential. However, the reversal potential and the outward current were unaltered by changes in the pH. Under the hypothesis that the channel senses the surface potential, all these results, as well as the nernstian behavior of the reversal potential with respect to Ca^{2+}, observed in previous studies, are accounted for by a three-barrier, two-ion model for a channel, provided it is assumed that the potential in the channel drops almost entirely across the barrier adjacent to the external solution.**

Voltage-gated Ca channels are found in hybridoma cell lines constructed by fusion of mouse myeloma cell line S194 and mouse splenic B lymphocytes (1). Voltage-clamp type analyses revealed that the current through the channel is carried in the inward direction by external Ca ions and in the outward direction by internal alkali cations. No other types of voltage-gated ion channels were found in these cells (2).

In the present experiments, the pH of the external solution was changed keeping all other external and internal ion concentrations constant. The gating kinetics of the membrane current shifted in the positive direction along the voltage axis when the external pH was reduced. This indicates that the surface potential is altered by the external H^+ concentration and that the potential changes are sensed by the gating mechanism. The aim of this work is to investigate whether or not a similar surface potential is also sensed by the channel itself. In studies of Ca channels some investigators have simply assumed that surface potential changes alter the field in the channel as well as in the surrounding lipid area, whereas others have asserted that only gating is altered, not the channel conductance. In most cases the assumptions had no clear experimental support. The same question has also been raised for the Na channel, leading to different conclusions depending on the type of preparation (3, 4). The conclusion that the Na channel in the node of Ranvier does not sense the surface potential (4) has been deduced from the behavior of the outward current, which was found to be insensitive to the ionic strength and the external pH. Difficulties in isolating the outward current have been the major obstacle to performing equally significant experiments with Ca channels. However, in the present preparation we can record clear outward currents flowing through the Ca channel, which constitutes a great advantage for studying these currents as a function of the external pH.

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METHODS

A hybridoma cell line (mAb-7B) constructed by fusion of S194 mouse myeloma cells and mouse splenic B lymphocytes was used. The cell line secretes immunoglobulin M. The whole-cell variation of the patch-electrode voltage clamp was used. The details of the technique were similar to those described (2, 5). The whole-cell-clamp condition was always established in the external solution at pH 7. After altering the external pH, recordings were made again at pH 7 to confirm a reasonable recovery. Recording was started at least 10 min after the whole-cell-clamp condition was established. The external solution had the following composition: 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM Hepes, 10 mM Mes, 10 mM 2-(N-Cyclohexylamino)ethanesulfonic acid (Ches), and the pH was adjusted to 9, 8, 7, 6, or 5 by adding either NaOH or HCl. For pHs below 5, Mes and Ches were replaced by 20 mM sodium hydrogen phthalate, and the required pH was obtained by adding NaOH. The composition of the internal solution was 155 mM NaOH, 40 mM HCl, 95 mM aspartic acid, 2 mM ATP-Mg, 0.1 mM cyclic AMP, 2 mM theophylline, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes, with the pH adjusted to 7.3 by adding HCl. All experiments were performed at room temperature (24–26°C).

RESULTS

pH Alters the Surface Potential. Membrane currents, produced by positive voltage pulses of varying amplitude by using an external solution at pH 7, are shown by the traces in Fig. 1*B*. Recordings in Fig. 1 *A*, *C*, and *D* were obtained from the same cell when the pH was altered to 6, 8, and 9, respectively. These effects were reversible. Current–voltage relations at the peak of the membrane current are shown in Fig. 2. The results show: (*i*) Both the beginning and the peak of the inward current were displaced toward less negative membrane potentials as the pH was reduced. This indicates that the voltage dependence of the gating-kinetic shifts along the voltage axis as the external pH is altered. (*ii*) The reversal potential and the current–voltage relations at the peak outward current were unaffected by pH in this range.

