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Evolution of Eukaryotic Ion Channels: Principles Underlying the Conversion of Ca^{2+}-Selective to Na^+-Selective Channels

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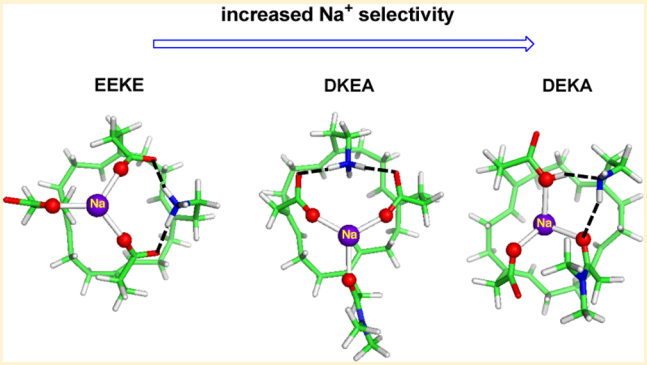
ABSTRACT: Ion selectivity of four-domain voltage-gated Ca^{2+} and Na^+ channels, which is controlled by the selectivity filter (the narrowest region of an open pore), is crucial for electrical signaling. Over billions of years of evolution, mutation of the Glu from domain II/III in the **EEEE/DEEA** selectivity filters of Ca^{2+}-selective channels to Lys made these channels Na^+-selective. Why Lys is sufficient for Na^+ selectivity and why the **DKEA** selectivity filter is less Na^+-selective than the **DEKA** one are intriguing, fundamental questions. By computing the free energy for replacing Ca^{2+} inside model selectivity filters with Na^+, we find that the nonmetal-ligating Lys in the **DKEA/DEKA** selectivity filter attenuates metal−protein interactions to such an extent that solvation effects become dominant, favoring Na^+. It constricts and rigidifies the **DEKA** pore to bind Na^+ optimally, highlighting the importance of lysine's nonobvious structural role, in addition to its electrostatic role, in the selectivity of Na^+ over Ca^{2+}.



INTRODUCTION

Eukaryotic voltage-gated sodium (Na_v) and calcium (Ca_v) channels are instrumental in regulating muscular excitation and contraction, gene expression, signal transduction, epithelial transport of nutrients and ions, taste and pain sensation, and release of hormones and neurotransmitters.[1] They belong to the superfamily of 4×6TM ion channels[2] containing four homologous domains (I−IV) with each domain composed of six transmembrane (6TM) segments. These channels selectively transport the cognate ion from the extracellular to intracellular compartments along a concentration gradient and efficiently discriminate between the "native" ion and its rival cations in the channel's selectivity filter (SF), the narrowest region of an open pore. Elucidating the principles underlying ion selectivity of these channels is important, as some Na_v and Ca_v channels are major targets for drugs to treat various diseases including arrhythmias, Dravet syndrome, epilepsy, hypertension, and pain. In this work, we focus on the competition between two ions of similar size, Ca^{2+} and Na^+, in the SFs of eukaryotic (as opposed to prokaryotic) Na_v and Ca_v channels.

Although high-resolution X-ray structures of metal-bound eukaryotic Ca_v and Na_v channels are not yet available, biochemical and site-directed mutagenesis experiments have revealed their SF compositions. The SF of the high-voltage-activated Ca_v1 (L-type) or Ca_v2 channel comprises four conserved Glu residues (**EEEE** locus) donated by each of the four homologous domains,[3−5] whereas that of the low-voltage-activated Ca_v3 (T-type) channel comprises two Glu and two Asp residues (**EEDD** locus).[6] The Ca_v1 and Ca_v2 channels are highly Ca^{2+}/Na^+-selective with a Ca^{2+}:Na^+ permeability ratio, $P_{Ca^{2+}}/P_{Na^+} > 1000{:}1$,[7] whereas the Ca_v3 channels are less selective[5] with $P_{Ca^{2+}}/P_{Na^+} \sim 87-234$.[8] Other types of tetrameric channels (e.g., invertebrate Na_v2) with **DEEA** SFs are even less Ca^{2+}-selective[9,10] with $P_{Ca^{2+}}/P_{Na^+} \leq 22$.[9] Although these channels conduct Ca^{2+}, their sequences are closer to those of Na_v1 rather than Ca_v channels. Furthermore, sodium leak conductance channel (NALCN) isoforms with an **EEEE** or **EDEE** SF also preferably transport Ca^{2+} rather than Na^+.[2]

