Aspartate Substitutions Establish the Concerted Action of P-region Glutamates in Repeats I and III in Forming the Protonation Site of L-type Ca\(^{2+}\) Channels*  

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Hydrogen ions reduce ion flux through voltage-gated Ca\(^{2+}\) channels by binding to a single protonation site with an unusually high pK\(_a\). Recent evidence localizes the protonation site to the same locus that supports high affinity Ca\(^{2+}\) binding and selectivity, a set of four conserved glutamate residues near the external mouth of the pore. Remaining controversy concerns the question of whether the protonation site arises from a single glutamate, Glu-1086 (EI1), or a combination of Glu-1086 and Glu-334 (EI) working in concert. We tested these hypotheses with individual Glu \(\rightarrow\) Asp substitutions. The Glu \(\rightarrow\) Asp replacements in repeats I and III stood out in two ways. First, in both EID and EIIDD, protonation was destabilized relative to wild type, whereas it was unchanged in EIIDD and stabilized in EIYD. The changes in affinity were entirely due to alterations in H\(^+\) off-rate. Second, the ratio of protonated conductance to deprotonated conductance was significantly closer to unity for EID and EIIDD than for wild-type channels or other Asp mutants. Both results support the idea that EI and EI111 act together to stabilize a single titratable H\(^+\) ion and behave nearly symmetrically in influencing pore conductance. Neutralization of EI111 by alanine replacement clearly failed to abolish susceptibility to protonation, indicating that no single glutamate was absolutely required. Taken together, all the evidence supports a model in which multiple carboxylates work in concert to form a single high affinity protonation site.

The pore of voltage-gated Ca\(^{2+}\) channels is capable of high affinity interactions with either calcium or hydrogen ions (1–3). These interactions are functionally important for the pore’s exquisite Ca\(^{2+}\) selectivity (4) and its sensitivity to blockade by acidification of the external medium in physiological or pathologic settings (5–7). The kinetics of proton block and unblock of single channels can be resolved in recordings of individual blocking events with monovalent ions as charge carriers (2, 3, 8, 9). Under such experimental conditions, half-maximal block by H\(^+\) occurs at pH \(
\approx
\) 8.5, three to four pH units greater than the pK\(_a\) of an individual glutamate carboxylate (3, 8, 10). All evidence supports the idea that block by protons occurs at the same locus where selectivity for Ca\(^{2+}\) takes place, where carboxylic acid side chains of a set of conserved glutamate residues come into close proximity (8, 10–14). However, there is sharp disagreement about the nature of the titratable group (8, 10). Klockner et al. (10) proposed that the protonation site consisted of only a single glutamate, Glu-1086, in repeat III (here designated simply as EI111). This conclusion was based on a series of glutamate to alanine replacements in which only EI111 appeared to abolish the ability of protons to cause flickering block of unitary flux. In contrast, Chen et al. (8) suggested that the protonation site arises from multiple glutamate side chains, acting in concert (Fig. 1A). In one particular version of this hypothesis, carboxylate groups from EI and EI111 were proposed to share the titratable H\(^+\), in a stable hydrogen-bonding configuration (Fig. 1B). Although more complex than that of Klockner et al. (10), this model readily accounts for the existence of only a single binding site for H\(^+\), not one for each Glu or Glu pair. It also provides an explanation for the finding of half-block at pH > 8, in line with the well documented properties of carboxylic acid-carboxylate complexes (15, 16), not at pH < 5, characteristic for a single carboxylate.

Aspartate substitutions for glutamate offered a useful approach for testing these conflicting models (e.g. Refs. 17 and 18). Unlike Glu \(\rightarrow\) Gln replacements, which alter side chain charge, or Glu \(\rightarrow\) Ala mutations, which change both charge and side chain bulk, Glu \(\rightarrow\) Asp substitutions preserve the acidity of the relevant groups while merely decreasing side chain length. Thus, aspartate substitutions would be expected to perturb the protonation of the channel in relatively subtle yet informative ways. Indeed, we found that each of the four aspartate mutants displayed a unique phenotype distinct from the wild-type channel and that their behavior in all cases conformed closely to the predictions of the model in Fig. 1. Therefore, this study provides new evidence for the coordinated interaction of multiple glutamates in forming the protonation site of L-type Ca\(^{2+}\) channels.

