Molecular Basis of Proton Block of L-Type Ca\textsuperscript{2+} Channels

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Abstract

Hydrogen ions are important regulators of ion flux through voltage-gated Ca\textsuperscript{2+} channels but their site of action has been controversial. To identify molecular determinants of proton block of L-type Ca\textsuperscript{2+} channels, we combined site-directed mutagenesis and unitary current recordings from wild-type (WT) and mutant L-type Ca\textsuperscript{2+} channels expressed in Xenopus oocytes. WT channels in 150 mM K\textsuperscript{+} displayed two conductance states, deprotonated (140 pS) and protonated (45 pS), as found previously in native L-type Ca\textsuperscript{2+} channels. Proton block was altered in a unique fashion by mutation of each of the four P-region glutamates (EII-E/IV) that form the locus of high affinity Ca\textsuperscript{2+} interaction. Glu(E) \textrightarrow{} Gln(Q) substitution in either repeats I or III abolished the high-conductance state, as if the titration site had become permanently protonated. While the EIQ mutant displayed only an \textsim{}40 pS conductance, the EIIQ mutant showed the \textsim{}40 pS conductance plus additional pH-sensitive transitions to an even lower conductance level. The EIVQ mutant exhibited the same deprotonated and protonated conductance states as WT, but with an accelerated rate of deprotonation. The EIHQ mutant was unusual in exhibiting three conductance states (\textsim{}145, 102, 50 pS, respectively). Occupancy of the low conductance state increased with external acidification, albeit much higher proton concentration was required than for WT. In contrast, the equilibrium between medium and high conductance levels was apparently pH-insensitive. We concluded that the protonation site in L-type Ca\textsuperscript{2+} channels lies within the pore and is formed by a combination of conserved P-region glutamates in repeats I, II, and III acting in concert. EIVQ lies to the cytoplasmic side of the site but exerts an additional stabilizing influence on protonation, most likely via electrostatic interaction. These findings are likely to hold for all voltage-gated Ca\textsuperscript{2+} channels and provide a simple molecular explanation for the modulatory effect of H\textsuperscript{+} ions on open channel flux and the competition between H\textsuperscript{+} ions and permeant divalent cations. The characteristics of H\textsuperscript{+} interactions advanced our picture of the functional interplay between P-region glutamates, with important implications for the mechanism of Ca\textsuperscript{2+} selectivity and permeation.

Key Words: ion channels \textbullet{} protonation \textbullet{} P-region \textbullet{} permeation \textbullet{} Xenopus oocytes

Introduction

Extracellular pH falls sharply during episodes of intense neuronal activity (Chesler and Kaila, 1992) or with ischemia in brain or heart (Katz, 1992; Siesjo et al., 1993). The change in pH\textsubscript{e} has a significant effect on many kinds of ion channels (Hille, 1992; Traynelis, 1996). However, the mechanism of pH-dependent control of channel function is not completely understood at the molecular level. Voltage-gated calcium channels are particularly interesting targets of hydrogen ion regulation because of their biological importance as a delivery system for a key intracellular messenger. Increased [H\textsuperscript{+}] strongly inhibits ion permeation through open Ca\textsuperscript{2+} channels as well as reducing channel opening (Prod'hom et al., 1987; Krafte and Kass, 1988; Klöckner and Isenberg, 1994). The inhibitory effect of extracellular acidification on voltage-gated Ca\textsuperscript{2+} channels helps limit Ca\textsuperscript{2+} overload and subsequent damage during a metabolic insult (e.g., Ou-Yang et al., 1994).

The interaction between protons and Ca\textsuperscript{2+} channels also shows intriguing biophysical properties. Recordings of unitary currents through L-type Ca\textsuperscript{2+} channels by the late Peter Hess and colleagues provided the first direct measurements of the protonation/deprotonation rates of a single molecule (Prod'hom et al., 1987). When probed with various monovalent cations as charge carriers, these channels were protonated at a single site with an anomalously high affinity for H\textsuperscript{+} (pK\textsubscript{a} > 7.0), resulting in an unusual subconductance state (Prod'hom et al., 1987; Pietrobon et al., 1989; Prod'hom et al., 1989). Protons also reduced the unitary fluxes of Ca\textsuperscript{2+} and other divalent cations, although much greater acidification was required for block (Krafte and Kass, 1988; Kuo and Hess, 1993; Klöckner and Isenberg, 1994).

Despite extensive study, disagreement remains about the locus of H\textsuperscript{+} block of Ca\textsuperscript{2+} channels and the mechanism of inhibition of ion flux. In the prevailing hypothesis, Hess and colleagues proposed that protons titrate an external histidine residue, outside of the permeation pathway, and reduce channel conductance by an allosteric mechanism (Pietrobon et al., 1989). Mutagenesis studies have provided direct support for such an allosteric mechanism in the case of proton block of Ca\textsuperscript{2+} channels.
inward rectifier K⁺ channels (Coulter et al., 1995). However, an alternative possibility of H⁺ block of Ca²⁺ channels is that the protonation site resides within the pore itself and that ion movements are reduced by a straightforward blocking mechanism (Kuo and Hess, 1993). This hypothesis gains plausibility by analogy to cyclic nucleotide gated (CNG) channels (Root and MacKinnon, 1994). H⁺ block of CNG channels involves glutamates in the pore-forming region (Root and MacKinnon, 1994), homologous to residues that form the high-affinity Ca²⁺ binding site in Ca²⁺ channels (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995).

