Interactions of Protons with Single Open L-Type Calcium Channels

pH Dependence of Proton-induced Current Fluctuations with Cs⁺, K⁺, and Na⁺ as Permeant Ions

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ABSTRACT We studied the pH dependence of the proton-induced current fluctuations that appear in single open L-type Ca channels when monovalent ions are the charge carriers. We used different methods of analysis to obtain kinetic measurements even under conditions where the individual transitions were too fast to be resolved directly as discrete current steps between two conductance levels. The reciprocal of the dwell times at the high conductance level increased linearly with the pipette proton activity, with a slope that was similar for Cs, K, and Na as permeant ions. Contrary to the expectation for a simple model in which the high and low conductances represent the unprotonated and protonated states of the channel, respectively, the dwell times at the low conductance level were also pH dependent and lengthened with increasing proton activity. At all pH values the dwell times at the low conductance level were longest with Cs as permeant ion and shortened in the order Cs > K > Na. We introduce a more general model of the protonation cycle in which the channel is represented by four states and can be protonated and deprotonated both at the high and low conductance levels. The conductance change is represented by a conformational change of the channel protein. We discuss the validity of this model and its implications for the mechanism by which protons interact with ion permeation through L-type Ca channels.

INTRODUCTION

We have recently shown (Prod’hom et al., 1987; Pietrobon et al., 1988) that under certain experimental conditions the interactions of individual protons with a site on
the dihydropyridine (DHP)-sensitive (L-type) Ca channel lead to fluctuations of the open channel current between two non-zero conductance levels. With increasing external proton activity the frequency of transitions from the high conductance to the low conductance state increases. We have previously interpreted the high conductance as the unprotonated state and the low conductance as the protonated state of the channel (Prod'hom et al., 1987). This simple two-state model predicts that the mean lifetime of the low conductance state should be independent of pH. In this paper we present a quantitative analysis of the pH dependence of the transitions between the two conductance levels. We use several analytical methods to extend the analysis to different permeant ions and to conditions under which the individual transitions are too rapid to be fully resolved at the bandwidth of our recording system. The results show that contrary to the expectations of a model with only two states, the lifetimes of both the high and low conductance states are pH dependent. This observation, taken together with our more recent evidence (Pietrobon et al., 1988) implying that the conductance change represents a conformational change of the channel, leads us to consider a more general model with four states, in which the channel can be protonated both in the high conductance and low conductance state. This model reproduces well the observed pH dependence of the dwell times at each conductance. In a companion paper (Prod'hom et al., 1989) we present additional evidence for the localization of the protonation site, its interaction with the permeant ion, and an attempt at a physical interpretation of the model presented in this paper.

METH ODS

Single-Cell Preparations

Single ventricular myocytes were obtained by enzymatic dissociation of adult guinea pig hearts as previously described (see Lee and Tsien, 1984 and references therein).

Pheochromocytoma cells (PC-12) were grown in the absence of nerve growth factor in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum, 7.5% horse serum, and 1% penicillin-streptomycin. The cells were grown on glass coverslips that were cut into small pieces for the transfer into the experimental chamber.

Patch-Clamp Recordings

Unitary Ca channel recordings were obtained as described by Hamill et al. (1981). Patch pipettes were pulled from Boralex hematocrit micropipettes (P5251; USA Scientific Plastics, Ocala, FL), coated with Sylgard (Dow Corning Corp., Midland, MI), and firepolished. Current signals were recorded with a LIST EPC-7 patch clamp amplifier (LIST-Electronic, Darmstadt, FRG). The currents were filtered with an eight-pole Bessel filter and sampled at a rate five times the corner frequency (−3 dB) of the filter setting. The signals were stored and analyzed on a PDP 11-73 laboratory computer.

To null the membrane potential outside the patch (Hess et al., 1986), the cells were superfused with a bathing solution of the following composition (in millimolar): 140 K-aspartate, 10 EGTA, 10 HEPES, titrated to pH 7.4 with KOH. To promote the formation of high resistance electrical seals, 20 mM MgCl₂ was added to the bath solution. This was necessary because the pipette solutions (see below) were free of divalent ions. However, even in the presence of Mg in the bath, the seal resistances were significantly lower than in the presence
of millimolar Ca or Ba on either side of the pipette. The values ranged from 2 to 20 GΩ, and they decreased with the following order of cations in the pipette: Li > Na > K > Cs. The DHP Ca channel agonist (+)-S-202-791 (a gift from Dr. Hof, Sandoz Co., Basle, Switzerland) was present at 0.5–1 μM in the bathing solution to promote long-lasting channel openings of the L-type Ca channel (Hess et al., 1984; Kokubun and Reuter, 1984; Kokubun et al., 1986).

The pipette solution contained 150 mM of the Cl-salt of Na, K, or Cs, 5 mM EDTA and 5 mM of the pH buffer MES (pK 6.15), HEPES (pK 7.55), or TAPS (pK 8.4). The solutions were titrated to the desired pH with Na-, K-, or CsOH. Thus the final pipette concentration of cation was ~160 mM. All experiments were performed at room temperature (21–23°C).

Identification of L-Type Ca Channels

In the absence of divalent ions, monovalent cations can carry single-channel current through L-type (Hess et al., 1986; Levi and DeFelice, 1986; Matsuda, 1986) and T-type (Carbone and Lux, 1987; Chen and Hess, 1987) Ca channels, as well as other cation channels. We distinguished L-type Ca channels from the other cation-permeable channels by three criteria: (a) the voltage dependence and kinetics of activation, inactivation, and deactivation are similar for Na and Ba (Hess et al., 1986), except for a negative shift along the voltage axis expected from the more negative surface potential in the absence of divalent ions. (b) The long channel openings (mean open time, 10–20 ms), promoted by dihydropyridine agonists, identify L-type Ca channels. (c) Micromolar concentrations of Ca in the pipette lead to resolvable discrete blocking events of L-type Ca channels (Lansman et al., 1986; Pietrobon et al., 1988). Once we had recognized the pattern of proton-induced current fluctuations as a characteristic property of L-type Ca channels, the channel identification became very easy even with K as the charge carrier.

