Mechanisms of Sodium/Calcium Selectivity in Sodium Channels Probed by Cysteine Mutagenesis and Sulfhydryl Modification

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ABSTRACT A conserved lysine residue in the "P loop" of domain III renders sodium channels highly selective. Conversion of this residue to glutamate, to mimic the homologous position in calcium channels, enables Ca^{2+} to permeate sodium channels. Because the lysine-to-glutamate mutation converts a positively charged side chain to a negative one, it has been proposed that a positive charge at this position suffices for Na^+ selectivity. We tested this idea by converting the critical lysine to cysteine (K1237C) in μ1 rat skeletal sodium channels expressed in *Xenopus* oocytes. Selectivity of the mutant channels was then characterized before and after chemical modification to alter side-chain charge. Wild-type channels are highly selective for Na^+ over Ca^{2+} ($P_{Ca}/P_{Na} < 0.01$). The K1237C mutation significantly increases permeability to Ca^{2+} ($P_{Ca}/P_{Na} = 0.6$) and Sr^{2+}. Analogous mutations in domains I (D400C), II (E755C), and IV (A1529C) did not alter the selectivity for Na^+ over Ca^{2+}, nor did any of the domain IV mutations (G1530C, W1531C, and D1532C) that are known to affect monovalent selectivity. Interestingly, the increase in permeability to Ca^{2+} in K1237C cannot be reversed by simply restoring the positive charge to the side chain by using the sulfhydryl modifying reagent methanethiosulfonate ethylammonium. Single-channel studies confirmed that modified K1237C channels, which exhibit a reduced unitary conductance, remain permeable to Ca^{2+}, with a P_{Ca}/P_{Na} of 0.6. We conclude that the chemical identity of the residue at position 1237 is crucial for channel selectivity. Simply rendering the 1237 side chain positive does not suffice to restore selectivity to the channel.

INTRODUCTION

The mechanism of selectivity in voltage-dependent sodium channels is unknown. What we have learned is largely by analogy to calcium channels (Yang et al., 1993; Mikala et al., 1993). These channels contain four glutamate residues in the putative selectivity filter, one contributed by each of the P segments. Neutralization of these glutamates (particularly those in domains I and III) undermines the calcium channel's exquisite ability to select for divalent cations (Yang et al., 1993). The sodium channel contains aspartate, glutamate, lysine, and alanine residues (DEKA) at the homologous positions within the pore. Conversion of the lysine in domain III to glutamate renders the sodium channel permeable to calcium (Heinemann et al., 1992); the charge at the other positions seems not to be as critical, as long as at least one negative charge is preserved. These findings have led to the concept that multiple negatively charged side chains confer calcium selectivity, with the charge at the K/E position in domain III being particularly critical. In the sodium channel, if this position is negatively charged, calcium can permeate; if it is positive, the channels are sodium-selective.

To test this idea, we have converted the lysine residue in the third domain to cysteine (K1237C) in the μ1 sodium channel. Our previous work has shown that this mutant loses its ability to select among monovalent cations, but divalent permeability was not examined (Chiamvimonvat et al., 1996a). The thiol side chain of cysteine confers upon the mutant channels a number of useful properties. First, the charge is neutralized; second, the chemical reactivity of the thiol can be exploited to restore a positively charged side chain by exposure to the sulfhydryl-modifying reagent methanethiosulfonate ethylammonium (MTSEA) (Akabas et al., 1992, 1994; Chiamvimonvat et al., 1996b). We find that K1237C is significantly calcium-permeable. Modification of the side chain reduces single-channel conductance but does not restore selectivity for sodium over calcium. Our findings necessitate revision of prevailing concepts regarding the requirements for monovalent/divalent cation selectivity in sodium channels.

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METHODS

Molecular genetics and mutagenesis

A 1.9-kb *Bam*HI-*Sph*I or 2.5-kb *Sph*I-*Kpn*I fragment of the μ1 skeletal-muscle sodium channel cDNA (Trimmer et al., 1989) was subcloned into pGEM-11Zf$^+$ and pGEM-7Zf$^+$ (Promega, Madison, WI), respectively. The 1.9-kb and 2.5-kb cassettes were used for oligonucleotide-directed mutagenesis (Kunkel, 1985) in domains I or II–IV, respectively. Each mutation was confirmed by sequencing the mutagenic cassette, which was then subcloned back into pSP64T (Krieg and Melton, 1984). Complementary RNA was prepared and injected into stage V–VI oocytes as described

(Pérez-García et al., 1996). *Xenopus laevis* were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (0.17% in water) in a protocol approved by the institutional committee on animal care.

Whole-cell current recordings

All experiments were performed at room temperature (22–23°C). Macroscopic Na^+ currents were recorded using two-microelectrode voltage clamp. For studies of the anomalous mole fraction effect between Na^+ and Ca^{2+}, the bath solutions contained (in mM): 80 NaOH, 5 HEPES, pH 7.6, with methane sulfonic acid or 40 $Ca(OH)_2$, 5 HEPES, pH 7.6, with methane sulfonic acid or combinations of different ratios of the two cations, keeping the total cation charge constant. These combinations included (in mM) 79 Na^+/0.5 Ca^{2+}, 78 Na^+/1 Ca^{2+}, 70 Na^+/5 Ca^{2+}, 60 Na^+/10 Ca^{2+}, 40 Na^+/20 Ca^{2+}, 20 Na^+/30 Ca^{2+}. These external solutions were Cl^- free, to minimize the Ca^{2+}-activated Cl^- current. In addition, oocytes were incubated for 1 h before electrophysiological study with 100 μM BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetoxymethyl ester, a cell membrane-permeant Ca^{2+} chelator; Molecular Probes, Eugene, OR), to eliminate the endogenous Ca^{2+}-activated Cl^- current.

