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On the Structural Basis for Ionic Selectivity Among Na^+, K^+, and Ca^{2+} in the Voltage-Gated Sodium Channel

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ABSTRACT Voltage-sensitive sodium channels and calcium channels are homologous proteins with distinctly different selectivity for permeation of inorganic cations. This difference in function is specified by amino acid residues located within P-region segments that link presumed transmembrane elements S5 and S6 in each of four repetitive Domains I, II, III, and IV. By analyzing the selective permeability of Na^+, K^+, and Ca^{2+} in various mutants of the μ1 rat muscle sodium channel, the results in this paper support the concept that a conserved motif of four residues contributed by each of the Domains I–IV, termed the DEKA locus in sodium channels and the EEEE locus in calcium channels, determines the ionic selectivity of these channels. Furthermore, the results indicate that the Lys residue in Domain III of the sodium channel is the critical determinant that specifies both the impermeability of Ca^{2+} and the selective permeability of Na^+ over K^+. We propose that the alkylammonium ion of the Lys(III) residue acts as an endogenous cation within the ion binding site/selectivity filter of the sodium channel to tune the kinetics and affinity of inorganic cation binding within the pore in a manner analogous to ion-ion interactions that occur in the process of multi-ion channel conduction.

INTRODUCTION

Sodium channels fulfill a unique physiological role as members of the superfamily of voltage-gated ion channels (Catterall, 1992; 1995). Although their primary function is to mediate the depolarizing phase of fast action potentials that propagate along nerve axons and skeletal muscle fibers, they are also involved in the initiation and spread of electrical excitation in cardiac myocytes, neuroendocrine cells, and cell bodies and processes of neurons. Apart from their finely tuned voltage sensitivity and complex gating kinetics, sodium channels are noted for their special type of discrimination of the major physiological inorganic cations, Na^+, K^+, and Ca^{2+}. They exhibit a high permeability ratio in favor of Na^+ over K^+, and are virtually impermeable to Ca^{2+} under physiological conditions. This particular ionic selectivity provides an effective mechanism for cell membrane depolarization without significant entry of Ca^{2+}. Because Ca^{2+} itself triggers a wide array of biochemical events, the independent influx of Ca^{2+} through structurally homologous calcium channels allows independent regulation of these processes.

The channel-forming polypeptide (α-subunit) of sodium channels and calcium channels is a large integral membrane protein with pseudo-tetrameric structure defined by a fourfold internal repeat of the S1-S6 core structural motif that is characteristic of the channel subunit of voltage-gated potassium channels (Goldin, 1995). The four internally homologous Domains I–IV of sodium channels can be aligned with the corresponding segments of calcium channels with ~32–37% sequence identity (Tanabe et al., 1987), indicating that these two channel families arose from a common evolutionary ancestor (Strong et al., 1993). This homology raises the question of how sodium channels and calcium channels achieve their characteristic selectivity for inorganic cations.

This question has begun to be addressed by mutagenesis experiments that have identified critical residues determining channel conductance and ion selectivity. Such residues have been found to reside within extracellular pore loops, or P-region segments, that link the four S5-S6 transmembrane-spanning elements of homologous Domains I–IV. Using pharmacological sensitivity to tetrodotoxin and saxitoxin as an assay, Terlau et al. (1991) identified residues in each of the four P-regions of the rat brain II sodium channel that determine the affinity for these classic pore-blocking toxins. Some of these residues were also found to affect sodium channel conductance. This led to the recognition that a cluster of four highly conserved amino acids in Domains (I–IV), Asp(I), Glu(II), Lys(III), and Ala(IV), or DEKA, is a critical component of the ion selectivity filter of sodium channels. In all known voltage-gated calcium channels, positions corresponding to the DEKA residues of sodium channels consist of four glutamate residues, or the EEEE locus (Fig. 1). The functional significance attributed to the DEKA and EEEE motifs is based on the key finding of Heinemann et al., (1992) that transmutation of the positively charged Lys residue and the neutral Ala residue in Domains III and IV of the sodium channel to negatively charged Glu residues found in calcium channels, conferred characteristic

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Na-channel					
I(398-402)	T	Q	D	Y	W
II(753-757)	C	G	E	W	I
III(1235-1239)	T	F	K	G	W
IV(1527-1531)	T	S	A	G	W
Ca-channel					
I(290-294)	T	M	E	G	W
II(612-616)	T	G	E	D	W
III(1012-1016)	T	F	E	G	W
IV(1321-1325)	T	G	E	A	W

FIGURE 1 Amino-acid sequence alignment of a highly conserved motif in the P-regions of homologous Domains I to IV of sodium channels and calcium channels. Sequence numbers correspond to the rat skeletal muscle μ1 sodium channel (Trimmer et al., 1989) and the rabbit muscle L-type calcium channel (Tanabe et al., 1987). Conserved flanking Thr and Trp residues are highlighted and the DEKA/EEEE loci that play a critical role in ionic selectivity are boxed.

ionic selectivity properties of the calcium channel to the sodium channel. Furthermore, mutagenesis studies of the EEEE locus of calcium channels have shown each of these four Glu residues appears to participate in the formation of at least one high-affinity binding site for Ca^{2+} or Cd^{2+}, as determined by blocking assay of Li^+-current and Ba^{2+}-current (Kim et al., 1993; Yang et al., 1993; Parent and Gopalakrishnan, 1995; Ellinor et al., 1995).

In view of the structural and functional homology of sodium channels and calcium channels outlined above, we have extended the initial study of Heinemann et al. (1992) to focus on the role of specific amino acid functional groups in allowing the selective permeation of Na^+ versus K^+ or Ca^{2+}. By using the *Xenopus laevis* oocyte expression system, we have measured the ionic selectivity of mutant μ1 sodium channels of rat skeletal muscle (Trimmer et al., 1989) containing various substitutions of the charged residues in the first three domains of the DEKA locus. Our results confirm that these residues are indeed crucial for discrimination of the major physiological inorganic cations. We find that the positively charged Lys residue in the Domain III position of the DEKA locus is required to prevent permeation of Ca^{2+} ions through the sodium channel. The phenomenon of Ca^{2+} exclusion attributed to the Lys(III) residue appears to arise by an electrostatic repulsion mechanism, inasmuch as positively charged Arg and His residues can substitute in this respect. Furthermore, the presence of a Lys residue in the Domain III position of the DEKA locus also appears to be specifically required for the high selectivity of Na^+ over K^+ characteristic of native sodium channels. This information leads us to propose a hypothesis for the mechanistic and structural contribution of residues of the DEKA locus to the process of ionic selectivity and permeation. A preliminary version of these results has been presented in abstract form (Favre et al., 1996).

MATERIALS AND METHODS

Generation of μ1 channel mutants

A recombinant plasmid containing the rat muscle μ1 sodium channel cDNA cloned into the *Eco*RI site of pBluescript SK^+ was generously provided by Dr. W. S. Agnew (Dept. of Physiology, Johns Hopkins University School of Medicine). The pBluescript SK^+ vector was modified to eliminate the *Acc*I, *Cla*II, *Kpn*I, *Xho*I, and *Ksp*I restriction sites. Site-specific mutations were introduced by PCR as described previously (Favre et al., 1995). Mutations in the P-region of the first homologous Domain I were introduced in a 413-bp DNA fragment between the two *Nsi*I restriction sites. For mutations in the second homologous Domain II, a unique *Nhe*I site was introduced by silent mutation of the Ala-806 codon, to allow mutations to be introduced in a 341-bp DNA fragment between *BssH*II and *Nhe*I restriction site. Mutations of the Lys-1237 codon in Domain III were introduced in a 128-bp cDNA fragment between a unique *Acc*I restriction site and a *Xho*I site introduced by silent mutations in codons 1249 to 1251. All mutations were verified by complete sequencing of the mutagenesis cassette. Complementary RNA was synthesized by in vitro transcription of linearized cDNA templates using T7 RNA polymerase.

Electrophysiology

Stage V to VI oocytes from *Xenopus laevis* were isolated and injected with 2–50 ng cRNA. Oocytes were incubated in a standard Barth's medium. Macroscopic Na^+ currents (I_{Na}) were measured 12–36 h after injection using the standard two-electrode voltage-clamp technique at room temperature (~20–23°C). Intracellular electrodes had resistances below 1 MΩ when filled with 3 M KCl. Occytes were maintained at a holding potential of −80 mV; Na^+ currents were elicited every 2 s by 20–25 depolarizing pulses lasting 30 ms from −50 mV to voltages as high as +70 mV. Leakage and capacitive currents were substracted using a P/4 protocol delivered at −120 mV. Current records were filtered at 1.8 kHz and digitized at 10 kHz for analysis.

To estimate relative ionic permeabilities of channel mutants, currents were measured consecutively in a control solution containing Na^+ ions as a major cation, then in a high K^+ solution, and finally in a high Ca^{2+} solution. The control Na^+ solution contained in mM: 115 NaCl, 0.2 $CaCl_2$, 2.5 KCl, 10 HEPES-NaOH, pH 7.2. The K^+ solution contained in mM: 117.5 KCl, 0.2 $CaCl_2$, 10 HEPES-KOH, pH 7.2. The Ca^{2+} solution contained in mM: 85 $CaCl_2$, 2.5 KCl, 10 HEPES-$Ca(0H)_2$, pH 7.2.

Changing from the control Na^+ solution to the K^+ or Ca^{2+} solutions generated small changes in liquid junction potentials, essentially between the agar salt bridge used as reference and the test solution. The agar bridge placed in the recording chamber was filled with 3 M KCl. The changes in the liquid junction potentials following ionic substitutions were measured in the recording chamber using a flowing 3-M KCl electrode and the 3-M KCl agar bridge used in our experiments. The recording chamber was first filled with the control solution and the offset adjusted such that the voltage of the reference appears zero. Substitution of control Na^+ solution with the high-K^+ solution resulted in a change in the junctional potential between the agar salt bridge and the solution of −3.1 ± 0.1 mV (n = 15), and +5.4 ± 0.05 mV (n = 7) for the Ca^{2+} solution. All measured reversal potentials were corrected for these changes in liquid junction potentials.

