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A Single Amino Acid Change in $Ca_V1.2$ Channels Eliminates the Permeation and Gating Differences Between Ca^{2+} and Ba^{2+}

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Abstract Glutamate scanning mutagenesis was used to assess the role of the calcicludine binding segment in regulating channel permeation and gating using both Ca^{2+} and Ba^{2+} as charge carriers. As expected, wild-type $Ca_V1.2$ channels had a Ba^{2+} conductance $\sim 2\times$ that in Ca^{2+} ($G_{Ba}/G_{Ca} = 2$) and activation was ~ 10 mV more positive in Ca^{2+} vs. Ba^{2+}. Of the 11 mutants tested, F1126E was the only one that showed unique permeation and gating properties compared to the wild type. F1126E equalized the $Ca_V1.2$ channel conductance ($G_{Ba}/G_{Ca} = 1$) and activation voltage dependence between Ca^{2+} and Ba^{2+}. Ba^{2+} permeation was reduced because the interactions among multiple Ba^{2+} ions and the pore were specifically altered for F1126E, which resulted in Ca^{2+}-like ionic conductance and unitary current. However, the high-affinity block of monovalent cation flux was not altered for either Ca^{2+} or Ba^{2+}. The half-activation voltage of F1126E in Ba^{2+} was depolarized to match that in Ca^{2+}, which was unchanged from that in the wild type. As a result, the voltages for half-activation and half-inactivation of F1126E in Ba^{2+} and Ca^{2+} were similar to those of wild-type in Ca^{2+}. This effect was specific to F1126E since F1126A did not affect the half-activation voltage in either Ca^{2+} or Ba^{2+}. These results indicate that residues in the outer vestibule of the $Ca_V1.2$ channel pore are major determinants of channel gating, selectivity, and permeation.

Keywords Mutation · Voltage dependent · Surface charge · Outer vestibule · Selectivity filter · Pore · Electrophysiology

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L-type calcium channels ($Ca_V1.2$) regulate Ca^{2+} influx into myocytes and neurons and are critical for regulating excitation-contraction coupling, neuronal excitability, and gene expression (Catterall et al. 2005). Ion permeation and gating are two essential biophysical properties of calcium channels and are controlled by distinct channel protein domains (Hille 2001) (Fig. 1), yet both properties are considered to be dependent on the identity of the charge carrier. For example, ionic conductance tends to be approximately two times higher and voltage-dependent activation and inactivation are shifted ~ 10 mV more hyperpolarized when Ca^{2+} is replaced by Ba^{2+} as the charge carrier. The preference for passing Ba^{2+} ions has been explained by modeling the selectivity filter as a single-file, multi-ion pore that has a higher affinity for Ca^{2+} than for Ba^{2+} (Sather and McCleskey 2003). Stronger Ca^{2+} binding with the polar head groups of the lipid bilayer (i.e., surface charge screening) has been proposed to explain the ~ 10-mV positive shift in activation voltage in Ca^{2+} vs. Ba^{2+} for sodium and calcium channels (Hille 2001).

However, an increasing number of studies have found that permeation and gating are not entirely independent: for

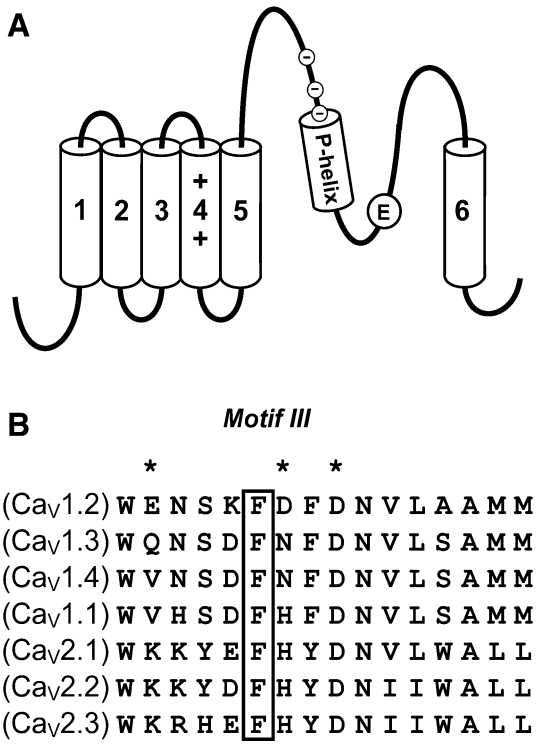

Fig. 1 The calcicludine binding region is highly conserved among voltage-gated calcium channel family members. **a** The membrane topology of the α1 subunit consists of four homologous repeats (repeat III is shown), each consisting of six transmembrane segments (S1–S6). The selectivity filter is formed by four conserved glutamate residues (E), each residing on one of the four S5–S6 connecting loops. The intracellular portions of all four S6 transmembrane segments form the inner lining of the permeation pathway and the activation gate. The S4 segment from each repeat is an amphipathic helix consisting of several positively charged lysine or arginine residues and functions as the voltage sensor that couples membrane depolarization to channel activation. Upstream of the pore-glutamate is the putative pore-helix (P-helix), which is juxtaposed with Phe-1126 and the calcicludine binding site residues (circled minus signs). **b** Amino acid alignment (residues 1121 to 1136) of the calcicludine binding region. Phe-1126 is indicated by a box and Glu-1122, Asp-1127, and Asp-1129 are indicated by asterisks

example, activation gating kinetics have been shown to influence ion selectivity of voltage-dependent potassium channels (Zheng and Sigworth 1997); the binding of alkaloid neurotoxins like veratridine and batrachotoxin to sodium channels promotes long open times and alters the selectivity properties of voltage-dependent sodium channels (Corbett and Vanderklok 1994; Naranjo and Latorre 1993); and, unlike L-type channels, ionic conductance and the voltage dependence for activation and inactivation of T-type (Ca_V3) calcium channels are similar in Ba^{2+} and Ca^{2+} (Kaku et al. 2003; Khan et al. 2008; Klugbauer et al. 1999). The latter result suggests that the screening of membrane surface charge has a smaller effect on channel gating than previously believed.

The relationship between gating and ion permeation of L-type channels has not been studied in great detail. We have previously used pharmacological agents such as dihydropyridine (DHP) antagonists and the peptide toxin calcicludine to study structural changes in the pore that are associated with permeation and gating (Peterson and Catterall 2006; Wang et al. 2007). Recently, we found that calcicludine functions as a positive allosteric modulator of DHP binding and binds to three acidic residues (Glu-1122, Asp-1127, and Asp-1129) in the S5-P-helix segment in the outer vestibule of domain III (Peterson and Catterall 2006; Wang et al. 2007). These findings suggest that calcicludine can be used as a molecular tool to gain a deeper understanding of the relationship among the pore structure, permeation, and channel gating.