For tests of selectivity, NaOH was replaced by 40 mM $Ca(OH)_2$, $Ba(OH)_2$, $CdCl_2$, $SrCl_2$, or 27 mM $LaCl_3$. MTSEA, a sulfhydryl-specific modifying reagent, was used to modify the side chain of the K1237C mutant. For quantification of susceptibility to MTSEA, peak Na^+ currents at −30 mV were compared before and after ~2 min of bath application at saturating concentrations (2.5 mM of MTSEA). The selectivity for di- and trivalent cations was retested after sulfhydryl modification. To minimize leak currents, whole-cell currents at various test potentials were measured as the difference between peak currents and currents measured at 50 ms. Whole-cell current-voltage relations were fit by using a function combining the Boltzmann distribution describing steady-state activation and the Goldman-Hodgkin-Katz constant-field equation to obtain the whole-cell conductance (Tomaselli et al., 1995). Statistical significance was determined using Student's *t*-test, with $p < 0.05$ representing significance.

Single-channel recordings

The excised inside-out variants of the patch-clamp recording technique (Hamill et al., 1981) were used to further quantify the permeability of Ca^{2+} over Na^+ (P_{Ca}/P_{Na}) in wild-type and K1237C mutant channels before and after sulfhydryl modification. Oocytes were prepared for patch recordings as described (Backx et al., 1992). Patch electrodes contained (in mM): 40 NaCl, 40 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH). The bath solution contained (in mM) 140 NaCl, 1 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH). The liquid junction potential in these experiments was 0.42 ± 0.08 mV (n = 6 measurements) and was not corrected for. Currents were recorded in patches from oocytes with and without prior exposure to 2.5 mM MTSEA. The currents were filtered at 2 kHz and sampled at 10 kHz. Unitary current amplitude was determined from long-lasting openings obtained in the presence of fenvalerate (2–20 μM; Backx et al., 1992). Amplitude histograms at a given test potential were generated and fitted to a single Gaussian distribution using a Levenberg-Marquardt algorithm to obtain the mean unitary currents. The permeability ratio for Ca^{2+} over Na^+ (P_{Ca}/P_{Na}) was calculated from the Goldman-Hodgkin-Katz equation as follows (Lewis, 1979):

$$V_{rev} = \frac{RT}{F} \ln \frac{[Na]_o + 4(P_{Ca}/P_{Na})[Ca]_o}{[Na]_i + 4(P_{Ca}/P_{Na})[Ca]_i \exp^{FV_{rev}/RT}}$$

where V_{rev} is the reversal membrane potential; $[Na]_i$, $[Na]_o$, $[Ca]_i$, and $[Ca]_o$ are concentrations of Na^+ and Ca^{2+} inside and outside, respectively; and R, T, and F have their usual meanings.

Molecular modeling

The modified model for the structure of the P loops of the Na^+ channel as reported in Chiamvimonvat et al. (1996a) was used to assess changes in pore architecture for wild-type, K1237C mutant channel before and after modification by MTSEA. The side chain of the lysine residue ($CH_2CH_2CH_2CH_2NH_3^+$) at the 1237 position was replaced by cysteine (CH_2SH) and modified by the addition of a mixed disulfide bond and the side chain of MTSEA ($SCH_2CH_2NH_3^+$), using Insight and Discover molecular modeling software (Biosym/MSI, San Diego, CA) on a Silicon Graphics Indy workstation (Silicon Graphics, Mountain View, CA). Volumes of the various side chains were calculated using the Insight software.

RESULTS

Fig. 1 shows whole-cell currents and current-voltage relations through wild-type and cysteine-substituted K1237C mutant channels. Wild-type channels (Fig. 1, *A* and *B*) showed no current in the presence of Ca^{2+} as the sole external charge carrier. In these experiments, oocytes were not pretreated with BAPTA-AM. Therefore, one would expect to observe current through the Ca^{2+}-activated Cl^- current if the wild-type channels were appreciably permeable to Ca^{2+}. However, Ca^{2+}-activated Cl^- current was never observed (Fig. 1 *A*). At more positive potentials, a small outward Na^+ current could be detected, as shown by the open circles in Fig. 1 *B*. In contrast, conversion of the