Data analysis

Macroscopic I-V parameters were obtained from peak current-voltage data fitted to the following transform of a Boltzmann function:

$$I_{Na} = \frac{G_{max}(V - V_{rev})}{1 + \exp\{(V - V_{0.5})/k\}} \quad (1)$$

where I_{Na} is the peak Na^+ current, V is the test potential, V_{rev} is the reversal potential, G_{max} is the maximal conductance, $V_{0.5}$ is the voltage activation midpoint, and k corresponds to a slope factor. In some experiments where

current reversal was not obviously detected by the pulse protocol, V_{rev} was obtained from linear extrapolation of I-V data nearest to the voltage axis, as discussed in the text.

Ionic permeability ratios were calculated from changes in the reversal potentials following cation substitutions in the external solution. In going from control Na^+ solution (subscript 1) to K^+ solution (subscript 2), the K^+ to Na^+ permeability ratio was calculated according to (Hille, 1971):

$$\frac{P_K}{P_{Na}} = \frac{[Na]_1}{[K]_2} \exp\left(\frac{\Delta V}{\alpha}\right) \tag{2.1}$$

where $\Delta V = V_2 - V_1$ and $\alpha = RT/F = 25.42$ mV at 22°C. It can be shown that the contribution of 2.5 mM K^+ in the control Na^+ solution has an insignificant effect on the determination of P_K/P_{Na} using Eq. 2.1.

In solutions containing Na^+, K^+, and Ca^{2+}, the extended Goldman-Hodgkin-Katz equation for V_{rev} as a function of ionic concentrations (activities) can be expressed as follows (Lewis 1979):

$$V_{rev} = \alpha \ln\left\{\frac{[Na]_o + \frac{P_K}{P_{Na}}[K]_o + 4\frac{P_{Ca}}{P_{Na}}[Ca]_o\left[\frac{1}{1+\exp(V_{rev}/\alpha)}\right]}{[Na]_i + \frac{P_K}{P_{Na}}[K]_i + 4\frac{P_{Ca}}{P_{Na}}[Ca]_i\left[\frac{\exp(V_{rev}/\alpha)}{1+\exp(V_{rev}/\alpha)}\right]}\right\} \tag{2.2}$$

where subscript o refers to solution outside, and i to solution inside the cell, and α has the same meaning as above.

The ionic permeability ratio P_{Ca}/P_{Na} can be obtained from changes in V_{rev} in going from control extracellular Na^+ solution (subscript 1) to a Ca^{2+} solution (subscript 2). Assuming that intracellular Na^+ and K^+ concentrations remain constant following external cation substitution, and that intracellular $[Ca^{2+}]_i$ is typically very small ($<10^{-7}$ M), and can be neglected, then changes in V_{rev} (ΔV) can be expressed as a function of P_{Ca}/P_{Na} as follows:

$$\Delta V = V_2 - V_1 = \alpha \ln\left\{\frac{[Na]_2 + \frac{P_K}{P_{Na}}[K]_2 + 4\frac{P_{Ca}}{P_{Na}}[Ca]_2\left[\frac{1}{1+\exp(V_2/\alpha)}\right]}{[Na]_1 + \frac{P_K}{P_{Na}}[K]_1 + 4\frac{P_{Ca}}{P_{Na}}[Ca]_1\left[\frac{1}{1+\exp(V_1/\alpha)}\right]}\right\} \tag{2.3}$$

Solving Eq. 2.3 for the permeability ratio P_{Ca}/P_{Na} as a function of ΔV yields:

$$\frac{P_{Ca}}{P_{Na}} = \frac{\left\{[Na]_1 + \frac{P_K}{P_{Na}}[K]_1\right\}\{\exp(\alpha/\Delta V)\} - [Na]_2 - \frac{P_K}{P_{Na}}[K]_2}{4\left\{\frac{[Ca]_2}{1+\exp(V_2/\alpha)} - \frac{[Ca]_1\exp(\Delta V/\alpha)}{1+\exp(V_1/\alpha)}\right\}} \tag{2.4}$$

In going from the K^+ solution (subscript 1) to the Ca^{2+} solution (subscript 2), a similar equation can be derived to calculate P_{Ca}/P_K:

$$\frac{P_{Ca}}{P_K} = \frac{\left\{\frac{P_{Na}}{P_K}[Na]_1 + [K]_1\right\}\{\exp(\alpha/\Delta V)\} - \frac{P_{Na}}{P_K}[Na]_2 - [K]_2}{4\left\{\frac{[Ca]_2}{1+\exp(V_2/\alpha)} - \frac{[Ca]_1\exp(\Delta V/\alpha)}{1+\exp(V_1/\alpha)}\right\}} \tag{2.5}$$

For channel mutants with low selectivity between Na^+ and K^+, a similar approach was used to determine the permeability ratio P_{Ca}/P_K by measurements of the shift in V_{rev} as a function of increasing Ca^{2+} concentrations in the range of 0.2 to 20 mM achieved by addition of $CaCl_2$ to the standard K^+ solution. In the absence of external Na^+, and assuming that intracellular Ca^{2+} concentration is negligible, Eq. 2.3 becomes:

$$\Delta V = V_2 - V_1 = \alpha \ln\left\{\frac{[K]_o + 4\frac{P_{Ca}}{P_K}[Ca]_2\left(\frac{1}{1+\exp(V_2/\alpha)}\right)}{[K]_o + 4\frac{P_{Ca}}{P_K}[Ca]_1\left(\frac{1}{1+\exp(V_1/\alpha)}\right)}\right\} \tag{2.6}$$

The above equations are strictly valid only if the activity of ions is used for all the concentrations terms $[x]$. The activity a for any species i in solution is given by the product of the activity coefficient f_i and its molar concentration $[x]$:

$$a_i = f_i[x] \tag{3.1}$$

At an ionic strength <0.5 M, the Davies equation can be used to calculate the activity coefficient f_i for any species i of valence z (Spalding et al., 1990; Butler, 1964):

$$\log_{10} f_i = -0.509z^2\left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2I\right) \tag{3.2}$$

The ionic strength, I, is defined as the half sum of the product $c_i z_i^2$ for all ionic species in the solution where c_i is the concentration and z_i the valence of the species i.

Statistics

Results are expressed as mean ± standard deviation (SD). Statistical significance was determined using an unpaired t-test.

RESULTS

Effect of alanine substitution in Domains I, II, and III

Fig. 1 is an abbreviated sequence alignment that underlies the basic premise of this study. The P-regions of homologous Domains I–IV of calcium channels, and Domains I, III, and IV of nearly all sodium channels that have been sequenced to date, contain a highly conserved motif of five residues flanked by a Thr residue on the N-terminal side, and a Trp residue on the C-terminal side. The middle positions of this motif, termed the EEEE locus in calcium channels and the DEKA locus in sodium channels, have been variously found to play an important functional role in determining conductance, cation selectivity, and divalent cation blocking properties of calcium channels and sodium channels (Heinemann et al., 1992; Kim et al., 1993; Mikala et al., 1993; Parent and Gopalakrishnan, 1995; Tang et al., 1993; Yang et al., 1993). In particular, Heinemann et al. (1992) found that substitution of Lys by Glu at the third position of the DEKA locus of a brain sodium channel conferred calcium channel-like ionic selectivity to the sodium channel. The question specifically addressed in this study is: What is the individual contribution of residues in the DEKA locus to the unique ionic selectivity of sodium channels? If the functional groups of these residues participate in the formation of a binding site or selectivity filter

for inorganic cations, the charged, polar groups would be expected to make the greatest contribution to ion binding energy by virtue of their potential electrostatic interactions, and the fact that crystallographically determined protein binding sites for Ca^{2+}, K^+, and Na^+ are generally formed by direct coordination of oxygen ligands (i.e., the functional groups of Asp, Glu, Asn, Gln, Ser, Thr, the carbonyl oxygen of the peptide bond, and oxygen of H_2O) with these metal ions (Glusker, 1991; McPhalen et al., 1991; Falke et al., 1994). Thus, we focused our attention primarily on the first three residues of the DEKA locus, inasmuch as the neutral methyl group of Ala in sodium channel Domain IV cannot coordinate directly to metal ions or form hydrogen bonds to other residues.

Fig. 2 shows records and peak I-V relationships of macroscopic currents of sodium channels expressed in *Xenopus* oocytes, as measured by the two-electrode voltage-clamp technique with an extracellular solution containing Na^+ (120 mM) as the predominant cation. This figure compares data obtained for the wild-type μ1 sodium channel and three mutants in which the D400(I), E755(II), and K1237(III) residues in the first three domains of the DEKA locus were individually replaced by Ala (mutants AEKA, DAKA, and DEAA). The magnitude of peak I_{Na}, the time course, midpoint voltage ($V_{0.5}$), and slope factor for voltage activation are all comparable for wild-type and mutant channels (Table 1), indicating that these particular Ala substitutions did not reduce expression or seriously alter the gating function of the channel. However, the K1237A (DEAA) channel clearly exhibits a reversal of the peak I-V relationship and outward currents at low positive voltages, where the other channels still exhibit inward currents. Under these ionic conditions, currents of the wild-type μ1 channel reverse direction at high positive voltage (~+70 mV) where it is difficult to maintain adequate voltage control with the oocyte voltage clamp. The extrapolated reversal potential (V_{rev}) of the wild-type and D400A (AEKA) mutant is close to the expected Nernst potential for Na^+, indicating high selectivity for Na^+ over K^+. The data of Fig. 2 and Table 1 indicate a significant negative shift of V_{rev}, by ~19 mV for E755A (DAKA), and ~60 mV for K1237A (DEAA), implying that these channels suffer a reduction in their ability to discriminate cations. This points to a prominent role of the residues in Domains II and III in cation selectivity. These results also indicate that a negatively charged residue (Asp) in Domain I of the DEKA locus is not absolutely required for Na^+ permeation and selectivity. This conclusion is further supported by our finding that the D400C mutation exhibits selectivity behavior very similar to that described for D400A (data not shown).