In remarkable contrast to the SF composition of Ca_v channels, the SFs of eukaryotic Na^+-selective Na_v channels all possess a Lys that is critical for Na^+/Ca^{2+} selectivity. The SFs of Na_v1 channels of higher (bilaterian) animals (e.g., vertebrates, cephalochordates, urochordates, mollusks, annelids, and arthropods) are composed of conserved Asp, Glu, Lys, and Ala from domains I−IV forming a **DEKA** locus.[11−13] These Na^+-selective channels exclude Ca^{2+} under physiological conditions.[1,14] The **DEKA** Lys in Na_v1 channels is sufficient to yield Na^+/Ca^{2+} selectivity, as its mutation to a negatively charged Asp or Glu rendered the channel Ca^{2+}/Na^+-selective.[14−17] Conversely, mutation of the domain III Glu from the **EEEE** SF of human cardiac Ca_v channel and the **DEEA** SF of the Ca^{2+}-selective BSC1 channel to Lys made the channel more permeable to Na^+ than to Ba^{2+}.[5,9] The same four residues comprising the SFs of Na_v1 channels line the SFs of $Na_v2.5$ channels in cnidaria (sea anemones, corals, hydras, and jellyfish), but the Glu and Lys belong to domain III and II,

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respectively. This **DKEA** motif[2,10] is less Na^+/Ca^{2+} selective than its **DEKA** counterpart: Whereas the **DEKA** SF of rat brain Na_v channel is impermeable to Ca^{2+}, the swopped **DKEA** SF of the mutant channel allows Ca^{2+} through.[14] In analogy, substituting the Glu from the **DEEA** SF of the *Nematostella vectensis* $Na_v2.1$ channel with Lys resulted in a Ca^{2+}-impermeable **DEKA** SF but a Ca^{2+}-permeable **DKEA** SF.[10] Notably, NALCN isoforms with **EEKE** or **EKEE** SF that are present in many eukaryotes are more permeable to Na^+ than Ca^{2+} ($P_{Na^+}/P_{Ca^{2+}} \approx 3$).[2]

Understanding the evolutionary origin of eukaryotic Ca_v, Na_v, and NALCN channels helps to elucidate how their SFs became selective for their cognate ion. The origin of these channels has been suggested to have evolved from single domain (1×6TM) ancestors (probably homotetrameric voltage-gated K^+ channels) through domain duplication and subsequent domain divergence.[17,18] Early in eukaryotic evolution, the gene for a 6TM channel duplicated yielding a protein with two domains (2×6TM). The gene for the two-domain protein in turn duplicated to create a protein with four domains that can form a 4×6TM ion channel on its own. Such a 4×6TM channel evolved permeability to Ca^{2+}, which conveniently became an intracellular signaling messenger. The simplest and oldest animals and their eukaryote relatives (choanoflgellates, sponges, protists, poriferans, and placozoans), which lack nervous systems, possess only Ca^{2+}-selective channels comprising **EEEE** or **DEEA** SFs.[8,9,19]

Eukaryotic Na_v channels are believed to have evolved from eukaryotic Ca_v channels,[1,18] as the four homologous domains of Na_v channels are more similar to those of Ca_v channels than to each other. Their relationship to single 6TM Na^+-selective bacterial channels with **EEEE** SFs is unclear. The appearance of Na^+-selective eukaryotic Na_v channels might be related to the evolution of more complex nervous systems in eukaryotes where separation between Ca^{2+}- and Na^+-dependent signaling in the cell was required: Na^+ currents, enabling fast and accurate signaling, are better suited to generate membrane excitability in complex nervous systems than Ca^{2+} currents, which may interfere with intracellular Ca^{2+} signaling and exert cytotoxicity.[1] The Na^+-selective cnidarian $Na_v2.5$ and bilaterian Na_v1 channels with **DKEA** and **DEKA** SFs, respectively, have been proposed to evolve independently from ancestral Ca^{2+} channels with **DEEA** SFs,[10] whereas the Na^+-selective NALCN channels with **EKEE**/**EEKE** pores have evolved independently from ancestral Ca^{2+} channels with **EEEE** SFs.[2]

The above summary of our current knowledge shows (see Table 1) that Ca^{2+}-selective channels have **EEEE**, **EDEE**, **EEDD**, and **DEEA** SFs lined by three or four carboxylates, whereas Na^+-selective channels have **EKEE**, **EEKE**, **DKEA**, and **DEKA** SFs with an invariant lysine from the second or third domain. Among the 4×6TM channels, eukaryotic Ca_v channels with an **EEEE** pore exhibit the highest Ca^{2+}/Na^+ selectivity, whereas Na_v1 channels with the **DEKA** SF show the highest Na^+/Ca^{2+} selectivity.[1,2,14,16] Previous theoretical studies have focused on the competition between (i) Ca^{2+} and Na^+ in model **EEEE**, **DDDD**, or **DEEA** SFs[19−25] as well as (ii) Na^+ and K^+[26−29] or Ca^{2+}[26,30] in model **DEKA SFs**. Notably, the transition from Na^+ selectivity to Ca^{2+} selectivity has been studied in **DEKA**, **DEKE**, **DEEA**, and **DEEE** SFs, whose pores were modeled as water-filled cylinders of radius 3.5 Å, whereas the Asp/Glu and Lys side chains (assumed to be infinitely flexible) were modeled as two half-charged oxygen ions and a positively charged ammonium ion, respectively, whereas alanine was not represented.[26,31] Because the different SF pores, which are assumed to have the same radius, only detect radii and charges of ions, the specific role of lysine in generating Na^+-selective **EKEE**, **EEKE**, **DKEA**, or **DEKA** SFs remains unclear. Furthermore, no studies (to our knowledge) have addressed the following puzzling question: Why is the **DEKA** SF more selective for Na^+ over Ca^{2+} than the **DKEA** one, even though both motifs have identical composition and the same net charge?

