Topology of the P Segments in the Sodium Channel Pore Revealed by Cysteine Mutagenesis

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ABSTRACT The P segments of the voltage-dependent Na^+ channel line the outer mouth and selectivity filter of the pore. The residues that form the cytoplasmic mouth of the pore of the channel have not been identified. To study the structure of the inner pore mouth, the presumed selectivity filter residues (D400, E755, K1237, and A1529), and three amino acids just amino-terminal to each of these residues in the rat skeletal muscle Na^+ channel, were mutated to cysteine and expressed in tsA 201cells. These amino acids are predicted (by analogy to K^+ channels) to be on the cytoplasmic side of the putative selectivity filter residues. Inward and outward Na^+ currents were measured with the whole-cell configuration of the patch-clamp technique. Cysteinyl side-chain accessibility was gauged by sensitivity to Cd^{2+} block and by reactivity with methanethiosulfonate (MTS) reagents applied to both the inside and the outside of the cell. Outward currents through the wild-type and all of the mutant channels were unaffected by internal Cd^{2+} (100 μM). Similarly, 1 mM methanethiosulfonate ethylammonium (MTSEA) applied to the inside of the membrane did not affect wild-type or mutant outward currents. However, two mutants amino-terminal to the selectivity position in domain III (F1236C and T1235C) and one in domain IV (S1528C) were blocked with high affinity by external Cd^{2+}. The Na^+ current through F1236C and S1528C channels was inhibited by MTSEA applied to the outside of the cell. The accessibility of these mutants to externally applied cysteinyl ligands indicates that the side chains of the mutated residues face outward rather than inward. The K^+ channel model of the P segments as protein loops that span the selectivity region is not applicable to the Na^+ channel.

INTRODUCTION

Voltage-gated ion channels have the exceptional property of supporting very high rates of ion flux, and yet they do so selectively. Recent experiments have identified a number of residues in the Na^+ channel that play crucial roles in ion permeation and selectivity (Heinemann et al., 1992; Pérez-García et al., 1996, 1997; Chiamvimonvat et al., 1996). The pore of the voltage-dependent Na^+ channel is composed of P segments, one donated from each of the four internally homologous domains. The extracellular mouth of the pore has been studied extensively by using site-directed mutagenesis, with toxin or metal cation blockers as structural probes (Terlau et al., 1991; Heinemann et al., 1992; Backx et al., 1992; Satin et al., 1992; Dudley et al., 1995; Chiamvimonvat et al., 1996; Bénitah et al., 1996). Amino acid residues in the carboxy-terminal portion of the P segments line the extracellular aspect of the Na^+ channel pore. This region of the permeation pathway is composed of random coils rather than periodic structures such as α-helices or β-strands (Chiamvimonvat et al., 1996; Bénitah et al., 1996; Marban and Tomaselli, 1997).

In contrast to the wealth of information regarding the extracellular aspect of the pore, little is known about the residues that form the cytoplasmic end of the Na^+ channel pore. In K^+ channels, residues on the amino- and carboxy-terminal sides of the P segments (known as SS1 and SS2, respectively) form parts of the extracellular pore region (Fig. 1 *B*). Amino acids between SS1 and SS2 face intracellularly (MacKinnon and Yellen, 1990; Yellen et al., 1991; Hartmann et al., 1991; Yool and Schwarz, 1991; Kirsch et al., 1992; Lü and Miller, 1995; Kürz et al., 1995; Pascual et al., 1995). Based on homology with a ring of glutamic acids in the Ca^{2+} channel and mutagenesis experiments (Heinemann et al., 1992), the sequence-aligned residues in the Na^+ channel (D400, E755, K1237, and A1529; DEKA) have been suggested to compose the selectivity filter. Contemporary models of the Na^+ channel pore predict that the DEKA selectivity filter residues occupy a turn between two α-helices (Guy and Durell, 1995) or β-strands (Lipkind and Fozzard, 1994); this implies an alternating pattern of accessibility of side chains to the intracellular and extracellular surfaces of the channel.

To determine whether the topological arrangement of the P segments in the Na^+ channel is similar to that in K^+ channels, we combined serial cysteine mutagenesis and electrophysiological recording. In each of the four P segments of the Na^+ channel, the DEKA amino acids and three residues amino-terminal to them were replaced with cysteine (Fig. 1). We then determined the sensitivity of the mutant channels to block by Cd^{2+} and methanethiosulfonate (MTS) reagents applied intra- and extracellularly. No cysteine mutant was susceptible to internal Cd^{2+} block or modification by internally applied MTS reagents. In contrast, two cysteine mutants in domain III and one in domain IV amino terminal to the DEKA ring exhibited enhanced

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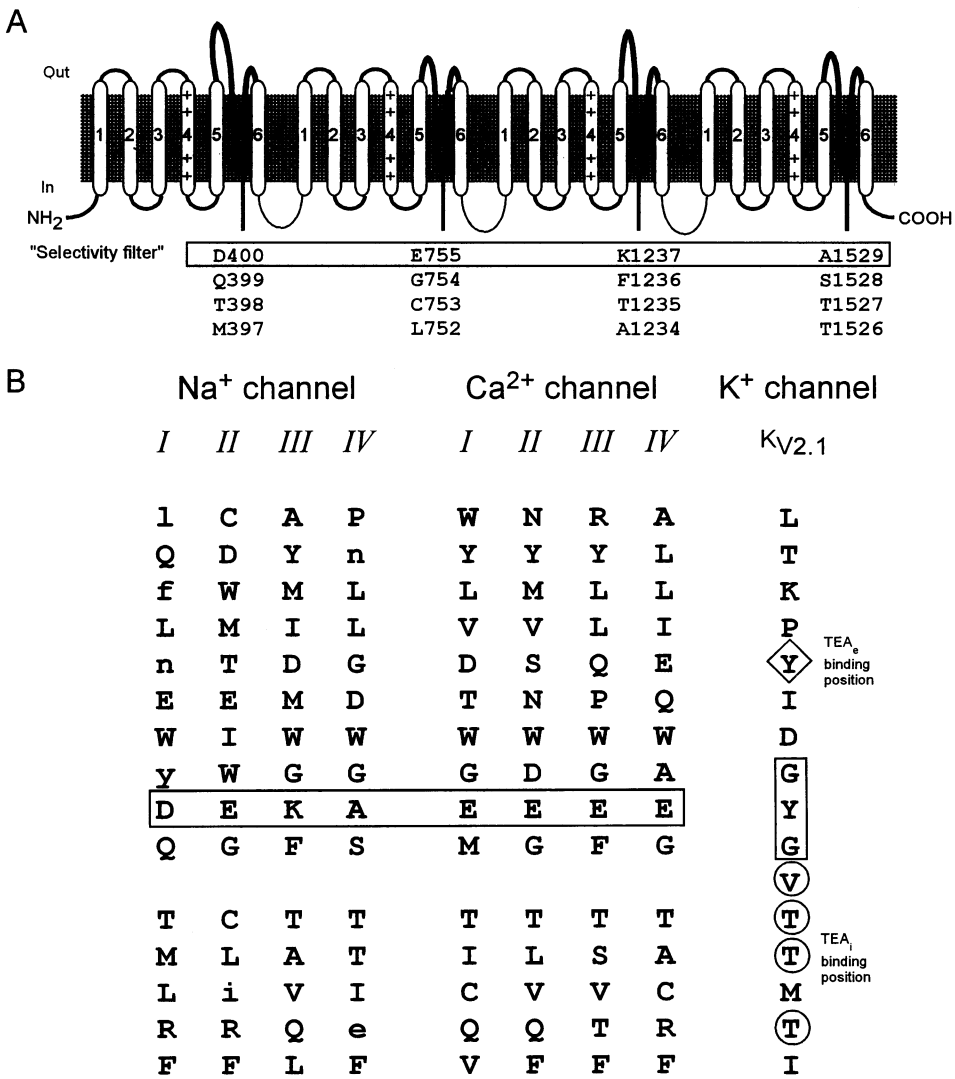