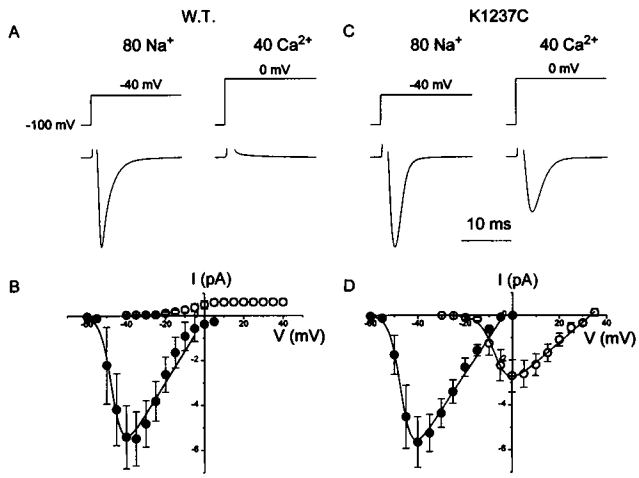

FIGURE 1 Ca^{2+} permeability in the cysteine substituted mutant in domain III (K1237C). (*A, C*) Whole-cell current traces recorded in the presence of 80 mM Na^+ or 40 mM Ca^{2+} from wild-type channel and K1237C mutant channels. Voltage-clamp protocols are indicated above the current traces. Current records obtained in the presence of 40 mM Ca^{2+} were normalized to the records obtained in the presence of 80 mM Na^+. Current amplitudes obtained in Na^+ were −3.2 and −4.5 μA for wild-type and K1237C, respectively. (*B, D*) Current-voltage relations from six different oocytes in the presence of 80 mM Na^+ (●) or 40 mM Ca^{2+} (○) from wild-type and K1237C channels. Data represent mean ± SEM. Only oocytes expressing K1237C mutant channel were pretreated with BAPTA-AM to eliminate the Ca^{2+}-activated Cl^- current. There is no evidence of Ca^{2+} current through the wild-type channels; note in particular the absence of the Ca^{2+}-activated Cl^- current. The small outward currents in *B* likely represent outward Na^+ currents. In contrast, a significant Ca^{2+} permeability is seen through K1237C channels (*D*). The solid lines represent the fit of the whole-cell current-voltage relations to a function combining the Boltzmann distribution describing steady-state activation and the Goldman-Hodgkin-Katz constant field equation (Hille, 1992). The depolarizing shift in the current-voltage relation in the presence of Ca^{2+} is expected from surface charge screening.

lysine at the presumed selectivity filter site in domain III to cysteine (K1237C) produced a channel with sizable permeability to Ca^{2+}, although macroscopic gating was unaffected (Fig. 1, *C* and *D*). Large Ca^{2+}-activated Cl^- currents were evident in oocytes not originally loaded with BAPTA-AM (data not shown).

The selectivity filter in the Na^+ channel was thought to be formed by four residues: aspartate D400 from domain I, glutamate E755 from domain II, lysine K1237 from domain III, and alanine A1529 from domain IV. In addition, our previous data have shown that three other consecutive residues that are carboxy-terminal to A1529 in domain IV (G1530, W1531, and D1532) also contribute significantly to the selectivity of the channel (Chiamvimonvat et al., 1996a). Therefore, these residues were individually mutated to cysteine and tested for Ca^{2+} permeability. Fig. 2 shows the predicted membrane topology for the Na^+ channel and the relative positions of these residues in the P-segments, together with the observed whole-cell conductances (in μS) in the presence of 80 mM Na^+ as compared to 40 mM Ca^{2+}. Only the K1237C mutant in domain III measurably conducts Ca^{2+}.

The divalent cation permeability of the K1237C mutant is selective and exhibits a unique pattern. Unlike the divalent cation permeability of the Ca^{2+} channel, K1237C is not permeable to Ba^{2+}. The channel is also impermeable to Cd^{2+} and La^{3+}, but conducts Sr^{3+} well (Fig. 3 *A*). Fig. 3 *B* shows the permeability of the mutant channel to various cations together with the Pauling radii of these ions (in Fig. 3 *A*). These results show that the K1237C is permeable to cations of intermediate size (Ca^{2+} and Sr^{2+}) and excludes larger cations (La^{3+} and Ba^{2+}). However, the channel also excludes the smaller Cd^{2+} ion.

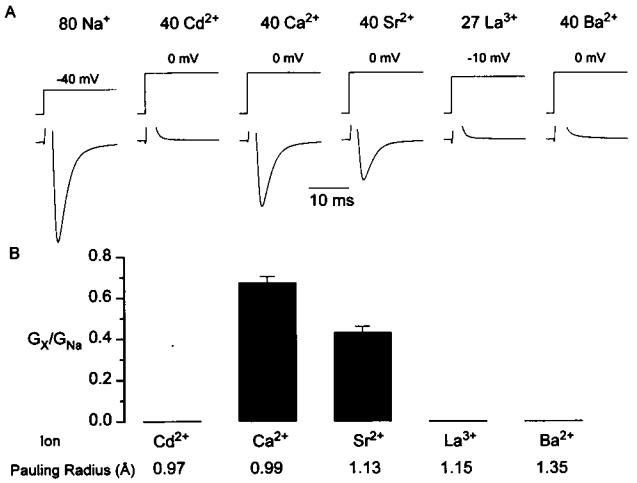

FIGURE 3 Di- and trivalent cation permeability through K1237C. (*A*) Whole-cell current traces recorded in one oocyte in the presence of 80 mM Na^+, 40 mM Cd^{2+}, 40 mM Ca^{2+}, 40 mM Sr^{2+}, 27 mM La^{3+}, or 40 mM Ba^{2+} through K1237C mutant channels. Voltage-clamp protocols are indicated above the current traces. (*B*) Whole-cell conductance calculated in the presence of di- or trivalent cations (G_X) normalized to the conductance obtained using 80 mM Na^+ (G_{Na}). Conductance data were normalized to G_{Na} before data were averaged. Data plotted represent mean ± SEM of three to six oocytes. The Pauling radius (in *A*) for each ion is indicated.