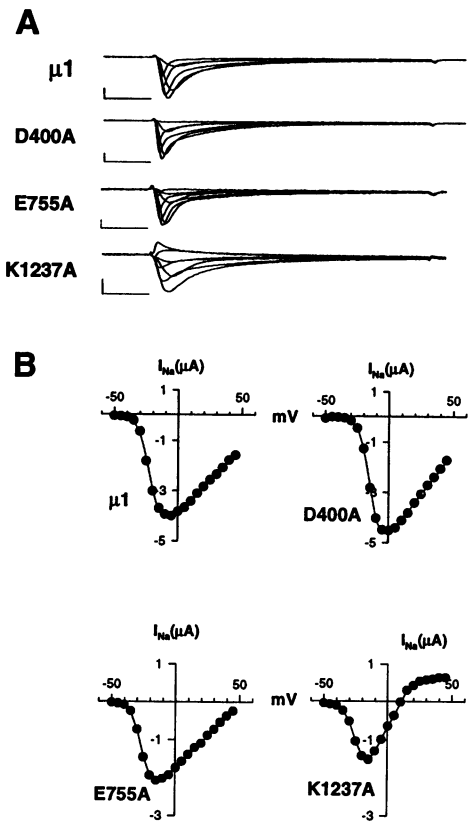

FIGURE 2 Effect of individual Ala substitution of the three charged residues in the DEKA locus on macroscopic Na^+ currents. (*A*) Current records were elicited by test potentials of −30, −20, −10, 0, 15, 30 mV for the μ1 wild-type and D400A mutant, and −35, −30, −20, −10, 0 15, 40 mV for the E755A and K1237A mutant. Vertical and horizontal calibration bars represent 1 μA and 5 ms, respectively. (*B*) Representative peak I-V relationships obtained in control solution with Na^+ as the major cation.

While the behavior of these particular Ala substitutions indicates that the sodium channel can accept some structural modifications at these positions of the DEKA locus, other mutations are not well tolerated. For example, D400K and D400H mutations either do not pass current or are otherwise nonfunctional because these mutations failed to express in the oocyte assay system (data not shown). Similarly, a double Ala mutation D400A/E755A (AAKA) also did not yield detectable currents. These results also support the notion that the DEKA locus is a critical region for channel function. In their original investigation, Terlau et al. (1991) also reported dramatically reduced unitary conductance for analogous NEKA and DQKA mutants in a rat brain sodium channel, which are rather conservative $D > N$ and $E > Q$ substitutions in Domains I and II, respectively.

Minimal structural requirements for selectivity of Na^+ over K^+

To quantitate changes in ionic selectivity of the DAKA and DEAA mutants more precisely, macroscopic currents were recorded before and after complete replacement of external Na^+ by K^+. As illustrated by the current records of Fig. 3, in extracellular K^+ solution inward current through the wild-type μ1 channel is virtually undetectable, and only a small outward current carried by efflux of intracellular Na^+ out of the cell is observed. Under the same conditions, the

TABLE 1 Macroscopic functional parameters of the μ1 Na^+-channel and Ala substitution mutants

		G_{max} (μS)	V_{rev} (mV)	$V_{0.5}$ (mV)	k (mV)	n
μ1	(DEKA)	45 ± 18	70 ± 10	−24 ± 4	−4.2 ± 0.8	13
D400A	(AEKA)	36 ± 17	79 ± 9	−19 ± 5	−4.5 ± 1.0	8
E755A	(DAKA)	32 ± 7	54 ± 9[a]	−23 ± 3	−4.5 ± 0.5	6
K1237A	(DEAA)	167 ± 114	12 ± 3[a]	−27 ± 3	−3.6 ± 0.7	17

[a]Denotes statistical significance ($p < 0.05$) with respect to values obtained for wild μ1.
Values are mean ± SD.

E755A mutant (DAKA) exhibited a small but reproducible inward K^+ current, and the K1237A mutant (DEAA) exhibited a large inward K^+ current in the μA range. The I-V data of Fig. 3 *B* show that the DAKA mutant allows K^+ to enter the cell, but the maximal K^+ conductance is much lower than the Na^+ conductance. The I-V relationship of the DEAA mutant shows that Na^+ and K^+ are equally permeant considering the identical conductances (G_{max}) and V_{rev} in Na^+ versus K^+ medium. The DEAA mutant also showed a reproducible ~10 mV hyperpolarizing shift of the voltage activation midpoint $V_{0.5}$ in the presence of extracellular K^+, but this phenomenon was not studied further in this work.

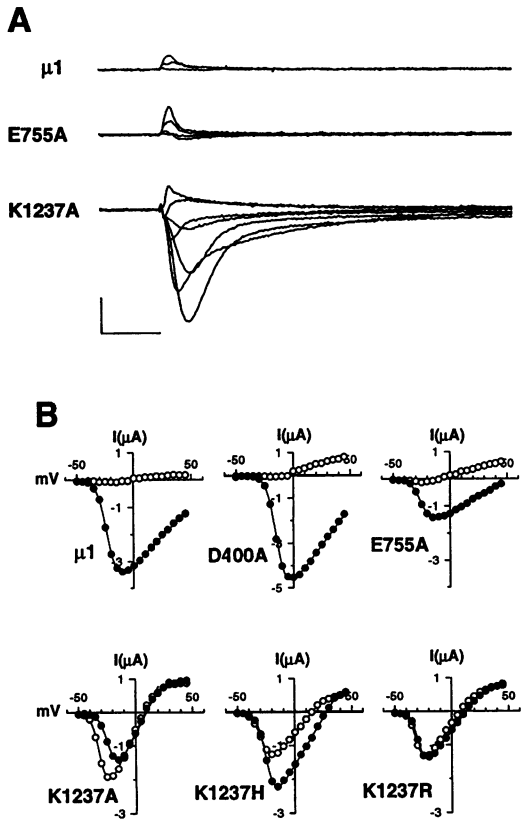

FIGURE 3 Effect of mutations in the DEKA locus on selectivity for Na^+ and K^+. (*A*) Current records were obtained in extracellular K^+ solution by test pulses of −40, 15, 45 mV for μ1, −40, −30, 15, 45 mV for the E755A mutant and −45, −35, −30, −15, 0, 15, 45 mV for the K1237A mutant. Vertical and horizontal scale bars denote 1 μA and 5 ms, respectively. (*B*) Representative peak I-V relationships in control extracellular Na^+ solution (●) and after replacement by K^+ solution (○).

Table 2 lists measured V_{rev} values in Na^+ and K^+ external media along with calculated permeability ratios of K^+ relative to Na^+, P_K/P_{Na} (see Methods section). The reversal potential for the wild-type μ1 channel was estimated by linear regression of the outward currents measured in K^+ solution at test potentials greater than 0 mV, as required for channel activation. The estimated value of $P_K/P_{Na} = 0.03$ for the wild-type μ1 channel is in good agreement with the reported value for the rat brain II channel expressed in *Xenopus* oocytes, $P_K/P_{Na} = 0.03$ (Heinemann et al., 1992), and also for native Na^+ currents in rat skeletal muscle fibers $P_K/P_{Na} = 0.045$ (Pappone, 1980). The D400A mutation in Domain I did not measurably affect Na^+/K^+ permeability compared to the wild type, indicating that the carboxylate functional group of the Asp residue in this position is surprisingly not essential for the ionic selectivity function of the channel. In contrast, the E755A mutation in Domain II resulted in a three- to fourfold increase in P_K/P_{Na}, and the K1237A mutant is completely nonselective for Na^+ versus K^+. These results show that Ala substitutions of the charged residues of the DEKA locus do not have equivalent effects on Na^+/K^+ selectivity, but point to special importance of the K(III) residue. Considering the observation of Heinemann et al. (1992) that the mutation A1714E in Domain IV of the rat brain channel significantly reduces Na^+/K^+ selectivity with respect to wild-type ($P_K/P_{Na} = 0.15$ for DEKE versus 0.03 for native DEKA), it appears that the neutral Ala in Domain IV of the DEKA locus also has a stabilizing role in native sodium channel selectivity.

Given the prominent contribution of K1237 in Domain III with respect to Na^+/K^+ selectivity, we studied a series of amino acid substitutions at this position to investigate the specific amino acid requirement for Na^+-selective permeability. I-V data in Fig. 3 *B* and the calculated permeability ratios of Table 2 indicate that substitution of K1237 by a hydrophobic residue (Met), a polar sulfhydryl group (Cys), a large aromatic residue (Phe), and other positively charged residues (Arg and His) all dramatically increased the wild-type P_K/P_{Na} ratio, resulting in a virtually complete lack of discrimination of Na^+ versus K^+. Thus, the requirement for K1237 in Na^+/K^+ selectivity does not seem to be related to the positive charge of the Lys group or size of its side chain. This conserved Lys residue apparently fulfills a specific structural or chemical requirement for this selectivity function of the sodium channel.

