Molecular Studies of the Asymmetric Pore Structure of the Human Cardiac Voltage-dependent Ca\textsuperscript{2+} Channel

CONSERVED RESIDUE, GLU-1086, REGULATES PROTON-DEPENDENT ION PERMEATION\textsuperscript{a}

(Received for publication, June 4, 1996, and in revised form, July 12, 1996)

Udo Klöckner†, Gabor Mikalás§, Arnold Schwartz‡, and Gyula Varadi¶

From the \textsuperscript{a}Department of Physiology, University of Cologne, Robert-Koch-Strasse 39, 50931 Cologne, Germany and the \textsuperscript{b}Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267-0828

Proton transfer to calcium channels results in rapid fluctuations between two non-zero conductance levels when the current is carried by monovalent cations. A combination of site-directed mutagenesis and single-channel recording techniques were used to identify the unique proton acceptor site as Glu-1086, a conserved glutamate residue located in the S5-S6 linker of motif III of calcium channels. Glu-1086 is part of an array of four glutamate residues in the pore-lining region of the channel conferring the high selectivity of calcium channels. Titration of Glu-1086 yielded a $pK_a$ value of 7.91 which is different from that expected for a free glutamic acid side-chain carboxyl. Proposed electrostatic interactions between charged nearby residues can account only in part for this phenomenon since individual elimination of the other three glutamate residues only slightly decreased the $pK_a$ of Glu-1086. These data, in addition to identifying the proton acceptor site, provide evidence for the influence of the microenvironment in forming the asymmetry of the conducting pathway of calcium channels.

Voltage-gated calcium channels are of pivotal importance in translating electrical events produced by membrane potential changes into biological activity such as secretion, contraction, and modulation of gene expression (1). The primary characteristic of calcium channels, \textit{viz.} high selectivity of Ca\textsuperscript{2+} over monovalent ions, is fundamental to biological function. In recent years, numerous observations have been provided bearing on the molecular nature of the high selectivity filters of calcium channels. Thus, it appears now well-established that the region responsible for the high selectivity filtering is in the S5-S6 linker of each motif and comprised by four non-identical glutamate residues (2–7) probably asymmetrically positioned. Permeation of ions through these channels can be modulated by extracellular pH, and the existence of a single proton-sensitive group located close to the external mouth of the calcium channel has been postulated for several years (8–11). Functionally, the interaction of protons with the binding site results in two conductance levels of the open channel current, when monovalent ions are used as the charge carrier (8–11). The voltage independence of protonation and deprotonation events and the exclusive accessibility of protons to the site from the exterior of the channel strongly suggest a location of the protonation site on the external surface of the channel protein, outside the transmembrane electric field (8, 10, 11). It is thought that proton blockage of calcium channels is associated with a distinct conformational change in the channel structure, rather than with any specific interaction with elements of the ion-conducting pathway (8–11).

Root and MacKinnon (12) recently showed that in the pore of the cyclic nucleotide-gated (CNG)\textsuperscript{2} channel, four glutamate residues give rise to two identical and independent proton binding sites. If the CNG channel consists of four identical subunits to form the ion conducting pathway, the four glutamate side chains have to be arranged in a precise way to form only two sites. On the other hand, for calcium channels, there is evidence for the existence of one proton binding site. This suggests that either all four glutamate residues will participate equally in the binding of a proton, or that only one glutamate residue is able to interact with a proton.

Recent advances in the molecular identification and characterization of the elements of high selectivity filter(s) of voltage-gated calcium channels (2–7) has opened the possibility of determining whether the proton block directly targets the mechanism of ion permeation or whether other pH-dependent conformational changes are responsible for the phenomenon.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—The construction of plasmids carrying cDNAs that encode the wild type (hHT-1) and the mutant Ca\textsuperscript{2+} channel α1 subunits E677A, E1086A, and E1387A has been described previously (3, 5, 13). The mutant E334A (A2411C) was constructed within the EcoRI (2098)/ClaI (2663) cassette using the Sculptor kit (Amersham). The sequence-verified EcoRI/BamHI (2498) cassette was then ligated into hHT-1 to replace the corresponding wild type fragment. cRNAs specific for α1, α1\textsubscript{B}, and β2 (17, 18) subunits were synthesized by \textit{in vitro} run-off transcription, as described previously (13).