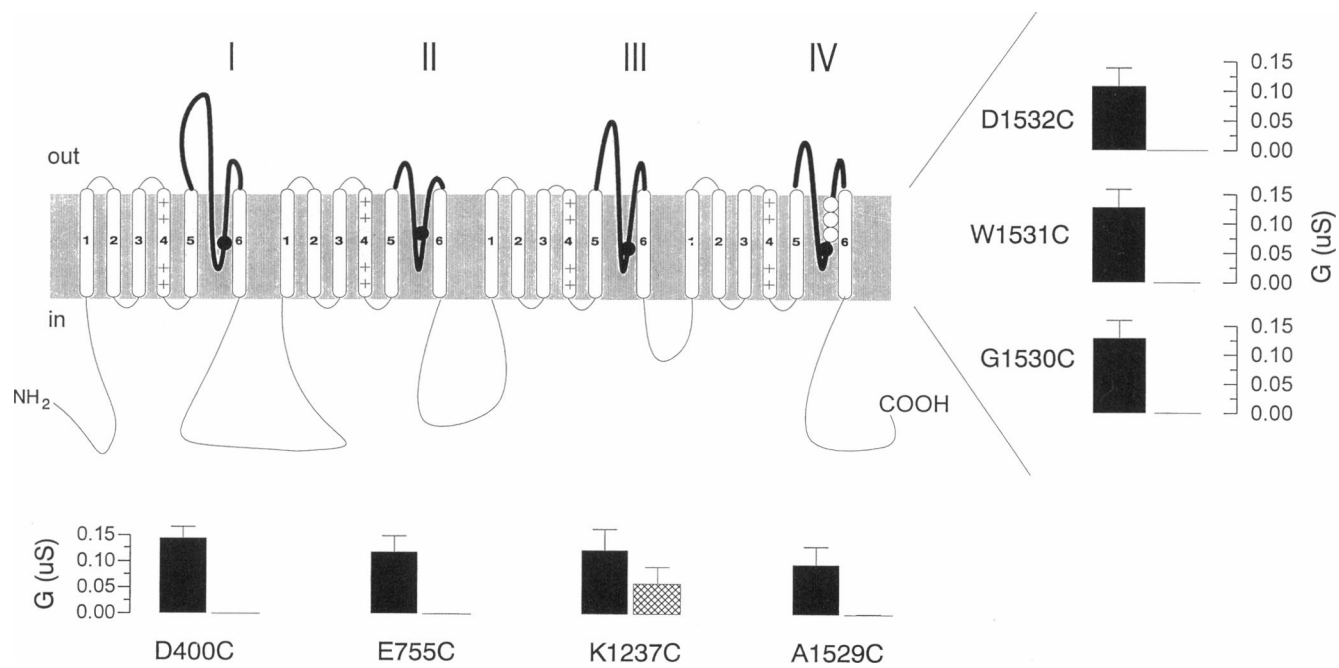

FIGURE 2 Ca^{2+} permeability in the putative selectivity filter and domain IV mutants. Predicted transmembrane topology of the Na^+ channel, with the four residues from the presumed selectivity filter and three residues in domain IV P segment that influence monovalent cation selectivity highlighted (Chiamvimonvat et al., 1996a). Whole-cell conductance obtained in the presence of 80 mM Na^+ (□) are compared to that in the presence of 40 mM of Ca^{2+} (▩) from individual mutant channels. Data represent mean ± SEM from three to six oocytes. Only K1237C shows appreciable permeability to Ca^{2+}. Abbreviations for each of the amino acids: D, aspartate; E, glutamate; K, lysine; A, alanine; G, glycine; W, tryptophan; C, cysteine.

To probe further the properties of permeation in the mutant channel, we analyzed the effects of varying the relative fraction of Na^+ and Ca^{2+} (Fig. 4). Fig. 4 *A* shows representative whole-cell current traces recorded in the presence of 80 mM Na^+, or a combination of Na^+ and Ca^{2+} using 70 mM Na^+/5 mM Ca^{2+} and 40 mM Na^+/20 mM Ca^{2+}. The peak current shifted to more depolarized potentials in the presence of increasing concentrations of Ca^{2+} due to surface charge screening. In addition, peak whole-cell currents decreased as concentrations of Ca^{2+} were increased up to 5 mM, but started to increase once again as the concentration of Ca^{2+} was raised. At very high Ca^{2+} concentration (>20 mM), a secondary decrease in current amplitude was observed. Fig. 4 *B* shows whole-cell conductance (*G*) data for increasing Ca^{2+} and decreasing Na^+ concentrations relative to the total conductance measured in 80 mM Na^+/0 mM Ca^{2+} (G_{max}). The conductance passes through a local minimum at 70 mM Na^+/5 mM Ca^{2+}, showing an anomalous mole fraction effect, consistent with the predicted behavior of a multi-ion pore (Hess and Tsien, 1984; Yue and Marban, 1990). The magnitude of the anomalous mole fraction effect is much smaller than that seen with the homologous K→E mutation in the rat brain II Na^+ channel (Heinemann et al., 1992), hinting that Ca^{2+} binds to the K→C channel with low affinity. The second minimum in the total conductance curve at $Na^+ < 40$ mM/$Ca^{2+} > 20$ mM may be related to outward conductance from K^+ ions at low $[Na^+]_o$. This idea seems reasonable, given that K1237C renders Na^+ channels quite permeable to K^+ (Chiamvimonvat et al., 1996a, and Fig. 5 *C*). Similar outward K^+ current would also be expected at low $[Ca^{2+}]_o$. However, surface charge screening by Ca^{2+} shifts the peak conductance to more depolarized potentials, increasing the driving force for K^+ efflux, and thus favoring outward K^+ current.

Previous reports have suggested that selectivity of Na^+ over Ca^{2+} ions is determined by the positively charged residue lysine in the presumed selectivity filter (Heinemann et al., 1992). The selectivity of Na^+ over Ca^{2+} ions can be decreased by neutralization of the lysine residue to alanine, and this selectivity can be restored by reintroduction of the