All records shown are segments of currents recorded in response to an appropriate voltage-clamp protocol designed to activate L-type Ca channels. Openings at negative potentials were obtained by a 10–30 ms long activating prepulse to a positive potential followed by repolarization to the desired test potential. The presence of the DHP agonist (+)-S-202-791 greatly slowed channel deactivation at negative potentials as expected from single-channel recordings with Ba as the charge carrier (Hess et al., 1984; Kokubun and Reuter, 1984; Ochi et al., 1984; Kokubun et al., 1986). This made it possible to record unitary “tail currents” at potentials as negative as −150 mV.

The unitary conductances and kinetics of the proton-channel interactions in L-type channels from cardiac and PC-12 cells are indistinguishable. We have therefore pooled the results from the two preparations.

Use of DHP Agonists to Study Permeation Properties of L-Type Ca Channels

We will assume that the presence of the DHP agonist does not alter the permeation properties of the L-type Ca channel. While we have no absolute proof for this assumption, it seems reasonable, because all previous studies of permeation mechanism obtained in the presence of DHP agonists (Hess et al., 1986; Lansman et al., 1986) have yielded results on conductance, reversal potentials, and sensitivities to block by multivalent ions that are consistent with single-channel and whole-cell experiments obtained in the absence of DHP agonists. The proton-induced fluctuations, which we could only analyze quantitatively in the presence of the DHP agonist, can nevertheless also be observed in the absence of the drug, as evidenced by a pH-dependent decrease of the elementary current with Na as charge carrier (data not shown).
We preferred (+)-(S)-202-791 to Bay K 8644 because it is an optically pure compound (Hof et al., 1985). The duration of the (+)-(S)-202-791–promoted openings was not markedly different from those described with Bay K 8644 (unpublished results from our laboratory and from S. Kokubun, B. Prod'hom, and H. Reuter). Consistent with the conclusions of Krafte and Kass (1988) of a pH-induced shift of channel gating, we found that the mean open times increased with increasing pH. With Li as charge carrier, the mean open times of the long openings at -40 mV were 9 ms at pH 6 and 15 ms at pH 9. pH-induced effects on DHP binding should not have affected our measurements, because only the pipette pH was different and the agonist was added to the bath solution. Furthermore, our analysis was only carried out on DHP-promoted long openings, which presumably reflect predominantly the DHP-bound state of the channel.

Data Analysis

The kinetics of transitions between the two conductance levels within a channel opening were idealized by fitting manually controlled cursors to the two levels and setting a discriminator at 50% of the current between the levels. Sampling points above and below the discriminator were considered sojourns at the high and low conductance level, respectively (Colquhoun and Sigworth, 1983). Histograms of the durations of sojourns at either level were fitted with single decaying exponential curves by a nonlinear chi-square minimization routine (Bevington, 1969).

Amplitude histograms were obtained from the data directly, with the bin size equal to the maximal resolution (204.8 points/pA at the highest gain). For display, bins were combined as needed to give the clearest presentation.

Power spectra were obtained as Fourier transforms from segments of open or closed channels of 1,024 consecutive sampling points filtered at 5 kHz and sampled at 25 kHz. Power spectra from several segments were averaged. The difference spectra (open minus closed channel) were fitted by eye to a single Lorentzian component. A fit by eye was preferred to a chi-square minimization because such a procedure would have weighted the high frequency part of the spectrum preferentially (many more data points).

Simulations of Stochastic Fluctuations and Accuracy of Kinetic Measurements

The validity of our interpretation of some of the data in this paper depends on our capacity to accurately measure the dwell times of the channel at the two conductance levels. Rather than adopting published methods to correct for the effects of missed events (e.g., Colquhoun and Sigworth, 1983; Neher, 1983; Roux and Sauve, 1985; McManus et al., 1987), we choose to assess the effects of noise and filtering directly in our system by simulating ideal stochastic events with known rate constants. Exponential distributions with known time constants were created and a pseudo-random number generator was used to choose durations of individual events. The resulting signals were then fed to the D/A converter, filtered by the same filter used for the experiments, sampled, and added to traces of experimental noise recorded in the absence of channel openings. The simulated currents were then passed through our analysis programs and the extracted rate constants were compared with the ones used to generate the ideal traces.

The maximal rate of our D/A converter is 125 kHz. To simulate rate constants > ~10 kHz we simulated slower transitions and reduced the filter cut-off frequency and A/D sampling rate accordingly. For example, to reproduce transitions with an on-rate of 40 kHz and an off-rate of 20 kHz, filtered at 5 kHz and sampled at 25 kHz, we simulated events with rate constants of 4 and 2 kHz, respectively, filtered them at 500 Hz, and sampled at 2.5 kHz. This procedure should faithfully reproduce the desired currents, provided the filter response $f_r/f_c$, (where $f_r$ is the signal frequency and $f_c$ is the corner frequency of the filter) is frequency...
independent. This is true for our 8-pole Bessel filter in the range of frequencies used (500–5,000 Hz).