To address these questions, we evaluated how the Ca^{2+} vs Na^+ competition in a model SF is affected by changing the composition, overall charge, dielectric constant, size, and rigidity of the SF. The metal ions and their ligands, which play a key role in the Ca^{2+} vs Na^+ competition, were treated using density functional theory to account for electronic effects such as polarization of the participating entities and differential amounts of charge transfer from the ligands to Ca^{2+} vs Na^+; various environments created by the protein matrix and surroundings were represented by an effective dielectric constant varying from 10 to 30.[23,24] The outcome of the competition between the bulk solvent and the protein ligands for the native cation in a SF was assessed by computing the free energy ΔG^x for replacing Ca^{2+} bound inside the SF, [Ca^{2+}-filter], with Na^+

$$[Na(H_2O)_6]^+ + [Ca^{2+}\text{-filter}] + H_2O \rightarrow [Na^+\text{-filter}] + [Ca(H_2O)_7]^{2+} \quad (1)$$

The ion exchange free energy for eq 1 was computed as a sum of (i) the gas-phase free energy (electronic effects) and (ii) the solvation free energy difference between the products and reactants (solvation effects), as described in the Methods section. A positive ΔG^x implies a Ca^{2+}-selective filter, whereas a negative ΔG^x implies a Na^+-selective one. Our aim is to yield reliable trends in the free energy changes with varying parameters in order to identify the key factors favoring the native ion in various Ca^{2+} and Na^+-selective SFs. The methodology used has yielded trends in the free energy changes that agree with experimental findings in previous works[23,24,28,32,33] and herein.

Table 1. Ca^{2+}:Na^+ Permeability Ratios, $P_{Ca^{2+}}/P_{Na^+}$, of Eukaryotic Ca^{2+}- and Na^+-selective Ion Channels

ion channel type	SF	$P_{Ca^{2+}}/P_{Na^+}$
	Ca^{2+}-selective	
Ca_v1 (L-type)/Ca_v2	**EEEE**	∼1000[7]
NALCN	**EEEE**/**EDEE**	NA[a]
Ca_v3 (T-type)	**EEDD**	87−234[8]
Na_v2	**DEEA**	≤22[9]
	Na^+-selective	
NALCN	**EKEE**/**EEKE**	∼0.33[2]
$Na_v2.5$	**DKEA**	NA[a]
Na_v1	**DEKA**	<0.09[17]

[a]"NA" means $P_{Ca^{2+}}/P_{Na^+}$ value is not available.

METHODS

Selectivity Filter Models. Because biochemical and mutagenesis[3−5,14−17,34] studies indicate monolayered tetrameric SFs in voltage-gated sodium (Na_v) and calcium (Ca_v) channels, SFs containing four metal-ligating groups such as $-CH_2-COO^-$ (modeling the Asp side chain), $-CH_2-CH_2-COO^-$

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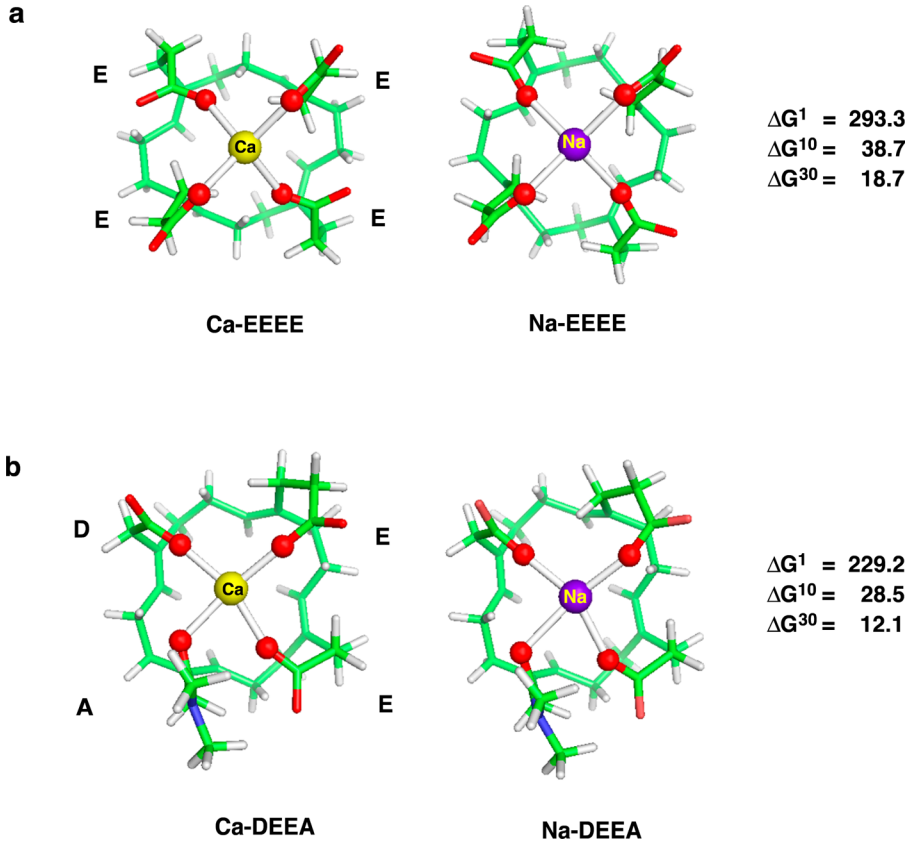