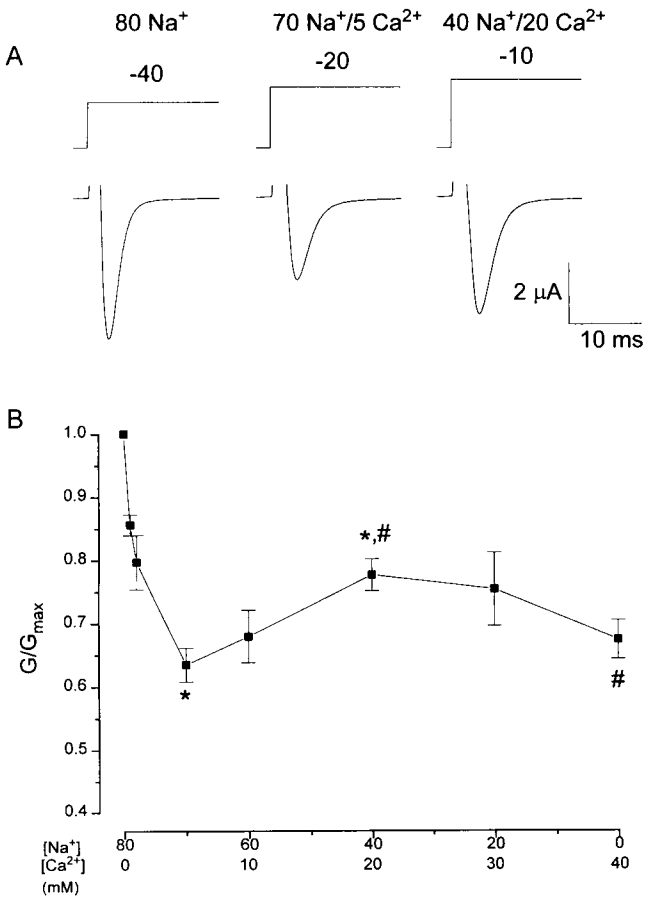

FIGURE 4 Anomalous mole fraction effect between Na^+ and Ca^{2+} through K1237C mutant channels. (*A*) Representative whole-cell current traces recorded in the presence of 80 mM Na^+, 70 mM Na^+/5 mM Ca^{2+}, or 40 mM Na^+/20 mM Ca^{2+} through K1237C mutant channels. Voltage-clamp protocols are indicated above the current traces. (*B*) The solid squares indicate the measured whole-cell conductance (*G*) from 12 oocytes in the presence of different mole fractions of Na^+ and Ca^{2+} normalized to the measurement in 80 mM Na^+/0 mM Ca^{2+} (G_{max}). $^{*,\#}$, $p < 0.05$ between the two conductances indicated.

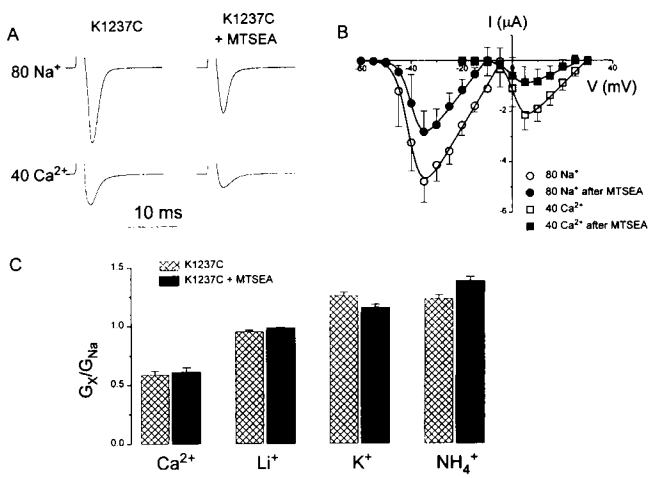

FIGURE 5 Modification of the charge of cysteine-substituted Na^+ channel mutant K1237C using methanethiosulfonate derivative MTSEA. The reaction of MTSEA with a cysteine residue adds $-SCH_2CH_2NH_3^+$ to the thiol side chain via a mixed disulfide bond. (*A*) Representative whole-cell currents elicited by a test potential of −40 mV (in the presence of 80 mM Na^+) and 0 mV (in the presence of 40 mM Ca^{2+}) from a holding potential of −100 mV before and after modification with 2.5 mM MTSEA. All current traces are normalized to that in the presence of Na^+ before modification. (*B*) Current-voltage relationships obtained before (○, □) and after modification with MTSEA (●, ■) in the presence of Na^+ (○, ●) versus Ca^{2+} (□, ■). The data are the mean ± SEM from four oocytes. Whole-cell conductances were obtained from a fit of the whole-cell current-voltage relations to a function combining the Boltzmann distribution describing steady-state activation and the Goldman-Hodgkin-Katz constant field equation (——). (*C*) Whole-cell conductances in the presence of Ca^{2+}, Li^+, K^+, or NH_4^+ before (▩) and after sulfhydryl modication by MTSEA (■). The data are the mean ± SEM from four to eight oocytes. The conductance ratios before and after modification are not statistically different.

positive charge in the form of arginine (Favre et al., 1996). We further tested this idea in our cysteine mutant, K1237C. A major advantage of cysteine mutagenesis is the ability to modify the charge of the side chain covalently by using sulfhydryl-specific reagents (Akabas et al., 1992, 1994; Chiamvimonvat et al., 1996a,b). We have exploited this by exposing oocytes to MTSEA, which adds the positively charged ethyl ammonium moiety to the thiol side chain, to compare the permeation properties of the mutant channels before and after restoration of the physiological charge. We have previously shown that the Na^+ conductance through the K1237C mutant is reduced by MTSEA, whereas the wild-type channel is unaffected (Chiamvimonvat et al., 1996a). Fig. 5 *A* shows representative Na^+ and Ca^{2+} current records through the K1237C mutant channel before and after modification with MTSEA. Both Na^+ and Ca^{2+} conductances were reduced after sulfhydryl modification. The pooled data in Fig. 5 *B* show that the MTSEA-induced changes were voltage-independent; MTSEA reduced the current through the mutant channel at all voltages. More importantly, the modified channel remained permeable to Ca^{2+}, as shown in Fig. 5, *A* and *B*. In fact, the modified K1237C shows a permeability profile similar to that of the unmodified channel. Fig. 5 *C* shows pooled data from a group of cells before and after treatment with MTSEA. The whole-cell conductance in the presence of Ca^{2+}, Li^+, K^+, and NH_4^+ (G_X), normalized to that in the presence of Na^+ (G_{Na}), remains unchanged in the modified channels as compared to unmodified K1237C mutant channels.