We set out to resolve these questions, using a combination of site-directed mutagenesis and single channel recordings from *Xenopus* oocytes expressing wild-type (WT) and mutated L-type Ca²⁺ channels. Our experiments provided direct evidence that protons block the Ca²⁺ channel by interacting with a site along the permeation pathway, rather than an external regulatory site outside of the pore. The observations allowed us to identify a specific subset of P-region glutamates that make up the protonation site and led to a simple explanation of how carboxylate side chains of these glutamates work in concert to form a single titration site with extremely high H⁺ affinity. The proton interactions gave fresh perspective on the asymmetrical disposition of the P-region glutamates, and their possible conformational flexibility, relevant to fundamental mechanisms of Ca²⁺ channel selectivity and ion permeation.

**METHODS**

**Expression of the Wild-type and Mutant L-type Ca²⁺ Channels in *Xenopus* Oocytes**

L-type channels were expressed in *Xenopus* oocytes in the subunit combination α₁Cβᵢ₂δ₂ as described previously (Yang et al., 1993; Ellinor et al., 1995). Briefly, the corresponding cRNAs were generated by in vitro transcription using rabbit clones for α₁C (Mikami et al., 1989), βᵢ₂ (Hullin et al., 1992), and δ₂ (Mikami et al., 1989), and injected in *Xenopus* oocytes in approximately equal molar ratio. Expression of the L-type Ca²⁺ channels was confirmed by the appearance of FPL 64176-sensitive Ba²⁺ current in whole oocyte recordings using the two-electrode voltage clamp. The experiments were usually performed 4–5 d after the cRNA injection. The single glutamate to glutamine mutations in the P-region of L-type Ca²⁺ were generated as previously described by Yang et al. (1993). Using the original constructs (Yang et al., 1993), single channel recordings for several mutants were difficult due to the relatively low density of L-type channels expressed on the surface of *Xenopus* oocytes. We increased the level of channel expression more than threefold by truncating part of the 5’-untranslated region. Thus, most of the single channel recordings were performed with the modified constructs.

**Single Channel Recordings**

The vitelline membrane was manually removed from the oocytes expressing wild-type or mutant L-type channels immediately before patch-clamp experiments using a published procedure (Methfessel et al., 1986; Sather et al., 1993). Cell-attached patch-clamp recording was performed on oocytes bathed in ~100 mM K⁺ solution (in mM, 100 KCl, 10 HEPES, 10 EGTA, 11 MgCl₂, pH 7.2 with KOH). In most experiments, the patch-clamp pipette was filled with KCl solution prepared in 3H₂O (in mM, 150 KCl, 5 EDTA). In some cases, 150 mM NaCl solution prepared in either 3H₂O or H₂O was used instead of KCl. The pipette solution also contained 5 mM pH buffer, either HEPES (for pH 7.5 and 8.5) or CHES (for pH 9.75), and was titrated with HCl to the desired pH. The pH value was obtained as the pH meter reading + 0.4 (Perrin and Dempsey, 1974). In all the experiments, the L-type Ca²⁺ channel agonist FPL 64176 (~5 μM) was included in both pipette and bath solutions to prolong channel openings.

The unitary currents were recorded using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz and sampled at 25 kHz. Membrane potential was held at −100 mV, and stepped to various test voltages ranging from −40 to −120 mV. In some experiments, a brief prepulse to a strongly positive voltage was applied before the test pulse to facilitate channel opening. Single channel recordings were corrected offline for linear leak and capacity current by subtracting averaged blank records. Computer programs for data acquisition and analysis were written in AxoBasic. All experiments were conducted at room temperature (21–23°C).

**Data Analysis**

Long channel openings were manually selected and pooled together from multiple sweeps for generation of the open-state all-points amplitude histograms (bin size 0.2 pA). The resulting peaks on the histogram were fit with Gaussian functions. For kinetic analysis, current records were idealized using a two-point crossing criterion, with the threshold set by eye halfway between blocked and unblocked states. Dwell-time distributions of blocked and unblocked states were generated based on idealized records and fit with a single exponential. The time constants were calculated from the mean dwell time using a method to correct for instrumental dead-time and missed events (Colquhoun and Hawkes, 1995). In the analysis of EIQQ mutant, the longest transitions to low and high conductance levels observed in several experiments were used to estimate the corresponding current levels. Once determined, these values were used for analysis of all records obtained at the same voltage. The idealized records for the EIQQ mutant were obtained by a two-point crossing criterion, using two thresholds, one halfway between L and M and another halfway between M and H.

To estimate the frequency of direct H⁺ transitions in EIQQ mutant, we performed the following analysis. We reasoned that due to limited frequency resolution in our recording setup (10–90% rise time of 67 ms), direct transitions between H and L levels would appear as H-M-L or L-M-H sequences on idealized records, with just one sampling point (40 ms/point) at the medium level. Quantitative analysis revealed that of all the occurrences of the sequences H-M-L or L-M-H, ~10% contained only one sample point at the medium level. Based on the overall dwell-time distribution of the medium state, at least half of these events could be accounted for by short-lived sojourns in the M state in a sequen-
tial H ↔ M ↔ L model. Thus, direct H-L transitions cannot be completely excluded but certainly do not occur frequently.