EXPERIMENTAL PROCEDURES

Materials—The cDNAs for rabbit \(\alpha_{1C}\) and \(\alpha_{2,\delta}\) subunits of L-type Ca\(^{2+}\) channels were kindly provided by Dr. T. Tanabe (University of Tokyo), and the cDNA for rabbit \(\beta_2\) subunit was a gift from Drs. V. Flockerzi and F. Hofmann (Technische Universität, München, Germany). FPL 64176¹ was purchased from RBI (Natick, MA); \(^2\)H\(_2\)O from Aldrich, and other chemicals were from Sigma.

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¹The abbreviations used are: FPL 64176, 2,3-dimethyl-4-[2-(phenylmethyl)benzoyl]-3-pyrrole-3-carboxylic acid methyl ester; WT, wild type; EID, glutamate to aspartate mutation at position 383 of rabbit \(\alpha_{1C}\); EI111, glutamate to aspartate mutation at position 736 of rabbit \(\alpha_{1C}\); EIYD, glutamate to aspartate mutation at position 1145 of rabbit
Expression of \( \text{Ca}^{2+} \) Channels in Xenopus Oocytes—Wild-type and mutant L-type channels were expressed in the subunit combination \( \alpha_1 \beta_2 \alpha_2 \) in Xenopus oocytes, as described previously (8, 12, 19). Expression of the L-type \( \text{Ca}^{2+} \) channels was confirmed by the appearance of FPL64176-sensitive \( \text{Ba}^{2+} \) current in whole oocyte recordings using the two-electrode voltage clamp. The single glutamate to aspartate mutations in the P-region of L-type \( \text{Ca}^{2+} \) channels were generated as described previously (12). In all mutant channels, the S untranslated region of \( \alpha_1 \) cDNA was truncated to increase the expression level (8).

Single Channel Recordings—Unitary currents carried by WT and mutant L-type channels were recorded as described previously (8, 20).

Briefly, cell-attached patch-clamp recordings were performed on stripped oocytes bathed in 100 mM KCl solution (in mM, 100 KCl, 5 EDTA, 5 HEPES) prepared in 2H2O. Under these conditions, the average time in the deprotonated state (see Ref. 8 for details).

The behavior of the E mutant differed from WT in several respects (Fig. 2B). Although transitions between high and low conductance states were retained, both levels of unitary current were increased relative to their WT values, as illustrated in the all-points histograms (Fig. 2B, right panel) and in analysis of the unitary current-voltage relationships. Over the range between −50 to −120 mV, the slope conductance of the protonated state \( \gamma_P \) was significantly increased in the E mutant relative to WT (94 ± 10 pS, \( n = 4 \) versus 46 ± 9 pS, \( n = 6, p < 0.001 \)). As for the deprotonated state \( \gamma_D \), unitary currents were consistently greater in E mutant than in control over the same voltage range. The slope conductances also may have increased slightly relative to WT, although not in a statistically significant way (150 ± 5.3 pS, \( n = 3 \) versus 132 ± 10 pS, \( n = 6 \) in WT, \( p > 0.05 \)). In addition to the increase in single channel current levels, the E mutant also altered the balance between protonated and deprotonated states (Fig. 2B, right). In the case of the mutant channels, the proportion of time spent in the protonated state was only 31 ± 1.5% (\( n = 3 \)) at pH 8.5, significantly less than the 50% observed in WT channels (49.5 ± 3%, \( n = 6, p < 0.001 \)). This change in the \( \gamma_P \) mutant corresponded to a 2-fold reduction in the affinity of \( \text{H}^+ \) ions for the protonation site relative to WT. The decrease of \( \text{pK}_a \) in the E mutant was due primarily to acceleration of the off-rate for protons, as revealed by kinetic analysis of single channel records (Fig. 2D). The time constant for the protonated state was reduced significantly, from 0.50 ± 0.001 ms in WT to 0.23 ± 0.001 ms in E mutant (\( p < 0.001 \)). On the other hand, the time constant for the deprotonated state remained unchanged in E mutant compared with WT (\( \tau_D = 0.42 ± 0.03 \) ms in E mutant versus 0.48 ± 0.005 ms in WT, \( p > 0.05 \), unpaired t test). The present data establish that protonation can be destabilized without altering the charge of the side chain, merely by shortening it by a methylene group (−1.4 Å), in strong support of the importance of \( \text{E} \) in the formation of the native proton site.