Since calcicludine is a highly basic peptide, consisting of 13 positively charged lysine or arginine residues and only 2 negatively charged glutamate residues, we used glutamate-scanning mutagenesis of the calcicludine binding region (Fig. 1) to determine whether the introduction of additional negative amino acids would affect L-type calcium channel calcicludine binding. Unfortunately, none of the mutant channels exhibited an affinity for calcicludine significantly higher than that of wild-type L-type calcium channels. However, in the process of characterizing the functional properties of the mutant channels, we found that the permeation and gating properties of F1126E measured in Ba^{2+} became "Ca^{2+}-like." The conductance, half-activation voltage, and half-inactivation voltage of the mutant F1126E measured in Ba^{2+} and Ca^{2+} were the same and resembled those of the wild-type L-type calcium channel in Ca^{2+}. These findings are consistent with previous reports suggesting that differences in the effects of divalent cation screening on channel gating are located in or near the pore (Kass and Krafte 1987; Wilson et al. 1983; Zamponi and Snutch 1996). Our results further suggest that common structural elements within the outer vestibule of the pore modulate both permeation and gating of L-type calcium channels.

Materials and Methods

Glutamate-Scanning Mutagenesis

Site-directed mutagenesis was used to sequentially replace 13 amino acid residues in the calcicludine binding segment (Trp-1121 to Met-1136) with glutamate. Mutant fragments of the channel were generated by polymerase chain reaction (PCR) using mutagenic primers designed to overlap with an engineered *Nhe*I site at positions 1131/1132 in the P-loop of repeat III of the L-type calcium channels (Wei et al. 1991). The mutant PCR products were gel-purified, digested using

*Eco*RI/*Nhe*I (W1121E through V1131E) or *Nhe*I/*Bse*EII (L1132E through M1136E) restriction endonucleases and subcloned into *Eco*RI/*Nhe*I- or *Nhe*I/*Bst*EII-digested $Ca_V1.2$ vector. The presence of the mutation and the integrity of each mutant were confirmed by qualitative restriction map analysis and directional DNA sequence analysis of the entire subcloned region. Functional expression of the mutant cDNAs was confirmed by Western blot analysis and whole-cell patch-clamp electrophysiology.

Cell Culture and Transfections

HEK293 cells were grown at 37°C and 6% CO_2 in DMEM-F12 medium supplemented with 10% fetal bovine serum and 1% Penn Strep antibiotics. cDNAs encoding wild-type and mutant $Ca_V1.2$ channels were cotransfected with $\alpha_2\delta$ (Tomlinson et al. 1993), β_{2a} (Perez-Reyes et al. 1992), and CaM_{1234} (Peterson et al. 1999) into HEK293 cells by calcium phosphate precipitation as described previously (Peterson et al. 1999; Wang et al. 2005). CaM_{1234} was used to eliminate complications that could arise from Ca^{2+}/CaM-dependent changes in channel gating, since it encodes an inactive form of calmodulin that has been shown to eliminate Ca^{2+}-dependent inactivation and facilitation (Peterson et al. 1999, 2000; Qin et al. 1999; Wang et al. 2005; Zuhlke et al. 1999). All cDNAs were expressed using pcDNA3 mammalian expression plasmids (Invitrogen, Carlsbad, CA).

Patch-Clamp Electrophysiology

Whole-cell patch-clamp recordings were acquired as described previously (Wang et al. 2005). Briefly, whole-cell currents were recorded at room temperature 2–3 days after transfection. Pipettes were pulled from borosilicate glass using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA) and fire-polished on a MF200 microforge (World Precision Instruments, Sarasota, FL). Five types of external solutions were used for whole-cell recordings. (1) External solutions containing 30 mM Ba^{2+} or Ca^{2+} were used in the initial screen of all the mutant channels to increase the probability that currents could be recorded from poorly expressing mutant channels (mM): *N*-methyl-D-glutamine (NMG)-aspartate, 130; HEPES, 10; 4-aminopyridine, 10; and $BaCl_2$ or $CaCl_2$, 30. The osmolarity was adjusted to 300 mmol/kg with glucose and the pH was adjusted to 7.4 using a 1 mM NMG base solution. (2) Concentrations of $BaCl_2$ and $CaCl_2$ were reduced to 10 mM to assure proper voltage-clamp of currents used in more detailed studies described in Figs. 3, 4, and 5d–f. (3) Bath solutions used to measure Ba^{2+}- or Ca^{2+}- dependent block of Li^+ currents (Fig. 7) contained (mM): LiCl, 100; HEPES, 10; TEA-Cl, 14; EDTA, 5; HEDTA, 5; and $BaCl_2$ or $CaCl_2$, as needed based on published binding constants (WINMAXC; Chris Patton, Stanford University). The indicated concentrations of free divalent cations were (μM) 0.3, 1, 3, 10, 30, and 100 for [Ba^{2+}] and (nM) 10, 30, 100, 300, 1000, and 10,000 for[Ca^{2+}]. The pH was adjusted using TEA-OH, and the osmolarity was adjusted to 300 mmol/kg using TEA-Cl. (4) External solutions similar to those used in No. 1 were used in experiments that assess current–concentration relationships (Fig. 8), except that the concentrations of $BaCl_2$ or $CaCl_2$ were 3, 10, 30, and 100 mM and glucose and NMG were added or removed as necessary to maintain the desired osmolarity of 300 mmol/kg. (5) The 10 mM Ba^{2+} external solution described under No. 2 was used in experiments to determine the response of each mutant channel to calcicludine. Each mutant was tested using calcicludine concentrations of 50 (<10% wild-type block) and 500 (approx. wild-type IC_{50}) nM. The internal solution for all experiments contained (mM): NMG-$MeSO_3$, 140; EGTA, 10; $MgCl_2$, 1; Mg-ATP, 4; and HEPES, 10. The osmolarity was adjusted to 300 mmol/kg with glucose and the pH was adjusted to 7.4 with NMG base. Pipettes had resistances of 2.5–3.0 MΩ when filled with internal solution.

Data Acquisition and Analysis

Data were acquired using a HEKA EPC-9/2 amplifier and PULSE/PULSEFIT software (HEKA Electronic, Lambrecht, Germany). Leak and capacitive transients were corrected by −P/4 compensation. Series resistance was <6 MΩ and compensated to 70%. Tail currents and data acquired for nonstationary noise analysis were sampled at 50 kHz and filtered at 5.0 and 10 kHz, respectively. All the other currents were sampled at 20 kHz and filtered at 3.0 kHz. Pulse protocols are described in the figure legends.

