A highly conserved position of negatively charged amino acids is present in the SS2 segments of the S5-S6 linker regions among calcium channels. We report here that replacing Glu residues at this position alters the ion selectivity of the human cardiac calcium channel. Substituting Glu334 in motif I or Glu1086 in motif III with Lys produced mutant calcium channels that permeated sodium ions 10-fold more effectively than barium ions. More conservative changes such as substitution of Glu1086 with Gln or substitution of Glu1087 with Ala also increased sodium permeation through the mutant calcium channels. Sodium currents through the mutant calcium channels could be modulated by dihydropyridines and blocked by external divalent cations. These results suggest that Glu334, Glu1086, and Glu1087 are part of a ring of glutamate residues formed in the pore-lining SS1-SS2 region and are critical in determining ion selectivity and permeability of a human cardiac calcium channel.

Calcium channels are members of the structurally homologous superfamily of voltage-gated ion channels. In Na+ and K+ channels, the SS1-SS2 region of the S5-S6 linker region has been postulated to line the channel pore (1, 2) and therefore play a major role in ion selectivity. Single amino acid substitutions in this region altered ion selectivity (3-6), conductance (7, 8), as well as toxin and Cd2+ sensitivity (9-11). Analysis of the amino acid sequences of the SS2 and flanking regions of the four repeating domains (motifs) of Ca2+ channels (12-16) reveals a highly conserved position of negatively charged Glu residues (Fig. 1). In Na+ channels (4), negatively charged residues occupy equivalent positions in motifs I and II, but a positively charged residue, Lys, occupies the equivalent position in motif III and a neutral residue, Ala, occupies the equivalent position in motif IV (Fig. 1). These 2 residues, Lys1422 and Ala1714, proved to be critical in determining the ion selectivity of the Na+ channel (4). In Ca2+ channels these positions are occupied by Glu in all four motifs. We investigated whether the homologous region in the human cardiac Ca2+ channel lines the channel pore and is critical in determining ion selectivity. Our hypothesis is that these 4 Glu residues may form a Ca2+ binding site or ion selectivity filter in the pore of the Ca2+-channel.

We examined the permeation of Ba2+ and Na+ ions through the wild-type normal human cardiac Ca2+ channel and through mutant channels in which Glu was substituted by Lys (E334K in motif I, E1086K in motif III) or by Ala (E1387A in motif IV). In addition, a double mutant (E1086K.E1387A) was examined in which 2 Glu residues were substituted to mimic the Na+ channel at the equivalent positions. We also altered the charge of a residue within the SS2 region (D1391K), which may be positioned near the extracellular mouth of the pore.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutant Ca2+-Channels—** The full-length cDNA of the human heart Ca2+- channel α1 subunit (hHT) (16) was engineered in the plasmid pBluescript SK−. Mutants E334K and E1086K were constructed by first subcloning Mus11732/SphI(2718) or AliII(3923)/SphI(5598) fragments of hHT into M13mp18 or M13b20, respectively, for production of single-stranded templates. The desired mutations were then made according to the method of Kunkel et al. (17). Cassettes carrying the desired mutations were then ligated into hHT in pBluescript SK−. Other mutants in motif III and motif IV were synthesized within BasBl(4664)/BciI(5720) or BasBl(4664)/SphI(5598) cassettes, respectively, using the polymerase chain reaction (PCR; Hoffman-LaRoche) method (18). Oligonucleotides encoding BasBl or SphI recognition sites and carrying the designed base mismatches served as forward primers, and oligonucleotides covering BciI or BasBl sites served as reverse primers. PCR products were sequenced to verify the presence of the desired mutation and then ligated into hHT to replace the corresponding BasBl(4664)/BciI(5720) or BasBl(4664)/SphI(5598) segment. Double mutants E1086K.E1387A and E1086K.D1391K were constructed by replacing the AalII(5123)/BciI(5720) fragment from the mutant E1086K with the corresponding fragment from E1387A or D1391K. After mutagenesis, cassettes were sequenced to verify the presence of the desired mutation and to check for undesired random mutations. cRNAs specific for the wild-type Ca2+- channel α1 subunit, mutant Ca2+- channel α1 subunits, skeletal muscle Ca2+- channel α1 subunit (19), and Ca2+- channel β subunit from human heart, designated β2 (20), were synthesized by in vitro run-off transcription.