The Ca channel in the present preparation shows voltage-dependent inactivation (2). It has been shown that the relationship between steady-state inactivation, h_∞, and the holding membrane potential, V, is expressed by $h_\infty = [1 + \exp(V - V_h)/v)]^{-1}$, where V_h is the membrane potential at which h_∞ becomes 1/2 and v is about 4 mV. The voltage dependence of h_∞ was obtained in the same cell at pH 6, 7, 8, and 9 and is shown in Fig. 3*A*. The curves were calculated from the above equation with v equal to 3.8 mV. As the pH is low-

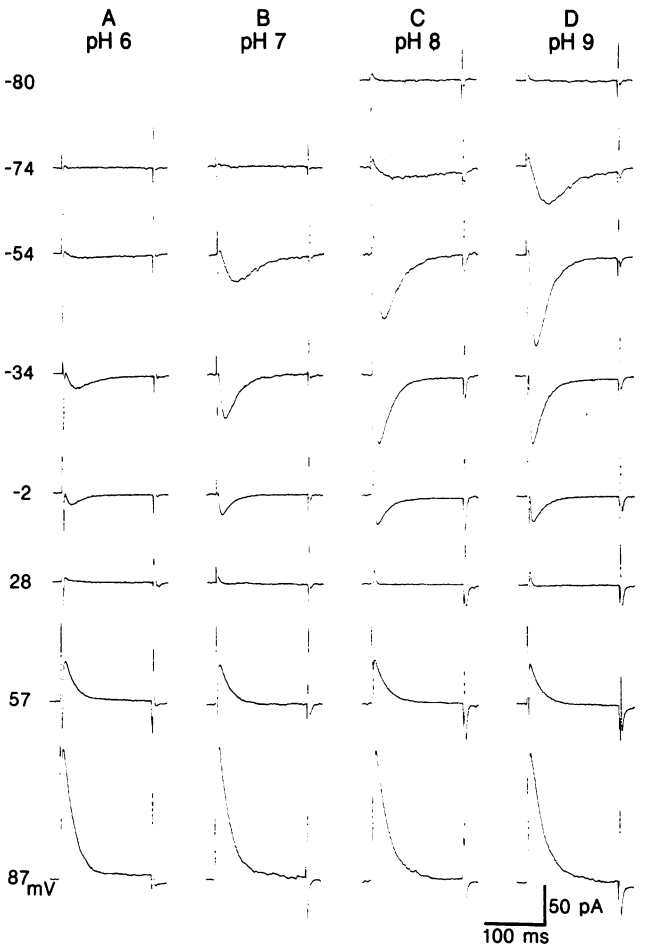

FIG. 1. Effects of the external pH on membrane currents. The records were obtained from the same cell at pH 6, 7, 8, and 9. Holding potential was −103 mV. The numbers on the left of the traces denote membrane potentials during the voltage pulse. The cell diameter was 14 μm. Traces at pH 8 and 9 are slightly distorted due to imperfect capacity compensation.

ered, the curve shifts along the voltage axis in the positive direction.

The current–voltage relation at the peak of the inward current also shifts along the voltage axis as the pH is altered (Fig. 2). The potential $V_{1/2}$ is defined as the membrane potential at which the peak inward current becomes 1/2 of its maximum value. In order to compare the shift of the activation to that of inactivation, V_h was plotted against $V_{1/2}$ in Fig. 3*B*, showing a linear relation with a slope of 1. These results indicate that protonation reduces the amplitude of the negative surface potential and that this effect is sensed by the gating mechanism.

Reduction of the Inward Current. The amplitude of the inward current, carried by the external Ca^{2+} at a given membrane potential, decreased as the external pH was reduced. In the pH range studied, 6 to 9, this effect is likely due to reduction in the amplitude of the negative surface potential rather than to blockage of the Ca channel, since no reduction is seen for the outward current.*

*The outward current started to decrease only when the pH was below 5.5. When it was 4.5, the voltage-gated current became unrecognizable, and the leakage conductance increased significantly. This change, however, could be reversed by raising the pH to higher values, implying that some other type of conductance increase occurs when the pH is lowered below 4.5. It was thus impossible to obtain a titration curve of the Ca channel and to determine the pK; although it seems reasonable to conclude that it must be lower than 5.