Data analysis was performed using FITMASTER and PULSETOOLS (HEKA Electronic) and Origin 7 (Originlab, Northampton, MA). Nonstationary noise analysis (Sigworth 1977, 1980) was performed to obtain information on single-channel parameters such as the unitary current amplitude (i), maximal open probability (P_o), and number of active channels (N). A series of 100 × 15-ms depolarization steps was evoked from a holding potential of −120 mV to 0 mV for both wild-type and F1126E channels with Ca^{2+} or Ba^{2+} as the charge carrier. The ensemble variance (σ^2) plotted against the mean current (I) was fitted with the parabolic function:

$$\sigma^2 = i \times \mathrm{I} - \mathrm{I}^2/N + \sigma_b^2$$

where σ_b^2 is the baseline variance.

All data are reported as mean ± SE. Statistically significant results ($P < 0.05$) are indicated in the figures by an

asterisk for comparisons between wild-type and mutant channels and a pound sign (#) for comparisons of a particular channel variant (wild type or mutant) in Ca^{2+} vs. Ba^{2+}.

Results

F1126E Conducts Ba^{2+} Ions as if they were Ca^{2+}

An alignment of the calcicludine binding segment in domain III of $Ca_V1.2$ with the corresponding segments from other members of the high-voltage-gated calcium channel family indicates that this segment is highly conserved (Fig. 1b). Each $Ca_V1.2$ residue depicted in Fig. 1b was replaced with glutamate, and of the 13 positions investigated (1121–1136) in this study, only F1128E and A1134E failed to produce measurable currents. As previously observed (Wang et al. 2005), switching from Ba^{2+} to Ca^{2+} produces a twofold decrease in the wild-type currents at all potentials between −30 and +30 mV (Fig. 2a). Of all the mutant channels screened, only F1126E yielded an $I_{Ba(max)}/I_{Ca(max)}$ that differed from that of the wild type (Fig. 2b and c). In fact, Ca^{2+} currents through F1126E tended to be larger than Ba^{2+} currents (Fig. 2b).

The normalization of Ca^{2+} and Ba^{2+} currents by F1126E could be explained if Ca^{2+} permeation became more Ba^{2+}-like, or vice versa. This was assessed by measuring instantaneous current–voltage relationships from tail currents, from which we determined whole-cell conductance (Fig. 3), and nonstationary noise analysis, from which we estimated single-channel properties (Fig. 4). As expected, the normalized maximum slope conductance (G) for the wild type is approximately twofold higher in Ba^{2+} vs. Ca^{2+} (Fig. 3c and e). In contrast, G_{Ba} is dramatically reduced for F1126E, while G_{Ca} for F1126E and wild-type do not differ (Fig. 3c and d). The summarized data in Fig. 3e indicate that Ba^{2+} conductance for F1126E is reduced such that it is indistinguishable from Ca^{2+} conductance for the wild type or F1126E.

The above results support a decreased unitary current for F1126E in Ba^{2+}, but other possibilities such as decreased P_o and/or the number of active channels cannot be excluded. As shown previously (Bean 1985), nonstationary noise analysis is well suited for estimating single-channel properties from whole-cell voltage-clamp recordings because of the low background noise associated with this technique. Therefore, we used nonstationary noise analysis to obtain unitary current amplitude (i) for wild-type and F1126E currents in both Ca^{2+} and Ba^{2+} (Fig. 4). Mean current and variance were determined as described under Materials and Methods. For the wild type, i was approximately twofold higher in Ba^{2+} vs. Ca^{2+}. In contrast, i_{Ba} was selectively

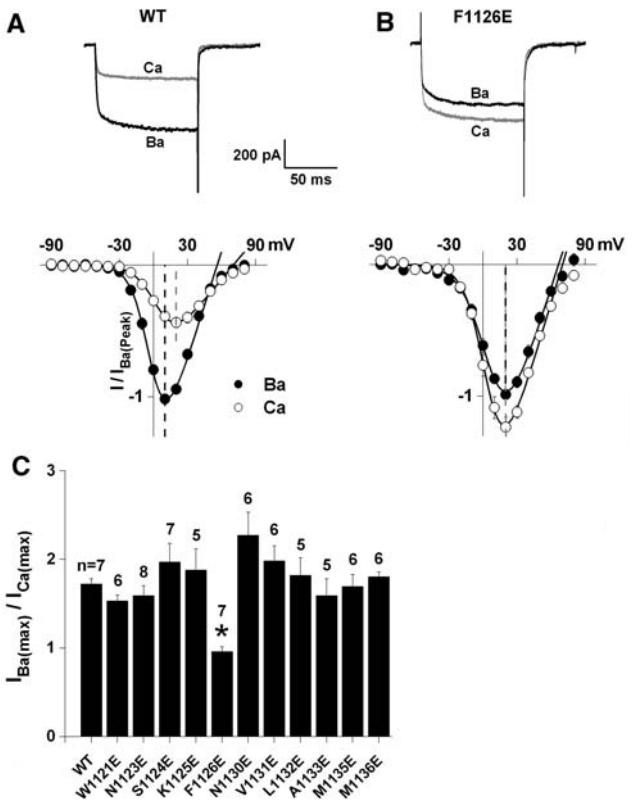

Fig. 2 Substitution of glutamate for Phe-1126 selectively alters L-type calcium channel permeation properties in Ba^{2+}. Wild-type (**a**) and F1126E (**b**) currents were evoked by 100-ms step depolarizations to +20 mV from a holding potential of −90 mV in 30 mM Ba^{2+} and Ca^{2+}. *I–V* relationships were normalized to peak Ba^{2+} currents recorded from the same cell. Dashed lines indicate peak current voltages for Ba^{2+} (*black*) and Ca^{2+} (*gray*). **c** Peak tail currents were measured at −50 mV following 100-ms depolarizing steps ranging from −90 to +80 mV in 30 mM Ba^{2+} and Ca^{2+}. Data were fit with a Boltzmann equation through the activation-vs.-voltage data and normalized to maximal Ba^{2+} currents [$I_{Ba(max)}$]. $I_{Ba(max)}/I_{Ca(max)}$ are plotted for wild-type and all mutant channels tested. Of all the mutants investigated in this study, only F1126E was significantly different from the wild type (*)

reduced for F1126E, since i_{Ca} was not significantly different from that of the wild type (Fig. 4e). Thus, the data in Figs. 3 and 4 indicate that the F1126E mutation selectively alters Ba^{2+} conductance so that it becomes indistinguishable from that in Ca^{2+}.