**Electrophysiology—** Oocytes were injected with a total of 40 nl of cRNA from wild-type human heart α1 subunit or mutant α1 subunit (0.1 μg/μl) together with skeletal muscle α1 subunit (0.1 μg/μl) and heart β subunit (0.1 μg/μl) and incubated in modified Barth’s solution for 3-7 days. Whole cell currents were recorded with a two-microelectrode voltage-clamp amplifier (Axoclamp 2A). Voltage and current electrodes (1-2 mg/μl) were filled with 3 M KCl. The external solutions contained 40 mm Ba(OH)2, 50 mm N-methyl-d-glucamine, 2 mm KOH, and 5 mm HEPES (pH 7.3 with methanesulfonic acid) for the Ba2+-containing solution and 120 mm NaOH, 1 mm MgCl2, 2 mm KOH, 1 mm EGTA, and 5 mm HEPES (pH 7.3 with methanesulfonic acid) for the Ca2+-containing solution. Current records were sampled at 2-ms intervals after low-pass filtering at 0.2 kHz. Leakage and capacitative currents were subtracted using a P/4 protocol. All electrophysiological experiments were performed at room temperature (19-21 °C). In the Na+-containing external solution, current responses to potentials greater than +40 mV often showed biphasic or off-scale currents and are omitted from data.

**The abbreviations used are:** hHT, human heart Ca2+- channel α1 subunit; PCR, polymerase chain reaction; DHP, dihydropyridine.
RESULTS AND DISCUSSION

We investigated the permeation of Ba$^{2+}$ and Na$^+$ ions through the wild-type and mutant Ca$^{2+}$ channels. Fig. 2 shows a series of whole cell currents recorded from Xenopus oocytes injected with cRNA encoding wild-type human heart α subunit (A), mutant E1086K (B), mutant D1391K (C), and mutant E1387A (D), with Ba$^{2+}$-containing (a) or Na$^+$-containing (b) external solution. All oocytes were co-injected with skeletal muscle α and heart β Ca$^{2+}$-channel subunit cRNAs. Peak current-voltage relationships recorded in Ba$^{2+}$-containing external solution (open symbols) and Na$^+$-containing external solution (boxed symbols) are shown in c. Currents were measured with standard two-microelectrode voltage clamp, using depolarizing pulses from a holding potential of -80 mV to potentials between -40 and +60 mV (A), between -50 and +30 mV (B), between -50 and +60 mV (C), and between -50 and +30 mV (D), in 10-mV increments. Current traces to the indicated test potentials are shown in the upper panels. Scale bars, 100 nA and 500 ms.

The mutant Ca$^{2+}$ channels display higher permeability to Na$^+$ than Ba$^{2+}$. Whole cell currents recorded from Xenopus oocytes injected with cRNA encoding wild-type human heart α subunit (A), mutant E1086K (B), mutant D1391K (C), and mutant E1387A (D), with Ba$^{2+}$-containing (a) or Na$^+$-containing (b) external solution. All oocytes were co-injected with skeletal muscle α and heart β Ca$^{2+}$-channel subunit cRNAs. Peak current-voltage relationships recorded in Ba$^{2+}$-containing external solution (open symbols) and Na$^+$-containing external solution (boxed symbols) are shown in c. Currents were measured with standard two-microelectrode voltage clamp, using depolarizing pulses from a holding potential of -80 mV to potentials between -40 and +60 mV (A), between -50 and +30 mV (B), between -50 and +60 mV (C), and between -50 and +30 mV (D), in 10-mV increments. Current traces to the indicated test potentials are shown in the upper panels. Scale bars, 100 nA and 500 ms.
Ion Selectivity Sites in a Cardiac Calcium Channel