Figure 1. B3-LYP/6-31+G(3d,p) optimized structures of Ca^{2+} and Na^{+}-bound model SFs: (a) **EEEE** motif and (b) **DEEA** motif. The free energies ΔG^{x} (in kcal/mol) for replacing Ca^{2+} in the model SF characterized by dielectric constant x with Na^{+} are shown on the right. ΔG^{1} refers to metal exchange free energy in the gas phase, whereas ΔG^{10} and ΔG^{30} refer to metal exchange free energies in an environment characterized by an effective dielectric constant of 10 and 30, respectively.

(representing the Glu side chain), $-CH_2-CH_2-NH_3^+$ (modeling the Lys side chain), and $-CON(CH_3)_2$ (representing the Ala backbone peptide group) were modeled. The metal-ligating groups were coordinated to the permeating ion (Ca^{2+} or Na^{+}) and attached to a carbon–hydrogen ring scaffold via methylene spacers (Figures 1 and 2). Models of the SFs were built using GaussView version 3.09[35] following the guidelines from our previous work.[32] They were designed to maximize resemblance to the SFs of Ca_v and Na_v channels and were constructed on the basis of the following considerations:

- The ring mimics the tetrameric state of the ion channel pore.
- The ring scaffold mimics the role of the second shell in properly orienting the metal-ligating groups to interact with the passing cation without obstructing the permeation pathway. Detaching the metal ligands from the ring scaffold would lead to unrealistic structures with one or two metal-ligating groups occluding the ion passage pathway.[24]
- The metal-ligating groups and their connection to the ring are flexible enough to allow them to optimize their positions upon metal binding.
- The shape and C–H orientations of the ring do not obstruct the pore lumen and hamper the metal-ligating groups from coordinating to the metal ion.[32]

Gas Phase Free Energy Calculations. Among several combinations of different ab initio/DFT methods (HF, MP2, S-VWN, and B3-LYP) and basis sets (6-31+G(d,p), 6-31+G-(2d,2p), 6-31+G(3d,p), 6-31+G(3d,2p), 6-311++G(d,p), and 6-311++G(3df,3pd)), the B3-LYP/6-31+G(3d,p) method has been shown to be the most efficient in yielding dipole moments of the metal ligands that are closest to the respective experimental values; it can also reproduce (within experimental error) the metal–oxygen bond distances in aqua and crown-ether complexes, which resemble metal-occupied ion channel pores.[32] Hence, the B3-LYP/6-31+G(3d,p) method was used to optimize the geometry of each metal complex without any constraints and to compute the electronic energies, E_{el}, using the Gaussian 09 program.[36] The lowest-energy structure resulting from various trial starting configurations was chosen for evaluating the gas-phase free energy.

Frequency calculations for each optimized structure were performed at the same level of theory. No imaginary frequency was found in any of the optimized structures. The B3-LYP/6-31+G(3d,p) frequencies were scaled by an empirical factor of 0.9613[37] and used to compute the thermal energies including zero-point energy (E_{th}) and entropies (S). The differences ΔE_{el}, ΔE_{th}, ΔPV (work term), and ΔS between the products and reactants in eq 1 were used to calculate the gas-phase ΔG^{1} free energy at T = 298.15 K according to

$$\Delta G^{1} = \Delta E_{el} + \Delta E_{th} + \Delta PV - T\Delta S \quad (2)$$

The basis set superposition error had been found to be negligible for the type of ion exchange reactions described by eq 1;[32] hence, it was not considered in the present calculations.

Solvation Free Energy Calculations. The solvation free energies of products and reactants in eq 1, ΔG_{solv}^{x}, were