RESULTS

Clear Resolution of Individual Protonation Events in Expressed L-type Channels

cRNAs encoding L-type Ca^{2+} channels were coinjected into *Xenopus* oocytes as the subunit combination α_{1Cβ_{2δ}α_{2}}, and unitary current recordings were obtained using the cell-attached patch configuration 4–7 d after cRNA injection. Fig. 1 shows proton block of the expressed L-type Ca^{2+} channels, recorded with monovalent cations as current carriers. Consistent with published observations with native L-type channels (Prod'hom et al., 1987), the single channel current displayed fast flickery transitions between high conductance and low conductance levels when Na\(^+\) was used as a charge carrier at pH 7.4 (Fig. 1 A, top). The previous results indicated that these transitions correspond to binding and unbinding of individual protons to the L-type channel, with the high and low conductance levels corresponding to deprotonated and protonated states of the channel respectively (Prod'hom et al., 1987). Deviations from the predictions of the simple blocking model have been reported (Pietrobon et al., 1989; Prod'horn et al., 1989), but they most likely result from participation of the buffer in the proton transfer reaction, as was recently shown for proton block of the cyclic nucleotide-gated channels (Root and MacKinnon, 1994). Thus, in the following analysis, pH-dependent transitions between two conductance levels of L-type channel will be interpreted as transitions between protonated and deprotonated states of the channel, in accordance with a simple blocking mechanism.

The resolution of the two conductance states in our experiments was improved when H\(^+\) was replaced by D\(^+\), a heavier hydrogen ion, due to the isotope effect (Fig. 1 A, middle; see Prod'hom et al., 1987). Substitution of K\(^+\) for Na\(^+\) as a charge carrier also slowed down the transitions between these two states (data not shown; Pietrobon et al., 1988). Since we anticipated that some of the L-type channel mutants might produce severe changes in block or unblock rates, we chose to use these effects in combination to optimize the recording conditions for kinetic analysis. Thus, most of our experiments were performed with K\(^+\)-D\(_2\)O (Fig. 1 A, bottom). The average time in the protonated state in K\(^+\)-D\(_2\)O was two to threefold longer than that measured in K\(^+\)-H\(_2\)O, and greater than fivefold longer than that found with Na\(^+\)-H\(_2\)O. Under these conditions, the individual protonation/deprotonation events were clearly evident as abrupt transitions in the amplitude of the single channel current.

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**FIGURE 1.** Proton block of L-type Ca\(^{2+}\) channels expressed in *Xenopus* oocytes. (A) The kinetics of proton block were strongly affected by the species of the permeant and blocking ions. Unitary currents recorded in either Na\(^+\)-H\(_2\)O (−40 mV), Na\(^+\)-D\(_2\)O (−40 mV), or K\(^+\)-D\(_2\)O (−70 mV) are shown on compressed and expanded time scales. Dotted lines mark the closed state or the two conductance levels of the channels. Horizontal arrows indicate the high (H) and low (L) conductance levels recorded in K\(^+\)-D\(_2\)O (bottom trace). (B) The pH dependence of proton block of unitary L-type channel currents. The experiments were performed in K\(^+\)-D\(_2\)O with a −70 mV test potential at indicated external pH values. Representative current traces and open-state all-points amplitude histograms at each pH are shown. The solid curves represent the fitting of the histograms with the sum of two Gaussian functions. Note the shift of the relative areas of the two peaks according to the external pH values.
When the external pH was varied from 7.5 to 9.75, progressive changes were seen in the balance between the high conductance (deprotonated) level and the low conductance (protonated) level (Fig. 1 B). Lowering the proton concentration decreased the proportion of time spent in the low conductance state and increased the preponderance of the high conductance state, while not significantly affecting the unitary current amplitude at either level (Fig. 1 B, right). The slope conductances of the deprotonated and protonated states were 140 _+ 23 pS, n = 4 and 45 _+ 11 pS, n = 4 over the range between -120 and -50 mV (Fig. 2 C).

To describe the proton block quantitatively, the open-state all-points amplitude histograms were fitted with the sum of two Gaussian functions (Fig. 1 B, right, solid curves). The unfitted data points between the peaks are due in large part to poorly resolved fast transitions between the blocked and unblocked states. The percentage of block was estimated from the relative areas of the peaks corresponding to protonated (lower conductance) and deprotonated (high conductance) states. The average percentage of time spent in the deprotonated state was 17 ± 2% (n = 4) at pH 7.5; 53 ± 1.5% (n = 4) at pH 8.5; 85 ± 5% (n = 2) at pH 9.75 (Fig. 3 C, WT). These data are consistent with the presence of a single protonation site with pKa ~8.5 under our experimental conditions. Thus, proton block of L-type Ca^{2+} channels expressed in Xenopus oocytes (Fig. 1) shares major similarities with that described in guinea pig ventricular myocytes and PC-12 cells (Prod'hom et al., 1987; Pietrobon et al., 1988; Pietrobon et al., 1989; Prod'hom et al., 1989).