Table I

Properties of wild-type and mutant Ca\textsuperscript{2+} channels in Na\textsuperscript{+} and Ba\textsuperscript{2+}-containing solutions

| Channel | Amplitude | Peaks I-V | E<sub>rev</sub> | n | Ratio Peak Na+/peak Ba<sup>2+</sup> | n |
|---------|-----------|-----------|--------------|---|-----------------|---|
| In 120 mM Na\textsuperscript{+} | | | | | | |
| hHT | nA | 37.1 ± 21.1 | 134 ± 2.3 | 2.7 ± 2.3 | 6 | 447.0 ± 136.0 | 218 ± 1.1 | 67.3 ± 3.5 | 6 | 0.05 ± 0.01 | 5 |
| D1391K | nA | 28.6 ± 12.5 | 76 ± 0.5 | 4.1 ± 0.7 | 9 | 533.8 ± 71.2 | 21.0 ± 1.1 | 636 ± 0.6 | 9 | 0.06 ± 0.02 | 9 |
| E1387A | nA | 60.3 ± 14.1 | 133 ± 1.2 | 4.0 ± 1.1 | 11 | 576.6 ± 76.3 | 27.1 ± 0.8 | 610 ± 1.1 | 11 | 0.10 ± 0.01* | 22 |
| E1096Q | nA | 164.1 ± 9.4 | -82 ± 0.4 | 2.2 ± 0.3 | 4 | 175.3 ± 25.0 | 15.9 ± 0.4 | 36.9 ± 0.6 | 7 | 1.15 ± 0.11* | 8 |
| E1096K | nA | 62.5 ± 3.1 | -108 ± 1.0 | 0.6 ± 0.7 | 6 | 11.9 ± 2.4 | -2.8 ± 1.7 | 4.2 ± 1.0 | 6 | 8.25 ± 2.40* | 6 |
| E1334K | nA | 140.8 ± 28.1 | -129 ± 1.3 | 1.1 ± 1.1 | 8 | 167.6 ± 6.5 | -0.1 ± 0.8 | 3.4 ± 0.5 | 3 | 14.10 ± 3.80* | 5 |
| E1096K,D1391K | nA | 668.2 ± 99.7 | -128 ± 0.9 | 7.9 ± 1.2 | 16 | 589 ± 10.2 | +14.0 ± 1.2 | +12.4 ± 1.2 | 11 | 6.85 ± 0.49 | 11 |
| E1096K,E1387A | nA | 243.2 ± 63.4 | -11.5 ± 2.0 | 4.4 ± 0.8 | 6 | 18.1 ± 2.1 | -2.3 ± 1.4 | +3.1 ± 1.7 | 8 | 14.80 ± 2.25 | 11 |

| In 40 mM Ba\textsuperscript{2+} | | | | | | |
| A control | b PN200-110 | | | | | |
| B control | | | | | | |
| C control | b Cd\textsuperscript{2+} | | | | | |

* Significantly different from wild-type human cardiac Ca\textsuperscript{2+} channel (hHT); p < 0.05.

Fig. 3. Effects of the Ca\textsuperscript{2+} channel antagonist PN 200–110 at 2 μM (A), Ca\textsuperscript{2+} channel agonist BayK 8644 at 1 μM (B), and Cd\textsuperscript{2+} at 100 μM (C) on whole cell currents, in Na\textsuperscript{+}-containing external solution, in oocytes expressing the mutant E1686K,D1391K. All oocytes were co-injected with skeletal muscle α<sub>2</sub> and heart β Ca\textsuperscript{2+} channel subunit cRNAs. The holding potential was -60 mV, and currents were elicited by depolarizing pulses from -50 to +30 mV, in 10-mV increments. Currents were recorded in Na\textsuperscript{+}-containing external solution (A and B) or Na\textsuperscript{+}-containing solution that was EGTA-free (C). Whole cell currents are shown before (a) and after (b) superfusion with either PN 200–110, BayK 8644, or Cd\textsuperscript{2+}. Current-voltage relationships before (open symbols) and after (filled symbols) superfusion with PN 200–110, BayK 8644, or Cd\textsuperscript{2+} are shown in C, Scale bars: 200 nA and 500 ms.

The charge of a residue within the SS2 region that may be positioned near the extracellular mouth of the pore, Asp<sub>1391</sub>, is significantly different from wild-type human cardiac Ca\textsuperscript{2+} channel (hHT); p < 0.05.