Since the Glu → Gln mutants in both repeats I and III displayed a very similar phenotype (8), it was of great interest to compare the behavior of E mutant to that of the Glu → Asp mutation in repeat III (EIID) (Fig. 3). In EIID, both WT and mutant channels were increased relative to control. Once again, the slope conductance of the low conductance state \( \gamma_L \) was more than double the WT value, whereas the high conductance of EIID \( \gamma_H = 161 ± 4.5 \text{ pS, } n = 3 \) was only slightly increased if at all relative to WT (\( p > 0.05 \)). The percent blockade at pH 8.5 was 33 ± 2.7% (\( n = 5 \)), corresponding to a 2-fold reduction in proton affinity relative to WT. The altered \( \text{pK}_a \) was due to an accelerated rate of deprotonation \( k_{\text{off}} \) (Fig. 3D, right). In all respects, the properties of the EIID mutant were very much like those of E mutant, suggesting that the side

\[ \text{pK}_a \]

\[ \gamma_P \]

\[ \gamma_D \]

\[ \text{H}^+ \]

\[ \text{E} \]

\[ \text{pS} \]

\[ \mu \text{m} \]

\[ \text{KCl} \]

\[ \text{EDTA} \]

\[ \text{HEPES} \]

\[ \text{pS} \]

\[ \mu \text{m} \]

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\[ \text{ms} \]

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chains at these positions play similar roles in the formation of the protonation site.

The Glu to Asp substitution in repeat II generated more modest changes in channel behavior. On the one hand, $\gamma_h$ for $\text{EIID}$ was $86 \pm 3.2$ ($n = 5$), approximately twice the WT value, much the same as in $\text{EID}$ and $\text{EIID}$. In addition, $\gamma_l$ was $196 \pm$

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**FIG. 2.** Glu $\rightarrow$ Asp substitution in the P-region of repeat I ($\text{EID}$) significantly destabilized the protonation state of L-type Ca$^{2+}$ channels. Wild-type and mutant L-type Ca$^{2+}$ channels were expressed in Xenopus oocytes in the subunit combination $\alpha_1C\beta_2b\alpha_2d$. Single channel records were obtained by cell-attached patch using K$^+$-H$_2$O external solution, pH 8.5, in the pipette. All recordings were done at a test potential of $-70$ mV unless otherwise specified. **A**, representative unitary current trace (left) of WT channels. Dotted lines and the horizontal arrows mark the two open states: $h$, high conductance/deprotonated state; $l$, low conductance/protonated state. Solid line, current level of the closed state. **B**, corresponding all-points amplitude histogram for the open state, generated from multiple current sweeps of a representative experiment, fitted with the sum of two Gaussian functions (solid curve). The vertical arrows mark the mean current values for $h$- and $l$-states. **C**, representative current trace of $\text{EID}$ mutant (left), recorded under the same conditions as in **A**, and the corresponding all-points amplitude histogram (right). The histogram was fitted with the sum of two Gaussian functions (solid curve) and the individual Gaussians are shown as dotted curves. **D**, comparison of the current-voltage relationships of WT channels (circles) and the $\text{EID}$ mutant channels (triangles). Open and filled symbols denote $h$ and $l$ conductance states as marked. Horizontal axis indicates various test potentials where unitary currents were recorded. **EIID**

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**FIG. 3.** Behavior of $\text{EIID}$ closely resembles that of $\text{EID}$. **A**, representative example of $\text{EIID}$ current trace. **B**, corresponding open-state all-points amplitude histogram. **C**, unitary current-voltage relationships of $h$ and $l$ conductance states of $\text{EIID}$ (triangles, solid lines) compared with WT data, also at pH 8.5 (dotted lines show linear fits). **D**, dwell time distributions of the $h$-state (left) and $l$-state (right) of $\text{EIID}$. Dotted curves, exponential fits to the WT data.