**Elimination of the High Conductance State in EIQ and EIIIQ Mutants**

To determine whether protonation involves residues within the pore, we analyzed changes in single channel behavior arising from mutations in individual P-region glutamates. We focused on glutamine replacements because they are isosteric and can be regarded as functionally equivalent to permanent protonation. If the protonation site of L-type channels were indeed formed by P-region glutamates, then at least some of the E → Q mutations should reduce the single channel current by simulating the protonation of the wild-type channel and alter or occlude the effect of acidic pH by interfering with any additional protonation. These predictions were borne out by the E → Q mutation in repeat I (EIQ), which induced a dramatic change in channel properties (Fig. 2 A). Only one current level was apparent in this mutant, the current amplitude histogram conforming to a single Gaussian distribution at either pH 7.5 or 8.5. The corresponding slope conductance was 37 ± 2.5 pS (n = 4) over the range between -120 and -50 mV, in good agreement with the slope conductance of the protonated state of the wild-type channel (Fig. 2 C). This result was not restricted to the use of K^+ as a current carrier, as similar observations were made using Na^+ (data not shown). Thus, the EIQ mutation not only mimics the effect of protonation on wild-type channel conductance, but also prevents external H^+ from binding to its native site over the pH range tested.

Neutralization of the glutamate in repeat III (EIIIQ) also abolished the high conductance level that had been observed in the wild-type channel (Fig. 2 B). The major current level for the EIIIQ mutant was characterized by a conductance of 42 ± 3 pS (n = 3), similar to the value for EIQ and the low conductance level of...
FIGURE 3. \( E \rightarrow Q \) mutation in the P-region of repeat IV (EIVQ) reduces proton binding affinity without affecting the high (H) and low (L) conductance levels. (A) Representative current traces of EIVQ (top) and WT (bottom) recorded at −70 mV at pH 8.5. (B) Open-state all points amplitude histogram of EIVQ. Note difference in relative weights of high (H) and low (L) conductance states relative to WT at the same pH (Fig. 1B, right). (C) pH dependence of blockade by external \( H^+ \) of WT (open circles) and EIVQ (open triangles). The relative occupancy of the unblocked state (% unblocked, ordinate) was estimated from the fractional area under the corresponding Gaussian fit.

Destabilization of the Protonated State in the EIVQ Mutant

The \( E \rightarrow Q \) mutation in repeat IV (EIVQ) resulted in a much milder alteration in channel behavior (Fig. 3). This mutation did not prevent blockade by external protons or alter significantly the conductances of either deprotonated and protonated states relative to wild-type channels (Fig. 3A, and see Fig. 7B). However, the degree of blockade at pH 8.5 was only 30 ± 2.4% (n = 4) in the EIVQ mutant (Fig. 3B), compared to 47 ± 1.5% (n = 4) in WT (Fig. 1B). Compared to the WT channel, the \( pH \) dependence of the fraction of time spent in the unblocked state was shifted in the acidic direction in the EIVQ mutant (Fig. 3C), corresponding to a change in \( pK_a \) from 8.5 (WT) to 8.2 (EIVQ).

Kinetic analysis illuminated the mechanism of \( pK_a \) changes in the EIVQ mutant (Fig. 4). Dwell-time distributions of the unblocked and blocked states of WT channels and the EIVQ mutant were generated from idealized records and fitted with single exponentials. Fig. 4B shows a comparison of dwell time distributions and the corresponding exponential fits obtained for EIVQ (solid lines) and WT channels (dashed lines) at pH 8.5. It is evident that the mean blocked time was significantly reduced in the EIVQ mutant compared to WT, without a significant change in the mean unblocked time.

Using a conventional procedure to correct for instrumental dead-time and missed events (Colquhoun and Hawkes, 1995), we estimated the dwell time constants for the WT channel and the EIVQ mutant at two different external proton concentrations (Fig. 4C). At pH 8.5, the corrected dwell time in the deprotonated (unblocked) state was 0.48 ± 0.03 ms (\( n = 4 \)) for the WT channel and 0.40 ± 0.08 ms (\( n = 4 \)) for EIVQ, not significantly different (\( P > 0.05 \)). In contrast, comparison
We concluded from these results that the protonation site is essentially intact in the E/VQ mutant. While the side-chain carboxylate of the glutamate in repeat IV has no detectable influence on access of protons to the site (lack of effect on blocking rate), it helps to stabilize the bound proton, most likely via through-space electrostatic interaction. The stabilizing influence of E/VQ, revealed by an increase in proton off rate in the E/VQ mutant, can be interpreted in terms of an electrostatic interaction that deepens the energy well for the bound proton in WT channel compared with E/VQ. The energy of this interaction \( \Delta E \) may be simply estimated as 
\[
\Delta E = kT\ln(K_{\text{off}}^{\text{E/VQ}}/K_{\text{off}}^{\text{WT}}),
\]
where \( K_{\text{off}}^{\text{E/VQ}} \) and \( K_{\text{off}}^{\text{WT}} \) are the proton off rates for the E/VQ and WT mutants, respectively. For a Coulombic interaction, the 16 mV change would correspond to a distance between the proton and the E/V carboxylate of at least 11 Å, since the effective dielectric constant in the channel pore \( \epsilon \) must be <80 (Dudley et al., 1996).