The modulation by dihydropyridines (DHP) is characteristic of L-type cardiac Ca\textsuperscript{2+} channels. Therefore, we tested the effects of the DHP antagonist PN 200–110 and the DHP agonist BayK 8644 on the double mutant E1096K,D1391K (Fig. 3) as well as on all of the single point mutants (data not shown). Both inward and outward currents, in the Na\textsuperscript{+}-containing extracellular solution, were reduced in the presence of 2 μM PN 200–110 (Fig. 3A). The peak inward current was reduced by 86.7 ± 2.1% (n = 5). The agonist BayK 8644 (1 μM) increased peak inward current (Fig. 3B), in the Na\textsuperscript{+}-containing solution, by 85.8 ± 0.53% (n = 4). DHP sensitivity provides strong evidence that the inward current in the presence of the Na\textsuperscript{+}-containing extracellular solution, produced by expression of the double mu-

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occyte and due to partial block by external Mg\textsuperscript{2+}, these ratios cannot be used as a quantitative estimate of ion permeability of the channel. Nevertheless, the data provide a qualitative index of the degree of change in selectivity of the channel between divalent and monovalent cations. In addition, mutations E334K and E1096K exhibited a shift in reversal potential to more negative potentials in the presence of Ba\textsuperscript{2+}-containing external solutions (Table I), consistent with an increase in permeation of monovalent cations through the mutant Ca\textsuperscript{2+} channels resulting in an increased outward flux of intracellular K<sup>-</sup>. Therefore these mutations most likely induce a general loss of selectivity of the channel for divalent over monovalent cations.

The ratios of peak inward current, peak current potentials, and apparent reversal potentials in Na\textsuperscript{+}-containing and Ba\textsuperscript{2+}-containing external solutions for all mutants tested are summarized in Table I. Briefly, more conservative changes such as substitution of Glu<sub>1086</sub> with Gin (mutant E1096Q) or substitution of Glu<sub>1387</sub> with Ala (mutant E1387A) also increased the Na\textsuperscript{+} current relative to the Ba\textsuperscript{2+} current. Mutant E1096Q exhibited a peak inward current ratio of 1.51 ± 0.11:1 (n = 8), in the Na\textsuperscript{+}-containing versus the Ba\textsuperscript{2+}-containing external solution, which was less than that observed for the more drastic substitution of Lys for Glu at the same position. Also, the reversal potential for Ba\textsuperscript{2+} current was shifted to negative potentials to a lesser degree than that for mutant E1096K. Mutant E1387A exhibited only a slight increase in permeation of Na\textsuperscript{+} ions through the channel (Fig. 2D). Alteration of the
tant E1086K,D1391K, as well as by the single mutants, is the result of Na\textsuperscript{+} permeation through a mutant Ca\textsuperscript{2+} channel. In addition, the results suggest that the double mutation and the single mutations described induced no major conformational changes in channel structure outside of the pore region since the channel retained proper responses to dihydropyridines.

Cadmium is a potent blocker of native Ca\textsuperscript{2+} channels (24). The application of external Cd\textsuperscript{2+} (100 \mu M) blocked inward current in the double mutant E1086K,D1391K (Fig. 3), as well as in the single mutants (data not shown), in the Na\textsuperscript{+}-containing extracellular solution (Fig. 3C). Cadmium at a concentration of 10 \mu M produced a 37.5 \pm 3.5\% (n = 3) decrease in inward current, whereas 100 \mu M Cd\textsuperscript{2+} produced an 83.0 \pm 0.2\% (n = 3) block of the inward current. At similar concentrations of Cd\textsuperscript{2+}, Na\textsuperscript{+} permeation is completely blocked in native cardiac Ca\textsuperscript{2+} channels (24).

The present study provides evidence that the SS1-SS2 regions of voltage-gated ion channels form at least part of the pore-lining region. In voltage-dependent Ca\textsuperscript{2+} channels, ion permeation occurs through multiple-ion occupancy states (22, 25). The ability of the Ca\textsuperscript{2+} channels to discriminate between divalent and monovalent cations is determined by ion binding affinity and by ion-ion electrostatic interactions in the multiple occupancy state (22, 25). In this study, substitution of positively charged or neutral residues for the negatively charged Glu residues, at equivalent positions in motifs I, III, and IV, modified the permeation properties such that the channel discriminated poorly between divalent and monovalent cations. Similar mutants in motif II are being tested, and preliminary results are consistent with the importance of the Glu residues. These results, taken together, strongly suggest that Glu\textsuperscript{334}, Glu\textsuperscript{1086}, and Glu\textsuperscript{1387} are part of a “glutamate ring” formed in the putative pore-lining SS1-SS2 region and are critical in determining Ca\textsuperscript{2+} ion binding and ion selectivity of the cardiac Ca\textsuperscript{2+} channel.

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