**Functional Expression and Electrophysiological Measurements**—*Xenopus laevis* oocytes at stage V–VI were injected with 40–80 nl of the cRNA (0.1–1 μg/ml) solution (13). The oocytes were screened for the expression of calcium channel current by a two-electrode voltage clamp. In oocytes showing Ba\textsuperscript{2+} (40 mM) currents larger than −100 nA and responding to calcium agonists Bay K 8644 (1 μM) or FPL 64176 (3 μM) with an at least 3-fold increase in the peak current amplitude, the vitelline membrane was removed. Single-channel activity was recorded from cell-attached patches (14) using an Axon 200A amplifier (Axon Instruments). The membrane potential of the oocytes was “zeroed” with a high potassium medium composed of (in millimolar): 20 KCl, 100 potassium glutamate, 5 EGTA, 10 HEPES (pH 7.4 with KOH). The patch electrodes were pulled from thick walled borosilicate glass (Hilgenberg, Malsberg, Germany) and polished with a microforge to an

---

\textsuperscript{a} This work was supported by grants from the Deutsche Forschungsgemeinschaft (to U. K.) and by Grants HL22619-17 and HL43231-07 from the National Institutes of Health and a fund from Tanabe Seiyaku Pharmaceutical Co. Ltd. for Molecular Pharmacology and Biophysics (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{†} To whom correspondence should be addressed: Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, College of Medicine, P. O. Box 670828, 231 Bethesda Ave., Cincinnati, OH 45267-0082. Tel.: 513-558-2466; Fax: 513-558-1778.
inner diameter of 0.5–2 μm. They were coated with pyrolon-2 (15) and had resistances in the order of 2–10 megohms when filled with the recording solutions. The pipette solutions were composed of 150 mM K+, 5 mM EDTA, and 10 mM concentrations of the buffer MES (pKₐ 6.15), HEPES (pKₐ 7.55), and TRIZMA (pKₐ 8.1) used at the appropriate pH. We have calculated the affinity constants of EDTA-calcium complex (16) for pH range 6.0–9.0 and found that EDTA effectively chelates the trace amount of divalent cations (Ca²⁺, Mg²⁺) present in the solution.

The single-channel currents were filtered at 6–10 kHz, digitized at 25–50 kHz, and stored for off-line analysis. Leakage and capacitative currents were corrected by subtracting blank records (ISO2 software MFK, Germany). Single-channel current amplitude was obtained by fitting the amplitude histogram with the sum of Gaussian distributions. Current-voltage relationships were fitted by straight lines using a least squares procedure resulting in the slope as the conductance. Data are presented as mean values ± S.E. Only well resolved openings were measured; therefore, all single-channel experiments were conducted in the presence of 1 μM Bay K 8644 (gift from Bayer Pharmaceutical Co.) or 3 μM FPL 64176 (gift from Fisons, Plc.). Statistical significance was determined by Student’s t test. All recordings were done at room temperature (32 ± 2°C). The L-type calcium channel was identified by its sensitivity to calcium channel antagonists (almost complete block of channel activity by 50 μM nitrrendipine), agonists, and by its voltage dependence. Since the presence of calcium agonists does not alter the permeation properties of native L-type calcium channel (7–9) and certain pore mutant L-type calcium channels (3, 6), they were used to increase the amount of well-resolvable channel openings. The kinetics of the transitions between the two conductance states were idealized by setting a discriminator at 50% of the current between the levels.

RESULTS AND DISCUSSION

We detected two conductance states using a cell-attached configuration of the patch clamp technique and 150 mM K⁺ as the charge carrier in Xenopus oocytes expressing wild type human heart α₁ (hHTα₁), α₂δ, and hδ₃ subunits (Fig. 1A, upper part). The current fluctuations between three levels: the zero current level of the closed channel (indicated by the dashed line), a partially blocked, and the fully open levels. When the amplitude histogram is fitted with Gaussian curves, the three “humps” represent three conductance levels of the cloned L-type calcium channel. The fitted Gaussian curve of the second hump reached a maximum at ≈−1.12 pA, a value representing the mean current amplitude through the protonated channel. The current amplitude through the deprotonated channel exhibited a mean amplitude value of −3.04 pA (Fig. 1A, lower left). From measurements at different test potentials, conductance values of 36 pS for the protonated and 107 pS for the unprotonated conductance states were obtained (Fig. 1A, lower right). These values are comparable with those which have been reported for L-type calcium channels in cardiac myocytes or undifferentiated PC12 cells (8–11).