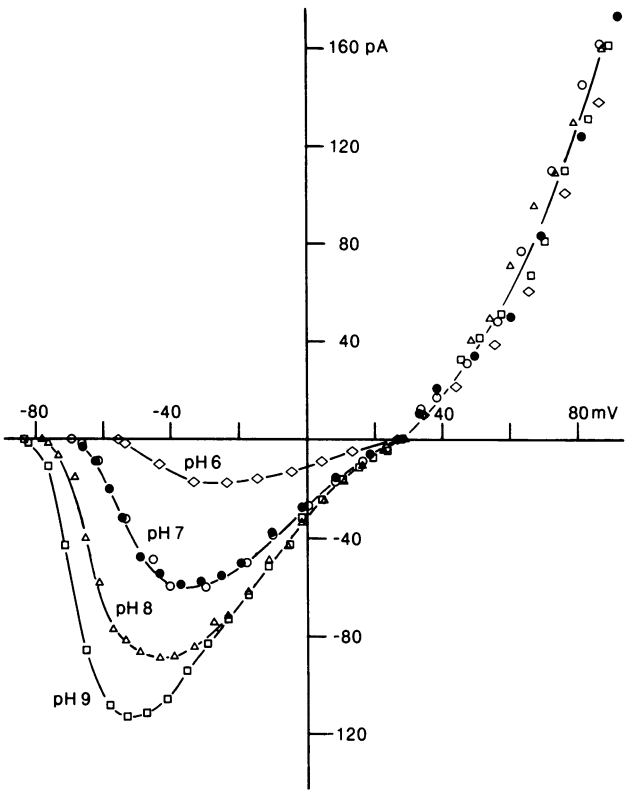

FIG. 2. Current–voltage relations at the peak of the membrane current. Data were obtained from the cell used to collect data for Fig. 1.

If one assumes that the channel does not sense the surface potential, this decrease of the inward current, as the pH is lowered, would simply be due to reduced activation. However, if the channel does sense the surface potential, a decrease of the Ca^{2+} concentration at the mouth of the channel, due to a shift of the surface potential in the positive direction, would also be expected to occur and contribute to the reduction of the current.

To distinguish between these two alternatives, it is pertinent to analyze the current at different pH values for V equal to $V_{1/2}$, or $V_{1/2} + \Delta V$, ΔV being a fixed potential increment. When the membrane potential is $V_{1/2}$ (or, in general, $V_{1/2} + \Delta V$), the number of open Ca channels is generally considered to be the same regardless of the surface potential, since the gating mechanism senses this potential. The current–voltage (*I–V*) relation at $V_{1/2}$ obtained from the same cell at different pH values would then be simply proportional to the *I–V* relation of the single open channel. Thus, if the surface potential is not sensed by the channel, different currents at $V_{1/2}$ would mainly reflect different potentials across the channel. However, if the channel does sense the surface potentials, the potential drop across the channel would always be the same when $V = V_{1/2}$; in which case, differences in the currents would be expected to reflect different ion concentrations at the entrance to the channel. If the Ca^{2+} concentration at the external mouth of the channel is related to that of the bulk solution, C_{Ca}, and to the surface potential, ϕ_s, by $C_{Ca}e^{-2\phi_s}$, and if the conductance is proportional to that concentration, a linear relation with a slope of $RT/2F$, or 12.5 mV, is expected between log I and ϕ_s where R is the molar gas constant, T is temperature, and F is the Faraday constant. Even though ϕ_s is not known, it can be realized that a similar relation is also expected between log I and $V_{1/2}$ (or $V_{1/2} + \Delta V$), since, when the channel senses the same potential as gating, differences in $V_{1/2}$ should correspond to equal differences in ϕ_s. In Fig. 3*C*, the logarithm of the membrane current at $V_{1/2}$, as well as at $V_{1/2} + 25$ mV, obtained from the

