A Selectivity Filter Gate Controls Voltage-Gated Calcium Channel Calcium-Dependent Inactivation

Graphical Abstract

**Highlights**
- CaV selectivity filter forms the calcium-dependent inactivation (CDI) endpoint
- Conserved CaV domain II selectivity filter (+1) aspartate plays an active role in CDI
- CaV selectivity filter asymmetry is important for CDI
- CaV's gating relies on an SF-based gating framework shared among the VGIC superfamily

**In Brief**
Calcium-dependent inactivation (CDI) is essential for voltage-gated calcium channel (CaV) autoregulation. Abderemane-Ali et al. demonstrate that the CaV selectivity filter (SF) forms the CDI gate, suggesting an SF-based inactivation paradigm shared with other voltage-gated ion channel (VGIC) superfamily members.

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A Selectivity Filter Gate Controls Voltage-Gated Calcium Channel Calcium-Dependent Inactivation

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SUMMARY
Calcium-dependent inactivation (CDI) is a fundamental autoregulatory mechanism in CaV1 and CaV2 voltage-gated calcium channels. Although CDI initiates with the cytoplasmic calcium sensor, how this event causes CDI has been elusive. Here, we show that a conserved selectivity filter (SF) domain II (DII) aspartate is essential for CDI. Mutation of this residue essentially eliminates CDI and leaves key channel biophysical characteristics untouched. DII mutants regain CDI by placing an aspartate at the analogous SF site in DIII or DIV, but not DI, indicating that CaV SF asymmetry is key to CDI. Together, our data establish that the CaV SF is the CDI endpoint. Discovery of this SF CDI gate recasts the CaV inactivation paradigm, placing it squarely in the framework of voltage-gated ion channel (VGIC) superfamily members in which SF-based gating is important. This commonality suggests that SF inactivation is an ancient process arising from the shared VGIC pore architecture.

INTRODUCTION
Voltage-gated calcium channels (CaVs) are multisubunit, macro-molecular complexes that control cellular calcium entry in response to membrane potential changes in the brain, nervous system, and heart (Catterall, 2011; Zamponi et al., 2015). Due to the central role of calcium in cellular signaling (Clapham, 2007) and the importance of CaVs as sources of Ca^{2+} influx that impact synaptic transmission, hormone release, vascular tone, muscle contraction, and gene expression (Nanou and Catterall, 2018; Simms and Zamponi, 2014; Zamponi et al., 2015), a multifaceted set of activity-dependent feedback regulation mechanisms shape CaV function. Chief among these is calcium-dependent inactivation (CDI), a process by which Ca^{2+} influx through CaV1 and CaV2 channels causes a cessation of ion conduction (Ben-Johny and Yue, 2014; Christel and Lee, 2011; Dunlap, 2007; Simms and Zamponi, 2014). Perturbations in CDI are involved in autism (Limpitikul et al., 2016), blindness (Singh et al., 2006), and cardiac arrhythmias (Aisekhan et al., 2002; Dick et al., 2016; Limpitikul et al., 2014, 2017; Mahajan et al., 2008; Morotti et al., 2012; Splitski et al., 2004, 2005), demonstrating that CDI is an important factor in diseases linked to CaV dysfunction.

The CaV pore-forming CaV_{1,2} subunit is a 24 transmembrane segment protein comprising four repeats (DI–DIV) that are each made from six transmembrane helices that form the voltage sensor domain (SI–S4) and the pore domain (S5–S6) (Catterall et al., 2017; Wu et al., 2015, 2016). CaV_{1,2} shares the overall architecture found throughout the voltage-gated ion channel (VGIC) superfamily but requires auxiliary subunits for proper function (Campiglio and Flucher, 2015; Dolphin, 2016). Complexes with two intracellular components that bind to the cytoplasmic I-II loop and C-terminal tail, the CaV_b subunit (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004) and calmodulin (CaM) (Fallon et al., 2009; Kim et al., 2008, 2010; Mori et al., 2008; Van Petegem et al., 2004), respectively, are particularly important for activity-dependent feedback modulation by the two principal CaV inactivation mechanisms, voltage-dependent inactivation (VDI) (Cens et al., 2006; Stotz et al., 2004) and CDI (Ben-Johny and Yue, 2014; Cens et al., 2006; Christel and Lee, 2011; Halling et al., 2006).