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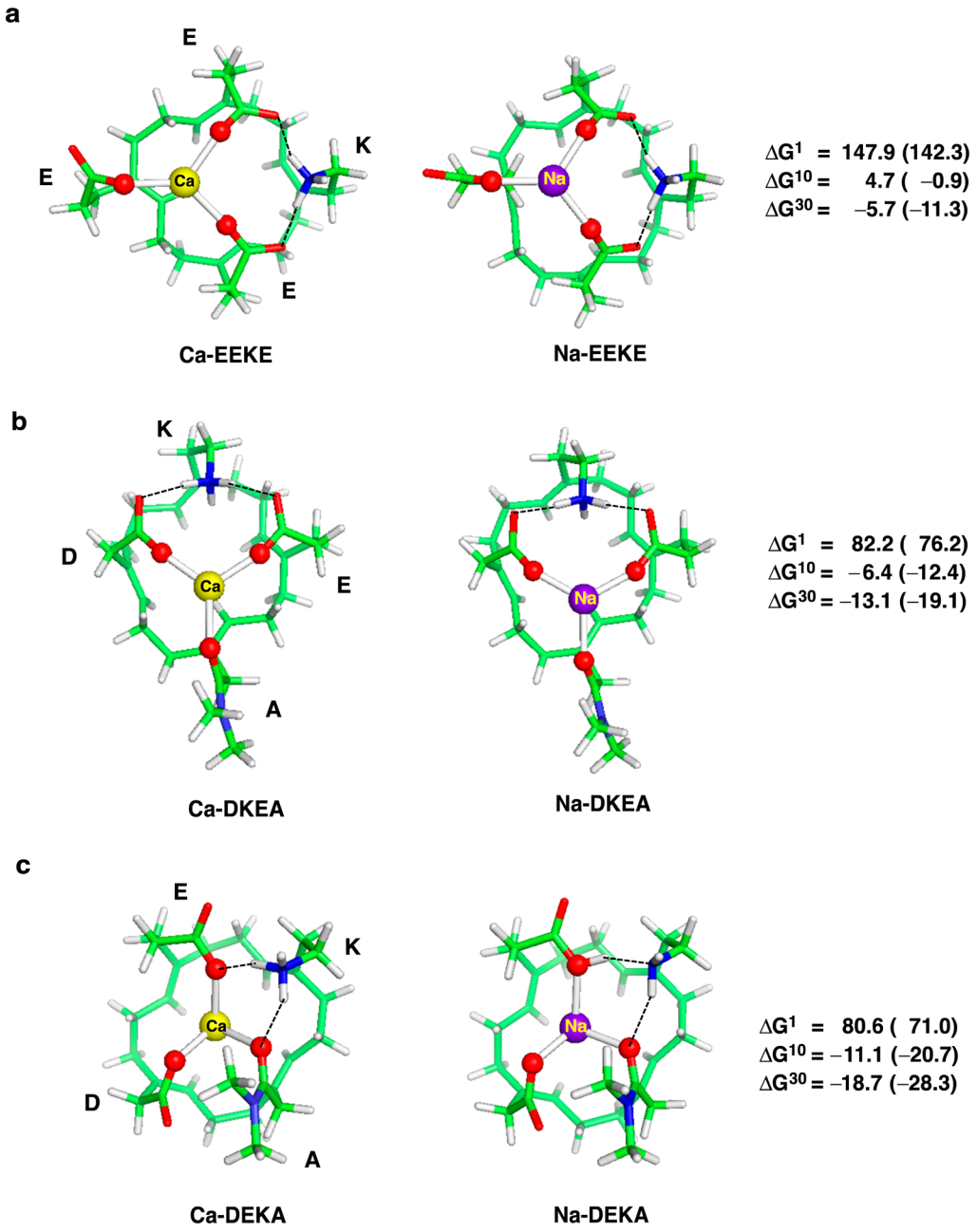

Figure 2. B3-LYP/6-31+G(3d,p) optimized structures of Ca^{2+} and Na^{+}-bound model SFs: (a) **EKEE**/**EEKE** motif, (b) **DKEA** motif and (c) **DEKA** motif. The free energies ΔG^{x} (in kcal/mol) for replacing Ca^{2+} in the SF characterized by dielectric constant x with Na^{+} are shown on the right. ΔG^{1} refers to metal exchange free energy in the gas phase, whereas ΔG^{10} and ΔG^{30} refer to metal exchange free energies in an environment characterized by an effective dielectric constant of 10 and 30, respectively. Free energies of metal exchange in a rigid Na^{+}-optimized pore prohibited from relaxing upon Ca^{2+} binding are given in parentheses.

estimated by solving Poisson's equation using finite difference methods[38,39] with the MEAD (Macroscopic Electrostatics with Atomic Detail) program,[40] as described in previous works.[32] Natural bond orbital atomic charges, which are known to be numerically quite stable with respect to basis set changes,[41] were employed in the calculations. The effective solute radii were obtained by adjusting the CHARMM[42] van der Waals radii to reproduce the experimental hydration free energies of Na^{+}, Ca^{2+}, and model ligand molecules to within 1 kcal/mol.[23,24] The resulting values (in Å) are: $R_{Na} = 1.72$, $R_{Ca} = 1.75$, $R_{C} = 1.95$, $R_{N} = 1.75$, $R_{O}(Na{-}H_2O) = 1.85$, $R_{O}(Ca{-}H_2O) = 1.84$, $R_{O}(-CON(CH_3)_2) = 1.72$, $R_{O}(Na{-}COO) = 1.40$, $R_{O}(Ca{-}COO) = 1.25$, $R_{H} = 1.50$, $R_{H}(H_2O{-}Na) = 1.26$, $R_{H}(H_2O{-}Ca) = 1.053$.