To further verify that the modified channels remain permeable to Ca^{2+}, we examined the relative permeabilities to Ca^{2+} and Na^+ (P_{Ca}/P_{Na}) more directly by performing single-channel recordings in excised inside-out patches. Unitary currents were recorded from excised patches expressing wild-type or K1237C channels, using 40 mM Na^+/40 mM Ca^{2+} in the external solution and 140 mM Na^+/1 mM Ca^{2+} on the internal surface. Fig. 6 *A* shows single-channel current records in the wild-type channels at test potentials of −100, 0, and +30 mV. Outward openings can be easily distinguished at both 0 and +30 mV. The lower panel shows the current-voltage relations from four different patches. Data were fit to the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949). The current reversed at −33 mV, the same value as the calculated E_{Na} (−32 mV). Fig. 6 *B* shows single-channel current traces at −100, −30, and +30 mV from an unmodified K1237C channel. Clear inward openings are observed at the predicted Na^+ reversal potential, −30 mV, consistent with a significant permeability to Ca^{2+}. The K1237C mutant channel also shows a significant increase in the single-channel current amplitude as compared to the wild type. The single-channel conductances calculated at $E = 0$ mV are 11.1 pS for wild type and 28.7 pS for K1237C. The current-voltage relation in the lower panel shows a V_{rev} of +12 mV. Fits of the data to the Goldman-Hodgkin-Katz equation indicate a P_{Ca}/P_{Na} of 0.58. To determine whether restoration of the positive charge suffices to restore selectivity, we recorded unitary current from K1237C channels from oocytes pretreated with 2.5 mM MTSEA. Fig. 4 *C* illustrates that such channels are smaller in amplitude than the unmodified K1237C, but still larger than wild type. The single-channel conductance of the modified K1237C calculated at $E = 0$ mV is 20.8 pS. Most interestingly, these modified channels remain permeable to Ca^{2+}, as indicated by the reversal potential of +8 mV ($P_{Ca}/P_{Na} = 0.56$). These data unequivocally demonstrate that the modified channel, with restoration of the positive charge of the side chain at the 1237 position, remains highly permeable to Ca^{2+}.

Finally, we sought to compare the pore structure of wild-type and K1237C mutant channels before and after modification by using an explicit model of the sodium channel pore (Chiamvimonvat et al., 1996a). The model was modified from Guy and Durell (1995) to be consistent with our results of cysteine scanning mutagenesis studies in the P segments (Chiamvimonvat et al., 1996a; Pérez-García et al., 1996). Using this modified structure, we replaced the lysine (Fig. 7 *A*) with cysteine (Fig. 7 *B*) and cysteine modified by MTSEA (Fig. 7 *C*). The K→C mutation decreased the side-chain volume from 79.2 Å^3 to 42.4 Å^3. In addition to restoring the physiological charge to the cysteine side chain, sulfhydryl modification by MTSEA also predicts a net increase in side-chain volume (Fig. 7, *right*). Because the lysine side chain at the 1237 position is accessible from the pore lumen (Chiamvimonvat et al., 1996a), a decrease in side-chain volume (as produced by the K→C mutation) is expected to increase the pore size, as is predicted from our model (Fig. 7, *A* and *B*). However, such steric considerations do not suffice to rationalize the observed changes in selectivity. Our data show that the modified K1237C channel still remains permeable to Ca^{2+}, in spite of the restoration of a fixed positive charge and an actual increase in side-chain volume compared to the wild-type channel. These data support the notion that the precise chemical identity at this site is at least as important for selectivity as the steric hindrance and the side-chain charge.

DISCUSSION

Mechanisms of Na^+ over Ca^{2+} selectivity

One remarkable feature of the Na^+ channel pore is the extremely high selectivity of the pore for Na^+ over other ions in the external milieu, despite a very high flux rate of Na^+ ions across the pore. Indeed, our measurments of the reversal potential in mixed Na^+/Ca^{2+} solutions show that wild-type channels have no detectable Ca^{2+} permeability, in contrast to quoted values of P_{Ca}/P_{Na} as high as 0.10 (Hille, 1992). The exact mechanisms for this selectivity are not understood. By analogy to the Ca^{2+} channel, where the four glutamate residues have been shown to determine the selectivity of the channel, the homologous positions in the Na^+ channel (DEKA) have been argued to be crucial for selectivity of the Na^+ channel. These four residues are absolutely conserved among Na^+ channels of different spe-