The molecular origins of CDI have been extensively investigated, especially from the vantage point of the intracellular, CaM-based sensor apparatus and the role of the CaM-binding IQ domain (Ben-Johny and Yue, 2014; Fallon et al., 2005; Minor and Findeisen, 2010; Kim et al., 2008, 2010; Mori et al., 2008; Van Petegem et al., 2005). Although the involvement of this CaM-based sensor in CDI is clear (Ben-Johny and Yue, 2014; Minor and Findeisen, 2010; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Van Petegem et al., 2005; Zühlke et al., 1999), the fact that the N-terminal cytoplasmic domain (Ben-Johny et al., 2013; Dick et al., 2008; Ivanina...
Figure 1. CaV1.2 Selectivity Filter Mutations in DII Affect CDI

(A) Sequence alignment of pore helices and selectivity filters of NaVAe1 (Shaya et al., 2014), calcium-selective BacNaVs CaVBh1 (Yue et al., 2002), CaVSp1p (Shaya et al., 2011), and human CaV1 and CaV2 exemplars: CaV1.1 (UniProtKB: Q13698), CaV1.2 (UniProtKB: Q13936), CaV1.3 (UniProtKB: Q01668), and CaV2.1 (UniProtKB: O00555). SF sites (0) and (+1) are highlighted in orange and red, respectively. Other SF sites are highlighted in gray. Sequences are NaVAe1 185-211, CaVBh1 179-205, CaVSp1p 164-190, CaV1.1 DI 280-306, CaV1.1 DII 602-628, CaV1.1 DIII 1002-1028, CaV1.1 DIV 1311-1337, CaV1.2 DI 351-377, CaV1.2-DII 694-720, CaV1.2-DIII 1103-1129, CaV1.2-DIV 1404-1430, CaV1.3-DI: 343-369, CaV1.3-DII 704-730, CaV1.3-DIII 1100-1126, CaV1.3 DIV 1390-1416, CaV2.1-DI 306-332, CaV2.1 DII 656-682, CaV2.1 DIII 1448-1474, CaV2.1 DIV 1744-1770.

(B) Structural comparison of the NaVAe1 (PDB: 5HK7) (Arrigoni et al., 2016) SF (marine) with the CaV1.1 (PDB: 5GJV) (Wu et al., 2016) DII SF (green). Ca2+ from NaVAe1 (PDB: 4LTO) (Shaya et al., 2014) is shown as a sphere. DI (blue), DII (green), DIII (cyan), and DIV (orange) domains of CaV1.1 are shown. CaV1.1 and NaVAe1p residues are labeled in plain and italics, respectively. CaV numbering follows the CaV1.2 equivalent residues. SF positions are shown in parentheses. Side view (top) omits DI for clarity. DI (+1) position is highlighted in yellow.

(C) Exemplar normalized recordings at +20 mV in Ca2+ (black) or Ba2+ (gray) from Xenopus oocytes expressing CaV1.2 or indicated mutants.

(legend continued on next page)
et al., 2000; Tadross et al., 2008) and the CaVβ/I-II loop complex (Almagor et al., 2012; Findesen and Minor, 2009) also impact CDI has left open the question of how conformational changes in these cytoplasmic parts of the channel complex terminate Ca2+ influx (Babich et al., 2007; Barrett and Tsien, 2008; Cens et al., 2006; Findesen and Minor, 2009; Kim et al., 2004; Tadross et al., 2010). The prevailing model suggests that the activation gate formed by the S6 helices of the channel pore acts as the CDI endpoint via an allosteric mechanism (Dick et al., 2016; Limpitikul et al., 2016; Tadross et al., 2010). Other studies have raised the idea of a close link between ion selectivity and CDI, based on the fact that CDI and selectivity properties are simultaneously affected by SF mutations or changes in extracellular Ca2+ concentrations (Babich et al., 2005; Zong et al., 1994) and on the observation that SF Gd3+ block appears mutually exclusive with inactivation (Babich et al., 2007). Further, the extent to which CDI and VDI share common pathways (Cens et al., 1999; Findesen and Minor, 2009; Kim et al., 2004) or act by different mechanisms (Barrett and Tsien, 2008; Tadross et al., 2010) has been unclear.