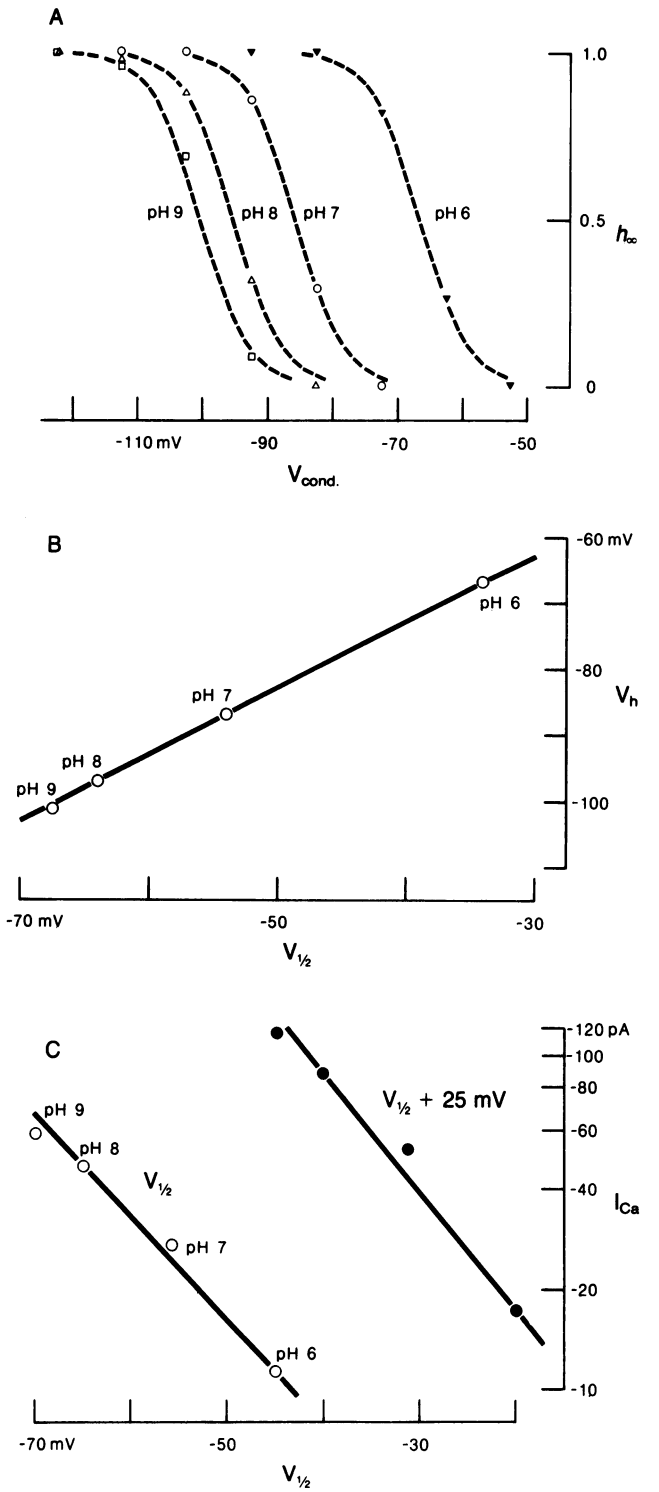

FIG. 3. (*A*) Steady-state inactivation curves obtained from the same cell at pH 6, 7, 8, and 9. Dashed lines were drawn according to $h_\infty = [1 + \exp(V - V_h)/3.8\ \text{mV}]^{-1}$. (*B*) V_h is plotted against $V_{1/2}$ for the corresponding pH values. The relation is fitted by $V_h = V_{1/2} - 33$ mV. (*C*) Relation between $V_{1/2}$ and ($V_{1/2}$ + 25 mV) and the peak amplitude of Ca current at this potential plotted on a logarithmic scale. An *e*-fold change of the current is found per 12.5 mV.

same cell at pH of 6, 7, 8, and 9, was plotted against the membrane potential, indicating that the behavior of the inward current is consistent with the assumption that the ion concentration near the channel varies with the surface potential.

In contrast, the reversal potential is unaltered by pH, and so is also the *I–V* relation at the peak of the outward current. One way to account for these two results is to postulate that only gating is sensitive to the pH, while the properties of permeation remain unchanged. In this case, the outward current would understandably become pH-independent when the potential is sufficiently positive that the probability of channel opening reaches saturation. However, we have shown that the reduction of the inward current at different pHs is remarkably consistent with the idea that the ion concentration at the channel mouth is sensitive to the surface potential.

Moreover, models (6, 7) have postulated that Ca channels have two internal binding sites for this ion and that the monovalent cations become permeant only when neither site is occupied by Ca^{2+}. Binding of Ca^{2+} to these sites is voltage-dependent, so that large positive membrane potentials would expel Ca^{2+} ions and create Ca^{2+}-free channels, permeable to univalent cations. These considerations suggest the possibility that shifts of the surface potential, induced by pH changes, might influence permeation as well as gating, although the net sum of their effects on the parameters of permeation, such as the field in the channel and the ion concentrations at the boundaries, would result in negligible changes for both the reversal potential and the outward current. A theoretical justification for this hypothesis is described below.

Theoretical Results for a Channel that Senses the Surface Potential. It will be postulated that altering the pH causes variations of the surface potential, ϕ_s, between the mouth of the channel and the external solution. The total measured potential, ϕ, will thus be written

$$\phi = \phi_c + \phi_s (\phi = FV/RT), \qquad [1]$$

where ϕ_c is the potential drop across the interior of the channel.