FIGURE 1 (*A*) Transmembrane topology of the Na^+ channel. The P segments are the regions between the fifth and sixth membrane-spanning repeats in each domain (*bold line*). The approximate position of the residues that were mutated are enclosed in the box and shown below the topology cartoon. The putative selectivity residues D400, E755, K1237, and A1529 are shown for reference. (*B*) Single-letter amino acid alignment of the residues in the P segments of the rat skeletal muscle μ1 Na^+ channel (Trimmer et al., 1989) are anchored by the DEKA selectivity filter (*enclosed in the box*). The uppercase letters in the Na^+ and Ca^{2+} channel sequences signify conservation of the residue across all channel isoforms. The Na^+ channel P segments show sequence homology to the Ca^{2+} channel (Tanabe et al., 1987), but little to the K^+ channel (Frech et al., 1989). The residues enclosed in the rectangle are the K^+ channel signature sequence; those enclosed in circles are accessible to MTSET from the intracellular side of the channel (Pascual et al., 1995), and the tyrosine (Y) inside the diamond and the threonine (T) in the circle are the extracellular and intracellular TEA binding sites, respectively (MacKinnon and Yellen, 1990).

sensitivity to block by externally applied Cd^{2+}. Two of these three mutants could be modified by methanethiosulfonate ethylammonium (MTSEA) applied to the outside of the cell. These data suggest that the third and fourth domain P segments have ascending and descending limbs in the extracellular pore, but in contrast to the topology of the K^+ channel, no residues are accessible to internally applied blockers.

MATERIALS AND METHODS

Mutagenesis and channel expression

An *Eco*RI fragment containing the entire coding region of the wild-type μ1 Na^+ channel cDNA (Trimmer et al., 1989) was cloned into the mammalian expression vector pGW1H (BBL, UK). The rat brain β1 subunit (Isom et al., 1992) was cloned into pCMV-5 (Phillipson et al., 1993). A plasmid containing the green fluorescent protein (GFP) (pGreenLantern; Gibco BRL, Bethesda, MD) was used as a transfection reporter.

Cysteine substitutions were introduced into the rat μ1 skeletal muscle channel α-subunit by oligonucleotide-directed mutagenesis as previously described (Pérez-García et al., 1996) or by polymerase chain reaction with overlapping mutagenic primers (Quickchange site-directed mutagenesis kit; Stratagene, La Jolla, CA). All mutations were performed in duplicate and confirmed by DNA sequencing.

TsA-201 cells, a transformed human embryonic kidney cell line (HEK 293) stably expressing the SV40 T-antigen, were used for channel expression. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco BRL), 1% penicillin/streptomycin (Gibco BRL), and 300 μg/ml geneticin (G418) at 37°C and 5% CO_2. The cells were trypsinized, plated at a concentration of 1.5×10^5 cells/ml in 2 ml of culture medium in 35-mm dishes, and transfected with a combination of of 2.5 μg of the wild-type or mutant α-subunit, 1 μg of the β1-subunit and 0.3 μg of the GFP cDNA-containing plasmids per dish. Transfection was performed by the calcium phosphate precipitation method for 6–12 h as previously described (Pérez-García et al., 1995). The cells were rinsed in fresh culture medium and incubated for 1–3 days before patch-clamp recording.

Electrophysiology and data analysis

Cells were transferred to the stage of an inverted microscope and superfused with external solution at a rate of 1–2 ml/min. Transfected cells were identified by epifluorescence microscopy and voltage-clamped using either the whole-cell or cell-attached configurations of the patch-clamp technique

(Hamill et al., 1981). All experiments were performed at room temperature (22–23°C). Patch electrodes were pulled from borosilicate glass and had 2–5 MΩ tip resistances for whole-cell recordings and 12–15 MΩ tip resistances for single-channel recordings. Cell-attached patches were formed with seal resistances of 20–100 GΩ. Currents were recorded by using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) interfaced to a personal computer. Voltage was controlled and data were collected with custom-written software. Cell capacitance was calculated by integrating the area under an uncompensated capacity transient elicited by a 20-mV hyperpolarizing test pulse from a holding potential of −80 mV. The tsA-201 cells had capacitances of 15 ± 1 pF (n = 24). Series resistance was then compensated as much as possible without ringing, typically 70–90%. Given the average series resistance of our electrodes, the maximum uncompensated voltage error was <|6 mV| for the largest currents studied.

Whole-cell currents were studied with a normal transmembrane Na^+ gradient, in symmetrical Na^+, or with the gradient reversed. The default pipette solution was (in mM): 35 NaCl, 105 CsF, 1 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.2; in some experiments, the intracellular Na^+ concentration was increased by an equimolar substitution of CsF with NaCl. The bath solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4. Cd^{2+} and MTS reagents were applied intracellularly by addition to the pipette solution at the designated concentration. When Cd^{2+} was added, EGTA was omitted from the pipette solution. In some experiments QX314 (Alomone Labs, Israel) was added to the pipette solution at a concentration of 250 μM to verify the adequacy of internal dialysis.

Dose-response curves for block of the mutant channels by extracellular Cd^{2+} were determined by adding the chloride salt of the blocker to the bath at concentrations between 1 μM and 5 mM, depending on the sensitivity of the particular mutant. Full current-voltage relationships were determined at each Cd^{2+} concentration. The half-blocking concentration (IC_{50}) was determined by a least-squares fit (Levenberg-Marquardt algorithm) of the data to the function $I/I_o = 1/\{1 + ([\text{blocker}]/IC_{50})^n\}$, where I and I_o are, respectively, the currents in the presence and absence of blocker.

Susceptibilities to extracellular MTSEA, methanethiosulfonate ethyltrimethylammonium (MTSET), and methanethiosulfonate ethylsulfonate (MTSES) (Toronto Research Chemicals, Toronto, Canada; Akabas et al., 1992; Stauffer and Karlin, 1994) were determined by bath application of saturating concentrations of each reagent (MTSEA, 2.5 mM; MTSET, 1 mM; MTSES, 10 mM). MTS modification of the current was verified by the irreversibility of the reduction in the current.