Solution Free Energy Calculations. The ion exchange free energy for eq 1 in an environment characterized by an effective dielectric constant x is given by

$$\Delta G^{x} = \Delta G^{1} + \Delta G_{solv}{}^{x}([Na - filter]) + \Delta G_{solv}{}^{x}([Ca(H_2O)_7]) - \Delta G_{solv}{}^{x}([Ca - filter]) - \Delta G_{solv}{}^{x}([Na(H_2O)_6]) - \Delta G_{solv}{}^{x}(H_2O) \quad (3)$$

where ΔG^{1} is the gas-phase free energy for eq 1 and $\Delta G_{solv}{}^{x}$ is the free energy for transferring a molecule in the gas phase to a

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medium characterized by an effective dielectric constant x. The methodology used to compute ΔG^x had been validated against experimental ion exchange free energies between biogenic metal cations such as Na^+, K^+, Mg^{2+}, and Ca^{2+} in crown ethers (resembling **SF** pores)[32] or in systems containing carboxylic ligands (nitrilotriacetic acid)[23] with interactions that are similar to the Asp/Glu carboxylates lining the **SF**s of ion channels. The computed metal exchange free energies are in line with the experimental estimates to within 1 kcal/mol, as shown in our previous work.[33]

RESULTS

In this work, we do not attempt to reproduce the *absolute* free energy for replacing Ca^{2+} bound inside the SF with Na^+. Therefore, we focus on the sign and the *relative* magnitude of the free energy change upon varying a given parameter in interpreting the results.

Comparison with Experiment. The predicted outcomes of the Ca^{2+} vs Na^+ competition in the "ancestral" Ca^{2+}-selective SFs agree with experimental observations: (1) The **EEEE** and **DEEA** SFs are selective for Ca^{2+} over Na^+ (Figure 1, positive ΔG^x), because compared to Na^+, dicationic Ca^{2+} with stronger charge-accepting ability interacts more favorably with the **EEEE** or **DEEA** residues, resulting in a free energy gain that outweighs its larger dehydration penalty. (2) The **EEEE** SF is more Ca^{2+}-selective than its **DEEA** counterpart (more positive ΔG^x in Figure 1a than in Figure 1b). This is mainly because Na^+ is more destabilized in the **EEEE** SF with a net charge Q of -4 than in the **DEEA** SF with $Q = -3$. (3) The **EEEE** SF (ΔG^1 = 293 kcal/mol) appears to be slightly more Ca^{2+}/Na^+-selective than its **EEDD** counterpart (not shown in Figure 1, ΔG^1 = 291 kcal/mol).

Since mutation of a Glu from domain II or III of the Ca^{2+}-selective **EEEE** or **DEEA** SF to a Lys resulted in a Na^+-selective filter, we evaluated the effect of these mutations on the Na^+ vs Ca^{2+} competition in model **EKEE**/**EEKE**, **DKEA**, and **DEKA** SFs. (Note that the SF model shown in Figure 2a cannot distinguish between **EKEE** and **EEKE** SFs, hence it represents **EKEE** or **EEKE** SF.) The predicted outcomes of the Na^+ vs Ca^{2+} competition in these Lys-containing SFs also agree with the experimental observations:[14] (i) The **DEKA** SF is more Na^+-selective than the **DKEA** SF with the same composition and net charge (more negative ΔG^x in Figure 2c than in Figure 2b). (ii) It is the most Na^+-selective with the most negative ΔG^x among the three SFs, whereas the **EEKE** SF is the least Na^+-selective.

Why a Lysine in the SF Could Generate Na^+- or Ca^{2+}-Selective Ion Channels. The results not only agree with experiments but also reveal why mutation of Glu from domain II or III in the Ca^{2+}-selective **EEEE** or **DEEA** SF to Lys converts the SF into a Na^+-selective one:

Metal Coordination Number (CN). Because Lys is *not* a metal ligand, the metal CN decreases from four in the Ca^{2+}-selective **EEEE** and **DEEA** SFs to three in the Na^+-selective **EKEE**/**EEKE** and **DKEA**/**DEKA** SFs, respectively. The decrease in metal CN decreases the net charge transfer from the ligands to Ca^{2+} more than that to Na^+ in a narrow, rigid pore, thus decreasing the competitiveness of Ca^{2+} over Na^+. The metal CN of three in the **DEKA** SF also favors Na^+ over other divalent metals such as Mg^{2+} ($P_{Mg^{2+}}/P_{Na^+} < 0.1$), Sr^{2+} ($P_{Sr^{2+}}/P_{Na^+} < 0.1$), and Ba^{2+} ($P_{Ba^{2+}}/P_{Na^+} < 0.09$).[17]

Filter's Net Charge Density. Exchanging a negatively charged Glu for a positively charged Lys reduces the net negative charge Q of the SF, leading to enhanced Na^+/Ca^{2+} selectivity: The gas-phase free energy ΔG^1 (Figure 3, red circles) for replacing Ca^{2+} with Na^+ in the **EEEE** ($Q = -4$) and **DEEA** ($Q = -3$) SFs decreased dramatically in the **EEKE** SF ($Q = -2$) and **DKEA**/**DEKA** SF ($Q = -1$), whereas the corresponding ΔG^{30} values (Figure 3, blue circles) exhibit a more gradual decrease with decreasing Q. Interestingly, both ΔG^1 and ΔG^{30} decreased almost linearly with decreasing Q, as evidenced by R^2 equal to 0.998 and 0.965, respectively. Compared to the Lys→Glu exchange, a charge-conserving Asp→Glu exchange decreases Ca^{2+}/Na^+ selectivity only slightly: The gas-phase free energy ΔG^1 for replacing Ca^{2+} with Na^+ in the **EEEE** SF is slightly more positive than its **EEDD** counterpart (by 2.1 kcal/mol).