**The Appearance of Three Conductance Levels in the E/VQ Mutant**

The effect of the E → Q mutation in repeat II was the most surprising. Unlike the wild-type channel and the other point mutants, the E/VQ mutant displayed three distinct current levels, high (H), medium (M), and low (L). These are readily apparent in the voltage range between \(-70 \) and \(-120\) mV (Fig. 5 A), and correspond to three distinct slope conductances in plots of the voltage-dependence of unitary current amplitude (Fig. 5 B). The medium conductance level (102 ± 2 pS, \( n = 3 \)) was not found with any of the other mutant channels or WT, but appeared to be predominant in the E/VQ mutant at pH 8.5 (Fig. 5 A). The low conductance level in this mutant (53 ± 1 pS, \( n = 3 \)) was close to the protonated state of the WT channel. Most of the transitions to the high conductance level were too brief to allow a precise measurement of the current amplitude (Fig. 5 A). However, a rough estimate of the conductance for this state based on current amplitudes of relatively long-lived events was \( \approx 145 \) pS, close to the value for the deprotonated state of the wild-type channel.

Detailed analysis of the kinetic relationships between three conductance states in E/VQ mutant was begun by idealization of unitary current recordings (Fig. 5 A, bottom trace; see METHODS for details). Examination of such records suggested that direct transitions between high and low conductance states occurred rarely compared to those in the sequence H ↔ M ↔ L (see METHODS for details).

To determine whether any of the transitions involved protonation/deprotonation steps, we examined the pH-dependence of the relative occupancy of the three conductance states (Fig. 6). Open channel all-points

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**Figure 4. Reduction of proton binding affinity in E/VQ mutant**

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to the H state were too brief and infrequent to justify fitting the corresponding bins on the amplitude histogram with a third Gaussian function. To compare relative occupancies of all three conductance states at different pH values, current records were idealized as previously described (Fig. 5 A), and the proportion of channel open time spent at various conductance levels was determined from individual idealized sweeps, then averaged across multiple sweeps and experiments. At pH 8.5, the EIIQ mutant channel spends 5.5 ± 0.6% of its open time at the high conductance level, 83.1 ± 0.5% at the medium level, and 11.4 ± 0.1% at the lowest level (n = 3, Fig. 6 B). The probability of the low conductance state was elevated to 34% at pH 7.5, and reduced to 3.3% at pH 9.75 (Fig. 6 B), whereas occupancy of the medium conductance state showed opposite effects. Thus, the low conductance is likely to correspond to a protonated state, and the medium conductance to a deprotonated state.

In contrast to the M ↔ L equilibrium, the ratio of H and M occupancies remained at ~0.06 over the pH range from 9.75 to 7.5. This indicates that transitions between high and medium states do not involve protonation/deprotonation steps. As an additional test, we compared the behavior of the EIIQ mutant in D2O and H2O solutions, but found no significant difference in kinetics of transitions between high and medium conductances (data not shown). This runs contrary to the isotope effect that would be expected if this transition corresponded to a protonation event. The lack of

Figure 5. Appearance of three conductance states in an E → Q mutation in the P-region of repeat II (EIIQ). (A) Examples of current traces of the EIIQ mutant obtained at indicated voltages from a representative experiment (pH 8.5). A portion of the record at -120 mV and its corresponding idealized trace are shown on an expanded time scale. The three open conductance levels (high, H; medium, M; low, L) are indicated by arrows. (B) Current-voltage relationships of the three conductance states.

amplitude histograms of the EIIQ mutant at -70 mV were generated at the pH values as indicated. The data were fitted with a sum of two Gaussian functions, corresponding to medium and low levels. The balance between M and L conductance states was clearly affected by variation of extracellular pH (Fig. 6 A). Transitions

Figure 6. The pH sensitivity of the three-conductance states in the EIIQ mutant. (A) Open-state all-points amplitude histograms at indicated values of pH. The amplitude histograms were fitted with a sum of two Gaussian functions (solid curves), corresponding to M and L conductance states. (B) The pH dependence of the percent occupancy of the three conductance states. (C) The dwell time distribution of the low conductance state, pH 8.5. The dashed line shows an exponential fit to WT data at the same pH.

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pH-dependence of the H ↔ M transition implies that the EIIQ mutant channel can undergo changes between these two conductance levels without net entry or exit of protons.

The affinity of the protonated (low conductance) state in the EIIQ mutant was substantially reduced (pK_a < 7.5) as compared to wild-type channel (pK_a ~ 8.5) under the same ionic conditions (Fig. 6, A and B). A kinetic analysis of the dwell-time distribution of the low conductance state revealed that the weaker proton affinity of the EIIQ mutant was associated with a greater than fivefold faster off-rate of bound H^+ as compared to WT (Fig. 6 C), based on values of the uncorrected mean blocked time. Correction for missed events was not possible because the observed mean blocked time was very close to instrumental dead time (80 µs). It is clear, nonetheless, that bound protons were destabilized much more severely in the EIIQ mutant than in EIQ. If the influence of EII on the bound proton were to be assumed purely electrostatic, a distance of > 4.4 Å between the bound proton and a fully ionized EII may be estimated according to the simple electrostatic considerations described above.

DISCUSSION

By combining the approaches of site-directed mutagenesis and single channel recording, we have demonstrated that proton block of Ca^2+ channels involves a subset of the same conserved P-region glutamates which govern Ca^2+ selectivity. Because these amino acids have been conclusively localized to the permeation pathway (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995), these experiments strongly support a direct interaction between protons and a site within the Ca^2+ channel pore, not outside as it has been previously proposed (Pietrobon et al., 1989). Our results highlight notable differences between proton block of Ca^2+ channels and other types of cation channels, as discussed below. The observed interactions between H^+ ions and pore mutants provide a novel perspective on the disposition of the P-region glutamates and their specific roles in supporting ion selectivity and permeation.