In mutant channels with enhanced sensitivity to block by Cd^{2+} at the whole-cell level, we measured single-channel currents in the cell-attached configuration (Hamill et al., 1981). The currents were sampled at 10 kHz and low-pass filtered at 2 kHz. The pipette solution contained (in mM): 140 NaCl, 10 HEPES, pH 7.4, 500 μM Cd^{2+} for T1235C and F1236C, and 800 μM Cd^{2+} for S1528C. The bath contained (in mM): 140 KCl, 1 $BaCl_2$, 10 HEPES, pH 7.4, and 20 μM fenvalerate (Holloway et al., 1989; DuPont, Wilmington, DE) to facilitate the analysis of open-channel blockade by Cd^{2+} (Backx et al., 1992; Bénitah et al., 1997). Well-resolved single-channel opening events were determined using a half-height criterion (Colquhoun and Sigworth, 1983). Amplitude histograms were fitted to the sum of Gaussians by using a nonlinear least-squares method. The voltage dependence of Cd^{2+} blockade of the pore was used to estimate the fractional electrical distance to the substituted cysteine residue as previously described (Woodhull, 1973; Hille, 1992; Backx et al., 1992; Chiamimonvat et al., 1996). The average current during long openings in the presence of Cd^{2+} was used to quantify the blocked unitary current amplitude.

Pooled data are presented as means ± SEM. Statistical comparisons were made by using analysis of variance (ANOVA), with $p < 0.05$ considered to be significant.

RESULTS

Accessibility of cysteine mutants to internal Cd^{2+} and MTS reagents

The schematic of the Na^+ channel α-subunit (Fig. 1 *A*) designates the residues substituted in each domain with reference to their predicted locations based on analogy to K^+ channels. Alignment of homologous positions in Na^+, Ca^{2+}, and K^+ channels is shown in Fig. 1 *B*. The GYG enclosed in the box is part of the K^+ channel signature sequence, the residues enclosed in circles are accessible to internal MTSET (Pascual et al., 1995), and the tyrosine enclosed in the diamond influences extracellular block by tetraethylammonium (TEA) (MacKinnon and Yellen, 1990). Amino acids predicted to form the selectivity filter based on positional homology with a ring of glutamates in the Ca^{2+} channel (D400, E755, K1237 and A1529) are highlighted. Mutation of the native cysteine at position 753 in domain II to glycine resulted in no current expression; substitution by serine produced small currents. However, the mutant C753A generated robust Na^+ currents with wild-type permeation and Cd^{2+} block properties. The other two positions in domain II, L752 and G754, did not express functional channels when substituted with cysteine, in either the wild-type or C753A backgrounds. Addition of dithiothreitol (DTT) to the intracellular or extracellular solution did not reveal any current that might have been obscured by the formation of an intramolecular disulfide bond (Bénitah et al., 1996). All other cysteine substitutions were performed on the wild-type background.

We assessed the sensitivity of the cysteine mutants to internal Cd^{2+} by dialyzing the cell with Cd^{2+}-containing pipette solutions. Wild-type channels show no significant reduction in the peak inward or outward current after 5 min of cell dialysis with a pipette solution containing 100 μM Cd^{2+} (Fig. 2 *A*). The insets (Fig. 2 *Aa–d*) show representative current records elicited by depolarizing voltage steps to −20 and +40 mV immediately and 5 min after break-in. The small reduction in peak current amplitude observed at +40 mV is the result of channel rundown and was observed without Cd^{2+} in the pipette solution. Fig. 2 *B* shows the current-voltage relationships recorded in cells with and without Cd^{2+} in the pipette 5 min after establishing the whole-cell configuration. The currents were normalized to the peak inward current; the outward currents are superimposable in the presence and absence of intracellular Cd^{2+} at test voltages more positive than −20 mV. A representative cysteine mutant in domain I, Q399C, exhibited a similar phenotype when Cd^{2+} was added to the pipette solution (Fig. 2 *Ce–h*). The normalized current-voltage relationship in symmetrical Na^+ with and without Cd^{2+} in the pipette revealed no significant change in outward current up to test potentials of +80 mV (Fig. 2 *D*). The percentage reduction of outward current at +40 mV after 5 min of dialysis with a Cd^{2+}-containing pipette solution is shown in Table 1; there is no significant difference in the reduction of the outward current through the wild-type or any of the mutant channels, including cysteine substitutions at the DEKA ring.

It is possible that internal Cd^{2+} does bind to free cysteinyl side chains introduced by the substitution mutations, but that Cd^{2+} binding has no effect on permeation. We therefore attempted to modify the substituted thiol side chains with hydrophilic, sulfhydryl-specific MTS reagents; if a