We discovered that mutations in the conserved aspartate at the CaV1.2 domain II (DII) SF (+1) position, a site that is important for CaV SF Ca2+ binding and that forms an outer ion binding site in bacterial voltage-gated sodium channels (BacNaVs) and mammalian CaVs (Shaya et al., 2014), affect CDI. Mutation of this conserved aspartate can effectively eliminate CDI in CaV1.2, while sparing core biophysical properties including voltage-dependent activation, VDI, and ion selectivity. Analogous SF DII (+1) mutations in two other CaVs, a CaV1.3 variant bearing the most robust CDI among all CaVs (Huang et al., 2013; Singh et al., 2008; Xu and Lipscombe, 2001) and CaV2.1, a channel in which CDI relies on a different CaM lobe than in CaV1.2 (DeMaria et al., 2001; Minor and Findesen, 2010; Lee et al., 2003; Liang et al., 2003), demonstrate that the role of the DII (+1) aspartate in controlling CDI is both conserved and independent of the details of how the intracellular Ca2+ sensor acts. We also show that, in channels lacking CDI because the DII (+1) aspartate is mutated, one can restore CDI by placing an aspartate at the analogous SF (+1) sites of DIII or DIV but not DI. This observation is concordant with asymmetric functional roles for the four glutamates at the SF (0) position (Ellinor et al., 1995; Parent and Gopalakrishnan, 1995; Yang et al., 1993) and indicates that in the SF that drives CDI is asymmetric. Together, our data establish that the CaV SF is the endpoint gate for CDI and change the paradigm for understanding CaV inactivation mechanisms by placing it squarely within the framework of the growing list of VGIC superfamily members in which SF-based gating is central to function (Autzen et al., 2018; Bagriantsev et al., 2011; Cao et al., 2013; Cohen et al., 2008; Cuello et al., 2010, 2017; Liu et al., 1996; Lolicato et al., 2017; Lopez-Barneo et al., 1993; Ogieskia and Aldrich, 1999; Pavlov et al., 2005; Peters et al., 2013; Plechotta et al., 2011; Schewe et al., 2016; Steinberg et al., 2017). These findings support the idea that SF inactivation is an ancient process arising from the conserved VGIC pore architecture (Catterall et al., 2017) that not only affects slow inactivation, but also controls CDI in CaVs.

**RESULTS**

**Single CaV1.2 Selectivity Filter Mutations Dramatically Reduce CDI in Xenopus Oocytes**

The SF sequences of each of the four CaV domains are highly similar to each other and to those found in BacNaVs (Payandeh and Minor, 2015; Ren et al., 2001; Shaya et al., 2014; Wu et al., 2015; Yue et al., 2002), which are thought to be a common ancestor of both voltage-gated sodium channels (NaVs) and CaVs (Koishi et al., 2004; Payandeh and Minor, 2015; Ren et al., 2001). All share the central glutamate residue (denoted position (0); Payandeh and Minor, 2015; Shaya et al., 2014) (Figure 1A) and a common architecture that supports the SF structure (Figure 1B) (Payandeh and Minor, 2015; Payandeh et al., 2011; Wu et al., 2015). The SF (0) position glutamates form a charged ring at the center of the CaV and BacNaV SFs (Payandeh and Minor, 2015; Payandeh et al., 2011; Wu et al., 2015, 2016) and play a key role in Ca2+ selectivity and ion permeation (Parent and Gopalakrishnan, 1995; Yang et al., 1993). In the case of CaVs, the four SF sequences are not identical. Further, even though the side-chain identity at the CaV SF position (0) is the same, each (0) position glutamate contributes differently to SF properties with the DIII (0) glutamate (CaV1.2 Glu 1115) being the most important contributor to ion selectivity (Ellinor et al., 1995; Kim et al., 1993; Mikala et al., 1993; Yang et al., 1993). A further asymmetry in the CaV SF occurs at DII position (+1). This site has a unique aspartate (CaV1.2 Asp 707) (Figure 1A) that is involved in the high-affinity binding of Ca2+ to the SF and whose neutralization by mutation has similar effects on Ca2+ binding as neutralization of the DIII position (0) glutamate (Shaya et al., 2014).

In the course of studying the functional properties of CaV1.2 SF mutants using two-electrode-voltage clamp of *Xenopus* oocytes (Figure S1A), we observed that replacement of the DII (+1) site, Asp 707, with alanine, glycine, asparagine, or glutamate decreased CDI. These effects manifested as varied degrees of reduction of the classic “U-shaped” response characteristic of CDI with D707A and D707N causing the greatest and mildest effects, respectively (Figures 1C and 1D).

To quantify changes in CDI, we measured the ratio between the current amplitudes at the peak and 300 ms post-depolarization (R300) for both Ba2+ and Ca2+ as a function of voltage (Figure 1D) and compared the difference between Ba2+ and Ca2+ R300 Values, f, for CaV1.2 and D707A/G/N/E mutants at the potential of maximal current, +20 mV (Peterson et al., 1999). This

**Table 1.**

**Voltage-dependent activation curves for CaV1.2 (black circles), D707A (purple triangles), D707G (inverted blue triangles), D707N (green diamonds), D707E (red circles), and E1115A (orange squares).**

(DE) Fractional current remaining 300 ms post-depolarization (R300) as a function of the membrane potential for channels in (C).