Representative segments of current traces recorded at the indicated pH values are shown in Fig. 1B. At a pH value of 9.0, the current also fluctuates between levels described above; however, the fractional lifetime of the low conductance state is very short. As the pH decreases, the time that the open channel spends in the partially blocked state increases. Inspection of the traces at the pH of 8.0 shows that the fraction of time the channel is in the low and the high conductance state is about equal. At more “acidic pH values,” the open channel spends an even longer time in the low conductance state. The equilibrium probability of the protonated and unprotonated states was determined by the relative areas under the corresponding Gaussian curves. These values, plotted against the pH and fitted with a Hill equation provided a pKₐ value of 7.91 (Fig. 1C). The Hill coefficient of 0.85 suggests a single class of proton binding sites. These values are similar to those reported for cardiac myocytes (10).

Very recently it has been shown that the high selectivity of calcium channels for Ca²⁺ over Na⁺ or K⁺ ions resides in conserved glutamate residues located in each of the four P regions of the channel (2–7). Furthermore, it has been reported that in the pore of the cyclic nucleotide-gated (CNG) channel, which has permeation properties similar to the calcium channel, specific glutamate residues give rise to two identical and independent proton-binding sites (12). Since the CNG channel consists of four identical subunits surrounding a central pore, the four glutamate side chains have to be arranged in a precise way to form only two sites. In our present study, the finding that in calcium channels there is only one protonated state suggests that there is either only one functionally important acidic group in the appropriate pKₐ value of approximately 8 or that more sites participate, equally or nonequally, in the proton binding. To identify the site(s) at which protonate, we individually replaced the four glutamate residues that are essen-
tial for high affinity Ca\(^{2+}\) binding (2–5, 7) with alanines. Alanine was chosen over glutamine because the strong electron delocalization in the amide group of glutamine makes this group capable of associating with protons (19, 20) and perhaps with cations while alanine cannot. Inspection of selected records (Fig. 2A) reveals that after replacement of the glutamates in motifs I (E334A), II (E677A), and IV (E1387A), current fluctuations between the two different conducting levels were observed that were similar to the wild type control. This finding demonstrates that replacement of these three glutamate residues with alanine has no major impact on the proton-induced current fluctuation of the channel. In contrast, when Glu-1086 (motif III) was replaced with alanine, only one conducting state of the calcium channel was observed. The value of this conductance state is 124.7 ± 6.2 \(\mu\)S, similar to that of the wild type (112.4 ± 3.6, \(n = 7\)) (\(p > 0.022\)), and represents the high conductance, unprotonated state of the channel. This result is very surprising since it has been reported in a recent abstract (21) that replacement of this glutamate residue with glutamine and similarly the same replacement in motif I, eliminates the high conductance state of the calcium channel. Our results, however, clearly demonstrate that the glutamate 1086 in motif III is the sole acceptor for extracellular protons in the tested pH range, and protonation at this site is responsible for the appearance of the low conductance state.

Although, there is clearly only one glutamate residue (Glu-1086, in motif III) that is essential for protonation of the channel, replacement of others has minor but distinct and important effects on the conductance of the protonated and unprotonated channel (Fig. 2B). The behavior of the mutant in which the glutamate in motif II was replaced by alanine is of particular interest. The conductances of the protonated and unprotonated states are identical with the wild type suggesting that the motif II glutamate residue is not a target for protonation at the pH range tested and represents a minor energy barrier in the conducting pathway for monovalent ions. Replacement of the glutamate residue in motif I affects both the high and low conductance states. Since both conductances increase about 2-fold, this suggests that the glutamate residue in motif I plays an important role in controlling the permeation of the channel at least for monovalent cations.