Fig. 1 assesses the accuracy of our kinetic measurements. Here, $k_{on}$ and $k_{off}$ represent the forward and backward rate constants of a two-state process. Simulations were performed for increasing values of $k_{on}$ at a fixed value of $k_{off}$. The value of $k_{off}$ was set at 7,900/s to represent the worst case, slightly higher than the highest experimental values found (see Fig. 3). The value of $k_{on}$ was varied from 900 to 9,000/s. We have plotted the values of the rate constants used to generate the simulated single-channel currents (solid lines) and the rate constants measured from the kinetic analysis of the simulated traces (shown as symbols) as a function of the simulated value of $k_{on}$. As expected, with increasing values of $k_{on}$, the extracted rate constants progressively underestimate the true values used for the simulation. However, it is clear that for values of $k_{on} < 4,000/s$ the error in $k_{off}$ is small, less than 10%. The largest errors, both of $k_{on}$ and $k_{off}$, occur at the highest values of $k_{on}$. At a simulated value of $k_{on} = 8,900/s$, the errors are ~25%.

In our initial report (Prod'hom et al., 1987), we have used the method of Neher (1983) to correct measured time constants for missed events. Our simulations have however shown that in our case this method actually overcompensates the values by more than the errors encountered (~20% over the whole range of rate constants), such that in Pietrobon et al. (1988) and in the present paper and its companion paper (Prod'hom et al., 1989) we have chosen to show the uncorrected data instead. We will use the simulation presented in Fig. 1 to assess the validity of the conclusions draw from our kinetic measurements (see Results).

**RESULTS**

**Kinetics of Proton-induced Current Fluctuations with K as the Charge Carrier**

Fig. 2 shows segments of current traces obtained from different cell-attached patches with 160 mM K$^+$ in the pipette and a pipette pH that varies from 9.2 (top trace) to 7.0. As we have previously shown (Pietrobon et al., 1988), the currents fluctuate between three levels: the zero-current level of the closed channel and two conductance levels when the channel is open. The fraction of time the open channel spends at the low conductance level increases with the proton concentration in the
pipette. This is further emphasized by the amplitude histograms associated with each recording. For clarity, the peak of the closed channel (at 0 pA) is not included in the histograms. As the pipette pH decreases, the peak resulting from current samples at the fully open level gradually decreases, while the peak associated with the reduced conductance increases. Inspection of the traces shows that the bias towards the low conductance state at low pH is mainly due to an increased frequency of transitions from the high to the low current level. Qualitatively, this is the expected result if each such transition results from the interaction of a single proton with a site on the channel. The simplest model, in which the high (H) and low (L) conductance states represent the unprotonated and protonated state of the channel, has the following form:

\[
\begin{align*}
C & \xleftarrow{k_c} H \\
C^* & \xleftarrow{k_s} L \\
C & \xleftarrow{k_{off}} a_H \cdot k_{on}'
\end{align*}
\]

(Scheme 1)
where C and C* denote closed channel conformations. During a channel opening we only have two states (H and L). If \( \tau_h \) and \( \tau_l \) are the mean times spent at the high and low conductance levels, respectively, then from the relationship:

\[
\frac{1}{\tau_h} = k_{on} + k_c, \quad \text{where} \quad k_{on} = a_{H}k'_{on},
\]

\[
\frac{1}{\tau_l} = k_{off} + k_c^*
\]

several quantitative features are predicted (see e.g., Neher and Steinbach, 1978; Yellen, 1984; Lansman et al., 1986): (a) A plot of \( 1/\tau_h \) vs. \( a_H \) is linear with a slope corresponding to the (second order) association rate constant \( k_{on} \) for protons. (b) This plot should intersect the y-axis at \( 1/\tau_h = 1/k_c \). In our case \( k_c \) is in the range 60–120/s, since under our experimental conditions the mean open time of the L-type Ca channel in the presence of (+)-(S)-202-791 varies between 9 and 15 ms in the pH range tested (see Methods sections). (c) \( 1/\tau_l \) is independent of pH.

For practical purposes, we ignore the closing rate constants, since channel closures occur very infrequently at our timescale of interest. In this case the values of \( k_{on} \) and \( k_{off} \) are obtained directly as the reciprocals of the mean dwell times at the two conductance levels.

The above predictions are tested in Fig. 3. The distributions of lifetimes at either conductance level were always well fitted by single decaying exponential components. Histograms of the durations of the high and low conductance levels are shown in Fig. 3A for two values of pipette pH. The dependence of \( k_{on} \) and \( k_{off} \) on the proton activity (\( a_H = 10^{-pH} \)) in the pipette is illustrated in Fig. 3B. As expected, the relation between \( k_{on} \) and \( a_H \) is linear, with a slope of \( 4.1 \times 10^{11}/(M \cdot s) \). However, the intersection with the y-axis, obtained by linear regression, indicates a value of 715/s, much higher than the predicted maximal value of \( \approx 120/s \). Moreover, and more significantly, contrary to the expectations from the simple model, \( k_{off} \) is not independent of \( a_H \) but decreases by more than 50% between pH 9 and 8. After consideration of the accuracy of our measurements (see Methods), we are convinced...
that the observed decrease of $k_{\text{off}}$ between pH 9 and 8 is real and cannot be explained by an increasing number of missed events at the high conductance level, because it occurs over a range of values of $k_{\text{on}}$ and $k_{\text{off}}$ where the errors due to frequency limitations are < 10% (see Fig. 1). On the other hand, at values of $k_{\text{on}} > 4,000/s$ (pH < 8), the expected underestimation of $k_{\text{off}}$ resulting from missed events is large enough to account for the slight further decrease of the measured values of $k_{\text{off}}$ over that pH range.

The values of $k_{\text{on}}$ are probably accurate to 10% or less over the whole range of pH values, since the decrease of $k_{\text{off}}$ with increasing proton activity must keep the error on $k_{\text{on}}$ considerably smaller than in our simulation, in which $k_{\text{off}}$ was kept constant at a high value over the whole range of simulated $k_{\text{on}}$ values.