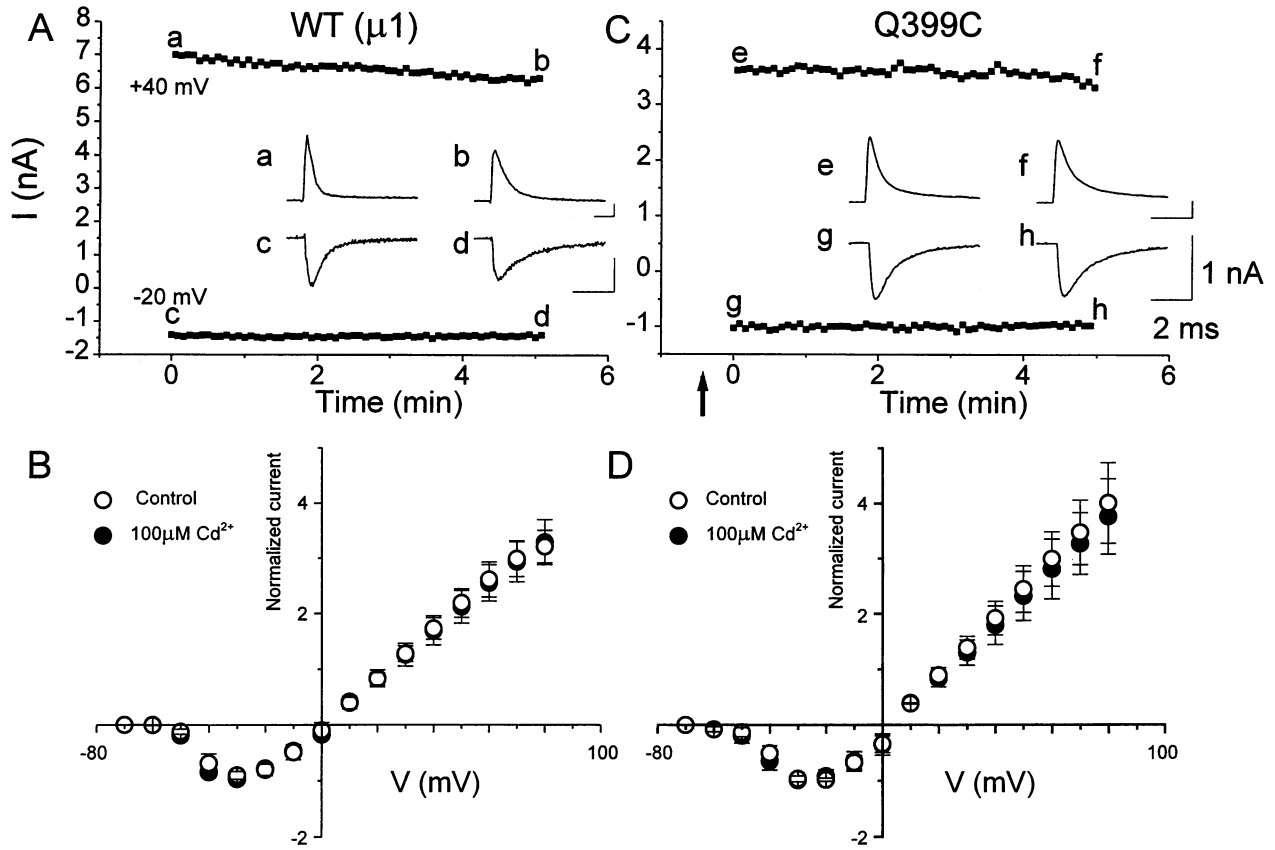

FIGURE 2 Internal Cd^{2+} has no effect on P segment mutants. (*A*) The time course of the peak current elicited by voltage steps from −100 mV to −20 and +40 mV after establishment of the whole-cell patch configuration (time 0) in the wild-type channel with 100 μM Cd^{2+} in the pipette, and symmetrical 140 mM Na^+ across the membrane. Representative current traces at time 0 (break-in; *a* and *c*) and after 5 min of dialysis (*b* and *d*). (*B*) Whole-cell current-voltage relationship normalized to the peak inward current in five cells with and four cells without Cd^{2+} in the pipette. The current-voltage relationships were recorded 5 min after break-in. (*C*) Time course of the peak current change for the Q399C mutant channel after cell dialysis with 100 μM Cd^{2+} in the pipette. The currents in the inset were recorded immediately after break-in (*e* and *g*) and at 5 min of dialysis (*f* and *h*). (*D*) Normalized current-voltage relationship as in *B*, from three cells with and without 100 μM Cd^{2+} in the pipette. The current-voltage relationships were recorded 5 min after break-in.

bulky mixed disulfide is formed in the inner mouth of the pore, there is good reason to expect a change in conductance (Pascual et al., 1995). Fig. 3 shows the peak current through the F1236C mutant elicited by depolarizing voltage steps to −20 and +60 mV after dialysis with 1 mM MTSEA in the pipette. A slow rundown of current is evident at the +60 mV test potential, similar to that observed in the wild-type channel. For all of the cysteine mutants, the percentage reduction of outward current at +40 mV after 5 min of dialysis with MTSEA in the pipette solution was not significantly different from that due solely to channel rundown (Table 1).

The application of Cd^{2+} or MTS reagents to the cytoplasmic face of the channel in the whole-cell configuration requires adequate cell dialysis. A hyperpolarizing shift in channel gating is routinely observed and implies that the cell contents are being adequately exchanged by the pipette solution (Fenwick et al., 1982). However, we assessed the dialysis of the cellular contents explicitly by adding the permanently charged quaternary lidocaine derivative QX314 (250 μM) to the pipette solution. QX314 is membrane impermeant and blocks the Na^+ channel from the cytoplasmic surface (Strichartz, 1973; Gingrich et al., 1993). Fig. 4 *A* demonstrates the development of QX314 block of the wild-type channel during voltage pulses from −100 mV to −20 and +40 mV in symmetrical 140 mM Na^+. The reduction in both inward and outward current begins immediately after patch rupture and reaches steady state within 2 min (Fig. 4 *A*). Block of the current by QX314 was voltage dependent, with more potent block at positive potentials. This is further demonstrated in the average current-voltage relationships (Fig. 4 *B*). QX314 and MTSEA are similar in size and charge; Cd^{2+} is substantially smaller than QX314 and, if anything, should diffuse more rapidly.

The MTS reagents are labile and their activity can be modified by the oxidation state of reaction environment. We examined the effect of internally applied MTSET on a cysteine substitution in the cytoplasmic S4-S5 linker in domain IV of the Na^+ channel (Filatov et al., 1997; Lerche et al., 1997). This mutation, F1466C, exhibited no change from the wild-type control in the baseline state, but the addition of MTSET increased the time constant of current

TABLE 1 No effects of internal Cd^{2+} or internal MTSEA on WT and P segment mutants

	n	Remaining currents at +40 mV after 5 min of break-in (%)
Control		
μ1 (WT)	4	83 ± 7
Internal 100 μM Cd^{2+}		
μ1 (WT)	6	81 ± 8
D400C	3	75 ± 12
Q399C	3	94 ± 4
T398C	4	82 ± 6
M397C	3	92 ± 7
E755C	3	78 ± 11
K1237C	3	84 ± 8
F1236C	3	87 ± 11
T1235C	3	86 ± 11
A1234C	3	82 ± 10
A1529C	3	78 ± 11
S1528C	5	79 ± 10
T1527C	3	91 ± 4
T1526C	4	91 ± 4
Internal 1 mM MTSEA		
μ1 (WT)	7	81 ± 5
D400C	3	75 ± 9
Q399C	3	86 ± 13
T398C	4	80 ± 11
M397C	3	79 ± 3
E755C	3	82 ± 9
K1237C	3	89 ± 8
F1236C	3	86 ± 8
T1235C	5	72 ± 6
A1234C	3	78 ± 6
A1529C	3	83 ± 7
S1528C	5	76 ± 9
T1527C	5	72 ± 4
T1526C	3	76 ± 14

decay in the mutant but not the wild-type channel (τh F1466C 1.33 ± 0.03 ms, τh F1466C + 1 mM MTSET 2.87 ± 0.08 ms, $n = 3$, $p = 0.001$). These results verify that the MTS reagents retain activity when applied intracellularly under our recording conditions, and attest to the adequacy of cellular dialysis. Thus the cells are quickly and adequately dialyzed under our recording conditions. These data suggest that unlike the "homologous" K^+ channel residues, amino acids predicted to be just inside the selectivity filter of the Na^+ channel are not accessible from the intracellular face of the membrane.

Accessibility of cysteine mutants to external Cd^{2+} and MTS reagents

The accessibility of residues on both sides of the K^+ channel signature sequence to extracellular blockers requires that the P segments dip into and exit from the same side of the membrane (MacKinnon and Yellen, 1990; Yellen et al., 1991; Hartmann et al., 1991; Kirsch et al., 1992; Lü and Miller, 1995; Kürz et al., 1995; Pascual et al., 1995). If the P segments of the Na^+ channel reverse direction in the membrane, we predict that some residues on the amino-