---

**Fig. 2.** Impact of mutations on P-loop glutamate residues on proton block of calcium channels. A, representative segments of current traces induced by depolarizations from −120 to −40 mV. The open protonated state (\(o, p\)) and the open unprotonated state (\(o, u\)) are indicated by dashed lines. The roman numerals on the right side indicate the motif in which the individual glutamate residues are located. B, mean ± S.E. conductances for the unprotonated and protonated states. Open bars, unprotonated; filled bars, protonated. C, fractional times the channels spent in the protonated and unprotonated states recorded at pH 8.0. Mean ± S.E. from 3–6 measurements.

**Fig. 3.** Influence of external pH on divalent cation conductance of calcium channels. A, the pH dependence of the conductance of wild type calcium channel with 10 mM Ba\(^{2+}\) as charge carrier. The curve-fitting was done according to a Hill equation using a least square algorithm. B, the invariability of conductance by pH for the point mutant E1086A. Upper panel, representative tail current events induced by repolarizations from +20 mV to the indicated potentials. Lower panel, corresponding current-voltage relationships.
implies that mutation at this site weakens the interaction with monovalent ions and increases the conductance values by lowering an energy barrier, compared to the wild type. Replacement of the glutamate residue in motif IV by alanine results in a conductance of the unprotonated state that was only slightly increased after the mutation, compared to the wild type, while the conductance value of the protonated state was nearly doubled. This suggests that an energy barrier of the protonated channel influencing the permeation of K+ has been lowered. Titration of the wild type channel (Glu-1086) yielded a $pK_a$ value close to 8 (Fig. 1C). This value is very high compared to 4.3 which is the $pK_a$ value of a free glutamic acid side-chain carboxyl (22). One explanation for this difference may reside in the local dielectric environment, which influences the intrinsic proton affinity of glutamate residues. It is known that in proteins the charge of a neighboring amino acid can alter the $pK_a$ value of glutamate and aspartate residues (22). Most likely, the three other glutamate residues which form the selectivity filter can exert electrostatic effects on the carboxyl group of Glu-1086, thereby also changing its $pK_a$ value.

In order to quantitate this effect, we compared the fractional times the channel spent in the protonated and unprotonated state at the fixed pH value of 8.0 for wild type and the three mutants (Fig. 2C).

Assuming a similar one to one binding as for the wild type, the measured values would correspond to a $pK_a$ for the mutants E334A, E677A, and E1387A of 7.42, 7.33, and 7.65, respectively. These data clearly show that the electrostatic contribution of the three glutamates, upon titration of Glu-1086, are moderate at best. Therefore, other factors must be (primarily) responsible for the unusually high $pK_a$ value. Nevertheless, the finding that there is an effect of mutating these glutamate residues on the $pK_a$ value suggests that these carboxyl side chains must be located closer than 7 Å in relation to the side chain of Glu-1086, since at distances larger than 7 Å, the interaction energies between two charged sites are too small to perturb each other (22).

Divalent cations such as Ca$^{2+}$ or Ba$^{2+}$ exhibit much stronger interaction with their intrapore binding sites than monovalent cations, as quantitated by the ability of low concentrations of divalent ions to block the current flow carried by monovalent ions through the calcium channel (2, 4, 7, 23). Therefore, we expected that divalent cations could be used as charge carrier, the conductance decreased by $\sim 50\%$ at $\sim pH 6.0$ compared to maximal conductance (Fig. 3A). A similar decrease in conductance has been demonstrated in native cells (25). Using the point mutant E1086A, no effect was observed on the conductance upon altering the pH (Fig. 3B). Additionally, the conductance level was close to the wild type. Thus, pH modulation of channel conductance through the wild type channel as determined by Glu-1086 is independent of the charge carrier.

In this study we demonstrate by analysis of the channel conductance states and by point mutations that there is only one glutamic acid side chain, among a number of candidates, that is predominantly protonated within the pH range of 6.0–9.0 thereby controlling ion permeation. This is the first study of a calcium channel pore that shows the influence of the microenvironment on forming the functional asymmetry of the conducting pathway. The unusually high $pK_a$ value of Glu-1086 does not arise exclusively from polar interactions, rather repulsive free energy interactions may contribute that exist between hydrophobic and carboxyl residues when placed sufficiently proximal to each other (26). Notably, residue Glu-1086 is preceded by a phenylalanine that is unique for motif III of this channel and has been shown to influence the calcium dependence of dihydropyridine binding (27). It is therefore possible that since in motif III, in addition to the spatially nearby carboxylate residues, there is the unique placement of a phenylalanine proximal to a glutamate that may confer the unique functional features of Glu-1086.