The observed pH dependence of $k_{\text{off}}$ is incompatible with the simple model proposed above and at the end of this paper we propose a more general model which can account for the data shown in Fig. 3. The rejection of the above model means that $k_{\text{on}}$ and $k_{\text{off}}$ no longer mean rate constants for protonation and deprotonation.

However, for the sake of simplicity we will continue to use the terms $k_{\text{on}}$ and $k_{\text{off}}$ to refer to the reciprocals of the measured mean durations of the high and low conductance levels, respectively.

**Kinetics of Proton-induced Current Fluctuations with Cs as the Charge Carrier**

When Cs ions carry the elementary current through L-type Ca channels, the kinetics of the proton-related transitions between the two conductance levels are slowed down, primarily because of lower values of $k_{\text{off}}$ (Pietrobon et al., 1988). Fig. 4 shows that the pH dependence of $k_{\text{on}}$ and $k_{\text{off}}$ is qualitatively similar to the case when K is the charge carrier. $k_{\text{on}}$ is linearly related to $a_H$, with a slope of $4.1 \times 10^{11} / (M \cdot s)$, and intersects the y-axis at a value of 1,062/s. The value of the slope is very similar to that obtained with K (Fig. 3). Although the values of $k_{\text{off}}$ at any pH are lower than those with K, a similar pH dependence is apparent, with a 45% decrease between pH 9 and 8.2.
Kinetics of Proton-induced Current Fluctuations with Na as the Charge Carrier

With Na as the charge carrier, the transitions between the two conducting states are so rapid that we can no longer resolve them kinetically (Prod'horn et al., 1987). Fig. 5A shows that instead of unitary transitions between two conductance levels, large open channel noise is apparent, and the amplitude of the elementary current decreases as the pH is changed from 9 to 7. The pH dependence of this effect is emphasized in Fig. 5B. Here we plot the elementary current after heavy filtering (50 Hz) as a function of pH. If the open channel noise represents very rapid current fluctuations between two conductance levels then filtering is expected to produce a "mean" current corresponding to the average proportion of time the channel spends at each conductance level. If we assume that the currents at pH 9 and 6 represent the high and low conductance levels, respectively, we can fit the curve well by a 1:1 titration curve with a Hill coefficient of 1 and a midpoint at pH 7.5.

To estimate the kinetics of the proton-induced transitions of currents carried by Na and their dependence on pH we used three different methods: (a) simulations of filtered rapid fluctuations, (b) an analytical method to fit amplitude histograms of filtered two-state processes (beta distributions, see Yellen, 1984), and (c) spectral analysis of the open channel noise with correction for the limited frequency response (Ogden and Colquhoun, 1985).

Simulations of Filtered Rapid Fluctuations

Fig. 6A shows the consecutive steps involved in the simulation of filtered first-order transitions between two states. Trace a is a simulated record of stochastic transitions between two current levels with forward and backward rate constants of 43,000/s and 14,700/s, respectively. The current amplitude of the fluctuations was chosen by assuming that the high and low conductance levels correspond to the currents at pH 9 and 6, respectively. Trace b shows the same record filtered at 5 kHz by the 8-pole Bessel filter. Trace c shows the result of the addition of background noise obtained from experimental records filtered at 5 kHz in the absence of channel openings.
Finally, the result of the simulation is compared with an actual recording (trace d). The very close similarity between experiment and simulation is shown in Fig. 6 B where amplitude histograms of experimental (scattered points) and simulated traces (smooth line) are superimposed. In practice we changed the rate constants for the simulation in increments of ±5% until we judged the fit of the experimental amplitude histogram by the simulated data satisfactory by eye. This method of simulation is quite straightforward, particularly because no assumptions about the filter characteristics are necessary. It is however extremely time consuming and we therefore could only apply it to a limited number of experiments (Prod'hom et al., 1987).

**Beta Distributions**

A much faster, analytical method is the one introduced by FitzHugh (1983) and Yellen (1984). It calculates the steady-state distribution of amplitudes \( f(i) \), equivalent to an amplitude histogram, which one obtains at the output of a first-order filter of time constant \( \tau \) when currents of amplitude \( i \) fluctuating between two states connected by first-order rate constants \( \alpha \) and \( \beta \) are applied to the filter input:

\[
f(i) = \left[ i^{a-1} \cdot (1 - i)^{b-1} \right] / B(a, b)
\]

where \( 0 < i < 1 \), \( a = \alpha \tau \), \( b = \beta \tau \), and \( B(a, b) = \int_0^1 i^{a-1} \cdot (1 - i)^{b-1} \cdot di \). \( f(i) \) is called a beta distribution, \( B(a, b) \) is the beta function that normalizes it. The main disadvantage of the beta distribution is that it only produces the correct output for a first-order filter. However, as pointed out by Yellen (1983), for heavy filtering, first-order and multi-stage filters produce similar outputs, and the beta distribution reproduces the output of an 8-pole Bessel filter well for values of \( a \) and \( b > 2 \). The -3-dB cutoff frequency of an 8-pole Bessel filter \( f_c \) is related empirically to the time constant \( \tau \) of an equivalent first-order filter by (Colquhoun and Sigworth, 1983):

\[
\tau = 0.228 / f_c.
\]
To include background noise, the noise of the closed channel is fitted to a Gaussian curve to determine its width. This Gaussian is then convolved with the beta distribution.

Fig. 7 shows that when the data of Fig. 6 is refiltered to give a $f_c = 1$ kHz, the resulting amplitude histogram is then more narrow and less skewed and it can be fitted by a beta distribution with the same rate constants that gave a good fit of the less heavily filtered data with the simulation method (Fig. 6).