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6 (n = 5), a significant increase compared with WT (p < 0.05, unpaired t test). On the other hand, EIID did not differ from WT in either the degree of proton block or in the kinetics of protonation and deprotonation. The proportion of channels in the l-state (49.5% ± 0.7, n = 5, pH 8.5) (Fig. 4B) was close to that found in WT, and the histograms of unblocked or blocked times (Fig. 4D) coincided perfectly with WT distributions. Evidently, shortening the side chain length of EII does not perturb the proton binding affinity, even though it can certainly influence the flux rates through high and low conductance states.

Replacement of the pore glutamate in repeat IV also produced changes in single channel behavior, some significantly different from the other Glu to Asp substitutions (Fig. 5). In EIVD, the slope conductances of protonated and deprotonated states were both increased compared with the wild-type channel. Estimates of the slope conductances γH (74 ± 7.3 pS, n = 4) and γD (174 ± 16 pS, n = 4) were ~60 and ~30% greater than WT values, respectively (p < 0.05 for both). More unusual was the finding that the EIVD mutation increased the proton affinity rather than weakening it. As illustrated in Fig. 5B, the percentage of time spent in the protonated state at pH 8.5 was 61 ± 1.5%, corresponding to approximately 50% increase in affinity relative to WT. The increased proton affinity was well accounted for by prolongation of the intervals in the protonated state (Fig. 5D, right). The time constants for τH in EIVD was 0.75 ± 0.01 ms (n = 7), an increase of 50% above WT (0.5 ± 0.001 ms, p < 0.001). In contrast, τD was 0.51 ± 0.005 ms (n = 7), no different than WT (0.48 ± 0.005 ms, p > 0.46).

Our observations with the Glu to Asp mutations support the idea that both E and EII act in concert to form a single proton binding site. This conclusion seems at odds with Klöckner et al. (10), who reported that replacement of EIII with alanine completely abolished susceptibility to proton block and concluded that EIII was the sole determinant of the proton binding site. Accordingly, we re-examined the behavior of the EIIIA mutant, using 2H2O rather than H2O, and the recordings from the EIIIA mutant were performed at a more negative potential (−20 mV) to increase driving force and unitary current size. The previously published records for EIIIA (10) display a significant degree of flickering in the open channel current, hinting at the existence of transitions between a predominant h level and a less prevalent l level, as is evident in our recordings (Fig. 6).

**DISCUSSION**

We have carried out critical tests of two different models of the protonation site within the pore of L-type Ca2+ channels. One model identified EIIIA (Glu-1086) as the unique proton acceptor site, solely responsible for the appearance of the low conductance state (10). This hypothesis has become untenable since it is now clear that replacement of this residue by alanine...
Figure 5. Glu → Asp substitution in the P-region of repeat IV stabilized the protonated state and increased slope conductances of both states. A and B, representative current trace (A) and corresponding open-state all-points amplitude histogram (B), pH 8.5. Note that in EIVD mutant the balance between the two conductance states is shifted toward the protonated (l) state relative to WT. C, current-voltage relationships of EIVD (triangles, solid lines) compared with WT (dotted lines denote linear fits for WT from Fig. 2C). D, dwell time distributions of the h-state (left) and l-state (right) of EIVD mutant. Exponential fits to WT data are shown as dotted curves. Note that mean lifetime of the l-state in the EIVD mutant is significantly increased compared with WT.

Actually spares responsiveness to H⁺ block (Fig. 6). When our results are considered along with previous evidence (10), it is evident that each of the four Glu → Ala substitutions shares the ability to destabilize the titratable proton. Estimated shifts in the pK₂ of the Glu → Ala mutants relative to wild-type ranged from −0.9 to −0.3 pH units, in the order EIII > EI > EI > EIV. The differences between the various Glu → Ala constructs are subtle enough to make it difficult to decipher the roles of the individual glutamates on the basis of these mutants alone.