Each E → Q Mutation Produces a Unique Effect on Proton Block

Each of the P-region glutamates appears to play a distinct role in promoting the intrapore protonation, in line with its asymmetrical contribution to selective interactions with Ca^2+ and other divalent cations (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995). The E/IQ construct showed the sharpest difference in behavior in comparison to native channels. Two findings are particularly interesting: first, E/IQ displayed only one current level, whose conductance was in close agreement with that of the protonated state of WT channels (Figs. 2 C and 7), and second, this mutant was completely unresponsive to changes in pH over a range that strongly affected WT channels (Fig. 2 A). These conclusions can be stated quite strongly because the single channel recordings would be capable of resolving even brief and infrequent sojourns at the high conductance level. The combination of results would be difficult to explain if the protonation were to take place at a site separate from EI, which the glutamine substitution had left intact. In such a scenario, mutations might be expected to shift the pH-dependence but not to abolish the deprotonated conductance level in entirety. On the other hand, the ability of the E/IQ mutant to mimic and occlude H^+-dependent block is exactly the result expected if EI were directly involved in forming the protonation site: the glutamine substitution for glutamate can be regarded as equivalent to permanently affixing a proton to the carboxylate side chain. This represents compelling evidence that E/I is titrated during the protonation event, and therefore, that the H^+ binding site lies within the pore. Accordingly, the well-described dependence of proton block on the permeant ion species (Pietrobon et al., 1989; Prod'hom et al., 1989; Kuo and Hess, 1993; Klockner and Isenberg, 1994) is readily explained without invoking an allosteric mechanism (Pietrobon et al., 1989).

Like E/IQ, E/I.IQ completely lacked the high conductance level corresponding to the deprotonated state in WT channels. Evidently, glutamine substitutions in motif III and motif I can exert a similar effect on monovalent cation permeation. However, one notable difference is that further protonation is possible for E/I.IQ, as indicated by flickery reductions in current to an even lower level (Fig. 2 B). We were unable to study these events in full detail, but it was clear that the reductions in conductance were pH-dependent, albeit over a more acidic range of pH than WT channels. One interpretation that we have considered is that the E/I.IQ replacement spares some possibility of protonation of EI, while markedly shifting the pK_a in the acidic direction.

In contrast to E/IQ and E/I.IQ, the glutamine replacement mutants in motifs II and IV both retained high and low conductance states, although the H^+ affinity of E/I.IQ and E/I.IQ was reduced relative to WT. The difference between E/IQ and WT was relatively mild, an approximately twofold decrease in proton affinity (Fig. 3 C), suggesting that E/I played only a limited role in stabilizing the bound H^+. The change in affinity was accounted for by an increased off-rate of the titratable proton, while the on-rate was hardly affected (Fig. 4). A change in the on-rate would have been expected on the basis of changes in local [H^+] if E/I were in ionized form and located on the extracellular side of the pro-
FIGURE 7. Comparison of behavior of individual E → Q mutants and a proposed structural model of how P-region glutamates form the proton-binding site of L-type Ca\(^{2+}\) channels. (A) Representative single channel recordings of WT and E → Q mutants at −100 mV and pH 8.5. (B) Summary of slope conductances of WT and the four E → Q mutants. The slope conductances of each mutant were obtained from l-V curves over the range between −50 and −120 mV at pH 8.5 and 7.5. Points are plotted as mean ± SD (n > 3). Open symbols, deprotonated states; filled symbols, protonated states. The existence of a protonated state with reduced conductance in the E///Q mutant is indicated by X. (C) A schematic drawing of a proposed arrangement of the four P-region glutamates and the structure of the protonation site in the L-type channel. (D) A three-centered H-bond arrangement as one possible configuration of the complex of E1, Eii, and Eiii in its deprotonated state.

The simplest explanation of our data is that E/V is positioned on the cytoplasmic side of the site. Other lines of evidence provide independent support for this interpretation. Parent and Gopalakrishnan (1995) replaced E/V with less bulky amino acids and found systematic increases in monovalent conductance, as if this residue acted as a final bottleneck for ion permeation. Furthermore, Sather et al. (1994) showed that alanine substitution for E/V has no detectable effect on the on-rate for block by external Cd\(^{2+}\). Thus, E/V appears to be less externally accessible than other P-region glutamates, being located further downstream along the permeation pathway (cf., Tang et al., 1993; Yang et al., 1993). If one presumes that E/V stabilizes protonation by a through-space electrostatic interaction, we calculate that its negative charge must be >10 Å from the titration site (see RESULTS). Notably, the alanine residue at the homologous position of repeat IV of Na\(^{+}\) channels has also been suggested to lie further along the permeation path than homologous P-region residues in other repeats (Chiamvimonvat et al., 1996), consistent with the overall structural similarity between voltage-gated Ca\(^{2+}\) and Na\(^{+}\) channels.