Spectral Analysis

The third method of analysis involves spectral analysis of the open channel noise. Fig. 8A shows superimposed power spectra of current segments from open and
closed channels. The difference (net) spectrum is shown in Fig. 8B. It has the form of a single Lorentzian curve with an apparent half-power frequency \( f_a \) of 2,056 Hz. We assume that this Lorentzian arises from the filtered fluctuations between the two current levels of the open channel. We can calculate the expected variance of the fluctuations \( \text{Var}(i) \), which is independent of the rate of the transitions and is given by (see e.g., Ogden and Colquhoun, 1983):

\[
\text{Var}(i) = m_i(i - m_i)
\]

where \( m_i \) is the mean current obtained by heavy filtering as in Fig. 6 and \( i \) is the amplitude of the fluctuations, taken as the difference between the currents at pH 9 and 6. In the example illustrated in Fig. 8 the expected variance is 2.43 pA\(^2\). The observed variance is the area under the net spectrum, which is 0.85 pA\(^2\). We only observe a fraction of the expected variance, obviously because of limited bandwidth.

As described by Ogden and Colquhoun (1985), at a given filter half power frequency \( f_c \), the ratio \( R \) between the predicted and observed variances can be used to estimate the true half-power frequency \( f_c \) of the underlying Lorentzian curve:

\[
R \approx (\pi \cdot 1n2)^{0.5}(f_c/f_t) = 1.476(f_c/f_t).
\]

This equation is an approximation for a Gaussian filter under conditions of heavy filtering. For the exact solution and a discussion of several filter types, see Ogden and Colquhoun (1983).

In the above example, \( f_c = 16.5 \) kHz. With

\[
1/\tau = 2\pi f_c = a_0k'_{on} + k_{off}
\]

and

\[
K_H = k_{off}/k_{on}
\]

we obtain the forward \( (k_{on}) \) and backward \( (k_{off}) \) rate constants of the unfiltered fluctuations. \( K_H \) is the equilibrium dissociation constant and is obtained as the midpoint of the titration curve shown in Fig. 5.

**pH Dependence of Kinetics of Fluctuations with Na as Charge Carrier**

Fig. 9 shows a plot of the combined kinetic results obtained by the three different methods. The method for each individual measurement is indicated by the shape of the symbol. Clearly, the three methods produce very similar results. As with K or Cs as charge carriers, the relation between \( k_{on} \) and \( a_0 \) is linear, with a slope of \( 3.55 \times 10^{11}/(\text{M} \cdot \text{s}) \). The values of \( k_{off} \) are much higher than with the other two ions, emphasizing the short duration (~55 \( \mu \)s) of the low conductance state, which escapes direct detection at our bandwidth of 5 kHz. There is no clear pH dependence of \( k_{off} \). A slight pH dependence cannot be excluded, since it might have been obscured by the scatter of the data. However, as we will show in the discussion, a more general model can also explain the pH independence of \( k_{off} \) at the lower pH values studied in the case of currents carried by Na.

**DISCUSSION**

In this paper, we have studied in detail the kinetics of the proton-induced fluctuations of the open L-type Ca channel when monovalent ions carry the current. We
could obtain the kinetic parameters directly for K and Cs as permeant ions, but we had to use several methods of signal reconstruction for the very rapid fluctuations in Na-carrying channels. The close agreement of the kinetic results obtained with the three different methods have convinced us that the current pattern underlying the Na currents is similar to the current patterns with K or Cs, except that the duration of the low conductance state is much shorter. This conclusion is inforced by our previously published observation (Prod'hom et al., 1987), that with Na in the presence of D$_2$O the current fluctuations are slowed enough to reveal individual transitions between a high and low conductance state.

A Four-State Model

The pH dependence of the dwell times at the low conductance state excludes a simple two-state model, in which the high and low conductance states are represented by the unprotonated and protonated states of the channel, respectively.

Perhaps more significandy, a two-state model would only be realistic in the case where the physical presence of the proton at its site directly interacts with the permeation pathway to cause the reduced conductance. Such direct interactions between protons and permeant ions could include competition for a common binding site at the entrance of the permeation pathway or the alteration of a local surface potential by the protonation of a site close enough to the permeation pathway such that ions entering the channel would feel the altered potential. We have several lines of evidence (see Pietrobon et al., 1988; Prod'hom et al., 1989) that make such direct interactions between the proton and the permeation path highly unlikely. Therefore, we have to assume that the reduced conductance results indirectly from some conformational change of the channel protein, promoted by protonation. Thus, for a quantitative description, we need at least one additional state following the protonation reaction. Since there is no reason to assume that the protonated channel in its low conductance conformation cannot deprotonate before returning to the high conductance, a minimal model is expected to include the following four states of the open channel. For reasons of simplicity, states of the closed channel.
have been left out of the discussion. This seems justified because closing transitions at our timescale of interest are rare.

\[ \text{Scheme 2} \]

\[
\begin{array}{c}
\text{Ch} \\
\text{High} \\
\text{Low} \\
\text{Ch*} \\
\text{ChH}
\end{array}
\]

\[
\begin{array}{c}
k_1 \\
k_{-1} \\
k_2 \\
k_{-2} \\
k_3 \\
k_{-3} \\
k_4 \\
k_{-4}
\end{array}
\]

\[
\begin{array}{c}
a_{H1} k_1 \\
a_{H2} k_2 \\
a_{H3} k_3 \\
a_{H4} k_4
\end{array}
\]

Ch and ChH represent the nonprotonated and protonated states of the high conductance channel, while Ch* and Ch*H are the corresponding states of the low conductance channel. The implication of this model is that the conductance changes are intrinsic properties of the channel and represent conformational changes of the channel protein.