TABLE 2 Ionic permeability ratios calculated from the shift in V_{rev} in $\mu 1$ Na^+-channel mutants after external ionic substitutions

		Na^+ bath V_{rev} (mV)	K^+ bath V_{rev} (mV)	Ca^{2+} bath V_{rev} (mV)	P_K/P_{Na}	P_{Ca}/P_{Na}	P_{Ca}/P_K
$\mu 1$	(DEKA)	73 ± 10 (12)	−18 ± 10 (12)	—	0.03 ± 0.01	—	—
D400A	(AEKA)	79 ± 9 (8)	−8 ± 5 (8)	—	0.03 ± 0.02	—	—
E755A	(DAKA)	54 ± 9 (6)	−6 ± 4 (6)	—	0.09 ± 0.03	—	—
K1237A	(DEAA)	13 ± 3 (22)	15 ± 4 (22)	41 ± 2 (10)	1.1 ± 0.06	18.7 ± 1.7	16.3 ± 2
K1237C	(DECA)	11 ± 3 (4)	16 ± 5 (4)	38 ± 2 (4)	1.2 ± 0.07	16.7 ± 2.5	13.9 ± 2.9
K1237F	(DEFA)	13 (1)	14 (1)	34 (1)	1.0	11.3	10.6
K1237H	(DEHA)	22 ± 4 (20)	13 ± 3 (20)	21 ± 2 (6)	0.7 ± 0.03	3.4 ± 0.7	4.7 ± 0.9
K1237M	(DEMA)	17 ± 3 (16)	13 ± 3 (16)	36 ± 3 (16)	0.8 ± 0.05	11 ± 1.8	12.9 ± 2.1
K1237R	(DERA)	14 ± 2 (14)	12 ± 2 (14)	—	0.9 ± 0.04	—	—

All experiments were carried out at pH 7.2. Permeability ratios were calculated according to equations 2.1, 2.4, and 2.5 (see Methods). Values are mean ± SD. The number of experiments is indicated in parentheses.

Effect of amino acid substitutions at K1237 in Domain III on the selective permeability of Na^+ and K^+ versus Ca^{2+}

In their original transmutation experiments, Heinemann et al. (1992) showed that substitution of Glu for Lys in the Domain III position of the DEKA locus resulted in a nonselective cation channel that is readily permeable to Na^+, K^+, Ca^{2+}, and Ba^{2+}. We have further examined the mechanism underlying the important physiological property of Ca^{2+} rejection in the wild-type sodium channel by measuring the permeability ratios, P_{Ca}/P_{Na} and P_{Ca}/P_K, for different amino acid substitutions at K1237.

Fig. 4 *A* shows current records for the $\mu 1$ wild-type and the K1237A (DEAA) mutation in an extracellular solution containing Ca^{2+} as the major cation. Inward Ca^{2+}-current is undetectable for the wild-type sodium channel, but a small, rapidly inactivating current of Na^+ ions moving out of the cell is observed at positive voltages. Under identical conditions, the K1237A mutant exhibits an obvious inward inactivating current in the early phase of the record that is indicative of inward Ca^{2+} movement through the mutant sodium channel. The large, slowly activating outward currents and tail currents at the end of these records reflect the activation of endogenous Ca^{2+}-activated Cl^- channels in the *Xenopus* oocyte, as also observed by Heinemann et al., (1992) for the Ca^{2+}-permeable mutant of the rat brain sodium channel, K1422E. An early inward Ca^{2+}-current and voltage-dependent activation of the oocyte Cl^--current is thus a reliable index for calcium permeability of sodium channel mutants in external calcium medium. Fig. 4 *B* shows peak I-V relations of the wild-type $\mu 1$ and mutant K1237A channels in sodium medium and calcium medium. In the wild-type channel, replacement of external 115 mM NaCl with 85 mM $CaCl_2$ results in a loss of inward current and activation of outward current at voltages more positive than +10 mV. The I-V relation of the wild-type $\mu 1$ channel in external calcium medium is indicative of a lack of Ca^{2+} permeation and a large depolarizing shift of the voltage activation midpoint. This latter phenomenon is a well known effect of external Ca^{2+} on sodium channel gating that is generally ascribed to a reduction in negative surface charge density due to screening and binding effects of Ca^{2+} and other divalent cations (Frankenhaeuser and Hodgkin, 1957; Hille et al., 1975; Cukierman and Krueger, 1990). Fig. 4 *B* shows that replacement of external Na^+ by Ca^{2+} in the K1237A mutant did not affect the magnitude of the peak inward current, but resulted in a large positive shift of both the voltage-activation midpoint and the reversal potential. The latter effect is indicative of a higher permeability for Ca^{2+} than Na^+ or K^+.

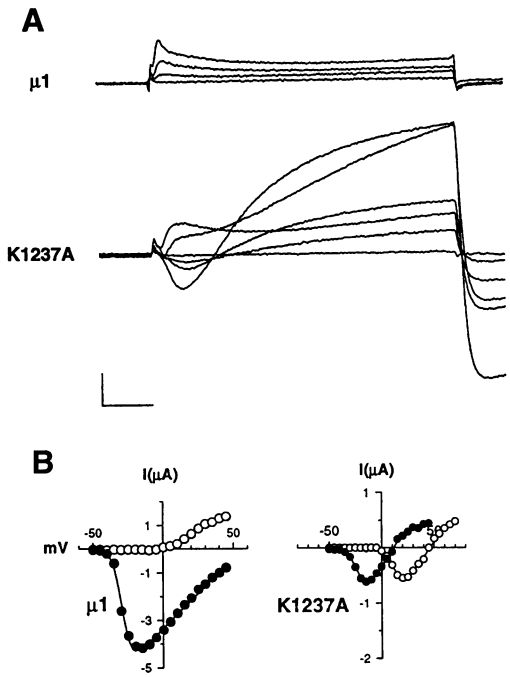

FIGURE 4 Effect of mutating the Lys residue in Domain III of the DEKA locus to Ala on permeability to Ca^{2+}. (*A*) Current records in extracellular Ca^{2+} solution for the $\mu 1$ wild-type sodium channel and K1237A mutant. Currents were elicited by test pulses of −10, 20, 30 40 mV, and −10, 5, 10, 20, 50, 70 mV for the $\mu 1$ and K1237A mutant, respectively. Vertical and horizontal scale bars represent 1 μA and 5 ms, respectively. (*B*) Representative peak I-V relationships of $\mu 1$ and K1237A mutant obtained in control Na^+ solution (●) and after replacement by Ca^{2+} solution (○)

The calcium permeability of the K1237A (DEAA) mutant (Fig. 4) demonstrates that a negatively charged residue such as Glu (III) found in the calcium channel EEEE locus is not an absolute requirement for the enhancement of Ca-permeation through the sodium channel as observed with the DEEA mutation of Heinemann et al. (1992). The residue requirements for permeation/exclusion of Ca^{2+} were further investigated using different substitution mutations at K1237 as shown in Fig. 5. The I-V relations of K1237C, K1237F, and K1237 M compared for external Na^+ and Ca^{2+} medium show that all of these mutations behave in a manner similar to K1237A (Fig. 4). Specifically, in the presence of external Ca^{2+}, a large inward current, a positive shift in the voltage-activation midpoint, and a reversal potential of about +40 mV is observed. In contrast, substitution of K1237 by a positively charged Arg residue did not give rise to a detectable inward Ca^{2+} current or a Ca^{2+}-activated Cl^- current. Also, the large outward current exhibited by the DERA channel in the presence of 85 mM external Ca^{2+} implies that outward K^+ and Na^+ efflux through this channel is not strongly blocked by extracellular Ca^{2+}.

In an external medium buffered at pH 6.0, the His imidazole group is expected to be partially protonated and positively charged. At this pH, the K1237H substitution does not exhibit detectable inward current and displays I-V behavior similar to the K1237R channel. However, the presence of a small, delayed Ca^{2+}-activated Cl^- current suggested that the K1237H mutation was slightly permeable to Ca^{2+}. A shown by the I-V data of Fig. 5, when the external pH is raised to 7.2, the K1237H mutation exhibits an obvious inward Ca^{2+} current and a positive shift in reversal potential as compared to pH 6.0. The pH-dependent behavior of the K1237H channel implies that protonation of the His residue in the Domain III position of the DEKA locus impairs the ability of Ca^{2+} to permeate. Taken together, our results with the K1237R and K1237H mutations lead to the conclusion that a positive charge at the position of K1237 is the critical structural feature of the pore that exludes Ca^{2+} from readily permeating through the native sodium channel.

The positive shift in V_{rev} that is observed in going from a sodium solution to a calcium solution for Ala, Cys, Phe, and Met substitutions of K1237 indicates that these mutant channels have a higher permeability for Ca^{2+} than for Na^+ or K^+. The measured values of V_{rev}, P_{Ca}/P_{Na}, and P_{Ca}/P_K for these mutations are listed in Table 2. The neutral amino acid substitutions result in channels with ~15-fold greater permeability to Ca^{2+} over Na^+ or K^+. The permeability profile of these mutants points to an essential role of the K1237 residue in both the selectivity for Na^+ over K^+ and the exclusion of Ca^{2+} from the channel pore. Our experiments suggest that this latter feature is dependent on the positive charge of the Lys side chain and implies that native Na^+ channels exclude Ca^{2+} by a mechanism based on electrostatic repulsion. These observations lead back to the question of what role do the negatively charged Asp and Glu residues of the DEKA locus play in cation permeation?

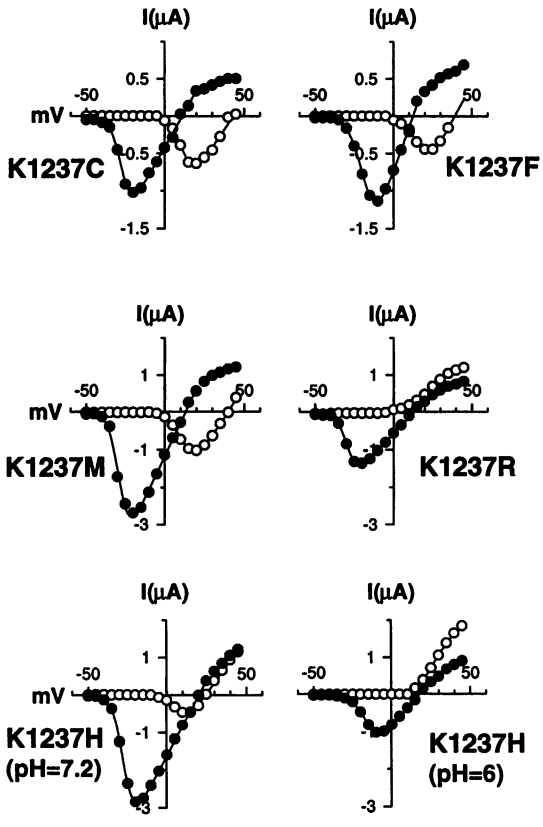

FIGURE 5 Effect of various amino acid substitutions of the Lys residue in Domain III of the DEKA locus (K1237) on Ca^{2+} permeability. Representative peak I-V relationships are shown for an oocyte expressing one of the labeled sodium channel mutants in control Na^+ solution (●) and in Ca^{2+} solution (○).