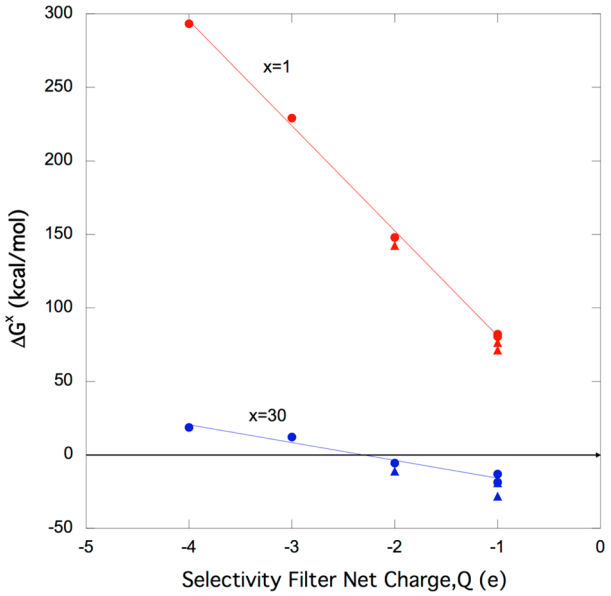

Figure 3. The free energies ΔG^x (in kcal/mol) for replacing Ca^{2+} in the SF characterized by dielectric constant x with Na^+ as a function of the SF net charge, Q (in e). ΔG^1 (in red circles) refers to metal exchange free energy in the gas phase, whereas ΔG^{30} (in blue circles) refers to metal exchange free energy in an environment characterized by an effective dielectric constant of 30. The respective metal exchange free energies in a rigid Na^+-optimized pore prohibited from relaxing upon Ca^{2+} binding are denoted by red or blue triangles, respectively. The free energies shown are taken from those in Figures 1 and 2.

Filter's Effective Dielectric Constant. Figure 3 also shows that increasing the effective dielectric constant (x) of the SF enhances Na^+/Ca^{2+} selectivity. This is mainly because the free energy gain upon releasing Ca^{2+} from the filter outweighs the free energy loss upon Na^+ binding to the filter (see eq 1). Thus, a high effective dielectric constant enhances solvation effects while reducing the net charge of the SF attenuates electronic effects. Consequently, in high-dielectric, Lys-containing pores, solvation effects become dominant and favor the ion with the smaller dehydration penalty (i.e., Na^+).

Pore Size and Rigidity. The Lys in the **EKEE**/**EEKE** and **DKEA**/**DEKA** SFs constricts and rigidifies the pore by forming hydrogen bonds/salt bridges with its neighbors. Such a narrow and rigid pore fits the native Na^+ better than Ca^{2+} and enhances Na^+/Ca^{2+} selectivity. An upper limit of this effect was estimated by computing the $Na^+ \rightarrow Ca^{2+}$ free energy in an absolutely rigid Na^+-optimized pore that was not allowed to relax upon Ca^{2+} binding (Figure 2, numbers in parentheses or Figure 3,

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numbers denoted by triangles). Comparison of the numbers with and without parentheses in Figure 2 show that rigidifying the pore increases Na^+/Ca^{2+} selectivity by 6−10 kcal/mol.

Why the DEKA SF Is More Na^+-Selective than the DKEA SF. The above results suggest that the **DEKA** SF of rat brain Na_v channel is more Na^+-selective than the swopped **DKEA** SF of the mutant channel[14] (see Introduction) due to differences in the SF pore size and rigidity since both channels have the same protein matrix and SFs with the same composition, net charge, and metal CN. Indeed, the calculations indicate that the Lys makes the **DEKA** pore more rigid and constricted compared to the **DKEA** one: In the Ca^{2+} or Na^+-bound **DKEA** SF, the Lys formed two salt bridges with the metal-free carboxylate oxygen atoms. However, in the **DEKA** SF, the Lys formed two hydrogen bonds with the metal-bound carboxylate and carbonyl oxygen atoms and when Na^+ was bound, one of its ammonium protons was transferred to the neighboring Glu carboxylate. Rigidifying the pore increases Na^+/Ca^{2+} selectivity by 10 kcal/mol for the **DEKA** SF and by 6 kcal/mol for the **DKEA** SF (see above). The Lys also makes the **DEKA** pore narrower than the **DKEA** one when Na^+ was bound: In the **DKEA** SF, Na^+ is nearly in the plane formed by the three metal-ligating oxygen atoms but in the **DEKA** SF, Na^+ has sunk below this plane; thus, the three metal-ligating oxygen atoms are closer to each other than those in the **DKEA** SF: the sum of the three O−O bond distances in the O(Asp)−O(Glu)−O(Ala) triangle (reflecting the pore size) in the **DEKA** SF (10.5 Å) is less than that in the **DKEA** SF (11.4 Å). As the Lys seems to constrict and rigidify the **DEKA** SF pore more than the **DKEA** one, the **DEKA** SF is more Na^+/Ca^{2+}-selective than the **DKEA** one.