The glutamate to aspartate mutations provided new perspectives on the protonation site. They are particularly useful in providing relatively subtle changes in the positions of key side chains without altering their net charge. Thus, Glu → Asp replacements provide information complementary to that derived from glutamine substitutions, which alter net charge with little change in geometry, thereby highlighting the importance of electrostatic effects on permeation (8). We used the aspartate mutants to test another model in which EI and EIII jointly coordinate the titratable H⁺ ion (8). Shortening an amino acid side chain by a single methylene group (−1.4 Å), while maintaining its carboxylic acid terminus, must be regarded as a relatively mild structural alteration. Yet, each of the Glu → Asp replacements was sufficient to cause a significant alteration in channel behavior. These effects took the form of changes in the balance between protonated and deprotonated states in some cases and changes in their conductance levels in all cases. Fig. 7 summarizes equilibrium and kinetic data for the protonation reaction, studied at pH 8.5. Of all the Glu → Ala constructs, only EIVD and EIIID displayed the same degree of proton block as WT (Fig. 7A), with no changes in either k₂ or k₉ (Fig. 7B and C). This seems compatible with the previous hypothesis, which proposed that EI has no direct interaction with the titratable H⁺, only an indirect effect through hydrogen bonding with EI and EIII. Since the Glu → Asp replacement caused no alteration of side chain charge, it is not surprising that it gave a very different effect from the Glu → Gln mutation at the same position, which resulted in a ~10-fold reduction in proton affinity. One might have expected that shortening the carboxylate side chain in domain II would cause some change in pK₂ merely through disturbance of the positioning of EI and EIII. On the other hand, it is possible that a slight tilting of EI and EIII would be sufficient to accommodate the Glu → Asp substitution or that other compensatory rearrangements take place, leaving the configuration of the proton binding site essentially unaltered.

In contrast to the very similar behavior of EIVD and EIIID, aspartate replacements in repeats II and IV each produced a different effect. EIIID displayed the same degree of proton block as WT (Fig. 7A), with no changes in either k₂ or k₉ (Fig. 7, B and C). This seems compatible with the previous hypothesis, which proposed that EI has no direct interaction with the titratable H⁺, only an indirect effect through hydrogen bonding with EI and EIII. Since the Glu → Asp replacement caused no alteration of side chain charge, it is not surprising that it gave a very different effect from the Glu → Gln mutation at the same position, which resulted in a ~10-fold reduction in proton affinity. One might have expected that shortening the carboxylate side chain in domain II would cause some change in pK₂ merely through disturbance of the positioning of EI and EIII. On the other hand, it is possible that a slight tilting of EI and EIII would be sufficient to accommodate the Glu → Asp substitution or that other compensatory rearrangements take place, leaving the configuration of the proton binding site essentially unaltered.

Unlike the other three constructs, the EIVD mutant displayed the striking property of accentuating the inhibition at pH 8.5, indicating an increase in proton affinity. This arose from a slowing of k₉ but, once again, no change in k₂ (Fig. 7). The stabilization of the bound proton might be partly attributed to a through-space electrostatic interaction of bound H⁺ ions with the carboxylate side chain of EIV, if it has been correctly pictured as projecting toward the cytoplasmic end of the pore (8, 24). Shortening the side chain would tend to bring the negative charge of its head group closer to the bound proton, thus promoting its retention.
Critical Roles of P-region Glutamates I and III

The conductances of the deprotonated (h) and protonated (l) states provided valuable information about the local environment within the channel pore (Fig. 6). In general, conductances of the aspartate mutants significantly exceeded those of WT channels. The low conductance was increased by Glu replacements at each of the four positions. A simple interpretation is that the various side chains protrude into the pore and thereby influence the energetic profile that a permeating ion encounters. Replacing a glutamate side chain with a smaller aspartate side chain would tend to widen the aperture for passage of monovalent ions. The Glu → Asp substitutions can be contrasted with the Glu → Gln substitutions, which did not significantly increase γ, relative to the protonated state of WT (8). This makes sense since glutamine is isosteric with glutamate and the Glu → Gln substitution may be expected to leave the geometry of the permeation path unchanged.