Contribution of the negatively charged residues of the DEKA locus to ion permeability

Substitution of K1237 by Ala produces a channel with two negatively charged carboxylate functional groups in Domains I and II of the DEKA locus and methyl groups at the other positions. To test the hypothesis that these two carboxyl groups represent a putative Ca^{2+}-binding site related to that proposed for the calcium channel, we first generated a channel mutant in which all of the charged residues within the DEKA locus are replaced by Ala (D400A/E755A/K1237A = AAAA). I-V relations for the AAAA mutant in external Na^+, K^+ or Ca^{2+} solution are shown in Fig. 6. In the presence of external Na^+, this mutant channel typically expresses whole-oocyte current in the range of ~0.5 μA. The activation voltage midpoint was unaffected by the mutations and the measured reversal potential of +12 mV indicates a loss of selectivity for Na^+ over K^+, as expected for a channel lacking K1237 in Domain III. Interestingly, this triple mutation reveals that Na^+ and K^+ permeation does not require the Asp(I) and Glu(II) residues. The ease of

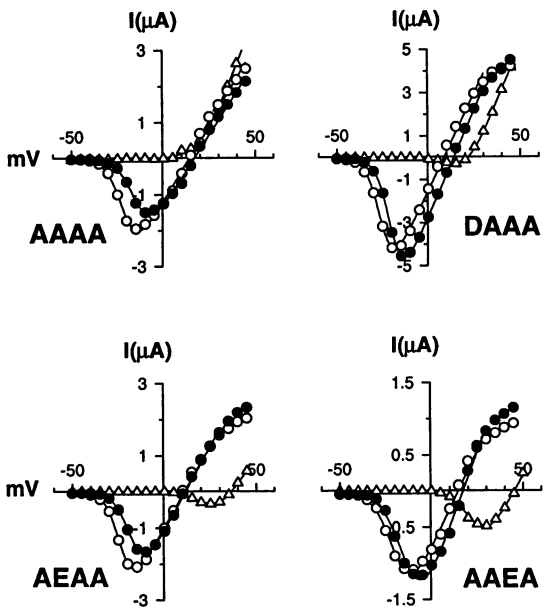

FIGURE 6 Distinct contributions of negatively charged residues in the DEKA locus to Ca^{2+} permeability. Peak I-V curves obtained in control Na^+ solution (●) and after successive replacement of Na^+ by K^+ (K^+ solution, ○) and Ca^{2+} (Ca^{2+} solution, △). The various mutations correspond to Ala substitutions at D400/E755/K1237 (AAAA), E755/K1237 (DAAA), and D400/K1237 (AEAA). The AAEA mutant corresponds to D400A/E755A/K1237E mutations.

expression of most of our mutations in the DEKA locus suggests that the absence of a measurable current for the AAKA double mutation noted earlier is due to the presence of Lys(III) as a significant energy barrier for Na^+ and K^+ permeation that cannot be overcome in the absence of Asp(I) or Glu(II) in Domains I and II. In calcium medium, the AAAA mutant showed neither an inward current nor Ca^{2+}-activated Cl^- currents, indicating that Ca^{2+} does not significantly permeate through this channel.

Compared to the AAAA channel, the DAAA double mutation with a native Asp residue in Domain I did not affect the poor Na^+/K^+ selectivity of the AAAA channel, but did result in a measurable inward current and a positive shift in V_{rev} when Na^+ was replaced by Ca^{2+}. Similarly, the AEAA and AAEA mutants exhibited corresponding larger inward Ca^{2+}-current and more positive shifts in V_{rev} for solution change to calcium medium. These results indicate that Ca^{2+} permeability progressively increases in the AAAA background with the introduction of carboxylate residues according to the sequence D(I) < E(II) < E(III). All of the examined mutants lacking K1237 are nonselective for Na^+ versus K^+, and the introduction of one carboxylic acid residue in either Domain I, II, or III is sufficient to confer higher permeability of Ca^{2+} versus Na^+ as indicated by the calculated permeability ratios listed in Table 3. The enhancement of Ca^{2+} permeability achieved by the introduction of acidic residues in the AAAA background is also additive, as shown by the comparison of the DEAA channel to DAAA and AEAA (Tables 2 and 3).

The higher permeability of Ca^{2+} versus Na^+ or K^+ in these mutants was verified in independent experiments in which the shift of V_{rev} was measured after increasing Ca^{2+} concentration from 0.2 to 20 mM in a bath solution with K^+ as the major cation. Representative I-V relationships for DAAA, AEAA, AAEA, and DEAA are shown in in Fig. 7 *A*. An increase in external Ca^{2+} concentration resulted in a typical positive shift in the midpoint of voltage activation that was virtually the same in each of the mutants. For the DAAA mutant, increasing the external Ca^{2+} concentration up to 20 mM was not sufficient to induce a significant shift in V_{rev}. However, increasing Ca^{2+} concentration resulted in a progressive positive shift of V_{rev}, with correspondingly larger shifts observed according to the sequence: DAAA < AEAA < AAEA < DEAA. The dependence of the change in V_{rev} versus external [Ca^{2+}] is plotted in Fig. 7 *B*. As shown in Table 3, fitting these data to an equation derived from the extended Goldman-Hodgkin-Katz equation (Lewis, 1979; see Methods) yields permeability ratios P_{Ca}/P_K for AEAA, AAEA, and DEAA mutants that are in good agreement with those independently calculated from experiments involving a single calcium solution change as presented in Fig. 6.

DISCUSSION

Since the remarkable demonstration of sodium channel → calcium channel transmutation by Heinemann et al. (1992), analysis of the structural basis for the ionic selectivity mechanism in voltage-activated sodium channels and homologous calcium channels has respectively focused on the specific role of the so-called DEKA and EEEE loci composed of four residues contributed by each of the four homologous P-regions (Kim et al., 1993; Mikala et al.,

TABLE 3 Contribution of carboxylate residues in homologous Domains I, II, and III to relative ionic permeabilities

	Na^+ bath V_{rev} (mV)	K^+ bath V_{rev} (mV)	Ca^{2+} bath V_{rev} (mV)	*n*	P_K/P_{Na}	P_{Ca}/P_{Na}	$P_{Ca}/P_K^{(1)}$	$P_{Ca}/P_K^{(2)}$
AAAA	12 ± 3	12 ± 2	—	9	1.0 ± 0.07	—	—	—
DAAA	14 ± 2	13 ± 2	16 ± 1	16	0.9 ± 0.03	3.0 ± 0.2	3.2 ± 0.3	—
AEAA	12 ± 2	14 ± 2	30 ± 2	8	1.1 ± 0.05	8.9 ± 0.5	7.8 ± 0.5	4.5 ± 0.5
AAEA	15 ± 2.0	15 ± 2	40 ± 2	12	1.0 ± 0.06	15.2 ± 1.8	15.2 ± 2	11.1 ± 0.8
DEAA			see Table 2				16.3 ± 2	15.6 ± 0.3

Permeability ratio $P_{Ca}/P_K^{(1)}$ was calculated according to Eq. 2.5 and $P_{Ca}/P_{Na}^{(2)}$ was obtained from fit of the data in Figure 7B according to Eq. 2.6. Values are mean ± SD; *n* is the number of experiments.

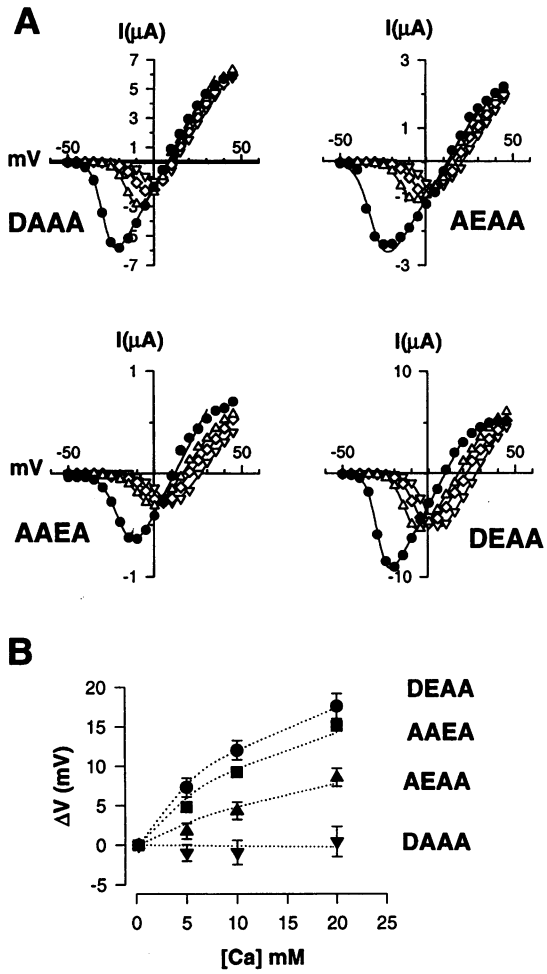

FIGURE 7 Measurements of P_{Ca}/P_{Na} permeability ratios in channel mutants possessing one or two negatively charged residues in the DEKA locus. (*A*) Peak I-V relations of DAAA, AEAA, AAEA and DEAA (K1237A) mutants in the presence of K^+ solution containing 0.2 mM Ca^{2+} (●) and after addition of 5 mM Ca^{2+} (△), 10 mM Ca^{2+} (◇) and 20 mM Ca^{2+} (▽). (*B*) Plot of the positive shift in V_{rev} (ΔV) versus external Ca^{2+} concentration. Data are fitted (·····) to Eq. 2.6 (see Methods). Data points indicate means ± SD.