Probably, a similar surface potential also exists at the inner mouth of the channel. However, since it may be relatively insensitive to changes in the external pH, we shall, for simplicity, ignore its effects and assume it is zero.

Using the Boltzman distribution, the concentration of ion i at the external mouth of the channel, C_i, will be related to that in the bulk, C_i'', by

$$C_i = C_i'' e^{-z_i \phi_s}. \qquad [2]$$

The assumptions we use, which include the principal features of models proposed (6, 7), can be thus summarized: (*i*) The channel has two internal binding sites for Ca^{2+} that can also be occupied at the same time by two Ca^{2+} ions. (*ii*) The binding constant of either site, $\overline{K}_{Ca}$, is large, so that $K_{Ca}C_{Ca} >> 1$ even in the submillimolar range. (*iii*) The rate constant for crossing the internal barrier, $\overline{\lambda}_{Ca}$, is much greater than that for exit from the channel, $\overline{\mu}_{Ca}$. (*iv*) The pore becomes permeable to univalent cations, with low selectivity among the alkali cations, only when neither of the two sites is occupied by Ca^{2+}. (*v*) The univalent cations sense the channel as a single energy barrier with the peak at the same location as that of the central barrier for Ca^{2+}. (*vi*) The interaction between the two Ca^{2+} ions in the channel is described by a repulsion factor, f, which multiplies the rate constant for exit and divides that for entry when one of the two sites is already occupied. (*vii*) The binding constants of the two sites for Ca^{2+} are equal, and so are also the rate constants for exit from the pore. (*viii*) The peaks of the three barriers for Ca^{2+} are in the middle between adjacent sites (although the sites need not be spaced evenly).

The number of open channels per unit area will be denoted by $P(\phi_c)N$, where N is the total number of channels and $P(\phi_c)$ is the probability of opening, considered as a function of the voltage drop across the interior of the membrane. The

fluxes through the open pores are calculated using Eyring rate theory and considering four possible states for the channel: two with one Ca^{2+} in either site, one with one Ca^{2+} in each site, and one empty (the channel being then permeable to the univalent cations). If I^+ the current density carried by the univalent ions, the total current per unit area will be

$$I = I_{Ca} + I^+. \quad [3]$$

With the aid of the assumptions stated above, using the notation specified in Fig. 4 for the rate and equilibrium constants for Ca^{2+}, and denoting by $\bar{\rho}^+$ the rate constant for the monovalent cations, one finds

$$\Delta I_{Ca} = -2FP(\phi_c)N\bar{\lambda}_{Ca}\bar{K}_{Ca}^2C_{Ca}^2e^{-\gamma\phi}e^{(\gamma-3)\phi_s}. \quad [4]$$

$$\Delta I^+ = FP(\phi_c)N\frac{\bar{\mu}_{Ca}}{\bar{\lambda}_{Ca}}\bar{\rho}^+e^{\beta(\phi-\phi_s)/2}(C^{+\prime}e^{\phi} - C^{+\prime\prime}) \times \left\{f\frac{\bar{\lambda}_{Ca}}{\bar{\mu}_{Ca}}[1 + e^{(\alpha+\gamma)(\phi-\phi_s)}][e^{(\beta+\gamma)(\phi-\phi_s)} + e^{-(\alpha+\beta)(\phi-\phi_s)}] + \bar{K}_{Ca}C_{Ca}e^{-2\phi_s}\right\}, \quad [5]$$

where

$$\Delta = 2f\bar{K}_{Ca}C_{Ca}e^{\beta\phi}e^{-(1+\beta)\phi_s} \times \left\{[1 + e^{(\alpha+\gamma)(\phi-\phi_s)}]\cosh[\beta(\phi - \phi_s)] + \left[\frac{\bar{\mu}_{Ca}}{2f\bar{\lambda}_{Ca}} + \frac{1}{2f^2}e^{(\alpha-\beta)(\phi-\phi_s)}\right]\bar{K}_{Ca}C_{Ca}e^{(\gamma-2)\phi_s}\right\}, \quad [6]$$

where α, β, and γ are the fractional potential drops between adjacent sites. It can be seen from these equations that, in general, both the current and the reversal potential will depend on the surface potential, ϕ_s, and hence on the pH. To obtain a better idea of this dependence, it is useful to analyze the limiting behavior of the outward current at high positive potentials. In this region, the inward Ca^{2+} current will be negligible, and the probability of channel opening will have reached its maximum value, P_{max}. By only keeping the steepest positive exponentials in Eqs. 5 and 6, one finds

$$\underset{\phi\to+\infty}{I} = FP_{max}N\frac{\bar{\rho}^+C^{+\prime}}{\bar{K}_{Ca}C_{Ca}}e^{(1+\gamma-\beta/2)\phi}e^{(1-\gamma+\beta/2)\phi_s}. \quad [7]$$