Similar considerations may apply to the high conductance state. Since glutamine replacements in domains I and III completely eliminate the high conductance state, these can only be directly compared for Glu → Asp and Glu → Gln replacements in domains II and IV (8). Here again, Glu → Asp replacements caused a clear increase in γ relative to WT (Fig. 6B), and the corresponding glutamine substitutions left γ unaltered (8).

To focus specifically on the incremental effects of protonation on channel conductance, we compared the ratio of low to high conductance values (γ/γ) for the various mutants (Fig. 6C). In EID and EIID, this ratio was significantly closer to unity than in WT or the other aspartate mutants. The similarity between EID and EIID provides further support for the idea that EII and EIID act as nearly equal partners in coordinating the titratable proton (8). For example, one might imagine that binding of H⁺ stabilizes EI and EIII side chains in a symmetrical carboxyl-carboxyl configuration, thereby impeding monovalent ion flux. The high value of γ/γ indicates that shortening the side chain of either EI or EIII has a disproportionate effect on the conductance of the protonated state. If the protonated EI-EIII complex posed the most severe impediment to monovalent ion flux (e.g., in forming the narrowest aperture), Glu → Asp replacement would be expected to yield a relatively large degree of relief (e.g., in widening such an aperture).

Fig. 6. Glu → Ala replacement in the P-region of repeat III (EIIIA) fails to abolish susceptibility to external proton block. A, representative current traces of EIIIA recorded at −70 mV in K₃HPO₄ solution at pH 8.5 (left) or pH 7.5 (right). A portion of the records at each pH is shown on an expanded time scale, and estimates of the l and h conductance levels are denoted by dotted lines and horizontal arrows. B, pH-dependent changes in prevalence of the two conductance states. Open-state all-points amplitude histograms of EIIIA mutant at pH 8.5 (left) and pH 7.5 (right) were each fitted as the sum of two Gaussian functions (solid curves), with individual Gaussians indicated by dotted curves. Note that the balance between the two peaks is shifted in favor of the l-state at pH 7.5 compared with pH 8.5. The all-points amplitude histogram of the closed state is included for comparison, along with the corresponding Gaussian fit (solid curve). C, unitary current-voltage relationships of the EIIIA mutant. Current amplitudes arising from h conductance state (open symbols) and l conductance state (closed symbols) at various testing potentials are shown. Current amplitudes for each state were no different at pH 8.5 (triangles) and at pH 7.5 (circles). The solid lines represent linear fits to the data obtained at pH 8.5.

Fig. 7. Comparison of the effects of Glu → Asp substitutions on the potency of proton block. A, comparison of the percent inhibition of WT channels and of the four Glu → Asp mutants at pH 8.5. Relative to WT, the reductions of proton block in EID and EIID mutants, and the potentiation of block in EIVD mutant are all statistically significant (p < 0.05). B, the effect of Glu → Asp substitution on the time constant of the l conductance (protonated) state (τ). Estimates of τ for EID, EIID, and EIVD are significantly different than the value for WT channels. C, lack of effect of Glu → Asp mutations on time constants of the h-state (τ). No statistically significant differences are detected between WT and mutant channels. The time constants for h-state (τ) and l-state (τ) were calculated from the mean dwell times using a method to correct for instrumental dead-time and missed events (22). * indicates that data are statistically different (p < 0.05) from that of WT.
Critical Roles of P-region Glutamates I and III

On the other hand, aspartate substitution at the EII and EIV positions increases $\gamma_l$ and $\gamma_h$ to nearly the same degree, suggesting a more general effect in favoring current flow. In the case of EIV, this fits well with the proposal that its side chain is positioned downstream of the point of greatest pore constriction (8, 24, 25).