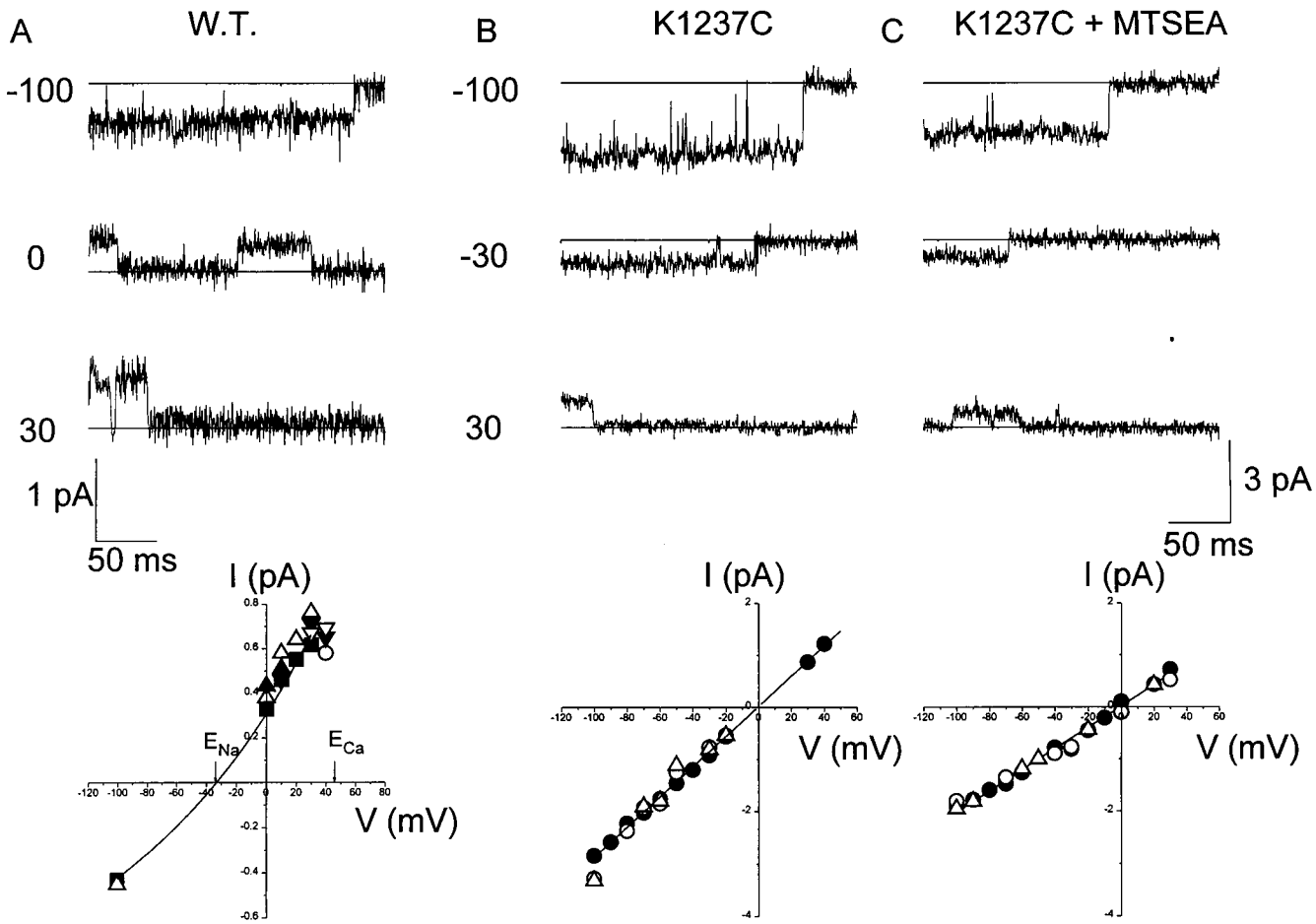

FIGURE 6 P_{Ca}/P_{Na} of the wild-type versus K1237C as calculated from the biionic experiments. (*A*) Representative single-channel currents through the wild-type channel at −100, 0, and +30 mV from a holding potential of −120 to −140 mV. Current records were obtained using excised inside-out patches with 40 mM Na^+/40 mM Ca^{2+} in the external solution and 140 mM Na^+/1 mM Ca^{2+} inside. Horizontal lines in each record represent the closed level. Single-channel current-voltage relations are shown below the current traces from four patches; each patch is represented by a different symbol. Solid lines represent the fit to the Goldman-Hodgkin-Katz equation with a calculated reversal potential of −33 mV. (*B, C*) Representative single-channel currents through K1237C mutant channel, before and after modification with MTSEA, at −100, −30, and +30 mV from a holding potential of −120 to −140 mV under the same conditions as in *A*. Single-channel current-voltage relations are shown below the current traces from different patches shown in different symbols. Modified channels (*C*) show a decrease in the single-channel current amplitude, but a reversal potential simialr to that of the unmodified channel (V_{rev} = +12 and +10 mV for unmodified and modified channels, respectively).

cies and tissues, except for the jellyfish, where there is a switch between the lysine and the glutamate residues in domains II and III (Anderson et al., 1993). These four residues do not contribute equally to the Na^+ selectivity, as is true for the four glutamate residues in the Ca^{2+} channel (Yang et al., 1993; Parent and Gopalakrishnan, 1995). In the Na^+ channel, the lysine residue plays a key role in the Na^+ versus Ca^{2+} selectivity. In addition, previous work has shown that by neutralizing this residue to alanine or reversing the positive charge to a negative one (K→E mutation), one can render the channel Ca^{2+}-permeable. The Na^+/Ca^{2+} selectivity can then be restored by mutation to arginine. However, our data would argue that the cation selectivity is not a simple function of the charge of the side chain at this position. Like Favre et al. (1996), we have shown that a fixed, negatively charged side chain at the 1237 position is not necessary for Ca^{2+} permeation. More importantly, our results demonstrate that a positively charged side chain at this position is not sufficient for Na^+ selectivity. We were not able to restore the Na^+/Ca^{2+} selectivity of the mutant K1237C channel by simply restoring the physiological positive charge using a methanethiosulfonate reagent. The results directly contradict the notion that a positive charge at the 1237 position plays a "sentry role" to exclude Ca^{2+} (Favre et al., 1996; Heginbotham, 1996). Therefore, distinct from the charge on the side chain, the chemical identity at this position is an important determinant of selectivity of the Na^+ channel.