(F) Average fraction CDI (f) at +20 mV, where f is the difference between Ca2+ and Ba2+ R300. *p < 0.015 and **p < 0.001 compared to CaV1.2. n values are in Table 1.

(G) I-V relationships for the indicated channels. Symbols are the same as (F).
The negative charge and the DII (+1) position side-chain geometry respectively; Table 1), suggesting the importance of both the negative charge are important for CDI. Experiments using a domain III position (0) mutant that reduces Ca²⁺ ion selectivity, E1115A (Ellinor et al., 1995; Mikala et al., 1993), also caused a large reduction in CDI (f = 0.14 ± 0.02) that was comparable to the Asp 707 mutant causing the most severe CDI reduction, D707A (Figures 1C–1E). None of the SF mutants that we tested had differing effects on CDI (D707A, D707E, and D707N, and D707G, respectively) (Figure 1G). Such a change in Erev was notably absent from the Asp 707 mutants, all of which behaved similarly to wild-type (Erev = 92.8 ± 4.8, 94.8 ± 4.0, 87.8 ± 2.5, and 88.0 ± 8.1 mV for D707A, D707G, D707N, and D707E, respectively) (Figure 1G).

The impact of the Asp 707 mutations on CDI, a result demonstrating that SF mutation effects on CDI are independent of Ca V subunit to CaV b₃, a subunit that allows fast VDI (De Waard and Campbell, 1995; Stea et al., 1994) did not alter the relative impact of the Asp 707 mutations on CDI, a result demonstrating that SF mutation effects on CDI are independent of CaV b₃ (Figure S3). Overall, these observations indicate that Asp 707 mutations affect CDI but leave other biophysical properties unchanged.

**SF Mutations Identify Elements that Selectively Affect CDI or VDI**

CaVs have two principal inactivation mechanisms, CDI (Ben-Joyne and Yue, 2014; Cens et al., 2006; Halling et al., 2006) and VDI (Cens et al., 2006; Stotz et al., 2004). Due to the strong slowing effects that CaV b₃ has on VDI (De Waard and Campbell, 1995; Olcese et al., 1994; Stea et al., 1994), VDI is largely absent in our initial experiments that uncovered the importance of the DII (+1) site in CDI (Figures 1C and 1D). Therefore, to examine the possible impact of changes at the DII (+1) SF position on VDI, we paired CaV 1.2 channels bearing a set of DII (+1) mutations that had differing effects on CDI (D707A, D707E, and D707G, respectively) with CaV 1.2 and CaV 2.1 channels bearing a set of DII (+1) mutations that had differing effects on VDI (D1420A, D1420A, and E1121A, respectively) (Table 1). The CaV 1.3 channel was co-expressed with the Asp 707 mutants to check for potential effects on the ability of these mutants to discriminate between Ba²⁺ and Ca²⁺.