The E///Q mutation displayed a third conductance level, between the H and L levels, the dominant conducting state of this construct over a wide range of pH. The emergence of this intermediate conductance was quite striking since it was not found in WT or any of the other glutamine substitution mutants. In interpreting the M conductance, a key finding was that while its prevalence relative to the low conductance was pH dependent, its prevalence relative to the high conductance was
not (Fig. 6 B). Thus, the relationship between the conductance states can be diagrammed as follows:

```
   H

pH-independent

   L

pH-dependent

M
```

We speculate that the H and M states correspond to two distinct configurations of the P-region glutamate side chains, rendered distinguishable by the EI/IIQ mutation. Since the proton affinity of EI/IIQ was >10-fold lower than WT, the EI side chain is also likely to be closely associated with the proton binding site, much more so than EI/IV.

**A Model to Explain Distinctive Features of the Ca\(^{2+}\) Channel Protonation Site**

Our experiments highlight notable distinctions between H\(^+\) block of Ca\(^{2+}\) channels, Na\(^+\) channels, and cyclic nucleotide-gated (CNG) channels. We found that H\(^+\) block of mutant or wild-type Ca\(^{2+}\) channels involved one protonation reaction at most (see also Prod'hom et al., 1987). In contrast, CNG channels display two independent and identical intrapore titration sites for protons (Root and MacKinnon, 1994). The difference in behavior is particularly intriguing because the CNG channel pore is thought to contain four glutamate residues in equivalent positions that form a high-affinity Ca\(^{2+}\) binding site (Root and MacKinnon, 1993), very much like the Ca\(^{2+}\) channel. The most striking difference between Ca\(^{2+}\) channels and Na\(^+\) channels lies in their apparent H\(^+\) affinity. The pK\(_a\) of the protonation site at the extracellular mouth of Na\(^+\) channels is 4.6–4.9 (Woodhull, 1973; Mozhaeva et al., 1981; Zhang and Siegelbaum, 1991; Daumas and Andersen, 1993), close to the pK\(_a\) expected for a single glutamate carboxylate (~4.4). In contrast, the pK\(_a\) of the protonation site within Ca\(^{2+}\) channels (and both of the sites in CNG channels) is more than three log units higher.

Here we propose a model to explain how the four glutamic acid side chains in Ca\(^{2+}\) channels give rise to single protonation site rather than two or more, with a H\(^+\) affinity >10\(^3\)-fold greater than a typical carboxylate. Our results lead us to hypothesize that the protonation site is formed by EI, acting in conjunction with EI/III and to a lesser extent, EI/II, while EI/IV influences protonation much less directly from a vantage point further along the permeation pathway (Fig. 7 C). In this model, the critical carboxylates take on H\(^+\) ions and are linked together by hydrogen bonds, which minimizes the energetic cost of positioning several negatively charged oxygen groups in close proximity in the absence of divalent cations. Hydrogen bonding networks among multiple carboxylates are quite common in proteins, often exerting a stabilizing force between adjacent subunits; multiple hydrogen-bonded carboxylates can exhibit a much higher pK\(_a\) than unpaired carboxylates (Sawyer and James, 1982). Hydrogen-bonding between neighboring oxygen groups has been proposed previously for CNG channels, albeit in the form of carboxylic acid-carboxylate pairs (Root and MacKinnon, 1994).

In contrast, concerted action of multiple carboxylate side chains is unlikely to occur in Na\(^+\) channels, in which the P-region positions corresponding to residues E/II/IV are occupied by Asp, Glu, Lys, and Ala (Heinemann et al., 1992). Pairing of the positively charged lysine with one of the acid residues would leave only one carboxylate free as a titratable group, thus accounting for the much lower H\(^+\) affinity of Na\(^+\) channels relative to Ca\(^{2+}\) channels.

We believe that the distinctive characteristics of Ca\(^{2+}\) channel protonation arise from the concerted action of the trio of E/I, E/II, and E/III and that this configuration provides a much more satisfactory account of our observations than arrangements in which EI and other glutamates act alone or pairwise. For illustrative purposes, we put forward a speculative scenario in Fig. 7 D. In the deprotonated state of the complex, the three carboxylate side chains are held together by a permanently shared H\(^+\) in a three-centered H-bond configuration (Jeffrey and Saenger, 1991). The complex bears a net charge of -2 and thus offers great attraction for entry of an external proton (indicated by arrow). This electrostatic interaction helps explain the unusually high pK\(_a\) of the Ca\(^{2+}\) channel site. In the deprotonated state, EI and E/III are likely to be transiently complexed to a monovalent cation or to be tilted out of plane due to electrostatic repulsion between the oxygens, which bear partial negative charge. Once H\(^+\) enters the site, it is shared between EI and E/III, allowing these side chains to become coplanar; the symmetry of the resulting carboxyl–carboxyl complex contributes further to the high pK\(_a\). The reduction in negative charge due to protonation would be expected to decrease the rate of monovalent cation flux, thus producing a subconductance state.

How might this model account for the EI/IIQ and EII/IIQ phenotypes? Substitution of an -NH\(_2\) for an -O on either EI and E/III would neutralize the side chain, acting in lieu of a titratable H\(^+\), resulting in formation of an N-H-O hydrogen bond or formation of alternative carboxyl–carboxylate pairs that were not present in the WT. We suggest that, for either EI or E/III, charge neutralization would mimic the effect of protonation in driving the channel out of the high conductance state. On the other hand, we speculate that EI and E/III might be expected to differ in whether the glutamine replace-
ment abolished all possibility of protonation, depending on geometrical position of these side chains in the channel pore or their chemical interaction with EII side chain or other neighboring residues. It is likely that E/ is involved in additional protonation in E/I/Q mutant, leading to the expectation that simultaneous glutamine substitution for both E/ and E/I/I would produce extremely small unitary currents, like those seen with the protonated state of E/I/Q. Indeed, oocytes expressing the E/Q-E/I/I/Q double mutant have shown small Ba\(^{2+}\) currents in whole-cell recordings and no clear unitary K\(^{+}\) currents in preliminary cell-attached recordings. This seems consistent with the hypothesis, but we cannot yet exclude the possibility that expression of the E/Q-E/I/I/Q construct is simply inefficient.