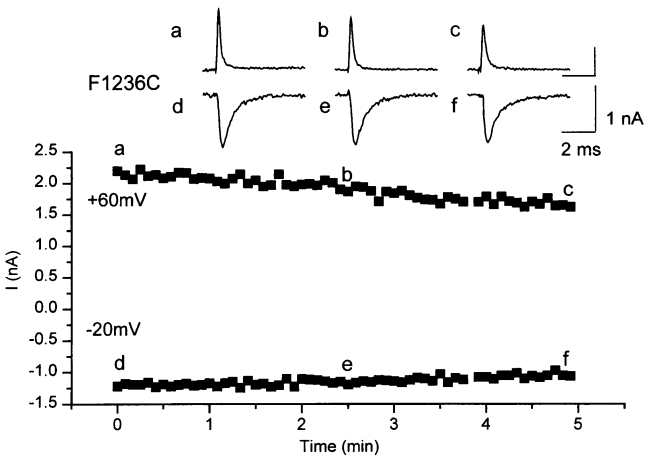

FIGURE 3 Internal MTSEA does not modify the current through the P segment mutants. The time course of the peak current through the F1236C mutant elicited by voltage steps from −100 mV to −20 and +60 mV after establishment of the whole-cell patch configuration (time 0) with 1 mM MTSEA in the pipette. The currents labeled *a–f* were acquired at the time of break-in (*a*, *d*) and at 2.5 (*b*, *e*) and 5 (*c*, *f*) min of cell dialysis. There is no change in the current amplitude after cell dialysis; the small reduction observed at the +60 mV test potential is the result of rundown.

terminal side of the DEKA ring should be accessible from the extracellular surface of the channel. We therefore examined the accessibility of the cysteine mutants listed in Table 1 to externally applied Ca^{2+} and MTS. Na^+ current through the F1236C mutant channel was more than 60% inhibited by 100 μM external Cd^{2+}; the effect was reversible (Fig. 5 *Aa–c*). Similarly, 2.5 mM MTSEA applied to the outside of the cell rapidly reduced the current elicited by depolarizing voltage steps to −20 mV (Fig. 5 *Ba–c*). Unlike Cd^{2+} block, MTS modification is irreversible upon washout, consistent with previous observations on other mutants (Pérez-García et al., 1996; Chiamvimonvat et al., 1996). In contrast, the wild-type channel and most of the mutants "internal" to the DEKA ring did not exhibit high-affinity block by bath-applied Cd^{2+} or modification by MTS reagents. Nevertheless, one other third-domain (T1235C) and one fourth-domain (S1528C) substitution could be potently blocked by external Cd^{2+} (Fig. 6 *A*; IC_{50}: T1235C 97 ± 36 μM, F1236C 43 ± 12 μM, S1528C 173 ± 25 μM, all $p <$ 0.05 versus wild-type). Two of these mutants, F1236C and S1528C, were 49 ± 15% and 80 ± 6% ($p < 0.05$) inhibited by 2.5 mM MTSEA (Fig. 6 *B*). The larger MTSET and the negatively charged MTSES had no effect on either F1236C (MTSET 1 ± 1% [$n = 4$]; MTSES 4 ± 5% [$n = 3$]) or S1528C (MTSET 1 ± 1% [$n = 4$]; MTSES 6 ± 2% [$n = 4$]). T1235C was not modified by any of the MTS reagents. This size and charge exclusion pattern resembles that previously reported for the DEKA mutants K1237C and A1529C (Chiamvimonvat et al., 1996).

The cysteine substitution mutants T1235C, F1236C, and S1528C were accessible from the extracellular surface of the channel by group IIB divalent cation block and/or covalent modification by MTS reagents. We therefore examined the voltage dependence of blockade of the unitary

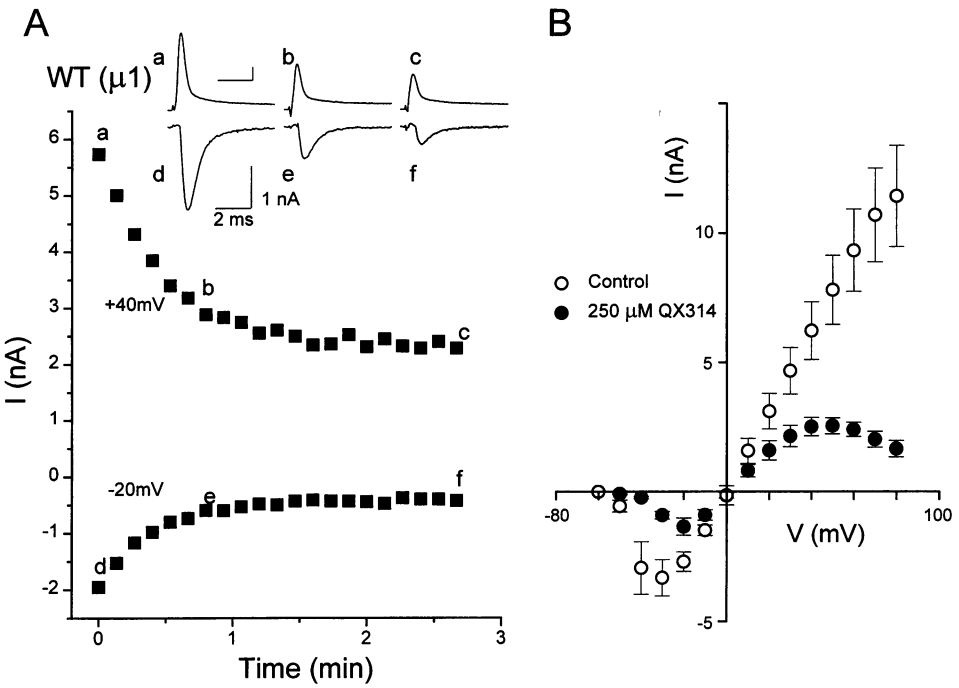

FIGURE 4 QX314 blocks the μ1 channel from the cytoplasmic surface. (*A*) The peak currents elicited by test potentials to +40 and −20 mV from a holding potential of −100 mV promptly decrease with cell dialysis by a pipette solution containing 250 μM QX314. The currents at break-in (time 0; *a*, *d*) after 45 s (*b*, *e*) and after 160 s (*c*, *f*) of cell dialysis. (*B*) The current-voltage relationship from four cells perfused with QX314 and five cells without. There is a voltage-dependent reduction of current in the presence of intracellular QX314. The estimated fractional electrical distance for QX314 block is 61% of the electrical field from the inside.

current by external Cd^{2+} to determine the relative locations of the substituted cysteines in the pore. Fig. 7 *A* shows unitary Na^+ currents for each of the three Cd^{2+}-sensitive mutants, in the presence and absence of Cd^{2+}. The block is too rapid to be resolved as discrete blocking events and appears as a reduction in the single-channel current amplitude. Fig. 7 *B* (*top*) shows the averaged single-channel current through each mutant in the presence and absence of Cd^{2+}, over the entire voltage range studied. In each case, the reduction in unitary current is more prominent at negative voltages. By plotting the ratio of the blocked and unblocked unitary currents and assuming a single-site model for Cd^{2+} binding (Woodhull, 1973; Backx et al., 1992), we estimated the fraction of the voltage field traversed by Cd^{2+} to reach its binding site (Fig. 7 *B*, *bottom*). The sequence-aligned positions in the third and fourth repeats (F1236C and S1528C) are blocked with a steeper voltage dependence than T1235C; the Cd^{2+}-binding sites of F1236C and S1528C are deeper in the transmembrane electrical field than that of T1235C. The Cd^{2+} block of T1235C predicts that this position is further out of the pore, yet this mutant is not modified by MTS.