**Table 1. Electrophysiological Parameters**

<table>
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<tr>
<th>Channel</th>
<th>f or f (_0)</th>
<th>IBa/ICa</th>
<th>Erev (mV)</th>
<th>V₁/₂ (mV)</th>
<th>k</th>
<th>n</th>
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<td>Oocytes</td>
<td></td>
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<tr>
<td>CaV 1.2</td>
<td>0.61 ± 0.02</td>
<td>1.72 ± 0.11</td>
<td>89.1 ± 4.0</td>
<td>5.4 ± 0.6</td>
<td>9.5 ± 0.3</td>
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<tr>
<td>D707A</td>
<td>0.10 ± 0.01</td>
<td>1.53 ± 0.05</td>
<td>92.8 ± 4.8</td>
<td>5.2 ± 0.9</td>
<td>9.8 ± 0.5</td>
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<td>D707G</td>
<td>0.24 ± 0.03</td>
<td>1.94 ± 0.13</td>
<td>94.8 ± 4.0</td>
<td>7.2 ± 0.8</td>
<td>9.0 ± 0.6</td>
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<td>D707N</td>
<td>0.49 ± 0.04</td>
<td>2.63 ± 0.03</td>
<td>87.8 ± 2.5</td>
<td>10.0 ± 0.6</td>
<td>9.8 ± 0.5</td>
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<td>D707E</td>
<td>0.35 ± 0.02</td>
<td>3.58 ± 0.24</td>
<td>88.0 ± 8.1</td>
<td>8.5 ± 1.0</td>
<td>8.7 ± 0.5</td>
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<tr>
<td>E1115A</td>
<td>0.14 ± 0.02</td>
<td>0.92 ± 0.01</td>
<td>49.8 ± 1.0</td>
<td>1.7 ± 0.3</td>
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<td>0.30 ± 0.02</td>
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<td>2.28 ± 0.29</td>
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<td>4.8 ± 3.3</td>
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<td>D707A-A1417D</td>
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<td>E1119A</td>
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<td>1.73 ± 0.18</td>
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<td>D1420A</td>
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<td>1.88 ± 0.23</td>
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<tr>
<td>CaV 1.2</td>
<td>0.59 ± 0.02</td>
<td>1.57 ± 0.38</td>
<td>77.5 ± 3.0</td>
<td>7.1 ± 2.0</td>
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<td>1.94 ± 0.21</td>
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<td>0.40 ± 0.04</td>
<td>2.86 ± 0.52</td>
<td>73.3 ± 2.9</td>
<td>11.3 ± 2.1</td>
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<td>E1115A</td>
<td>0.36 ± 0.04</td>
<td>1.00 ± 0.15</td>
<td>68.5 ± 1.5</td>
<td>0.5 ± 2.0</td>
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<td>CaV 1.3</td>
<td>0.73 ± 0.01</td>
<td>1.58 ± 0.11</td>
<td>75.6 ± 3.0</td>
<td>8.5 ± 1.1</td>
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<td>D726A</td>
<td>0.15 ± 0.05</td>
<td>1.85 ± 0.16</td>
<td>81.1 ± 1.3</td>
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<td>E1121A</td>
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<td>1.09 ± 0.08</td>
<td>61.9 ± 3.0</td>
<td>15.9 ± 1.7</td>
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<td>CaV 2.1</td>
<td>0.26 ± 0.03</td>
<td>1.64 ± 0.39</td>
<td>46.9 ± 7.0</td>
<td>7.7 ± 0.8</td>
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<td>D667A</td>
<td>0.04 ± 0.03</td>
<td>1.81 ± 0.12</td>
<td>56.4 ± 3.2</td>
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<td>1.07 ± 0.13</td>
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<td>8.8 ± 0.5</td>
<td>3.4 ± 0.2</td>
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</tbody>
</table>

f is defined as R₈₀₀ for Ba²⁺ – R₈₀₀ for Ca²⁺ where R₈₀₀ = I₈₀₀/I₀ where I₈₀₀ and I₀ are current amplitudes at 800 ms post-depolarization and at the peak current, respectively. f is defined as R₈₀₀ for Ba²⁺ – R₈₀₀ for Ca²⁺ where R₈₀₀ = I₈₀₀/I₀ where I₈₀₀ and I₀ are current amplitudes at 800 ms post-depolarization and at the peak current, respectively. f and f₀ were determined at membrane potential of +20 and +10 mV, respectively. IBa/ICa is the Ba²⁺ and Ca²⁺ peak current amplitude ratio. Erev is the reversal potential. V₁/₂ is the midpoint of activation. k is the slope factor of the activation curve. Erev, V₁/₂, and k were determined using Ca²⁺ as the charge carrier. n is the number of experiments. Data were fit to I = Gmax *( Vm /C₀ ) where I is the measured peak current at each test potential (Vm) and Gmax is the maximal macroscopic conductance. Data are expressed as mean values ± SEM.
were similarly expression system dependent, we measured the properties of the two mutants that had the strongest effects on CDI reduction of CaV1.2 in oocytes, D707A and E1115A, as well as the mutant that maintained the SF DII (+1) negative charge, D707E, using whole-cell recordings from transfected HEK293 cells (Figures 2A and S1B). As in oocytes, D707A, D707E, and E1115A caused a substantial reduction in CDI (Figures 2A–2C). The magnitude of the CDI diminishment was less pronounced in the E1115A mutant compared to oocytes, whereas this effect was comparable between the two different expression systems for D707A and D707E (Table 1). Similar to oocytes, voltage-dependent activation and I–V curves of CaV1.2 and Asp 707 mutants were identical (Figures 2D and 2E). E1115A caused a small hyperpolarizing shift (−8 mV) in voltage-dependent activation, not seen in oocytes (Figure 2E; Table 1), and perturbed the reversal potential, although this change is smaller in HEK293 cells (−9 versus −39 mV for HEK293 cells and Xenopus oocytes, respectively) (Figures 1G and 2E; Table 1). In further support of differential effects of the mutants on ion selectivity, similar to oocytes, the ability to discriminate Ca2+ over Ba2+, reflected by $I_{Ba}/I_{Ca}$, was conserved in the Asp 707 mutants but was eliminated in E1115A (Figure S2B; Table 1). Thus, in contrast to expression system-dependent differences in CDI at the level of the Ca2+ sensor (Barrett and Tsien, 2008), our results demonstrate that the SF mutation-induced CDI changes are expression system independent and provide additional support for the idea that the reduction in CDI caused by the DII (+1) mutants are not related to changes in the ion selectivity properties of the channel.