Substitution of Glutamate for Phe-1126 Alters the Gating Properties of $Ca_V1.2$ Channels

The voltage dependence of activation for the wild type is ion dependent, such that the peak current measured in Ca^{2+} occurs ~10 mV more positive than in Ba^{2+} (Fig. 2a). In contrast, switching from Ba^{2+} to Ca^{2+} failed to shift the *I–V* for F1126E channels (Fig. 2b) so that voltage-dependent activation in Ba^{2+} resembled that in Ca^{2+} (Fig. 2a;

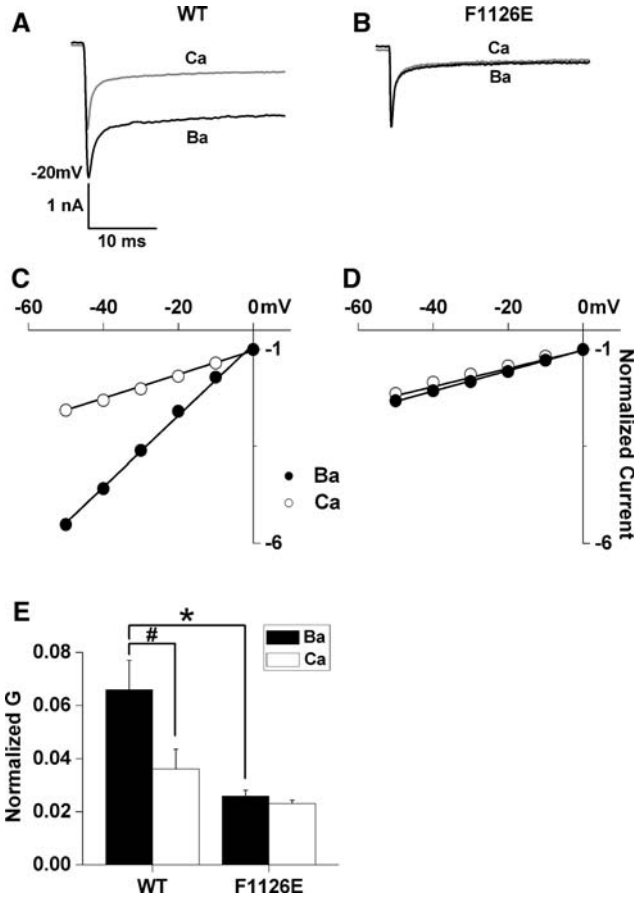

Fig. 3 F1126E conducts Ba^{2+} ions as if they were Ca^{2+}. Whole-cell conductance is a product of the single-channel current amplitude, the open probability (P_o), and the number of active channels. Peak tail currents were measured from −80 to +20 mV following 50-ms depolarizing steps to +50 mV which were used to maximally activate the wild type (**a**) and F1126E (**b**) in 10 mM Ba^{2+} and Ca^{2+} from the same cell, thus minimizing differences in P_o that could arise from shifts in the voltage dependence of activation. Linear portions of the current–voltage relationships (−50 to 0 mV) were normalized to amplitudes measured at 0 mV for the wild type (**c**) and F1126E (**d**). Normalized data were fit with the equation $I_{tail} = G \times (V - E_{rev})$, where I_{tail} is the peak tail current, E_{rev} the reversal potential, and G the maximal slope conductance. **e** Normalized slope conductances are plotted for the wild type ($n = 8$) and F1126E ($n = 5$)

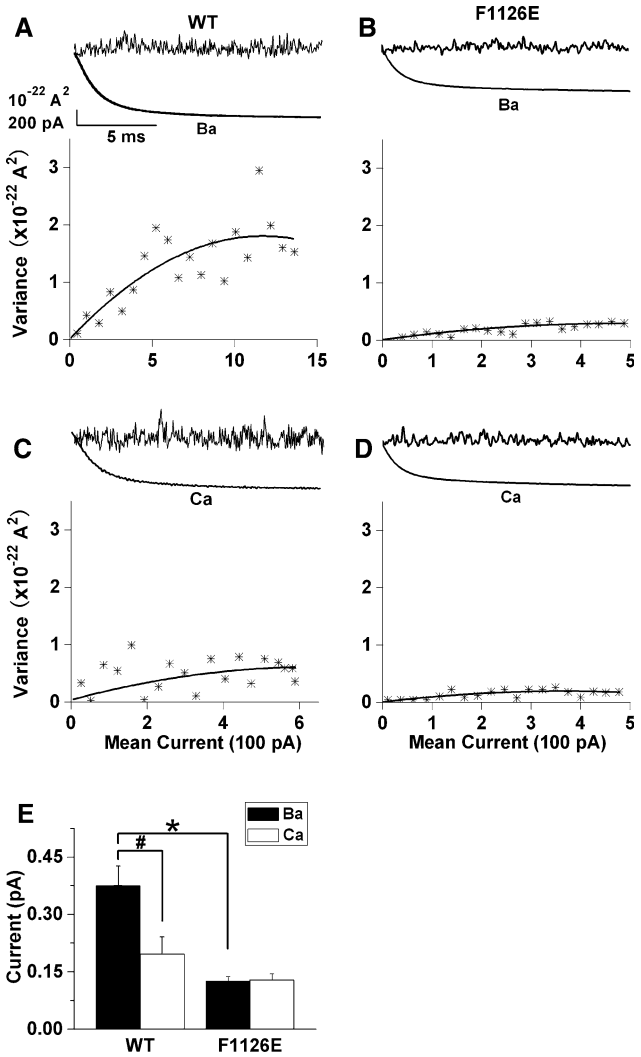

Fig. 4 F1126E reduces the unitary current amplitude in Ba^{2+} to match that in Ca^{2+}. Ba^{2+} (**a**, **b**) and Ca^{2+} (**c**, **d**) currents for the wild type and F1126E were evoked by a series of 100 voltage steps (15 ms) to 0 mV from a holding potential of −120 mV. Data were analyzed using PULSETOOLS nonstationary noise analysis software to acquire the variance and mean current of each cell (**a**–**d**, upper traces). Variance-mean current relationships (**a**–**d**, lower graphs) were fit with the equation $\sigma^2 = i \times I - I^2/N + \sigma_b^2$, where σ^2 is the variance, σ_b^2 the baseline variance or background noise, i the unitary current amplitude, I the mean current, and N the number of active channels: wild type (Ba^{2+}), $i = 0.290 \pm 0.019$ pA, $P_o = 53.8 \pm 6.1\%$, $n = 7$; wild type (Ca^{2+}), $i = 0.159 \pm 0.033$ pA, $P_o = 48.4 \pm 11.6\%$, $n = 7$; F1126E (Ba^{2+}), $i = 0.126 \pm 0.012$ pA, $P_o = 42.8 \pm 11.6\%$, $n = 6$; F1126E (Ca^{2+}), $i = 0.128 \pm 0.017$ pA, $P_o = 66.9 \pm 9.6\%$, $n = 7$. **e** Mean unitary current amplitudes are plotted for the wild type and F1126E