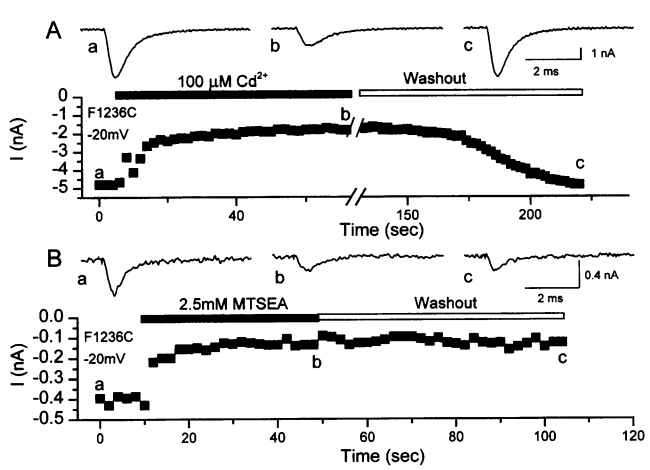

FIGURE 5 Effects of external Cd^{2+} and MTSEA on F1236C. (*A*) The current through F1236C is reversibly inhibited by 100 μM Cd^{2+} in the bath solution. Current records elicited by voltage steps from −100 to −20 mV before (*a*), during (*b*), and after (*c*) perfusion with 100 μM Cd^{2+}. (*B*) Time course of inhibition of F1236C current by bath application of 2.5 mM MTSEA. The current is irreversibly inhibited by MTS modification. The currents labeled *a*–*c* were elicited by depolarizing voltage steps to −20 mV before (*a*), during (*b*), and after (*c*) perfusion with an MTS-containing solution.

The single-channel conductances of the Cd^{2+}-sensitive mutants in the cell-attached configuration differed from the wild-type conductance (50 ± 0.3 pS in 140 mM $[Na^+]_o$) by less than 20%. Multiple residues in the domain IV P segment and K1237 in domain III influence the cation selectivity of the Na^+ channel (Chiamvimonvat et al., 1996; Favre et al., 1996; Pérez-García et al., 1997). We tested whether the mutants T1235C, F1236C, and S1528C altered the selectivity of the channel. We compared the whole-cell conductances in external solutions of different monovalent and divalent cation composition to the Na^+ conductance of T1235C, F1236C, and S1528C (Fig. 8). The selectivity pattern of all three mutations was similar to that of the wild-type channel recorded under identical conditions. The channels are comparably permeable to Li^+ (G_{Li}/G_{Na}: T1235C 0.72 ± 0.03, F1236C 0.89 ± 0.03, S1528C 0.73 ± 0.06, wild-type 0.83 ± 0.01), conduct small NH_4^+ currents (G_{NH4}/G_{Na}: T1235C 0.10 ± 0.03, F1236C 0.17 ± 0.06, S1528C 0.12 ± 0.05, wild-type 0.07 ± 0.01), and remain impermeable to K^+ and divalent cations. By comparison,

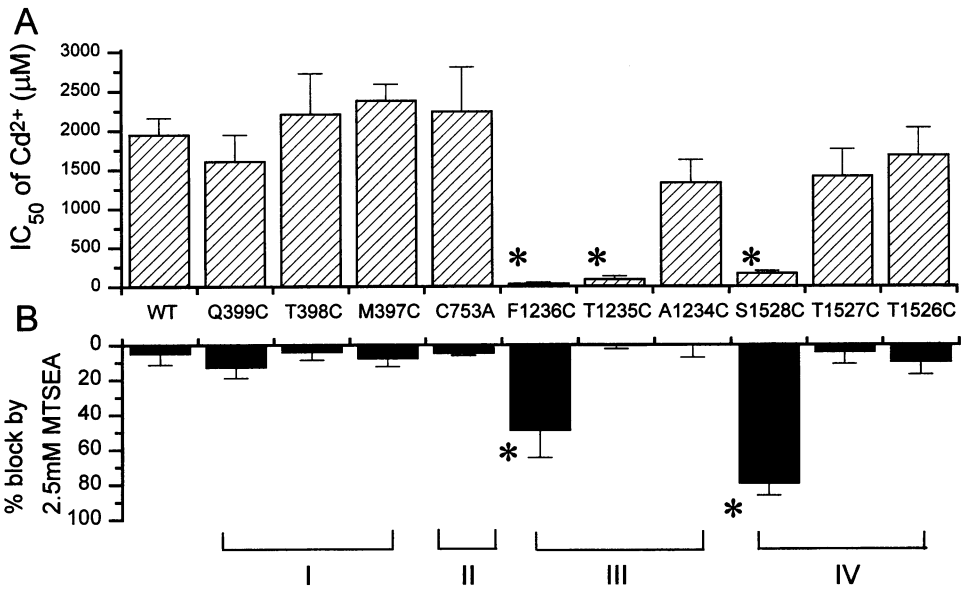

FIGURE 6 The effect of external Cd^{2+} and MTSEA on the cysteine substitution mutants. (*A*) Bar plot of the IC_{50}s for each of the mutants that express current. All but three of the cysteine substitution mutants and the cysteine neutralization (C753A) have an IC_{50} that is not significantly different from the wild-type channel. The mutants F1236C, T1235C, and S1528C have IC_{50}s of 43 ± 12, 97 ± 36, and 173 ± 25 μM ($p < 0.05$), respectively. (*B*) The percentage inhibition of the peak inward current by bath-applied 2.5 mM MTSEA. F1236C and S1528C currents are significantly reduced by modification with MTSEA.

the domain III DEKA mutant K1237C dramatically alters selectivity, supporting current carried by several monovalent and divalent cations (Chiamvimonvat et al., 1996; Favre et al., 1996; Pérez-García et al., 1997).

DISCUSSION

We combined electrophysiological recording with cysteine mutagenesis to explore the structure of the pore of the Na^+ channel. The unique reactivity of the thiol side chain of cysteine facilitates the formation of mixed disulfides with MTS-modifying reagents and the generation of binding sites for group IIB divalent cations, such as Cd^{2+}. The role of the P segments in the formation of the Na^+ channel pore contrasts significantly with that of K^+ channels. We have previously shown that cysteine mutants on the carboxy-terminal side of the DEKA "selectivity" ring are blocked by external Cd^{2+} with high affinity and are modified by extracellular MTS reagents (Chiamvimonvat et al., 1996; Pérez-García et al., 1995). The goal of this study was to determine the accessibility pattern of cysteine substitution mutants on the amino-terminal side of the DEKA ring.

Cysteine mutants are not accessible from the cytoplasmic face of the channel

The DEKA sites (Heinemann et al., 1992) and three residues immediately amino-terminal to each were substituted with cysteine (Fig. 1). In K^+ channels, residues on the amino- and carboxy-terminal sides of the GYG signature sequence affect block by tetraethylammonium added to the intracellular and extracellular surfaces of the channel, respectively (MacKinnon and Yellen, 1990; Yellen et al., 1991). In contrast, neither the wild-type skeletal muscle Na^+ channel nor any of the mutants studied were blocked by Cd^{2+} or MTS reagents added to the cytoplasmic side of the channel. Possible explanations for the absence of an effect of these blockers include side chains being buried in the protein; binding of Cd^{2+} or modification by MTS failing to alter the ion flux through the channel; or the P segments not fully spanning the selectivity region of the channel. Prolongation of the time constant of decay of current through the internal mutant F1466C demonstrates the ability to modify an intracellularly accessible cysteine with a functional effect on the current under our recording conditions. The observation that cysteine mutants on the amino-terminal side of the DEKA residues in domains III and IV are externally accessible suggests that these P segments descend and then ascend in the pore, but do not span the selectivity region.