1993; Parent and Gopalakrishnan, 1995; Tang et al., 1993; Yang et al., 1993). In this paper we have continued this effort by measuring the permeability properties of sodium channel mutations with substitutions in Domains I, II, and III of the DEKA motif. In addition to supporting the hypothesis that this motif is directly involved in ion permeation and selectivity, the major new finding is that the Lys residue of Domain III plays a central and specific role in two physiologically relevant selectivity processes: 1) discrimination between the two monovalent Group IA alkali cations, Na^+ and K^+, and 2) exclusion of the divalent Group IIA alkaline earth cation, Ca^{2+}.

The present experimental approach is based on a classic technique of measuring relative permeability ratios derived from the observed change in reversal potential in going from a solution containing a reference cation, Na^+, to a test cation, such as K^+ or Ca^{2+} (Hille, 1971; 1972). The advantage of this method is that it identifies the permeant ions by direct measurements of their inward current and yields relative permeability data that may be compared to known profiles of ionic selectivity for many different ion channels previously mapped by this technique (Hille, 1992). Quantitative estimates of *P*-ratios are interpretable in the context of the Nernst-Planck electrodiffusion theory (Levitt, 1986; Andersen, 1989), which assumes ion independence and a homogeneous membrane electric field; assumptions that are undoubtedly invalid for biological channel proteins. However, comparison of electrodiffusion theory with the Michaelis-Menten model of enzyme turnover shows that the ionic permeability, *P*, of a one-site channel is directly analogous to the catalytic efficiency parameter of an enzyme, k_{cat}/K_M, which is the most widely accepted measure of enzyme substrate discrimination (Fersht, 1985; Levitt, 1986; Andersen, 1989; Moss and Moczydlowski, 1995). Also, in relating ionic permeability to the simplest Eyring theory for a single-occupancy channel, it is found that relative permeability depends on the difference in peak heights or the energy barriers that different ions must surmount and not on well energies, or ion-binding affinities (Armstrong, 1975; Hille, 1975a; Levitt, 1986). Thus, permeability ratio data may be roughly interpreted in terms of the relative rate of association of Na^+, K^+, and Ca^{2+} with the channel (i.e., k_{cat}/K_M is an apparent second-order rate constant with units of $s^{-1}M^{-1}$) or in terms of the interactions of Na^+, K^+, and Ca^{2+} with the most unfavorable place (energy barrier) in the channel. Correspondingly, because relative permeability in a one-ion channel does not depend on the well depth, the present results provide little information on the relative equilibrium binding affinity of different cations. An assessment of this aspect of sodium channel behavior will require measurements of ion blocking affinity or characterization of the dependence of unitary conductance on ion concentration. This simple enzymological model may be justified for the sodium channel, inasmuch as under physiological conditions this channel appears to be primarily occupied by a single ion at a time (Ravindran et al., 1991), and the present results suggest that it operates by a single-ion exclusion mechanism.

Mechanism of Ca^{2+} exclusion by the sodium channel

Although some studies of invertebrate axons have reported small inward Ca^{2+}-currents attributed to TTX-sensitive sodium channels (Meves and Vogel, 1973), the absence of a measurable inward Ca^{2+} current has been widely documented in many sodium channel preparations: frog myelinated nerve (Hille, 1972), frog muscle (Campbell, 1976), rat muscle (Pappone, 1980), neuroblastoma cells (Yamamoto et al., 1984), guinea pig heart (Nilius, 1988), and batrachotoxin-modified (Ravindran et al., 1992). Some attempts to measure the P_{Ca}/P_{Na} ratio in native sodium channels have led to the tabulation of a maximal limit of $P_{Ca}/P_{Na} < 0.1$

(Hille, 1972; Campbell, 1976). This upper limit for relative Ca^{2+} permeability is based on an extrapolation of outward current to the voltage axis to estimate the reversal potential inasmuch as actual current reversal was not observed. Similar behavior with respect to Ca^{2+} permeation was observed in the present work for the wild-type rat muscle μ1 channel expressed in *Xenopus* oocytes, which also does not exhibit a measurable inward Ca^{2+}-current (Fig. 4 *B*). Not only are Ca^{2+} and other divalent metal cations practically impermeant through sodium channels of vertebrates, but they are also relatively weak extracellular blockers of Na^+ current. Single-channel experiments have measured a low affinity K_D of ~35 mM at 0 mV for external Ca^{2+}-block with a voltage-dependence equivalent to ~20-30% of the transmembrane field (Yamamoto et al., 1984; Nilius, 1988; Sheets and Hanck, 1992; Ravindran et al., 1991). Thus, Ca^{2+} exclusion and low-affinity block by Ca^{2+} are hallmark physiological properties of sodium channel function. This aspect of our results is discussed first, because it is easiest to rationalize from a physicochemical standpoint.

From the present results, it is clear that the only mutations of the DEKA locus that are permeable to external Ca^{2+} are those in which the Lys(III) residue is replaced by a neutral (Ala, Cys, Met, Phe) or negatively charged (Glu) functional group (Tables 2 and 3). None of the single-site mutations that we have studied at Asp(I) or Glu(II), nor the Domain IV mutation, A1714E, and others reported by Heinemann et al. (1992) exhibited evidence of Ca^{2+} permeability. Thus, we conclude that the Lys(III) residue is the essential structural feature responsible for Ca^{2+} rejection by the sodium channel. Furthermore, our results strongly suggest that the mechanism by which the Lys(III) residue excludes Ca^{2+} is based on an electrostatic interaction and not mere occlusion, because substitution of Lys(III) by a bulky Phe residue (DEFA channel) results in Ca^{2+} permeation (Table 2) and its substitution by a basic Arg retains a Ca^{2+}-impermeable phenotype. In further confirmation of this notion, the striking pH-dependence of the DEHA mutation (Fig. 5) implies that a channel with an imidazole group at the Domain III position is permeable to Ca^{2+} only in the neutral, deprotonated form, and not in the protonated, positively charged form. Thus, it appears that the positively charged ammonium group contributed by the Lys residue of the DEKA locus presents a nearly insurmountable energy barrier for the entry of Ca^{2+} and presumably other divalent metal cations, but does not prevent the influx of the smaller, permeant monovalent alkali cations, Li^+, Na^+ and K^+.

In its sentry role to guard against Ca^{2+} influx, the Lys(III) residue appears to be acting in opposition to two other members of the DEKA locus, the Asp(I) and Glu(II) residues. This is evident from the experiments of Figs. 6 and 7, where it is shown that these two acidic residues actually act to enhance Ca^{2+} permeability. The all-methyl form of the DEKA locus represented by the AAAA triple-mutation is quite a good nonselective Na^+/K^+ channel, but is impermeable to Ca^{2+} even though it lacks the Lys(III) residue. This shows that the sodium channel pore without the functional groups of Asp(I), Glu(II), and Lys(III) is an inherently unfavorable tunnel for the travel of Ca^{2+} across the membrane. Permeation of Ca^{2+} in the AAAA background is only observed when at least one a carboxylate-containing residue is introduced in Domains I, II, or III. The data of Fig. 7 *B* and Table 3 show that the permeability of Ca^{2+} smoothly increases for the mutation series: AAAA $\ll$ DAAA < AEAA < AAEA < DEAA. Thus, carboxylate residues at the DEKA locus confer the ability to catalyze Ca^{2+}-permeation upon an otherwise Ca^{2+}-impermeant cation channel. These data also show that the relative ability of carboxylate-containing residues to act in this manner depends on their relative location with the I-IV homologous domains in the order of effectiveness, I < II < III.

These results for the sodium channel are analogous to those described for similar studies of calcium channel permeation. Ellinor et al. (1995) observed that Ba^{2+}-permeation is almost completely suppressed in the QQQQ and AAAA quadruple mutations of the native EEEE locus of the rabbit cardiac L-type calcium channel. These mutations also have greatly reduced binding affinity for Ca^{2+} and Cd^{2+} as measured by assay of divalent-cation blockade of Li^+ current. Reintroduction of any combinations of pairs of Glu residues into the I-IV domains of the AAAA mutant calcium channel restores Ba^{2+}-current and enhances the affinity of Ca^{2+} for blocking Li^+ current (Ellinor et al., 1995). Such evidence further supports the conclusion that the respective DEKA and EEEE loci of sodium channels and calcium channels are equivalent to an "active site" of ion discrimination in these channels with ion selectivity arising from the molecular tuning of functional groups at this location. In its evolution, the calcium channel arrived at a configuration of four glutamate carboxyl groups that yield sufficient affinity to preferentially bind Ca^{2+} and exclude Na^+ and K^+ in the presence of millimolar Ca^{2+}. The sodium channel appears to have limited its affinity for Ca^{2+} by substitutions of both a neutral Ala residue in Domain IV and a Lys residue in Domain III, the latter of which prevents residual Ca^{2+}-permeation that would be effected by the combination of Asp(I) and Glu(II). Notwithstanding the simple beauty of this mechanism, a puzzling question is: Exactly how does the Lys(III) residue limit Ca^{2+} permeation?