dashed lines). The effect of replacing Phe-1126 with glutamate on the voltage dependence of activation was assessed in greater detail by measuring tail current amplitudes following a series of depolarizing steps ranging from −90 to +80 mV (Fig. 5a–c). As expected, the half-activation voltage (V_h) was right-shifted in Ca^{2+} vs. Ba^{2+} for wild-type L-type calcium channels. In contrast, no difference in V_h was observed in Ca^{2+} vs. Ba^{2+} for F1126E. Summarized data in Fig. 5c for F1126E indicate that V_h values determined in Ca^{2+} and Ba^{2+} were indistinguishable, and were not significantly different from the V_h determined for the wild type measured in Ca^{2+}. Only one other mutant (L1132E) showed a statistically similar V_h in Ba^{2+} and Ca^{2+}, but, unlike F1126E, the V_h values in neither Ba^{2+} nor Ca^{2+} differed from those for wild-type L-type calcium channels (Fig. 5c). Thus, the F1126E mutation alters the channel so that the activation gating mechanism no longer distinguishes Ba^{2+} from Ca^{2+}.

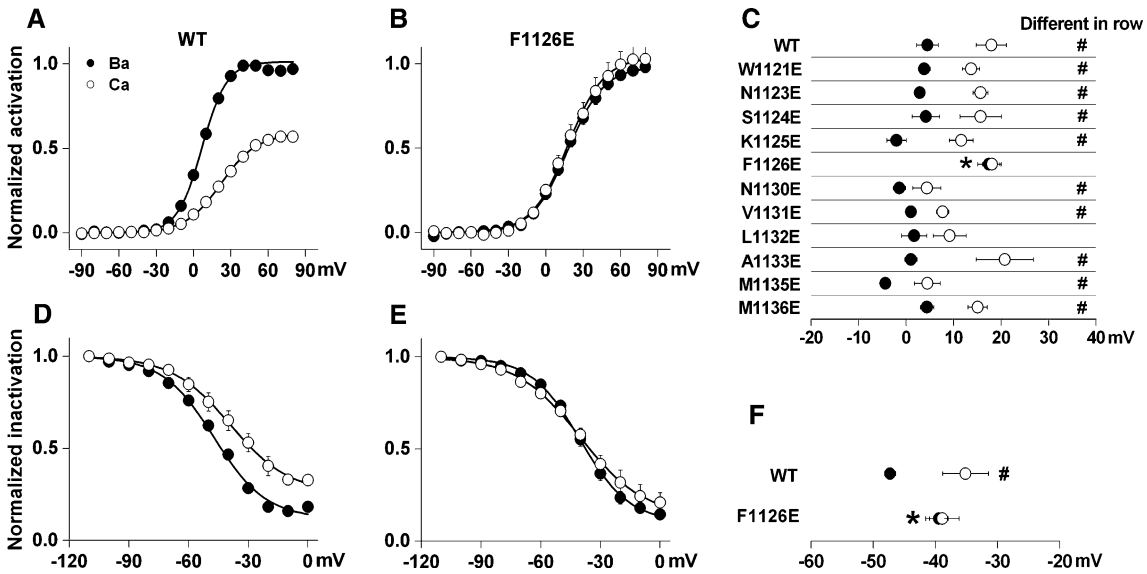

Fig. 5 Replacing Phe-1126 with glutamate selectively alters L-type calcium channel gating in Ba^{2+}. Peak tail currents were measured at −50 mV following 100-ms depolarizing steps ranging from −90 to +80 mV in 30 mM Ba^{2+} and Ca^{2+} for the wild type (**a**) and F1126E (**b**). Tail current amplitudes recorded in Ba^{2+} and Ca^{2+} were normalized to $I_{Ba(max)}$. Boltzmann fits were used to obtain half-activation voltages (V_h) and slope factors (k; mV): wild type (Ba^{2+}), $V_h = 4.5 \pm 2.3$, $k = 9.6 \pm 1.2$; wild type (Ca^{2+}), $V_h = 17.9 \pm 3.2$, $k = 14.8 \pm 0.8$ ($n = 7$); F1126E (Ba^{2+}), $V_h = 17.4 \pm 2.4$, $k = 14.6 \pm 2.1$; and F1126E (Ca^{2+}), $V_h = 18.1 \pm 2.0$, $k = 15.3 \pm 1.0$ ($n = 7$). **c** Mean V_h values derived from Boltzmann fits are plotted for the wild type and each mutant tested. The number of cells for each group is the same as that for Fig. 2c. **d**, **e** Steady-state inactivation was measured as the ratio of current elicited by 50-ms prepulses and postpulses to 0 mV separated by 10-s conditioning pulses ranging from −110 to 0 mV in 10 mM Ba^{2+} and Ca^{2+} for the wild type (**d**) and F1126E (**e**). Results from Boltzmann fits are as follows (mV): wild type (Ba^{2+}), $V_h = -47.3 \pm 0.9$, $k = 13.1 \pm 0.2$, $n = 7$; wild type (Ca^{2+}), $V_h = -35.2 \pm 3.7$, $k = 13.7 \pm 0.5$, $n = 6$; F1126E (Ba^{2+}), $V_h = -39.5 \pm 1.5$, $k = 12.7 \pm 1.3$, $n = 6$; and F1126E (Ca^{2+}), $V_h = -38.9 \pm 2.7$, $k = 16.3 \pm 1.3$, $n = 7$. **f** Mean V_h values for steady-state inactivation are plotted for the wild type and F1126E. #Significant difference in V_h between Ca^{2+} vs. Ba^{2+} (in row); * Significant difference between mutant and wild type (in column) in Ba^{2+}

V_h for steady-state inactivation of L-type calcium channels is typically 10 mV left-shifted in Ba^{2+} compared to Ca^{2+} (Hille 2001). Thus, we wanted to determine whether steady-state inactivation of F1126E in Ba^{2+} and Ca^{2+} differ from that of wild-type (Fig. 5d–f). As expected, V_h for steady-state inactivation was left-shifted in Ba^{2+} vs. Ca^{2+} for wild-type. However, no ion-dependent difference in V_h was observed for F1126E. In addition, V_h values for F1126E in Ba^{2+} and Ca^{2+} are not different from the V_h determined for wild-type in Ca^{2+} (Fig. 5f). Thus, as was observed for permeation and activation, the voltage-dependent inactivation gating mechanism of F1126E responds to Ba^{2+} as if it were Ca^{2+}.