In the case of the domain II P segment, a native cysteine at position 753 does not impart sensitivity to Cd^{2+} or MTS reagents applied from either the inside or outside of the channel. Replacement of C753 with alanine or serine does not change the blocking or permeation phenotype of the channel. Mutants at the other positions, L752C and G754C, did not express, so limited conclusions can be made about the transmembrane topology of the domain II P segments. However, cysteine substitution of residues in the P segments of domains I, III, and IV on the amino-terminal side of the DEKA ring are not altered by internally applied Cd^{2+} or MTS reagents.

External accessibility of cysteine mutants

If there is any similarity between the transmembrane dispositions of the P segments in Na^+ and K^+ channels, then residues on both sides of the selectivity regions should be accessible to extracellular blockers (MacKinnon and Yellen, 1990; Yellen et al., 1991; Hartmann et al., 1991). We have previously shown that residues that are on the carboxyl side of the DEKA ring form the outer mouth of the

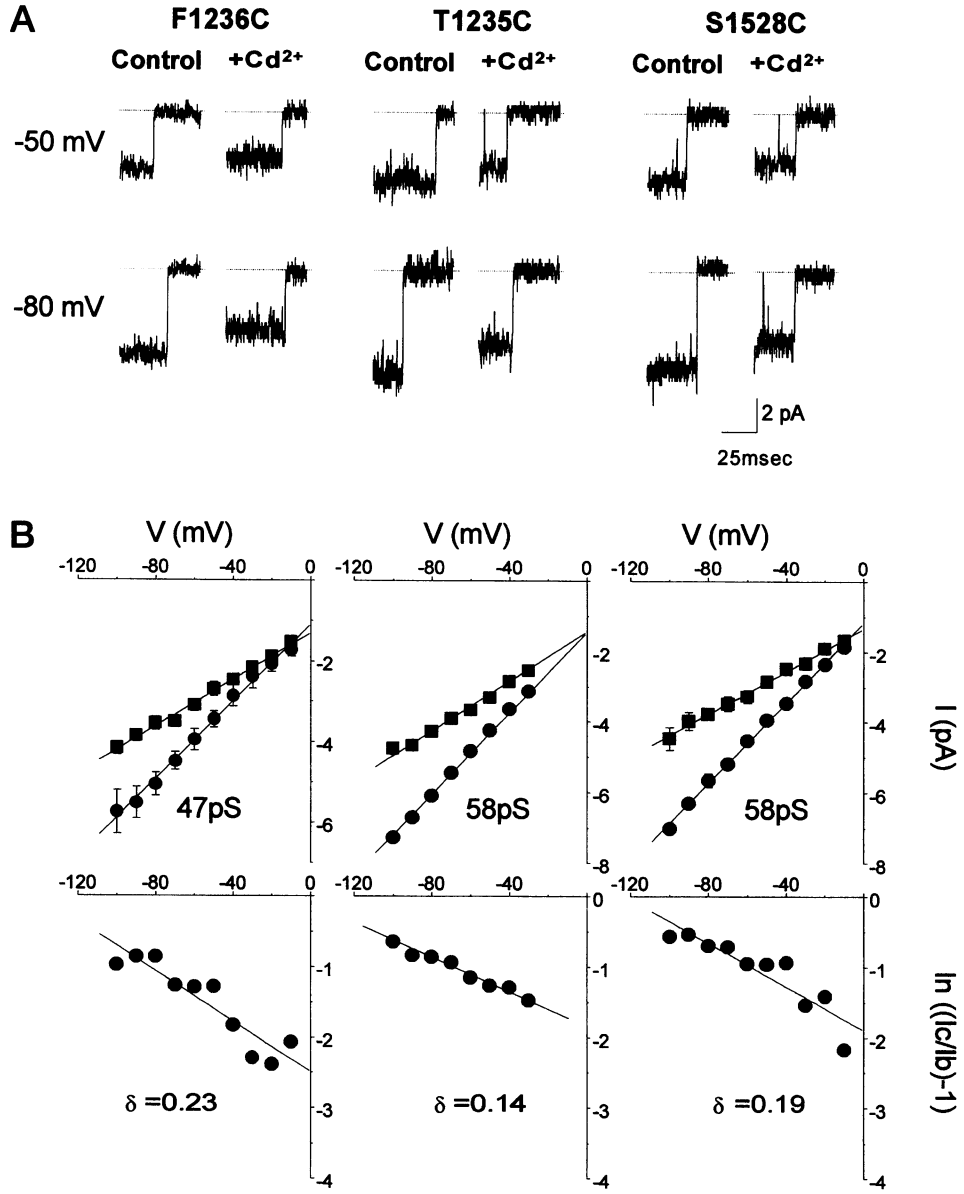

FIGURE 7 Single-channel Cd^{2+} block of cysteine mutants. (*A*) Representative single-channel currents after modification by 20 μM fenvalerate, elicited by voltage steps to −50 and −80 mV with 140 mM Na^+ in the pipette. The single-channel current is reduced in the presence of 500 μM (F1236C and T1235C) and 800 μM (S1528C) Cd^{2+}. (*B*) Single-channel current-voltage relationships for each of the mutant channels determined from at least three patches in the absence of Cd^{2+}, the slope conductances are within 20% of that of the wild-type channel (*top*). Cd^{2+} blocks each of the mutants in a voltage-dependent fashion. A plot of the logarithm of the ratio of the blocked and unblocked single-channel current amplitude versus voltage gives the fractional electrical distance (δ) for Cd^{2+} binding (*bottom*).

Na^+ channel (Chiamvimonvat et al., 1996; Pérez-García et al., 1996). Substituted cysteinyls on the amino-terminal sides of the ring in domains III and IV are also accessible from the extracellular side of the channel. The mutants T1235C, F1236C, and S1528C are blocked by extracellular Cd^{2+} with micromolar affinity. F1236C and S1528C are modified by MTSEA, resulting in reduction in Na^+ flux and reduced inward current. The larger (MTSET) and negatively charged (MTSES) reagents do not modify F1236C and T1528C, suggesting both size and charge exclusion from this region of the pore.

Single-channel recordings and analysis of Cd^{2+} block of the mutants in domains III and IV support the concept that these residues form the descending limb of a pore-forming hairpin loop. Fig. 9 summarizes the fractional electrical distances for Cd^{2+} block of the cysteine mutants in this study (T1235C, F1236C, and S1528C), and other previously reported cysteine mutants (Chiamvimonvat et al., 1996). The cysteinyls of the two mutations that can be modified by MTSEA are ~20% across the electrical field from the outside. Surprisingly, the mutant blocked by Cd^{2+} with a shallower fractional electrical distance, T1235C, is not modified by any MTS reagent. It is possible that a mixed disulfide is actually formed between MTSEA and the target cysteinyl, but Na^+ conductance is not changed; alternatively, the site created by this mutation could accommodate Cd^{2+}, but not the larger MTS reagents that require a 6 Å reaction sphere (Akabas et al., 1992).