### Lowered Ca2+ Influx in DII (+1) Mutants Does Not Cause Loss of CDI

Although the DII (+1) mutations did not cause ion selectivity changes, in principle, the effects on CDI could be caused by reduction of Ca2+ influx via a change in channel open probability or conductance. To investigate this possibility, we compared low-noise recordings from the DII (+1) mutants that exhibited the most severe, D707A, and the mildest, D707N, CDI loss with CaV1.2. Because of the challenges associated with single CaV channel recordings when Ca2+ is the charge carrier (Hess et al., 1984), we compared patches expressing CaV1.2, D707A, and D707N using Ba2+ as the charge carrier as in many prior studies (Adams et al., 2014; Bock et al., 2011; Dick et al., 2016; Doering et al., 2005; Hess et al., 1984; Tadross et al., 2008, 2013; Yang et al., 2015). These experiments showed single CaV1.2 channels having an ~1-pA current amplitude, consistent with prior reports (Dick et al., 2016). By contrast, in patches expressing either D707A or D707N, we could only observe measurable currents characteristic of multi-channel recordings with maximal current of ~2 pA (Figure 3A). These observations indicate that both the D707A and D707N decrease channel conductance relative to wild-type. To address whether there was a parallel reduction in Ca2+ conductance relative to the reduced level of CDI, we measured whole-cell Ca2+ currents of CaV1.2, D707A, and D707N (Figure 3B) and compared their current densities (Figure 3C). These data reveal similar current densities for D707A and D707N that are both reduced by ~80% relative to CaV1.2 (Figure 3C) and that are consistent with the reduced...
channel conductance assessed by the low-noise recordings (Figure 3A). The reduced single-channel conductance observed for D707A and D707N is in line with the ability of both mutants to cause similar reductions in Ca2+ binding to the channel pore (Shaya et al., 2014) and points to a key role of the DII (+1) site in ion conduction. Importantly, the data indicate that the shared decrease in ion conduction caused by the DII (+1) mutations cannot be the origin of the loss of CDI in D707A, as two channels having similar low conductances, D707A and D707N, have completely different CDI phenotypes (Figures 1C–1E).

To investigate whether reduced Ca2+ influx was the cause of CDI loss in D707A, we used the Ca2+ agonist Bay K8644 that increases single-channel open times (Hess et al., 1984) to enhance Ca2+ influx through the channel. Bay K8644 application to Xenopus oocytes expressing CaV1.2, D707A, D707N, or the mutant E1115A, in which changes in ion selectivity reduce Ca2+ influx (Ellinor et al., 1995; Mikala et al., 1993), showed clear augmentation in Ca2+ current amplitude for all cases, consistent with an increase in channel openings and higher Ca2+ influx (Hess et al., 1984) (Figures 3D and 3F). This Bay K8644-enhanced Ca2+ current was accompanied by a more complete inactivation caused by the DII (+1) mutations (Figures 3D and 3G). However, for CaV1.2 and D707N, the equivalent level of increased inactivation was also observed when Ba2+ was used as charge carrier, indicating that this effect is a consequence of Bay K8644 itself rather than a response to the increased Ca2+ influx due to an undersaturated CaM Ca2+ sensor (Figures 3D, 3H, and 3I). By contrast, for the mutant having compromised selectivity, E1115A, CDI was enhanced beyond the level expected from the Bay K8644 effect in Ba2+ (Figure 3I). Notably, Bay K8644-induced inactivation in CaV1.2 and D707N (Figure 3F), the enhanced Ca2+ influx was not able to induce CDI for D707A (Figures 3D, 3E, and 3G–3I). Hence, even though the magnitude of the Bay K8644-induced Ca2+ current increase was similar for CaV1.2, D707A, and D707N (Figure 3F), the enhanced Ca2+-influx was not able to induce CDI for D707A (Figures 3D, 3E, and 3G–3I). Taken together, these observations establish that CDI loss in the D707A mutant is not caused by a reduced Ca2+ influx and strongly suggest that SF DII (+1) position plays an active role in CDI.