The gating shift in wild-type channels induced by switching from Ca^{2+} to Ba^{2+} appears to be reflected in the kinetics of channel closing (deactivation) as well. To investigate this more closely, tail currents at voltages ranging from 0 to −80 mV (following a 50-ms +50-mV step) were fit using a single-exponential equation to yield the time constant (τ) of deactivation. Figure 6 shows that deactivation of wild-type channels in Ca^{2+} tended to be faster than that in Ba^{2+}, but this difference was not statistically significant at any voltage. Furthermore, τ values measured for F1126E channels were not different in Ca^{2+} versus Ba^{2+} and closely match the values for wild-type channels measured in Ca^{2+}. The small differences in deactivation are in the direction expected from changes in activation V_h.

Phe-1126 Does Not Form Part of a Ca^{2+} Binding Site in the Outer Vestibule of the Pore

One possible explanation of our results is that Phe-1126 forms part of a cation binding site capable of distinguishing between Ca^{2+} and Ba^{2+}, and the glutamate substitution could expand that site to permit Ba^{2+} binding. We constructed the mutant F1126A, which is predicted to disrupt Ca^{2+} binding to this site. If Phe-1126 contributes to a Ca^{2+}-selective binding site, the voltage dependence for activation in Ca^{2+} should shift in the hyperpolarizing direction such that it coincides with that determined for wild-type channels in Ba^{2+}. In other words, activation V_h for F1126A measured in Ca^{2+} should become Ba^{2+}-like. Results of these studies (Fig. 7) demonstrate that V_h for

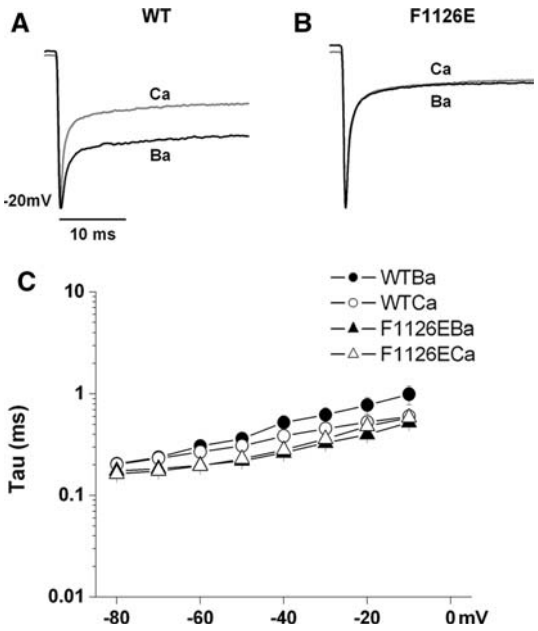

Fig. 6 Mutant F1126E does not affect deactivation time constants of the channel. Sample wild-type (**a**) and F1126E (**b**) tail currents were measured in 10 mM Ba^{2+} and 10 mM Ca^{2+} as indicated. Tail currents were elicited by repolarizing steps to −20 mV following 50-ms steps to +50 mV. Tail currents measured in Ca^{2+} were normalized to those measured in Ba^{2+} from the same cell. **c** To quantify deactivation time constants (τ) at the indicated voltages, raw data were fitted with a single-exponential equation. Average τ values at the indicated repolarization potentials are shown for the wild- type ($n = 8$) and F1126E ($n = 5$) in Ba^{2+} and Ca^{2+}

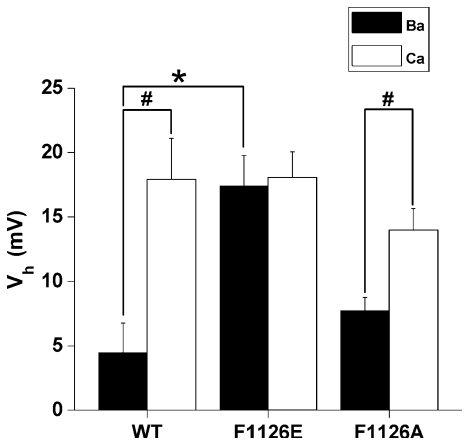

Fig. 7 Replacing Phe-1126 with alanine does not affect the L-type calcium channel V_h. Half-activation voltage (V_h) is plotted for wild-type, F1126E, and F1126A channels with Ba^{2+} and Ca^{2+} as the ion carriers. F1126A, (Ba^{2+}), $V_h = 7.7 \pm 1.0$ mV and (Ca^{2+}) $V_h = 14.0 \pm 1.7$ mV,; $n = 6$. See Fig. 5 for more details

activation measured in Ca^{2+} for F1126A does not differ from that for the wild type and F1126E, indicating that alanine substitution at 1126 does not disrupt Ca^{2+} binding to this putative site.

F1126E Alters Low- but not High-Affinity Cation Binding to the Selectivity Filter

Another possibility is that the introduction of a negative charge at position 1126 selectively alters pore configuration to normalize the binding of Ba^{2+} or Ca^{2+} within the selectivity filter, which shows a higher affinity for Ca^{2+} relative to Ba^{2+} for wild-type calcium channels (Kostyuk et al. 1983). We compared the properties of high- and low-affinity divalent cation binding to the pore for wild-type vs. F1126E channels. The IC_{50} for half-maximal block of Li^+ currents by Ca^{2+} (Fig. 8; open symbols) and Ba^{2+} (filled symbols) was used to estimate the dissociation constants for the high-affinity binding of Ca^{2+} and Ba^{2+} ions to the selectivity filter. Substituting Phe-1126 with glutamate does not alter the IC_{50} values for block of Li^+ currents by either Ca^{2+} or Ba^{2+} (Fig. 8). These findings indicate that the decreases in Ba^{2+} conductance exhibited by F1126E do not result from changes in the interactions of individual Ba^{2+} ions in the pore.

We next wanted to determine whether replacing Phe-1126 with glutamate alters the interactions of multiple divalent cations in the pore of the mutant channel. To test this, we compared peak tail currents generated in bath solutions containing 3, 10, 30, and 100 mM Ba^{2+} or Ca^{2+} in the same cell (Fig. 9). In Fig. 9a and b, tail currents were measured from wild-type or F1126E channels at the indicated Ba^{2+} and Ca^{2+} concentrations and the resulting current–concentration relationship was fit using the Michaelis-Menton equation. Figure 9b shows that normalized F1126E currents in Ba^{2+}