Cysteine substitutions at positions 1237 in domain III and 1530–1532 in domain IV alter monovalent and divalent cation selectivity of the Na^+ channel (Chiamvimonvat et al., 1996; Favre et al., 1996; Pérez-García et al., 1997). We observed no effect of any of the new domain IV mutants studied here on ionic selectivity of the channel. Therefore it

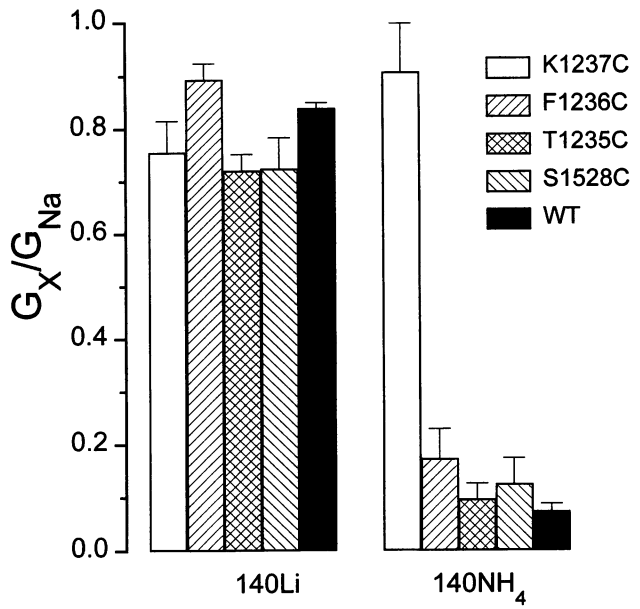

FIGURE 8 Selectivity of the cysteine mutants with altered Cd^{2+} affinity. Plot of the ratio of the whole-cell conductance in 140 mM Li^+ and NH_4^+ (G_x) compared to that in 140 mM Na^+ (G_{Na}). The selectivity of the wild-type channel and the selectivity filter mutant K1237C are shown for comparison. The mutants F1236C, T1235C, and S1528C carry no K^+ current and thus have a monovalent cation selectivity sequence similar to that of the wild-type channel. K1237C supports significant Ca^{2+} and Sr^{2+} current (Pérez-García et al., 1997); however, no significant inward current is observed through F1236C, T1235C, or S1528C in 70 mM Ca^{2+}- and Sr^{2+}-containing bath solution.

is unlikely that a structural change induced by cysteine substitution in the domain IV P-segment is the cause of altered selectivity of the mutants at positions 1530–1532. Instead, it appears that the identity of the side chains at the selectivity positions in domain IV determines the permeation phenotype (Chiamvimonvat et al., 1996; Favre et al., 1996).

Structural implications

Cysteine substitutions spanning five or six amino acids in the P segments of the Na^+ channel are accessible to block by extracellular Cd^{2+} and modification by MTS reagents. The fractional electrical distances for block of cysteine substitutions in domains III and IV are consistent with these protein segments descending, and then turning and ascending back toward the extracellular side of the pore (Fig. 9). Amino acid substitutions at the sequenced-aligned positions in the first and second domains produce significantly different results. In domain I, the amino acids on the amino-terminal side of D400 are not accessible from either the inside or the outside of the channel pore. It is possible that this part of the P segment is buried in the protein. The topology of domain II is even less certain. There is a naturally occurring cysteine at position 753 that is not modified by MTSEA added to the bath or pipette solution and does not mediate block of the pore by group IIB metals added to either side of the membrane. The cysteine substitutions on the amino-terminal side of E755 do not express in either the wild-type or C753A background in the presence or absence of a reducing environment. It is possible that short stretches of the P segments in domains I and II are inaccessible, but that the side chains of residues closer to the amino terminus will again become exposed to the pore. Defining such an arrangement of side-chain accessibility will require more extensive mutagenesis of residues in the P

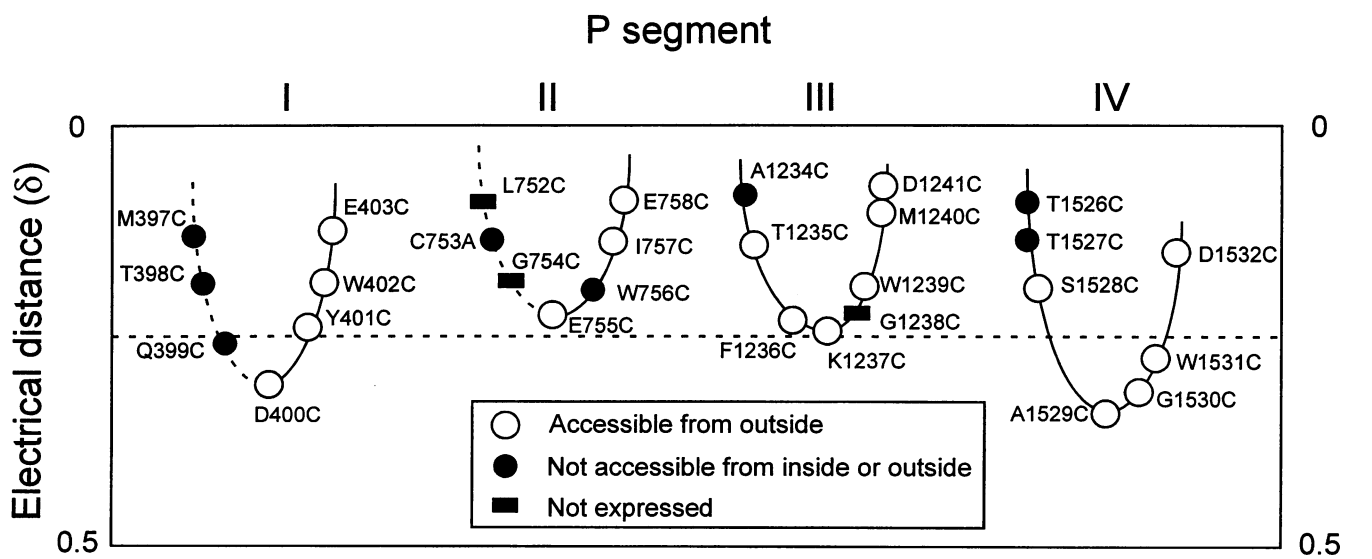

FIGURE 9 Plot of the fractional electrical distances (δ) for Cd^{2+} block of P segment cysteine mutants from the extracellular surface. The data in this plot are from this study and that of Chiamvimonvat et al. (1996). The open circles represent mutants that are accessible from the external surface of the channel. The filled circles are mutants that are not blocked or accessible from the inside or the outside of the pore. The filled squares are those mutants that do not express; they are positioned according to their location in the amino acid sequence.

segments. The unique topological arrangement of each of the P segments in the Na^+ channel highlights the asymmetry of the pore, and challenges contemporary structural models of the Na^+ channel.

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