A Single CaV1.3 SF DII (+1) Mutation Drastically Reduces CDI

The aspartate at the SF DII (+1) site is conserved among all CaVs (Figure 1A) (Payandeh and Minor, 2015; Shaya et al., 2014). CDI occurs in high voltage-activated CaVs (CaV1s and CaV2s) (Liang et al., 2003), and, among the L-type (CaV1) channels, the CaV1.3;2a variant exhibits the most pronounced CDI (Huang et al., 2013; Singh et al., 2008; Xu and Lipscombe, 2001). Therefore, to examine the general importance of the DII (+1) aspartate for CDI, we asked whether removal of this aspartate would affect a channel in which CDI is particularly strong. We compared CaV1.3;2a CDI with a DII (+1) mutant and a DII (0) mutant (D762A and E1121A, respectively) that correspond to the
mutants that were most effective at reducing CaV1.2 CDI. Measurement of the functional properties of these channels in HEK293 cells showed that, with the analogous CaV1.2 mutants, the DII (+1) mutation D726A drastically reduced CDI, whereas the DIII (0) mutation E1121A caused only a slight reduction in CDI (Δf = 0.73 ± 0.01, 0.15 ± 0.05, and 0.63 ± 0.02 for CaV1.3, D726A, and E1121A, respectively; Figures 4A–4C; Table 1). Similar to our observations for CaV1.2, the DII (+1) mutant D726A had no effect on channel selectivity or the activation voltage dependency (Figures 2D and 2E; Table 1), whereas the DIII (0) mutant, E1121A, caused a slight leftward shift of both the reversal potential and the activation curve, comparable to the effects of E1115A on CaV1.2 (Figures 2D, 2E, 4D, and 4E; Table 1). In addition, unlike the DII (+1) D726A change, the DIII (0) E1121A substitution caused a significant reduction in the peak current IBa/ICa ratio to ~1, indicating a loss in the ability to discriminate between Ba2+ and Ca2+ (Figure S2C; Table 1).

Hence, as with CaV1.2, the effects of neutralizing the DIII (0) glutamate with respect to the observed CDI reduction are consistent with a change in ion selectivity. By contrast, neutralization of the DII (+1) aspartate spared all of the tested CaV1.3 biophysical parameters, except CDI. These results demonstrate the general importance of the DII (+1) aspartate in CaV1 channels and lend further support to the hypothesis that changes in the SF have a direct role in CDI.

**SF DII (+1) Aspartate Is Generally Important for CDI**

CaV2 channels also have CDI (DeMaria et al., 2001; Lee et al., 2003; Liang et al., 2003), and, similar to CaV1s, this process relies on CaM/IQ domain interactions (Ben-Johny and Yue, 2014; Dunlap, 2007; Minor and Findelen, 2010). However, the roles of the CaM lobes are inverted between the two subfamilies, with the C-lobe controlling CDI in CaV1 (Peterson et al., 1999) but the N-lobe controlling CDI in CaV2s (DeMaria et al., 2001; Lee et al., 2003; Liang et al., 2003). To ask whether the conserved DII (+1) aspartate is important for CaV2 CDI regardless of which CaM lobe governs CDI, we measured the effects of mutants equivalent to CaV1.2 DII (+1) D707A and DIII (0) E1115A in a CaV2 family representative, CaV2.1 (D667A and E1461A, respectively). To quantify changes in CaV2.1 CDI, we measured the ratio between the current amplitude at peak and 800 ms post-depolarization (R800) for both Ba2+ and Ca2+ as a function of voltage (DeMaria et al., 2001) (Figures 4F and 4G) and compared the difference between Ba2+ and Ca2+ R800, f, for CaV2.1 and mutants at the potential of maximal current, +10 mV (DeMaria et al., 2001). As in the case of CaV1.2 and CaV1.3, DII (0) neutralization caused a CDI reduction (f = 0.26 ± 0.03 and 0.12 ± 0.02 for CaV2.1 and E1461A, respectively; Figure 4I) and did not affect activation voltage dependency. However, this site (0) mutation did induce a ~13 mV reversal potential shift and reduced peak current IBa/ICa ratio to ~1 (Figures 4H–4J and S2D; Table 1), consistent with an ion selectivity change. By contrast, neutralization of the CaV2.1 DII (+1) residue essentially eliminated CDI (f = 0.04 ± 0.03; Figure 4H) and did not affect the activation voltage dependency or the peak current IBa/ICa ratio (Figures 4J and S2D; Table 1), although it did cause a +10 mV reversal potential shift (Figure 4I). The clear loss of CaV2.1 CDI caused by the D667A mutation demonstrates the universal role of the CaV SF in CDI and establishes that this role is independent of the details of how the CDI is initiated by CaM. Together with the CaV1 results, these data support the idea that the SF is involved in the final step of the CaV CDI process.