This simple expression shows that the limiting outward current will become independent of ϕ_s if $(1 + \beta/2 - \gamma)$ is zero. Since $\alpha + \beta + \gamma = 1$, this is possible only if $\alpha = \beta = 0$ and $\gamma = 1$—namely, if the potential drop across the external barrier (the closest to the Ca-containing solution) is equal to that across the whole channel†. In this case, the effects of ϕ_s on the parameters of permeation cancel each other. For example, a decrease of the driving force for the inward fluxes, such as might be produced by making ϕ_s more negative, will be accompanied by an increase of the Ca^{2+} concentration near the external mouth of the channel, leaving the probability of channel occupancy unchanged.

It is now interesting also to examine the zero-current potential, when $\gamma = 1$. Considering that ϕ_o is positive and that ϕ_s is negative, and recalling that $\alpha = \beta = 0$, we shall use the following approximation in Eq. 5

$$[1 + e^{(\phi_o-\phi_s)}]^2 \simeq e^{2(\phi_o-\phi_s)}, \quad [8]$$

which holds approximately when $e^{(\phi_o-\phi_s)} \geq 10$.

In this case, the condition that $I_{Ca} + I^+ = 0$ yields

$$\left\{-2\bar{\lambda}_{Ca}\bar{K}_{Ca}^2C_{Ca}^2e^{-\phi_o} + \frac{\bar{\mu}_{Ca}}{\bar{\lambda}_{Ca}}\bar{\rho}^+(C^{+\prime}e^{\phi_o} - C^{+\prime\prime}) \times \left[\frac{f\bar{\lambda}_{Ca}}{\bar{\mu}_{Ca}}e^{2\phi_o} + \bar{K}_{Ca}C_{Ca}\right]\right\}e^{-2\phi_s} = 0, \quad [9]$$

showing that, when $\gamma = 1$, also the reversal potential, ϕ_o, is expected to be independent of ϕ_s. In contrast, it is easy to realize that the inward current is dependent on ϕ_s, and that the form of this dependence is consistent with the results of Fig. 3*C*. Neglecting the term in C_{Ca}^2 in Eq. 6—which is reasonable, considering that at 2.5 mM Ca^{2+} the inward current is still linearly dependent on external Ca^{2+}, and that linearity is predicted by Eqs. 4 and 6 only if that term is neglected—and substituting ϕ with $\phi_c + \phi_s$, Eqs. 4 and 6 give

$$I_{Ca} = -C_{Ca}e^{-2\phi_s}\left[\frac{F_P(\phi_c)N\bar{\lambda}_{Ca}\bar{K}_{Ca}e^{-\phi_c}}{f(1 + e^{\phi_c})}\right]. \quad [10]$$

This equation shows that, if the potential across the channel interior, ϕ_c, is constant, the logarithm of the current is proportional to V_s with a slope of $2F/RT$. In agreement with the data of Fig. 3*C*, the same proportionality is also expected with respect to $V_{1/2}$ or $V_{1/2} + \Delta V$, since fixed displacements from $V_{1/2}$ correspond to fixed values for ϕ_c.