It is important to note that charge neutralization at this position (K→C mutation) leads to a mutant channel that is different from the K→E substitution previously described by Heinemann et al. (1992). This acidic residue substitution

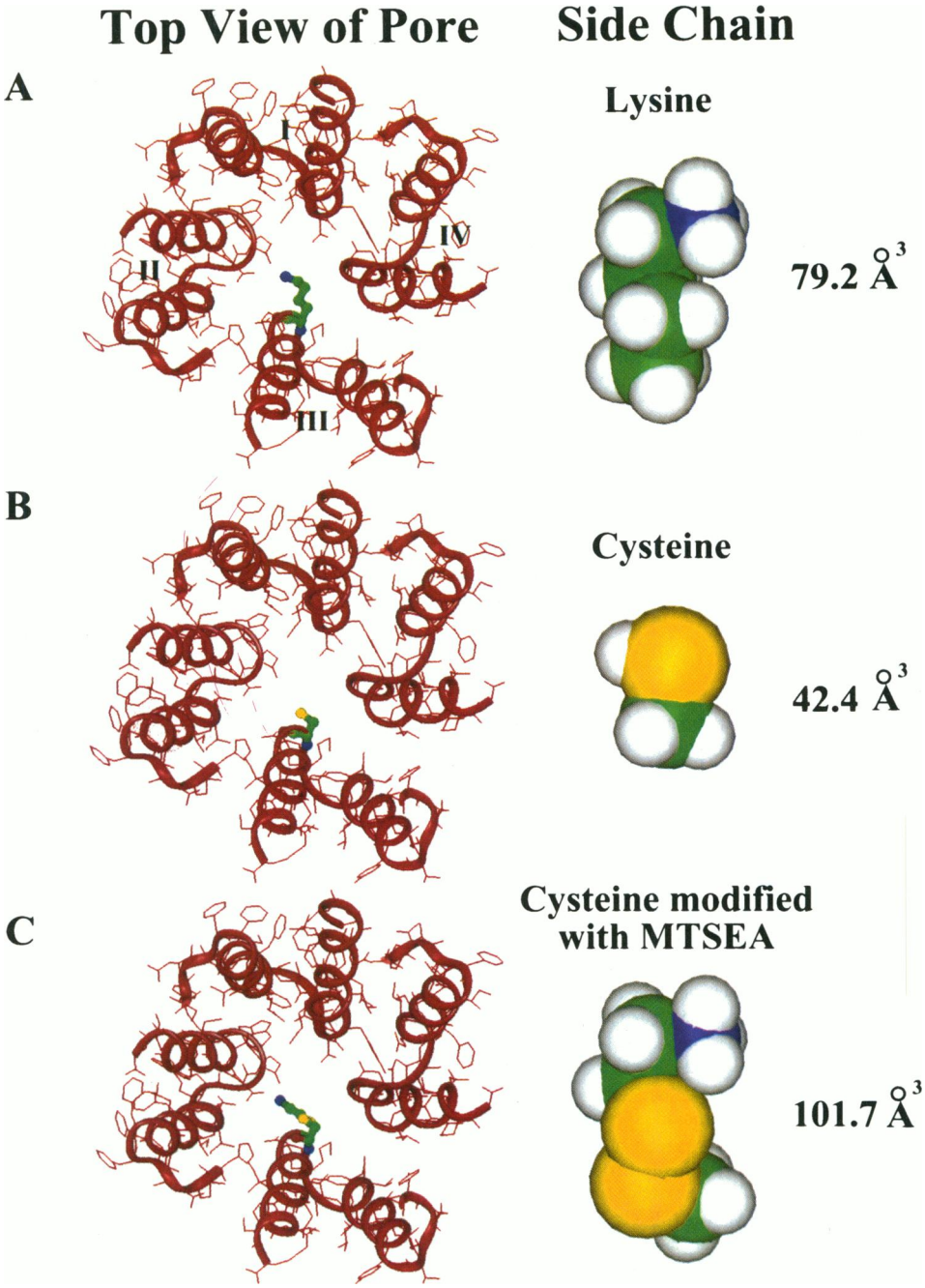

FIGURE 7 Structural model of the Na^+ channel pore and contributions of the various side chains at the 1237 position. (*A*) Top view of the structural model of the Na^+ channel pore as viewed from the extracellular surface from Chiamvimonvat et al. (1996a). The backbone structure is shown in a ribbon configuration, and the K1237 residue is shown in stick configuration. (*B, C*) The same model with the K1237 residue replaced by cysteine and cysteine modified by MTSEA, respectively. Right panels in *A, B,* and *C* show space-filling models of the side chains of amino acids lysine, cysteine, and cysteine modified by MTSEA, respectively, together with the calculated volumes in Å^3.

leads to a channel that is not only highly permeable to Ca^{2+}, but, like a Ca^{2+} channel, is also highly permeable to Ba^{2+}. In contrast, the K1237C channels are impermeable to Ba^{2+} and bind Ca^{2+} with relatively low affinity.

Evidence for multiple ion occupancy in the pore

Several observations hint that the Na^+ channel may behave like a multi-ion pore. These include voltage-dependent block by permeant and impermeant ions (French et al., 1994; Worley et al., 1992), permeability ratios that vary with the concentration of permeant ions (Ebert and Goldman, 1976; Begenisich and Cahalan, 1980), and two rising phases in the conductance-concentration relation (Ravindran et al., 1992; Naranjo and Latorre, 1993; Moczydlowski, 1993). Our data show that K1237C mutant channels also behave like multi-ion pores, with a definite but modest anomalous mole fraction effect between Na^+ and Ca^{2+} ions. These data on the mutant channel are consistent with previous results obtained in native channels (French et al., 1994).

Naturally occurring calcium-binding proteins containing cysteine residues

Several naturally occurring calcium-binding proteins have been shown to contain cysteine residues in the EF-hands, for

example, the light chains of some calpains (Miyake et al., 1986; Emori et al., 1986). Apart from neutralizing the positively charged side chain with our cysteine substitution, the introduced cysteine may help to form a specific binding site for the Ca^{2+} ions. However, this remains speculative, because the permeability of the modified cysteine mutant channel remains unchanged. In addition, the Ca^{2+}-binding affinity of the mutant channel is quite low, in contrast to that of native Ca^{2+} channels. The K1237C mutant channel also differs from native Ca^{2+} channels in being permeable only to Ca^{2+} and Sr^{2+}, but not Ba^{2+}. Among known biological proteins, the K1237C mutant Na^+ channels appear to be unique in exhibiting this particular pattern of divalent selectivity.

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