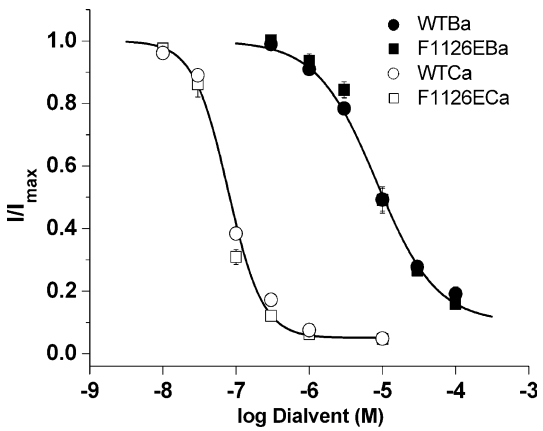

Fig. 8 High-affinity binding of single Ba^{2+} and Ca^{2+} ions to the selectivity filter is not altered by the F1126E mutation. Tail currents were evoked at −50 mV following 20-ms depolarizing steps to +30 mV to maximally activate wild-type and F1126E channels. Peak tail currents measured at the indicated concentrations of free Ba^{2+} and Ca^{2+} were normalized to the maximum current amplitude determined from the Hill equation fit to the dose-response data or each cell. IC_{50} values determined in Ca^{2+} (nM)—wild type, 80.0 ± 3.7 ($n = 6$); F1126E, 67.0 ± 5.6 ($n = 5$)—and in Ba^{2+} (μM)—wild type, 9.3 ± 1.0 ($n = 5$); F1126E, 10.5 ± 1.2 ($n = 6$)

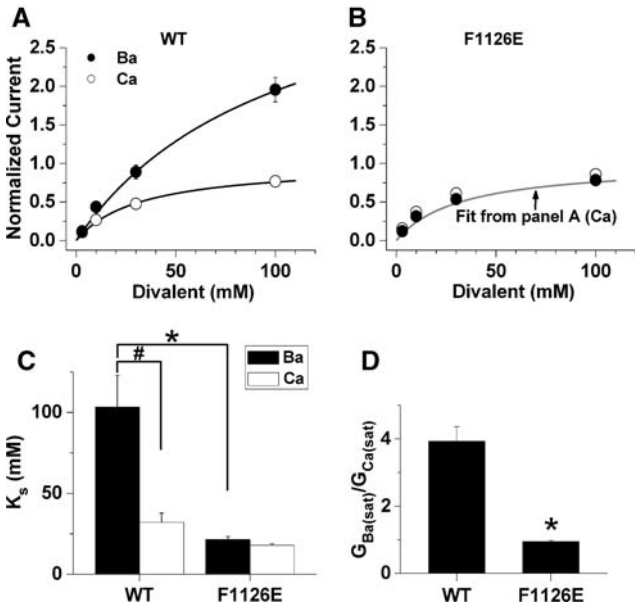

Fig. 9 F1126E alters the relationship between saturation and conductance of Ba^{2+}. Peak tail currents were recorded at 0 mV following 20-ms depolarizing steps to +70 mV, to maximally activate the wild type (**a**) or F1126E (**b**) in Ba^{2+} and Ca^{2+}. Tail currents were measured in 3, 10, 30, and 100 mM Ba^{2+} and Ca^{2+} from the same cell. Data were normalized to $G_{Ca(sat)}$ and fit by the Michaelis-Menton equation, $G = G_{sat}/[1 + (K_S/c)]$, where G_{sat} is the level of current at saturating concentrations of divalent cations, c is the concentration of divalent cation, and K_S is the divalent cation concentration that produces one-half G_{sat}. The fit for the wild type (Ca^{2+}) is shown in B (gray). $G_{Ba(sat)}$ (nS): wild type, 9.2 ± 2.9 ($n = 5$); F1126E, 3.6 ± 0.7 ($n = 6$). $G_{Ca(sat)}$: wild type, 2.7 ± 1.3; F1126E, 3.7 ± 0.6. **c** K_S values (mM) are plotted for the wild type and F1126E in Ba^{2+} and Ca^{2+}. **d** Data are plotted as $G_{Ba(sat)}/G_{Ca(sat)}$ for the wild type and F1126E

overlapped those in Ca^{2+}, resulting in a threefold decrease in K_S for F1126E in Ba^{2+} compared to the wild type. Consequently, the K_S for F1126E in Ba^{2+} did not differ from that determined for Ca^{2+} in either wild-type or F1126E channels (Fig. 9b and c). These results indicate that the identity of the residue at position 1126 determines the magnitude of G_{Ba}/G_{Ca} by specifically altering how multiple Ba^{2+} ions interact in the pore.

Discussion

We used glutamate-scanning mutagenesis in the S5/P-helix segment of repeat III to assess the role of amino acids within the calcicludine binding segment in determining the permeation and gating characteristics of the $Ca_V1.2$ channel. Of all the glutamate substitutions screened in this study, F1126E was unique in that permeation and gating in Ba^{2+} became Ca^{2+}-like, while neither of these parameters were altered when Ca^{2+} was the charge carrier. Ba^{2+} conductance was decreased approximately twofold and the voltage dependence of activation and inactivation was depolarized to match that measured in Ca^{2+}.

Replacement of Phe-1126 with Glutamate Alters $Ca_V1.2$ Channel Permeation

Amino acid substitutions within the highly conserved (EEEE) selectivity filter alter ion selectivity and permeation of $Ca_V1.2$ channels (Sather and McCleskey 2003). Others (Dilmac et al. 2003, 2004; Williamson and Sather 1999) and we (Wang et al. 2005) have studied the influence on permeation of amino acids proximal to the pore glutamates of $Ca_V1.2$ channels. In the rat L-type calcium channel, mutation of the threonine near the pore glutamate to alanine in domains II and III results in a loss of selectivity between Ba^{2+} and Ca^{2+} (Dilmac et al. 2003, 2004). Similar results have been observed for domain III phenylalanine-to-glycine mutation in both rat (F1117G) and rabbit (F1144G) L-type calcium channels (Dilmac et al. 2003, 2004; Wang et al. 2005). These mutations appear to disrupt Ca^{2+} affinity, suggesting that the selectivity filter configuration is affected. However, our finding that the F1126E mutation failed to alter half-maximal block of Li^+ currents by either Ca^{2+} or Ba^{2+} suggests that the selectivity filter is not altered when single divalent cations compete with Li^+ for occupancy of the pore. In our results, divalent ion permeation is affected so that the apparent Ba^{2+} affinity for the low-affinity permeation site(s) within the pore is increased by F1126E to match the apparently unchanged affinity of the channel for Ca^{2+}. The latter findings suggest that the interactions of multiple divalent cations in the pore of the channel are altered by F1126E.