DISCUSSION

Present Study. The above findings show that the interplay between electronic and solvation effects, which adapted to the specific physicochemical requirements of the cognate cation during evolution, regulates the selectivity of Ca_v, Na_v, and NALCN channels. They also show that the intrinsic properties of (i) the native ion, (ii) the ligands lining the SF, and (iii) the protein matrix all contribute to ion selectivity in eukaryotic Ca_v and Na_v channels. In Ca^{2+}-selective Ca_v channels, electronic effects dictate ion selectivity, favoring Ca^{2+} over Na^+: The stronger charge-accepting ability of Ca^{2+} compared to that of Na^+, the highly negative electrostatic field (net ligand charge of −4 or −3) and four metal-ligating residues of the **EEEE** or **DEEA** SF, as well as the relatively narrow and low-dielectric selectivity pore imposed by the protein matrix ensures stronger charge−charge interactions between the negatively charged carboxylates with divalent Ca^{2+} than univalent Na^+, yielding a free energy gain that can overcome the greater dehydration penalty of Ca^{2+} relative to that of Na^+. On the other hand, in Na^+-selective eukaryotic Na_v channels, solvation effects dictate ion selectivity, favoring Na^+ over Ca^{2+}: The non metal-ligating Lys in the **DKEA** or **DEKA** SF of Na_v channels attenuates electronic effects by reducing the net ligand charge and the number of metal-ligating residues. In addition to its electrostatic role, it also plays a structural role by constricting and rigidifying the pore, enabling the SF pore to select Na^+ over Ca^{2+}.

It is important to point out that an **EEEE** SF need not always produce high selectivity for Ca^{2+} over Na^+. The ryanodine receptor Ca^{2+} channels with **DDDD** SFs conduct both Ca^{2+} and Na^+ even when the Asp residues lining the SF are mutated to Glu.[43,44] Furthermore, bacterial Na_v channels with **EEEE** SFs are weakly selective for Na^+ over Ca^{2+} by a ratio of ∼15.[16] Why are these channels nonselective or Na^+-selective when their SFs have the same **EEEE** motif as the L-type Ca^{2+} channels? The answer lies in the protein matrix, which can affect the SF pore's solvent accessibility, size or rigidity, and Glu protonation state.[23,24,45,46] Our previous calculations[23,24,46] show that increasing the SF pore's solvent accessibility, size, and number of protonated Glu residues attenuates ion−*protein* interactions relative to ion−solvent interactions to such an extent that the **EEEE** SF becomes nonselective or Na^+-selective (see Figures 4 and 5 in ref 24). They predict that the **EEEE** SF is weakly Na^+-selective if the pore is solvent-accessible and two or more carboxylates are either protonated or bind the metal cation indirectly via water molecules.[23,24,46] Indeed, the crystal structure of the bacterial *Arcobacter butzleri* Na_v channel shows a wide, water-filled pore that can fit a Na^+ ion retaining two water molecules in the **EEEE** ring plane.[47]

Limitations and Future Work. Whereas the **DKEA** or **DEKA** SF with a net charge of −1 likely binds a single Na^+,[48] the **EEEE** SF with a net charge of −4 provides a high affinity Ca^{2+} site flanked by lower-affinity cation sites.[49−51] Nevertheless, the computed free energies for reaction 1 can reproduce the experimentally observed Ca^{2+} vs Na^+ selectivity in various SFs. Binding of other ions to lower-affinity sites is important to allow ion flux through the channel pore, where the strong electrostatic repulsion among the cations, especially doubly charged Ca^{2+}, can overcome the tight metal binding causing the Ca^{2+} to spend less time inside the SF (so-called "knock-off" mechanism of Ca^{2+} conduction).[7,51−53] When X-ray structures of metal-bound eukaryotic Ca_v, Na_v1, and $Na_v2.5$ channels become available, the influence from the surrounding protein matrix and other ions could be incorporated explicitly using all-atom free energy simulations.[54] Such calculations could help to elucidate the contribution of residues other than those lining the SF and the coupling between ions and kinetic barriers to metal selectivity in the ion channel. They could also help to elucidate how rigidity effects are finely tuned so that the channel protein can not only select but also permeate its cognate metal ions.

CONCLUSIONS

In conclusion, our results not only delineate the physical principles underlying the Ca^{2+} and Na^+-selective filters in Table 1 but also underscore the importance of the nonobvious structural role played by Lys in addition to its electrostatic role in reducing the net charge transfer to the metal cation in the SF. They also highlight the importance of the protein matrix, which can influence the size, flexibility, and solvent accessibility of the SF pore and, thus, contribute to metal ion selectivity, in addition to the conserved residues lining the SF.

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Notes

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