Possible Insights into the Mechanism of Ca\(^{2+}\) Permeation

H\(^{+}\) interactions provided a novel perspective on the behavior of the pore glutamates, useful in understanding their participation in Ca\(^{2+}\) selectivity and permeation. The proposed apposition of P-region glutamates I, II, and III is of great interest, not only as a possible high-affinity protonation site, but also because it represents an array of oxygen groups not unlike those found in high-affinity Ca\(^{2+}\) binding sites of known three-dimensional structure (Falke et al., 1994). There are clues that arrival of a divalent cation at the glutamate locus might cause some rearrangement in its configuration. For example, while E/IV has only mild influence on H\(^{+}\) block in the absence of Ca\(^{2+}\), mutations at this position affect Ca\(^{2+}\) and Cd\(^{2+}\) block to a far greater extent than could be explained by a through-space electrostatic interaction (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995). This disparity would make sense if Ca\(^{2+}\) interaction with the E/E/E/I/I/I complex allowed the E/IV carboxylate to swing into position to provide additional coordination for the divalent cation. As discussed above, an additional hint of conformational freedom in the P-region glutamates was provided by the behavior of the E/I/Q mutant, which displayed pH-independent transitions between high and medium conductances. The proposed rearrangements provide examples of what might be possible when the pore accommodates multiple Ca\(^{2+}\) ions. Conformational flexibility in the glutamate locus may be particularly important as a mechanism for mediating ion–ion interactions, thereby allowing the pore to support high rates of divalent cation transfer (Tsien et al., 1987; Armstrong and Neyton, 1992; Kuo and Hess, 1993; Yang et al., 1993).

Physiological Implications of Proton Regulation of Ca\(^{2+}\) Influx

These results provide a molecular basis for an important regulatory function of extracellular pH: the control of ion flux through open Ca\(^{2+}\) channels. The steeply pH\(_{o}\)-dependent reduction of unitary current will help decrease the overall Ca\(^{2+}\) influx, together with reduced channel open probability (Prod'hom et al., 1987; Krafte and Kass, 1988; Klockner and Isenberg, 1994). Our experiments provide a simple and satisfying explanation for the antagonism between H\(^{+}\) and Ca\(^{2+}\). At physiological [Ca\(^{2+}\)]\(_{o}\), competition for pore glutamates will strongly influence the pK\(_{a}\) for protons, thus explaining why changes in external pH in the vicinity of pH 7 produce such marked effects on voltage-gated Ca\(^{2+}\) current (Krafte and Kass, 1988; Pietrobon et al., 1989; Klockner and Isenberg, 1994) and K\(^{-}\)-induced Ca\(^{2+}\) entry (Ou-Yang et al., 1994). The inhibition of Ca\(^{2+}\) entry by H\(^{+}\) will exert a substantial effect in the modulation of cell excitability during vigorous neuronal activity (Chesler and Kaila, 1992) and the promotion of cell survival during epileptic seizures and short-term cerebral or cardiac ischemia (Katz, 1992; Siesjo et al., 1993).

Because the P-region glutamates are perfectly conserved in all known a\(_{1}\) subunits of voltage-gated Ca\(^{2+}\) channels, pronounced pH-sensitivity is likely to be a universal characteristic of this family of membrane proteins. This is in contrast to NMDA receptor channels, whose regulation by pH\(_{o}\) (Traynelis and Cull-Candy, 1991) is markedly dependent on a specific RNA splice complex. Thus, the two major pathways for synaptically driven Ca\(^{2+}\) entry show similar overall responsiveness to pH\(_{o}\) but by fundamentally different molecular mechanisms.

We thank R. Agin for technical assistance, T. Tanabe (Yale University) for a\(_{1}\) and a\(_{2}\) subunit cDNAs, J. Yang (University of California, San Francisco) and P.T. Ellinor (Stanford University) for mutant a\(_{1}\) subunit cDNAs, V. Flockerzi and F. Hofmann (Institut für Pharmakologie und Toxikologie, Technische Universität, München, Germany) for β\(_{2}\) subunit cDNA, R. Aldrich, K. Deisseroth, G. Liu, H. Bitto, T. Schwarz, and J.-F. Zhang for comments on the manuscript, W.A. Sather for instruction on patch-clamp methods, and J.J. Falke, D. Herschlag, R.Y. Tsien, J.W. Stocker, and W. Weis for advice on chemical structures.

This work was funded by research grants from National Institutes of Health, the Mathers Foundation, and the Silvio Conte-NIMH Center for Neuroscience Research at Stanford and training grants from the American Heart Association (California Affiliate (X.-H. Chen) and NIH (I. Bezprozvannaya). We are grateful to Jing Li and Svetlana Bezprozvannaya for support.

Original version received 4 June 1996 and accepted version received 29 July 1996.


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