The qualitatively important aspects of the pH dependence of the dwell times at the high and low conductance level can be summarized by simple inspection of the kinetic scheme:

(a) Because protons can also bind to the low conductance channel, the duration of the low conductance level becomes pH dependent. The value of \( k_{\text{off}} \) will vary from \( k_{-2} \) at high pH to \( k_{-3} \) at low pH. Thus, to simulate the observed decrease of \( k_{\text{off}} \) with increasing \( a_H \), we must set \( k_{-2} > k_{-3} \).

(b) In the absence of protons, we are left with the two states at the left (Ch and Ch*). Thus, transitions between the two levels should still be observed at very high pH. The extrapolated intersect of \( k_{\text{on}} \) with the y-axis yields the value of \( k_2 \). Our data suggest a value for \( k_2 \) of \( \sim 1,000/s \).

(c) The observed linearity of \( k_{\text{on}} \) and \( a_H \) is only expected if \( k_3 \gg a_{H1} k_1 \) over the pH range studied. On the other hand, except at very high pH, \( a_{H1} k_1 > k_2 \). This means that \( k_3 \gg k_2 \) and leads to the important conclusion that the presence of the proton greatly enhances the probability of the channel to change to its low conductance.

(d) A protonated high conductance channel (ChH) can either deprotonate (an event which is undetected, since Ch and ChH are electrically indistinguishable) or proceed to the low conductance. This choice of direction depends on the ratio \( k_{-1}/k_3 \). Unless \( k_3 \gg k_{-1} \), several undetected protonation/deprotonation cycles at the high conductance level may precede a detected conductance change. Thus the true protonation rate constant \( k_1 \) is likely to exceed the measured value of \( k_{\text{on}} \).

The quantitative dependence of the measured parameters \( (k_{\text{on}} \text{ and } k_{\text{off}}) \) on pH is not simple, because each dwell time measures the combined probability density of two electrically indistinguishable states. Each probability density represents the sum of two exponentials with amplitudes and time constants that are functions of many of the rate constants in the model. The derivation of the probability densities is presented in the appendix. In Fig. 10 we show calculated values of \( k_{\text{on}} \) and \( k_{\text{off}} \) as a function of \( a_H \) with rate constants listed in Table I that fit the data for Na, K, and Cs. In this plot the values of \( k_{\text{on}} \) and \( k_{\text{off}} \) are obtained as the reciprocal time constants.
of only the slower of the two exponential components describing the probability density function of each observable conductance level. This procedure will be justified in a following paragraph.

The following arguments governed the choice of rate constants. To satisfy linearity between $k_\text{on}$ and $a_{ii}$, we had to set $k_3 \gg a_{ii} k_1$. $k_2$ was set at 700/s, corresponding to the extrapolated intersect of the y-axis by the linear regression of the values of $k_\text{on}$ with K. $k_{-2}$, $k_{-3}$, and $k_{-4}$ were adjusted to fit the observed values of $k_\text{off}$ and their pH dependence. The protonation and deprotonation rate constants at the high conductance level ($a_{ii} k_3$ and $k_{-4}$) were chosen to give the desired slope of $k_\text{on}$ vs. $a_{ii}$. Because of the requirement of microscopic reversibility in this circular model, the value of

<table>
<thead>
<tr>
<th>$a_{ii}$ (M)</th>
<th>0</th>
<th>0.4</th>
<th>0.8</th>
<th>1.2</th>
<th>1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_\text{on}$ (s^{-1})</td>
<td>2,000</td>
<td>4,000</td>
<td>6,000</td>
<td>8,000</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>Cs(^+)</th>
<th>K(^+)</th>
<th>Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>$4.5 \times 10^{12}$/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>$2.25 \times 10^9$/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>700/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>$7,350$/s</td>
<td>$14,700$/s</td>
<td>$55,860$/s</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$2 \times 10^9$/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>$2,100$/s</td>
<td>$4,200$/s</td>
<td>$15,960$/s</td>
</tr>
<tr>
<td>$k_4$</td>
<td>$9 \times 10^{12}$/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{-4}$</td>
<td>4,500/s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rate constants used for calculations shown in Figs. 10 and 11. The rate constants not shown for K and Na are the same as for Cs.
was not freely adjustable, and it turned out that we needed a value that was two
times that of $a_H k_4$.

Fig. 10 shows that the pH dependence of $k_{off}$ with Cs and K is reproduced
between pH 8 and 9. At lower pH values $k_{off}$ becomes pH independent. This is also
consistent with our experimental results since the observed slight further decrease
of $k_{off}$ at pH < 8 can be fully explained by the frequency limitations of our system
(see Methods and compare Figs. 1 and 3). The pH independence of $k_{off}$ found with
Na as the charge carrier is also obtained without additional changes in the rate constants
other than those required to account for the much higher values of $k_{off}$ (see
below). $k_{off}$ is relatively insensitive to pH when current is carried by Na because
much of the pH dependence takes place at pH values above those studied. Thus in
the presence of Na at pH values ≤8 the model behaves like a two-state model, which
justifies in retrospect the use of techniques based on two-state processes for the
analysis of the current fluctuations in Na-carrying channels.