These findings resemble those of Wang et al., who also found that unitary Ba^{2+} conductance for F1144G decreases to match that of Ca^{2+} (Wang et al. 2005). In this work, the "volume exclusion/charge neutralization" model was used to explain the reduced conductance of Ba^{2+} but not Ca^{2+} through the pores of the $Ca_V1.2$ mutant channels F1144G, Y1152K, and FY/GK (Peterson and Catterall 2006; Wang et al. 2005). The crystal diameters of Ca^{2+} and Na^+ ions are nearly identical (2.00 vs. 2.04 Å, respectively), yet each Ca^{2+} ion carries twice as much countercharge as a Na^+ ion. Therefore, Ca^{2+} binds tightly to the selectivity filter because it is able to neutralize the highly charged EEEE locus without the overcrowding suspected for monovalent cations (i.e., too many ions in the pore attempting to neutralize the negative charges). Ba^{2+} and Ca^{2+} ions carry the same charge, but the ionic diameter of Ba^{2+} is ~36% larger than that of Ca^{2+}. Thus, for wild-type channels, Ba^{2+} ions are expected to exhibit a higher degree of crowding as multiple ions attempt to bind/neutralize the EEEE negative charges, which is thought to explain the lower binding affinity and consequently increasing exit rate

(i.e., higher conductance) relative to Ca^{2+}. Likewise, this model suggests that Ba^{2+} conductance is reduced for F1126E because Ba^{2+} ions in this pore are less prone to overcrowding. However, it is not clear how a change within the pore that would increase the apparent Ba^{2+} affinity would not impact Ca^{2+}.

Replacement of Phe-1126 with Glutamate Alters $Ca_V1.2$ Channel Gating

The mechanism by which Ca^{2+} or Ba^{2+} affects activation gating could result from effects on either closed-closed gating transitions (Yarotskyy et al. 2009) or closed-open transitions (Marks and Jones 1992). We reasoned that the gating effect of the F1126E mutation could be localized to either closed-closed or closed-open transitions by investigating the impact on closed-state inactivation. We found that the effect of the mutation on the closed-state inactivation V_h was similar to that measured for activation, which supports the idea that the mutation affects closed-closed transitions. Transitions between $Ca_V1.2$ channel closed states are voltage dependent, while closed-open transitions are voltage independent (Marks and Jones 1992). Thus, Ca^{2+} and Ba^{2+} appear to affect voltage sensor movement (i.e., the S4 segment). One scenario is that divalent cation binding electrostatically impedes voltage sensor movement. However, we cannot rule out other mechanisms such as allosterically induced conformational changes that impact channel activation.

Surface charge screening is the classical explanation for the effect of Ca^{2+} and Ba^{2+} on the gating of voltage-dependent channels. The idea is that local negative electric fields are set up by phosphate head groups at the lipid membrane surface, and these charges bias the transmembrane voltage sensed by the channel voltage sensors (Hille 2001). Stronger Ca^{2+} binding with the interface negative charges was the explanation for the $\sim$ 10 mV positive voltage shift observed with equimolar exchange of Ca^{2+} for Ba^{2+} for both sodium channels (Hanck and Sheets 1992; Hille et al. 1975; Ohmori and Yoshii 1977) and calcium channels (Byerly et al. 1985; Cota and Stefani 1984; Ganitkevich et al. 1988; Kass and Krafte 1987; Ohmori and Yoshii 1977; Smith et al. 1993; Wilson et al. 1983; Zamponi and Snutch 1996). However, this idea was recently challenged when it was found that T-type calcium channels fail to show such a voltage shift (Kaku et al. 2003, 2008; Klugbauer et al. 1999), which implied that the surface charge is specific to a particular ion channel. Our findings further support this idea by showing that the F1126E point mutation nullifies the L-type calcium channel gating difference between Ca^{2+} and Ba^{2+}. We previously identified a set of pore mutations that abrogated the Ca^{2+}–Ba^{2+} gating shift, F1144G/Y1152K (Wang et al. 2005). Neither F1144G nor Y1152K significantly affects L-type calcium channel gating in either Ca^{2+} or Ba^{2+}, but with both mutations together Ca^{2+} no longer right-shifted V_h. However, this change is opposite of what we have observed for F1126E, since V_h is significantly right-shifted by this mutation to equal that in Ca^{2+} for both wild-type and F1126E L-type calcium channels. The F1144G/Y1152K double mutant reduces Ba^{2+} conductance to that of Ca^{2+}, so that the effect on permeation (Ba^{2+} to Ca^{2+}) is opposite that for gating (Ca^{2+} to Ba^{2+}) (Wang et al. 2005). In the case of F1126E, the effects on permeation and gating are convergent in that the channel responds to Ba^{2+} as if it were Ca^{2+}.

This convergence suggests that Ca^{2+} binds to a site on Ca_V channels to affect both gating and permeation, but this site has little or no affinity for Ba^{2+}. The F1126E mutation alters the Ca^{2+} binding site so that it can accommodate Ba^{2+} as effectively as it does Ca^{2+}. Two possibilities are that the location of such a site could be the selectivity filter or formed amino acids in the outer vestibule of the pore. In support of the selectivity filter hypothesis, permeation models such as the size exclusion model discussed above provide a quantitative basis on which to understand that apparent increased affinity of the pore for Ba^{2+}. However, it is currently believed that Ba^{2+} binds within the pore of the wild-type channels (albeit with a lower affinity), which creates a problem for explaining the voltage-dependent shift in gating in Ca^{2+} vs. Ba^{2+}. In support of the second possibility, previous reports have suggested that differences in the effects of divalent cation surface charge screening on channel gating are dependent on a selective Ca^{2+} binding site in the outer vestibule of the channel pore (Kass and Krafte 1987; Wilson et al. 1983; Zamponi and Snutch 1996). However, our attempt to disrupt Ca^{2+} binding to this putative site was unsuccessful since the F1126A mutation failed to alter the response of the channel to Ca^{2+} (or Ba^{2+}). A scenario that is consistent with all of our results is that amino acids within the outer vestibule form a binding site that is selective for Ca^{2+}, but Phe-1126 is not involved in forming this binding site. However, the Phe-Glu substitution at position 1126 modifies that site, enabling it to accommodate the larger Ba^{2+} ions while not altering the affinity of the site for Ca^{2+}. Thus, the binding of both Ca^{2+} and Ba^{2+} to this site results in low conductance for both ions through the pore and a depolarizing shift in channel gating. Additional outer pore mutations within each $Ca_V1.2$ channel domain will be required to understand more completely the underlying mechanism by which F1126E affects Ba^{2+}-channel interaction.

Regardless of the precise mechanism, our results provide further support for an interaction between ion occupancy of the pore and calcium channel gating (Babich et al. 2007; Cens et al. 2007; Li et al. 2004, 2005; Wang et al.

2005). More specifically we have identified a single amino acid responsible for setting the differential gating and permeation response of the L-type calcium channel to Ca^{2+} vs. Ba^{2+}. The mutation of this site from phenylalanine to glutamate appears to slow Ba^{2+} flux through the pore so that it equals that of Ca^{2+}. Furthermore, this mutation specifically shifts channel activation to more depolarized voltages so that V_h equals that in Ca^{2+}, which was not affected by the mutation. The fact that mutation of amino acids on either side of Phe-1126 failed to significantly affect either permeation or gating further demonstrates the specificity of this site.

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