It is interesting to compare the properties of L-type $\text{Ca}^{2+}$ channels with the behavior of other systems where multiple carboxylates are involved in $\text{H}^+$ titration. The pore of the cyclic nucleotide-gated (CNG) channel also contains four glutamates, one from each of four monomers, which appear to cluster in pairs to form two independent protonation sites ($pK_a \approx 7.6$) (11). Although mutagenesis has not yet been reported at the level of individual glutamates, replacement of all four glutamates with aspartate was found to greatly weaken the $\text{H}^+$ affinity and to obscure the appearance of discrete conductance levels (11). Another interesting case is the photosynthetic reaction center of the purple bacterium, a well characterized $\text{H}^+$ transporter, whose $Q_b$ cluster contains a tightly coupled set of three glutamates. The carboxylate side chains of these residues strongly interact, giving a $pK_a$ as basic as 8.2 depending on oxidation state (16). In the $Q_b$ cluster, two Glu residues seem to share the titratable proton, whereas a third remains fully protonated over a wide experimental range, very much similar to the behavior of L-type $\text{Ca}^{2+}$ channels described here. In both of these systems, as in voltage-gated $\text{Ca}^{2+}$ channels, the protonation site arises from multiple glutamate residues, each originating from a distinct region of the transmembrane protein. The effects of the aspartate mutations in L-type channels provide a compelling basis for further clarification of how such carboxylate head groups might work in concert.

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REFERENCES

1. Hess, P., and Tsien, R. W. (1984) Nature 309, 453–456
2. Prod’hom, B., Petrobon, D., and Hess, P. (1987) Nature 329, 243–246
3. Petrobon, D., Prod’hom, B., and Hess, P. (1989) J. Gen. Physiol. 94, 1–21
4. Tsien, R. W., Hess, F., McCleskey, E. W., and Rosenberg, R. L. (1987) Annu. Rev. Biophys. Biophys. Chem. 16, 265–290
5. Krafter, D. S., and Kass, R. S. (1988) J. Gen. Physiol. 91, 641–657
6. Kloekner, U., and Isenberg, G. (1994) J. Gen. Physiol. 103, 665–678
7. Ou-Yang, Y., Kristian, T., Mellergaard, P., and Siejo, B. K. (1994) Brain Res. 646, 65–72
8. Chen, X.-H., Bezprozvanny, I. B., and Tsien, R. W. (1996) J. Gen. Physiol. 108, 363–374
9. Prod’hom, B., Petrobon, D., and Hess, P. (1989) J. Gen. Physiol. 94, 23–42
10. Kloekner, U., Mikala, G., Schwartz, A., and Varadi, G. (1996) J. Biol. Chem. 271, 22293–22296
11. Root, M. J., and MacKinnon, R. (1994) Science 265, 1852–1856
12. Yang, J., Elinor, P. T., Sather, W. A., Zhang, J.-F., and Tsien, R. W. (1993) Nature 366, 158–161
13. Kim, M. S., Morii, T., Sun, L. X., Imoto, K., and Mori, Y. (1993) FEBS Lett. 318, 145–148
14. Tang, S., Mikala, G., Bahinski, A., Yatani, A., Varadi, G., and Schwartz, A. (1993) J. Biol. Chem. 268, 13026–13029
15. Sawyer, L., and James, M. N. G. (1982) Nature 295, 79
16. Lancaster, C. R. D., Michel, H., Heneg, B., and Gunner, M. R. (1996) Biophys. J. 70, 2469–2492
17. Carrasco, N., Puttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Loikema, J. S., Roepe, P. D., and Kaback, H. R. (1989) Biochemistry 28, 2553–2559
18. Frillingos, S., and Kaback, H. R. (1996) Biochemistry 35, 1016–10171
19. Elinor, P. T., Yang, J., Sather, W. A., Zhang, J.-F., and Tsien, R. W. (1995) Neuron 15, 1121–1132
20. Sather, W. A., Tanabe, T., Zhang, J.-F., Morii, Y., Adams, M. E., and Tsien, R. W. (1993) Neuron 11, 291–303
21. Petrobon, D., Prod’hom, B., and Hess, P. (1988) Nature 333, 373–376
22. Colquhoun, D., and Hawkes, A. G. (1985) in Single Channel Recording (Sakmann, B. and Neher, E., eds) 2nd Ed., pp. 397–482. Plenum Publishing Corp., New York
23. Kim, C. C., and Hess, P. (1993) J. Physiol. (Lond.) 466, 657–682
24. Parent, L., and Gopalakrishnan, M. (1995) Biophys. J. 69, 1801–1813
25. Chaimvimonvat, N., Perez-Garcia, M. T., Ranjan, R., Marban, E., and Tomasselli, G. F. (1996) Neuron 16, 1037–1047