The only rate constants differing from one permeant ion to another are $k_{-2}$ and
$k_{-3}$. They determine the rate of the conformational change from the low to the high
conductance level and with respect to Cs they are accelerated by a factor of 2.0 in K
and a factor of 7.6 in Na. This is consistent with our hypothesis (Pietrobon et al.,
1988) that occupancy of the channel, which increases in the order Cs < K < Na
(Coronado and Smith, 1987; Pietrobon et al., 1988), progressively destabilizes the
conformation of the channel in which the proton is tightly bound to its binding site.
The greatly increased affinity for the proton of the low conductance channel can
best be seen when the protonation equilibria at each conductance are considered.
The pK values are $-\log(k_{-1}/k_1) = 6.3$ for the high conductance channel and $-\log
(k_{-4}/k_4) = 9.3$ for the low conductance channel. The protonation rate constants ($k_1
and k_4$, see Table I) exceed by a factor of ~100 the diffusion-controlled association
rate constants for protons with bases in aqueous solutions $[10^{10} - 10^{11}/(M \cdot s)]$, see Bell
1973]. This might indicate that the proton concentration near the channel surface
exceeds that in bulk water, because of net negative charge in the vicinity of the
protonation site. We will address this point in the companion paper (Prod’hom et
al., 1989).

At first sight the four-state model seems to be in conflict with our results because
the probability densities $H(t)$ (high conductance) and $L(t)$ (low conductance) are the
sum of two exponentials, contrary to the monoexponential distributions found
experimentally. The conflict is resolved by considering the time constants and amplitudes
of the two exponential components and their pH dependence. With the set of
rate constants used for Fig. 10 (see Table I), only one of the time constants is long
enough to result in measurable unitary events at our bandwidth. The other time
constant is < 60 μs with Cs, < 40 μs with K, and even faster with Na. However, we
must also consider what proportion of events in each distribution are fast and slow,
since although not measurable directly, the fast events in one distribution will artifi-
cially prolong the slow events in the other distribution by concatenating two or
more successive complementary events. The relative number of slow and fast events,
expressed as the relative areas under each exponential component, are shown in
Fig. 11 for both probability densities as a function of $a_{H}$.

The relative areas for $H(t)$ are shown by the open symbols. Over the entire pH
range the fast component accounts for < 1% of the events. Thus concatenation of

 figur 12

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low conductance events by missed fast high conductance events is negligible and measured values of \( k_{\text{off}} \) accurately reflect the slow exponential component of \( L(t) \).

The relative areas for \( L(t) \) are shown by filled symbols. The worst case is that of K, where the fast component accounts for 13.7% of the events at pH 9.2 and 7.2% at pH 9.0. Over the rest of the pH range with K, and over the entire pH range with Cs, the fast component never exceeds 5% of the events. With Na, over the pH range studied (pH 7–8.5) the fast component of \( L(t) \) never exceeds 3% of all events. Thus, the error introduced into the measurement of slow high conductance events by missed fast low conductance events will only be significant at pH 9.2 with K, and even there it will not exceed 15%.

Thus, with the set of rate constants chosen, the four-state model successfully reproduces the main experimental findings. Under our recording conditions it predicts essentially monoexponential distributions for \( H(t) \) and \( L(t) \) and allows us to interpret the measured values of \( k_{\text{on}} \) and \( k_{\text{off}} \) in terms of the slow exponential component of \( H(t) \) and \( L(t) \) without significant error.

A model that includes the interactions between channel occupancy and the protonation site would have eight states arranged as the corners of a cube with transitions allowed along all the edges of the cube. The bottom states of the cube would represent the four states of the protonation site in the empty channel while the four states at the top of the cube would represent the corresponding four states in the channel occupied by a permeant ion. \( k_2 \) and \( k_3 \) would be much faster for the top set of states (occupied channel) than for the bottom set. In practice, since the dwell time of an ion in the channel is much less than 100 ns, the two sets of states can be considered at equilibrium and represented by a single set of four states. In this case \( k_2 \) and \( k_3 \) are apparent rate constants that represent the weighted average of the true rate constants of the empty and occupied channel, respectively.

If the four-state model is correct, it necessitates a reinterpretation of the effects of deuterium oxide on the kinetics of the current fluctuations with Na as the charge carrier. We have previously interpreted the slowing down of the current fluctuations observed when D\textsubscript{2}O replaces H\textsubscript{2}O (Prod'hom et al., 1987) as an expression of the longer lifetime of the deuteronated state (deprotonation rate > dedeuteronation rate).
tion rate). However, in the above model, with sets of rate constants that fit our data, the deprotonation event is not the rate-limiting step for the termination of the duration of the low conductance state, and therefore decreasing the deprotonation rate constant has little effect on the value of $k_{on}$. Thus, within the framework of this model it would appear that D$_2$O exerts its effect directly on the rate constants governing the conformational change, stabilizing the low conductance states.

We believe that the model presented above represents a realistic and reasonable framework for the quantitative analysis of our data. We cannot, however, exclude alternative models, in which protons interact with more than one site. For example, the observed pH dependence of $k_{on}$ could be explained by an additional effect of some other protonation group with a pK value $\approx 9$. The apparent non-zero intercept of $k_{on}$ vs. $a_H$ could be caused by an increasingly negative surface potential in the vicinity of the protonation group at high pH, which could tend to accelerate the protonation rate constant and therefore $k_{on}$. However, we feel that the appeal of such alternative models is considerably lessened by the fact that extra assumptions and adjustable parameters are necessary for them to reproduce our results.

In the companion paper (Prod'hom et al., 1989) we present evidence for the location of the protonation site and its interaction with permeant cations. We also attempt to give a physical meaning to the model presented in this paper.