With the evidence for an electrostatic interaction given above, one way this could occur is by Coulombic repulsion. For example, we have previously shown that Zn^{2+} block of the cardiac sodium channel occurs as a subconductance event when monitored in single sodium channels that are modified by batrachotoxin to inhibit inactivation (Schild et al., 1991; Schild and Moczydlowski, 1994). Similar behavior is suggested in unmodified sodium channels from the Zn^{2+}-concentration dependence of macroscopic Na^+ conductance (Favre et al., 1995). It is now known that Zn^{2+} block of the cardiac sodium channel is mediated by Zn^{2+}-coordination to the sulfhydryl group of a unique Cys residue at a position corresponding to Tyr-401 in the μ1 sodium channel, directly adjacent to the D400 position in Domain

III of the DEKA motif (Schild and Moczydlowski, 1991; Satin et al., 1992; Backx et al., 1992; Favre et al., 1995). The observation of a Zn^{2+}-substate block directly demonstrates that positioning of a positive divalent charge near the DEKA locus strongly reduces Na^+ conductance but does not fully eliminate it (Schild and Moczydlowski, 1994). This suggests that ion-ion interactions in the vicinity of the DEKA locus are very important in determining which ions permeate and which do not. On this basis, the positively charged Lys group within the DEKA cluster may lower the unitary conductance of the doubly charged Ca^{2+} ion to an undetectable level while still allowing a reasonable conductance for Na^+. This idea predicts that the DEAA mutant, and possibly other neutral substitutions of the Lys(III) residue, should exhibit a higher unitary conductance for Na^+ than the wild-type channel.

Another interpretation of the role of the Lys^+ group in selecting for Na^+ against Ca^{2+} permeation can be devised on the basis of metal ion coordination chemistry and crystallographic studies of metal ion binding sites. According to the Lewis acid-base theory of metal ion ligand binding, Na^+, K^+, and Ca^{2+} ions are classified as hard acids, meaning that they have low polarizability or little tendency to distort their charge distribution (Glusker, 1991). Such hard acids strongly prefer nonpolarizable hard bases as ligands, resulting in a preference for oxygen ligands versus nitrogen or sulfur ligands. Thus, it is rather unlikely that the amino group of the Lys(III) residue would be directly involved as a ligand in coordinating Na^+, K^+, or Ca^{2+}. On the other hand, empty binding sites containing multiple uncomplexed carboxylate groups are considered to be structurally unstable due to electrostatic repulsion between neighboring oxygen anions (Glusker, 1991; McPhalen et al., 1991; Falke et al., 1994). Cations are needed to offset this repulsion. Protein carboxyl groups are often held in place by a network of hydrogen bonding interactions with —NH— groups of the peptide backbone or residue side chains. Thus, it seems likely that the function of the Lys(III) residue is to stabilize the structure of a metal ion binding site in the sodium channel by contributing to the overall charge compensation and hydrogen bonding to one or both carboxyl groups of Asp(I) or Glu(II). In this structural role, the Lys(III) residue could stabilize a metal binding cavity of an appropriate size, charge density, and ligand geometry required for ionic selectivity.

Metal ion binding sites in proteins generally tend to maintain electroneutrality in the ion-occupied state. Thus, the DEKA locus with a bound monovalent alkali cation contains two negative charges (Asp^-, Glu^-) and two positive charges (Lys^+, Na^+) adding up to zero net charge. Similarly, the EEEE locus of the calcium channel with four negative Glu^- residues would be electroneutral with two bound Ca^{2+} ions. Maintenance of these electroneutral states may be strongly influenced by pH. Metal ion binding sites are well known to be modulated by protonation equilibria, as exemplified by the sensitivity of wild-type sodium channel currents to block by H^+ with an apparent $pK_{0.5}$ of ~5 (Woodhull, 1973; Zhang and Siegelbaum, 1991; Daumas and Andersen, 1993) and the substrate block of L-type calcium channel by H^+ (Prod'hom et al., 1987). H^+ titration curves of inhibition of sodium channel conductance have been previously interpreted to suggest that protonation of one or more carboxylate groups in the pore inhibits Na^+ permeation. In the present context, the unfavorable uncompensated net charge of +1 of the DEKA locus containing a Ca^{2+} ion could theoretically be neutralized by the loss of a proton from Lys(III). Permeation of Ca^{2+} through native sodium channels at high pH (where Lys(III) might be deprotonated) has not been previously described, but in light of the present results with the DEHA channel, perhaps this point should be carefully examined. Thus, from a consideration of charge balance, pH-dependence, and hydrogen-bonding chemistry, it seems reasonable to suggest that the negatively charged Glu(II) and/or Asp(I) residue(s) interact with the positively charged Lys(III) residue, which functions in both an electrostatic and structural capacity to fine-tune physiological ion discrimination at this active site in favor of Na^+/K^+ versus Ca^{2+}.

Mechanism of selection of Na^+ over K^+ by the sodium channel

Na^+/K^+ selectivity is a crucial feature of sodium channel physiology because high selectivity for Na^+ allows the production of action potentials that transiently depolarize cell membranes by ~+130 mV. Na^+/K^+ permeability ratio measurements for sodium channel currents have been variously reported as $P_{Na}/P_K = 11.5$ (squid axon, Chandler and Meves, 1965), 11.6 (frog node, Hille, 1972), 10.0 (*Myxicola* axon, Ebert and Goldman, 1976), 20.8 (frog skeletal muscle, Campbell, 1976), and 22 (rat muscle, Pappone, 1980). In patch experiments on *Electrophorus* electrocytes, Shenkel and Sigworth (1991) observed a curious variability in P_{Na}/P_K ratios ranging from 8 to 40 in patch-to-patch, suggesting possible heterogeneity in ionic selectivity at the cellular level in this particular preparation. In the present work, our measurement of $P_{Na}/P_K \simeq 30$ for the rat muscle μ1 channel is similar to that reported by Heinemann et al. (1992) for the rat brain II sodium channel expressed in *Xenopus* oocytes. From these and many other studies, it may be generally concluded that sodium channels are able to select a smaller Na^+ ion (effective ionic diameter = 2.0 Å, Falke et al., 1994) for permeation in the range of 10–40 times more often than a K^+ ion (effective ionic diameter = 3.0 Å).

The unexpected finding here is that this important property appears to be absolutely dependent on the presence of a Lys residue in Domain III of the DEKA locus. The experiments of Fig. 3 and the data in Table 2 show that the only mutations that display a strong permeability preference for Na^+ over K^+ are those that contain the Lys(III) residue. Single-site mutations at Lys(III) to Ala, Cys, Met, Phe, His, and Arg yield P_K/P_{Na} ratios of 0.7–1.2, giving rise to a

channel that cannot discriminate Na^+ from K^+. Other residues in the DEKA locus also appear to have a modest influence on this property, inasmuch as the Glu(II) to Ala mutation increased P_K/P_{Na} by a factor of 3 from 0.03 to 0.09, and in the work of Heinemann et al. (1992), the Ala(IV) to Glu mutation increased P_K/P_{Na} by a factor of 5 from 0.03 to 0.15. In contrast, mutation of Asp(I) to Ala appears to have no effect at all on Na^+/K^+ selectivity (Table 2). Thus, Na^+/K^+ selectivity is dependent on the presence of Lys(III) in an all-or-nothing manner, with Glu(II) and Ala(IV) making secondary contributions. These observations present us with the challenge of attempting to explain how a Lys residue may give rise to selectivity for Na^+over K^+.

Given that a three-dimensional structure of the sodium channel is required to provide the intimate molecular details that are needed to answer this question, some guesswork can be offered based on current understanding of ion selectivity mechanisms. Perhaps the safest point is that only a small difference in free energy is necessary to generate the observed selectivity ratio of P_K/P_{Na}. For example, a ratio in the range of 10–40 for two kinetic or equilibrium constants corresponds to a free energy difference in the range of only 1.4–2.2 kcal/mol. Thus, the free energy difference necessary to generate the physiologically relevant difference in permeability of Na^+ versus K^+ is similar in magnitude to that contributed by a single hydrogen bond (Fersht, 1987). This recognition has been evident since Hille (1975b) formulated an Eyring rate theory model of sodium channel permeation that was based on three binding sites (energy wells) and four energy barriers (transition states) that could only contain one ion at a time. In Hille's 1975 model, the highest energy barrier was positioned close to the extracellular side of the membrane just after the first ion binding site. The difference between the energy profiles for K^+ and Na^+ found to satisfy the observed ratio of P_K/P_{Na} was 2.7 *RT* units or 1.6 kcal/mol. Similar differences in energy profiles for Na^+ and K^+ permeation derived from fitting extensive single-channel I-V data to two-ion occupancy models have also been obtained in more recent modeling studies (Ravindran et al., 1992; Naranjo and Latorre, 1993; French et al., 1994). From this perspective, the function of the Lys(III) residue may be to generate a small energy difference in the rates of Na^+ and K^+ association to and dissociation from their binding site.

As discussed above in reference to the mechanism of Ca^{2+} rejection, the ammonium form of the Lys(III) residue could potentially form an ion pair, actually a strong hydrogen bond, with either of the negatively carboxylate groups of the Asp(I) and Glu(II) residues. In addition to tuning the electrostatic environment in favor of a monovalent group IA cation versus a divalent group IIA cation, this same interaction could also shape the ion binding site to preferentially accommodate Na^+ instead of K^+. This effect could be achieved by defining a smaller-sized cavity that more readily accepts Na^+ than the larger K^+ ion, or by positioning the coordinating oxygen atoms to favor a preferred Na^+-coordination geometry. Either of these structural/chemical effects may underly the ability of Lys(III) to generate the small energy difference in ion interaction with the binding site versus Na^+/K^+ dehydration energy that is necessary to achieve selectivity of Na^+ over K^+ (Eisenman and Horn, 1983).

Implications for the active site of ion selectivity in sodium channels based on multi-ion conduction theory and examples of metal binding sites in proteins

It is recognized that protein mutagenesis experiments can lead to serious misinterpretations because the flexibility of protein structure can generate unsuspected structural changes at locations far removed from the site of the mutation. However, the new results of this paper, together with other mutagenesis studies of sodium channels and calcium channels, continue to support the notion that residues of the DEKA/EEEE loci are at the heart of the ionic selectivity mechanism of these two classes of ion channels. The overall results indicate that the ionic selectivity function is specifically targeted by the mutations in question inasmuch as the gating function of the various DEKA/EEEE mutant channels appears quite normal and the transfer of calcium channel-like selectivity to the sodium channel implies that catalytic specificity can be transferred across two homologous proteins of a protein family in a relatively seamless manner.