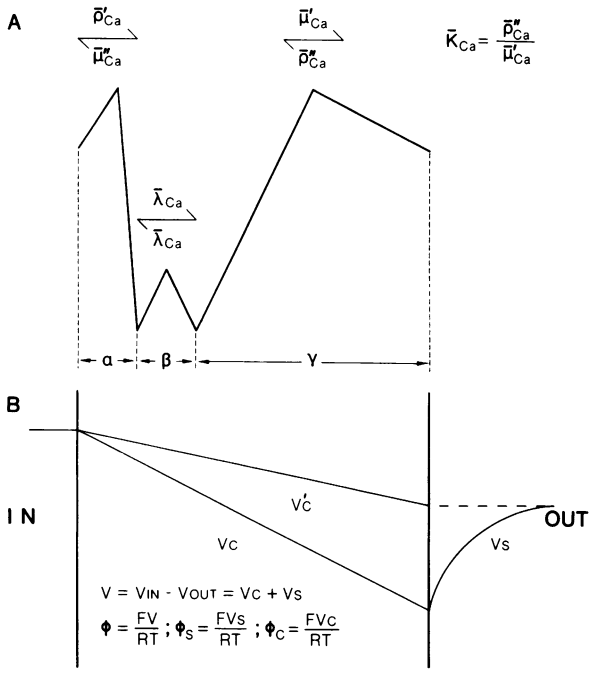

FIG. 4. (*A*) Three-barrier model for Ca^{2+} ions in Ca channels. α, β, and γ are fractions of the potential drop across the channel. The bars on the rate and equilibrium constants denote their values at zero-field in the channel ($V_c = 0$). (*B*) Schematic potential profiles. V_c is the potential drop across a channel that senses the surface potential. V_c' is the potential drop for a channel that does not sense it.

†This restrictive condition for γ is related to the arbitrary assumption that the position of the barrier peak for the monovalent cations, ε, coincides with that of the central barrier for Ca^{2+} ($\varepsilon = \alpha + \beta/2$). If this assumption is released, and ε is allowed to take any value between 0 and 1, the condition under which the outward current would be expected to be independent of the surface potential, ϕ_s, becomes $\gamma = 1 - \varepsilon/2$. Note that though this condition is less restrictive than $\gamma = 1$, it nevertheless still requires that $\gamma \geq 1/2$, implying that the "electrical distance" between the two sites for Ca^{2+} cannot be larger than 0.5.

DISCUSSION

The data shown in this paper describe some effects of the external pH on several properties of gating and permeation in Ca channels. Although it may seem that most results could be explained in terms of shifts of the potential profiles sensed only by the mechanism of gating, the analysis of the inward currents suggested that different currents at $V_{1/2}$, or $V_{1/2} + \Delta V$, for different pH values, reflected changes of the Ca^{2+} concentration at the external mouth of the channel. It then became interesting to consider the possibility that potential shifts are sensed also by the channel, and to investigate whether this hypothesis could be reconciled with the observation that both the outward current and the reversal potential are insensitive to changes in the external pH. The analysis in the previous section shows that, within the frame of the assumptions of our model, the outward current becomes indeed independent of the surface potential, ϕ_s, if $\gamma = 1$; namely, if the potential drop across the external barrier is almost equal to that across the whole channel. In this case, also the reversal potential, ϕ_o, is independent of ϕ_s, provided, however, that also the condition expressed by approximation **8** is satisfied. Since the reversal potential shown in Fig. 2 is about 28 mV for 2.5 mM Ca^{2+}, it is easy to realize that this condition will be satisfied for $C_{Ca} \geq 2.5$ mM if $V_s \leq RT/F(-\ln 10 + 1.1) = -30$ mV, which is a reasonable range for surface potentials in negatively charged membranes. Thus, if $\gamma = 1$ and $V_s \leq -30$ mV, the reversal potential becomes independent of V_s and can be calculated by solving Eq. **9**, which is of the fourth order in $e^{-\phi_o}$. An interesting solution is found if the external concentration of permeant monovalent cations is sufficiently low that the following approximation can be made: $(C^{+'}e^{\phi_o} - C^{+''}) \simeq C^{+'}e^{\phi_o}$. In this case, one finds

$$\phi_o = \frac{1}{2}\ln C_{Ca} + \frac{1}{2}\ln\left\{\frac{\bar{K}_{Ca}}{2}\left[-\frac{\bar{\mu}_{Ca}}{f\bar{\lambda}_{Ca}} + \left(\frac{\bar{\mu}_{Ca}^2}{f\bar{\lambda}_{Ca}^2} + \frac{8\bar{\lambda}_{Ca}}{f\bar{p}^+C^{+'}}\right)^{1/2}\right]\right\}. \quad [11]$$

Thus, the reversal potential is insensitive to ϕ_s, and hence to the pH, and also depends on external Ca^{2+} in a nernstian way. The first of these predictions is verified by the data in this paper, while the second is in good agreement with previous results (2). Eq. **11** also predicts a slow dependence of the potential on the internal concentration of the monovalent cations, and no dependence on that of the external ones; the latter prediction also being in agreement with previous studies (2).

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