REFERENCES

1. Hille, B. (1992) Ionic Channels of Excitable Membranes Sinauer Associates, Inc., Sunderland, MA
2. Kim, M. S. Morii, T., Sun, L. X, Imoto, K. & Mori, Y. (1993) FEBS Lett. 318, 145–148
3. Tang, S., Mikala, G., Bahinski, A., Yatani, A., Varadi, G. & Schwartz, A. (1993) J. Biol. Chem. 268, 10286–10292
4. Yang, J., Ellison, P. T., Sather, W. A., Zhang, J. F. & Tsien, R. W. (1993) Nature 366, 158–161
5. Mikala, G., Bahinski, A., Yatani, A., Tang, S. & Schwartz, A. (1993) FEBS Lett. 335, 265–269
6. Yatani, A., Bahinski, A., Mikala, G., Yamamoto, S. & Schwartz, A. (1994) Circ. Res. 75, 315–323
7. Ellison, P. T., Yang, J., Sather, W. A., Zhang, J. F. & Tsien, R. W. (1995) Neuron 11, 1121–1132
8. Prodrom, B., Pietrobon, D. & Hess, B. (1997) Nature 329, 243–246
9. Pietrobon, D., Prodrom, B. & Hess, B. (1988) Nature 333, 573–576
10. Pietrobon, D., Prodrom, B., & Hess, B. (1989) J. Gen. Physiol. 94, 1–21
11. Prodrom, B., Pietrobon, D., & Hess, B. (1989) J. Gen. Physiol. 94, 23–45
12. Root, M. J. & MacKinnon, R. (1994) Science 265, 1852–1856
13. Schultz, D., Mikala, G., Yatani, A., Engle, D. B., Ives, D. E., Seger, B., Old Weghuis, D., Klockner, U., Wakamori, N., Wang, J.-J., Melvin, D., Varadi, G. & Schwartz, A. (1993) Proc. Natl. Acad. Sci. USA 90, 6228–6232
14. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. I. (1981) J. Physiol. (Paris) 85, 431–453
15. Wendi-Gallitelli, M. F. & Isenberg, G. (1989) Am. J. Physiol. 256, H574–H583
16. Fabiato, A. & Fabiato, F. (1979) J. Physiol. (Paris) 73, 463–505
17. Klockner, U., Mikala, G., Varadi, M., Varadi, G. & Schwartz, A. (1995) J. Biol. Chem. 270, 17306–17310
18. Ellis, S. B., Williams, M. R., Ways, N., Brenner, R. N., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W. J., Hui, A. & Schwartz, A. (1988) Science 241, 1661–1664
19. Bash, H. & Tora, H. (1992) The Chemistry of Acid Derivatives, Suppl. B, Vol. 2, Part 2, pp. 1–50, John Wiley & Sons, New York
20. Zalewski, R. I. (1992) The Chemistry of Acid Derivatives, Suppl. B, Vol. 2, Part 1, pp. 305–369, John Wiley & Sons, New York
21. Chen, H.-H., Bezprozvanny, I. & Tsien, R. W. (1996) Biophys. J. 70, A78
22. Matthew, J. B. (1985) Annu. Rev. Biophys. Biophys. Chem. 14, 387–417
23. Varadi, G., Mori, Y., Mikala, G. & Schwartz, A. (1995) Trends Pharmacol. Sci. 16, 43–49
24. Kuo, C.-C. & Hess, P. (1993) J. Physiol. (Lond.) 466, 657–682
25. Klockner, U. & Isenberg, G. (1994) J. Gen. Physiol. 103, 663–678
26. Urry, D. W., Giedema, D. C., Peng, S. G. & Parker, T. M. & Harris, R. D. (1992) J. Am. Chem. Soc. 114, 8717–8719
27. Peterson, B. Z. & Catterall, W. A. (1995) J. Biol. Chem. 270, 18201–18204