The functional behavior of the various mutations thus leads to the conclusion that the DEKA/EEEE loci contribute to the formation of ion binding sites in sodium channels/calcium channels that principally determine ionic selectivity. In particular, the results of divalent cation blocking experiments in calcium channel mutations of the EEEE residues are consistent with behavior expected if each of the individual Glu carboxylate groups serve as direct ligands of Ca^{2+} and Cd^{2+} ions (Yang et al., 1993; Parent and Gopalakrishnan, 1995; Ellinor et al., 1995). Assuming that we are extrapolating to a protein of homologous architecture, we may interpret the results of our permeability measurements on mutant sodium channels as reflecting the perturbation of ion-binding interactions taking place at the DEKA residues. On this basis, we can discuss how these two particular clusters of four residues may explain principal aspects of ion permeation and selectivity.

Much evidence indicates that the families of voltage-dependent potassium channels, calcium channels, and sodium channels are pores that under particular conditions can simultaneously contain more than one ion, and that ion-ion interactions are very important in determining ionic selectivity and rates of conduction (Begenisich, 1987; Tsien et al., 1987; Yellen, 1987). A key feature of calcium channels is that they can conduct monvalent ions (e.g., Li^+, Na^+) only in the absence of Ca^{2+}. At least two Ca^{2+} ions can simultaneously bind to the channel, because titration of

monovalent ion current exhibits a biphasic inhibition by micromolar Ca^{2+} and a concentration-dependent increase of Ca^{2+}-current in the range of millimolar Ca^{2+} (Almers and McCleskey, 1984; Hess and Tsien, 1984). This fact, together with recent evidence that the interaction of two divalent cations, Ba^{2+} and Cd^{2+}, is weakened by neutral mutations of any of the EEEE residues, has been taken to indicate that two divalent metal cations bind in close proximity when coordinated by these same carboxylate groups (Ellinor et al., 1995). Thus, in the Ca^{2+} channel it is probable that all or some part of the Ca^{2+}-Ca^{2+} interaction underlying the multi-ion conduction mechanism occurs at the EEEE locus.

In considering how this picture might be adapted to the sodium channel, it is useful to consider data previously gathered on the minimum diameter of channel pores. Organic cations have been previously used to determine the size of the narrowest region of the pores of sodium channels and calcium channels, by finding the largest organic cation that is able to carry current and thus successfully traverse these channels. For the sodium channel the largest permeant cations are aminoguanidinium and hydroxyguanidinium ions (Hille, 1971), whereas the calcium channel can accept larger alkylammonium cations, with the largest permeant cation being tetramethylammonium, as measured in the absence of Ca^{2+} (McCleskey and Almers, 1985). These latter studies have determined that the most constricted region of the sodium channel is equivalent to a rectanglar area of 3.1 × 5.1 Å, and that for the calcium channel is a square area of 5.5 × 5.5 Å. If one accepts the general notion that ion selectivity in pores primarily occurs at the narrowest region where protein atoms can directly interact with preferred ions, then one would surmise that these minimum cutoff areas actually correspond to the cavities framed by the respective DEKA/EEEE residues. This supposition would be consistent with the idea that the larger size and opposite charge of the butylammonium group of the sodium channel Lys(III) residue, as compared to the proprionate group of a calcium channel Glu(III) residue, is the primary basis of the smaller cutoff diameter of the sodium channel versus the calcium channel as probed by organic cations.

Viewed from this perspective, the Lys(III) residue shown in this study to dictate ionic selectivity of the sodium channel acquires an obvious functional significance: the alkylammonium group of Lys(III) appears to be a resident pore cation specifically engaged in ion-ion interactions. In the doubly occupied state, the hypothetical active site of calcium channel selectivity, at 5.5 × 5.5 Å, is wide enough to contain two Ca^{2+} ions of ionic diameter, 2.1 Å. The sodium channel active site, at 3.1 × 5.1 Å, can easily fit only one Na^{+} ion of ionic diameter, 2.0 Å, and would be strained by ions larger than K^{+} with a diameter of 3.0 Å. With this picture in mind, it is easy to imagine that the endogenous alkylammonium ion of the Lys(III) residue performs a role similar to Ca^{2+} in the high-affinity binding site of the singly occupied Ca^{2+} channel. The lysine-ammonium ion of the sodium channel energetically tunes the affinity and kinetics of mobile ions that either pass through the channel or are rejected by the cooperating residues of the selectivity filter.

As also noted by Ellinor et al. (1995), such models of channel selectivity mediated by a handful of residues at a central location in a pore might be dismissed as fantasy without examples of metal ion binding sites in proteins. In the case of Ca^{2+}-binding sites, there are several striking examples that evoke the hypothetical structure we and others have envisioned for the calcium channel selectivity locus. In the atomic structure of the icosahedral bacteriophage PX174, a cation appears to be bound at the convergence of five Asp117 residues at the center of a fivefold symmetry axis of the phage G protein (McKenna et al., 1992). This appears to be a clear example of the convergence of radially symmetric carboxylate groups forming a Ca^{2+}-binding site, as proposed for the pseudotetrameric calcium channel. This structure is also interesting as a channel model, inasmuch as the pentameric viral protein subunit interface defines a hydrophilic pore with a diameter that varies from 6 to 26 Å. The narrow 6-Å constriction occurs near the convergence of the five Asp residues involved in Ca^{2+} binding. As also cited by Ellinor et al. (1995), the proteinase thermolysin contains two bound Ca^{2+} ions that are located at a distance of only 3.8 Å apart from each other (McPhalen et al., 1991). One of these Ca^{2+} ions is bound in a seven-coordinate pentagonyl bipyramid geometry and the other Ca^{2+} ion is bound in a six-coordinate octahedral geometry. This structure is largely stabilized by three acidic residues, one Asp and two Glu, whose oxygen atoms directly coordinate to both Ca^{2+} ions. A similar example is found in the crystal structure of the decameric serum amyloid *P* component, a protein that also contains two Ca^{2+} binding sites located 4.0–4.3 Å apart (Emsley et al., 1994). In this structure, also, one site is seven-coordinate and the other is six-coordinate with two carboxylate residues, one Glu and one Asp, plus one anion, acetate, all providing oxygen ligands to both Ca^{2+} ions. These and other examples of divalent cation binding sites in the database of known protein structures serve to demonstrate that a doubly occupied state with two closely bound Ca^{2+} ions is a structurally feasible intermediate state in the process of calcium channel permeation.

The literature contains fewer examples of Na^{+} and K^{+} binding sites in proteins, but one previously discussed example (Miller, 1993) merits special attention. The enzyme dialkylglycine decarboxylase is activated by K^{+} and inhibited by Na^{+} at an alkali cation binding site near the active site of catalysis. K^{+} binds at this site with an octahedral geometry and is coordinated to six oxygen atoms contributed by one Asp carboxylate group, one Ser hydroxyl group, three backbone carbonyl groups, and one H_2O molecule (Toney et al., 1993). Na^{+} can also bind at this location in a distorted trigonal bipyramidal geometry coordinated to five oxygen atoms contributed by one Asp carboxylate, two backbone carbonyl groups, and two water molecules. In a representation of the Li^{+}-complexed form of this enzyme,

the Asp residue coordinating Li^+ is directly hydrogen-bonded to two different His residues (Hohenester et al., 1994). These latter three structures of dialkylglycine decarboxylase complexed with different Group IA cations provide a good analogy to the picture that we propose of an alkali cation bound within a cage of residues at the DEKA locus with at least one carboxylate group, Asp(I) or Glu(II), coordinating to a Na^+ ion, while also hydrogen-bonding with Lys(III). Another interesting example of a Na^+-binding site is found in the crystal structure of two adjacent fibronectin type III repeats (Fn-III) from the neural cell adhesion molecule, neuroglian (Huber et al., 1994). In this structure, a five-coordinate square pyramidal site for Na^+ is formed at the interface of two Fn-III domains. Four residues on two different loops, one from each Fn-III domain provide oxygen ligands to Na^+: a Ser —OH, and amide oxygen of Asn, and backbone carbonyl oxygens of Ile and Pro. This is a particularly relevant model structure for comparison with the Na^+ channel site, since the DEKA residues in the P-region of homologous Domains I-IV are thought to reside on loop-like regions of the peptide backbone (Lipkind and Fozzard, 1994; Guy and Durell, 1995). Finally, the proteinase thrombin is an example of an enzyme that is allosterically activated by the binding of Na^+ (Wells and DiCera, 1992). Curiously, the octahedral Na^+-binding site in this enzyme is specified by a loop of residues in which the backbone carbonyl oxygens of two different basic residues, Arg and Lys, coordinate with the metal ion (Dang et al., 1995). This latter example lends credence to the counterintuitive idea of a basic residue, Lys(III), located in close proximity to a Na^+ ion bound to the DEKA locus of the sodium channel.

In closing, the notion of the butylammonium side chain of Lys(III) as a tethered cation within the sodium channel selectivity locus has certain biophysical implications. The positively charged Lys side chain (as well as the negatively charged Asp and Glu residues) may be subject to the influence of a certain fraction of transmembrane electric field. If it is mobile within the pore, the position of the Lys side chain would be expected to fluctuate within the channel. At negative voltages, the average position of the butylammonium group would be expected to shift slightly toward the intracellular side of the pore and, at positive voltages, its average location would move toward the extracellular side. This displacement might only be a fraction of an angstrom, but it could give rise to important functional properties. Movement of this charged group might facilitate the passage of permeant ions and result in structural changes central to ionic selectivity and I-V behavior. This kind of fluctuation corresponds to pore dynamics proposed to occur in generalized fluctuating barrier models of ion permeation through channel proteins (Läuger et al., 1980) and may be expected to generate current noise analogous to the "open channel noise" observed in many single-channel recordings (Heinemann and Sigworth, 1993). These other ideas, stemming from the results we have described, suggest a number of predictions that we hope to test in future experiments.

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