### Appendix

**Derivation of Equations for Four-State Model**

We will derive here the equation relating the mean dwell time at the low conductance to the rate constants in the system. Since we cannot experimentally distinguish between the two low conductance states, we must find the probability density function for the disappearance of the observable state L as:

\[
L(t) = C_{*}(t) + C_{*H}(t)
\]  

for the initial condition $L(t = 0) = 1$:

\[
dC_{*}(t)/dt = k_{-4}C_{*}(t)H(t) - (k_{-2} + a_Hk_4)C_{*}(t)
\]

\[
dC_{*H}(t)/dt = a_Hk_4C_{*}(t) - (k_{-3} + k_{-4})C_{*H}(t)
\]

We denote $C_{*}(t = 0) = C_{*}$ and $C_{*H}(t = 0) = C_{*H}$ and obtain the Laplace transforms of Eqs. 2A and 3A as

\[
s \cdot \tilde{C}_{*}(s) - C_{*} = k_{-4} \tilde{C}_{*H}(s) - (k_{-2} + a_Hk_4) \tilde{C}_{*}(s)
\]

\[
s \cdot \tilde{C}_{*H}(s) - C_{*H} = a_Hk_4 \tilde{C}_{*}(s) - (k_{-3} + k_{-4}) \tilde{C}_{*H}(s)
\]

Solving for $C_{*}(s)$ we obtain

\[
\tilde{C}_{*}(s) = C_{*} \cdot \{(s + \gamma)/(s + \alpha)(s + \beta)\}
\]

where

\[
\alpha + \beta = a_Hk_4 + k_{-4} + k_{-2} + k_{-3}
\]

\[
\alpha \cdot \beta = k_{-4}k_{-2} + k_{-3}k_{-5} + k_{-5}a_Hk_4
\]

\[
\gamma = k_{-3} + k_{-4}[1 + (C_{*H}/C_{*})]
\]

The inverse transform of Eq. 6A yields

\[
C_{*}(t) = C_{*}[(\gamma - \alpha)/(\beta - \alpha)] \exp(-\alpha t) + [(\gamma - \beta)/(\alpha - \beta)] \exp(-\beta t)
\]
Eqs. 4A and 5A can also be solved for \( \text{Ch}^*\text{H}(t) \). The result has a form similar to Eq. 7A with an additional constant, defined as \( \delta = k_{12} + a_i k_i (1 + Ch^*/Ch^*H_0) \). The sum of \( \text{Ch}^*(t) \) and \( \text{Ch}^*\text{H}(t) \) gives the desired probability density:

\[
L(t) = \left[ \left( Ch^*(\gamma - \alpha) + Ch^*H_0(\beta - \alpha) \right) / (\beta - \alpha) \right] \exp \left( -\alpha t \right)
+ \left[ \left( Ch^*(\gamma - \beta) + Ch^*H_0(\delta - \beta) \right) / (\alpha - \beta) \right] \exp \left( -\beta t \right)
\]

The two rate constants \( \alpha \) and \( \beta \) are obtained as solutions of the quadratic equation

\[
\alpha, \beta = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}
\]

where \( a = 1 \), \( b = \alpha + \beta \), and \( c = \alpha \beta \).

The initial conditions (or in terms of single-channel events the probability that a low conductance event will start at \( \text{Ch}^* \) or \( \text{Ch}^*\text{H} \)) are not important for the time constants but influence the amplitudes of the two components. We assume that in the observable high conductance state the channel spends most of its time in \( \text{Ch} \). This is justified given the rate constants that satisfy our results (\( k_{12} \gg k_{11} \) over the pH range studied).

The proportion of low conductance events starting at \( \text{Ch}^*\text{H} \) or at \( \text{Ch}^* \) was obtained by considering the probability that an event starting at \( \text{Ch} \) would first reach the low conductance state at \( \text{Ch}^* \) or at \( \text{Ch}^*\text{H} \). We solved the problem with an iterative algorithm. At the first transition away from \( \text{Ch} \), the probability to find the channel at \( \text{Ch}^* \) is \( P_{\text{Ch}^*} = 1 \), the probability that \( \text{Ch}^* \) is reached is \( P_{\text{Ch}^*} = P_{\text{Ch}^*} \times p_1 \), where \( p_1 = k_{21} / (k_{21} + a_i k_i) \). The probability that the first transition away from \( \text{Ch} \) reaches \( \text{Ch}^*\text{H} \) directly is \( P_{\text{Ch}^*\text{H}} = (P_{\text{Ch}^*} - P_{\text{Ch}^*}) \times p_2 \), where \( p_2 = k_{31} / (k_{31} + k_{12}) \). The probability that the channel returns from \( \text{Ch}^*\text{H} \) to \( \text{Ch} \) is \( P_{\text{Ch}^*} = (P_{\text{Ch}^*\text{H}} - P_{\text{Ch}^*}) \times P_{\text{Ch}^*\text{H}1} \). The cycle is now repeated with \( P_{\text{Ch}^*\text{H}} = P_{\text{Ch}^*\text{H}} \times p_1 \) and so on. After \( n \) iterations the desired initial conditions (\( \text{Ch}^* \) and \( \text{Ch}^*\text{H}_0 \)) are obtained as

\[
\text{Ch}^* = P_{\text{Ch}^*} = \Sigma P_{\text{Ch}^*}\text{a}
\]

and

\[
\text{Ch}^*\text{H}_0 = P_{\text{Ch}^*\text{H}} = \Sigma P_{\text{Ch}^*\text{H}\text{a}}
\]

Iteration was stopped when \( P_{\text{Ch}^*} + P_{\text{Ch}^*\text{H}} > 0.9999 \).

The same derivation as for \( L(t) \) is obtained for the probability density function of the high conductance state \( H(t) \), except that here we simply set the initial conditions to \( \text{Ch}_0 = 1 \) and \( \text{Ch}^*\text{H}_0 = 0 \), again because the equilibrium between these two states is very much biased toward \( \text{Ch} \) with the rate constants chosen.

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