**CDI Requires a Negative Charge at SF (+1) Position on DII, DIII, or DIV**

The four CaV SF domains contribute an identical set of negatively charged glutamate side chains at the (0) position but a set of non-equivalent residues at the (+1) site (G, D, G, and A for DI–DIV, respectively) (Figures 1A and 1B). Given the crucial role we found for the DI (+1) aspartate in CDI (Figures 5A and 5B), and the fact that it is the sole negatively charged amino acid at this CaV SF level, we asked whether the DII (+1) position had a special role in CDI or whether CDI would be preserved if the aspartate were moved to other (+1) positions around the SF. Hence, we created a set of mutants that placed an aspartate at the DI, DII, or DIV (+1) sites. To preserve the SF amino acid composition, each of these mutants exchanged the amino acid from the host site into the DI (+1) site to create the following swap mutants: DIAsp (G364D/D707G), DIIAsp (D707G/G1116D), and DIVAsp (D707A/A1417D) (Figure 5C). We recorded whole-cell currents from Xenopus oocytes injected with these constructs using Ca2+ and Ba2+ as charge carriers and compared these mutants with the corresponding single-point changes at DI (+1) (Figures 5C and 5D). Exchange of the SF (+1) aspartate between the DII and DI positions, DIAsp, yielded channels that had severely diminished CDI that was equivalent to the D707G
mutant (Figures 5C–5E), indicating that the DI (+1) site aspartate was unable to restore CDI in the face of the loss of the aspartate at the DII (+1) position. By contrast, exchange of the SF (+1) aspartate from the DII to DIII positions, DIIAsp, or from the DII to DIV positions, DIVAsp, resulted in complete or near complete CDI restoration, respectively (Figures 5C–5E). These results demonstrate the ability of a SF (+1) aspartate to preserve CDI when located on DIII or DIV but not DI. To test whether reduced CaV2.1 influx was the cause of the compromised CDI in DIAsp, we examined the consequences of Bay K8644-enhanced CaV2.1 influx on the SF (+1) swap mutants. Bay K8644 application increased CaV2.1 currents by ~2-fold in all three (+1) swap mutants, similar to results on other SF mutants (Figures 3C, 3F, and 3G), but failed to enhance CDI in the channel having reduced CDI, DIAsp (Figures S6A, S6C, and S6D). Studies using Ba2+ showed that, as with our other SF (+1) Bay K8644 experiments (Figure 3), inactivation is increased in a CaV2.1-independent manner (Figures S6B, S6E, and S6F). These results indicate that reduced CDI of DIAsp is not caused by insufficient CaV2.1 influx but is linked to a change in the ability of the SF to reach an inactivated state. The preservation of CDI in the DIIAsp and DIVAsp swaps demonstrates that the single changes at DI (+1), D707G and D707A, do not preclude CDI provided that either the DIII or DIV domain provides the SF position (+1) negative charge. The fact that CDI occurs only when the (+1) negative charge resides on DII, DIII, or DIV, but not DI, supports the idea of asymmetric functional roles for the four elements of the SF (Ellinor et al., 1995; Parent and Gopalakrishnan, 1995; Yang et al., 1993) and indicates that the mere presence of a negative charge at the SF (+1) level is insufficient to support CDI.

To probe whether the swap mutations affected the ability of the channel to bind Ca2+, we measured Ca2+ block of Li+ currents (Shaya et al., 2014; Yang et al., 1993). Interestingly, all three aspartate-swap mutants behaved identically to CaV1.2, indicating that the affinity for Ca2+ was preserved regardless of the domain that housed the (+1) aspartate (Figure 5F). This result contrasts the reduction in CaV2.1 block caused by the single-point mutants at the DI (+1) position (Figure 5F) (Shaya et al., 2014). The observation that CaV2.1 block is preserved when the aspartate is placed at any of the four possible site (+1) positions contrasts with the domain selective results for CDI that these mutants cause. This finding indicates that the involvement of the SF in CDI is not strictly related to its ability to bind Ca2+. These results taken together with the differential contributions of the individual (+1) sites to CDI support the idea that the SF has a direct role in CDI.

**DISCUSSION**

CDI is an essential feature of high voltage-activated CaVs (CaV1s and CaV2s) that serves as an activity-dependent autoregulatory mechanism for limiting CaV2.1 influx (Christel and Lee, 2011; Dunlap, 2007; Liang et al., 2003; Simms and Zamponi, 2014). This process contributes to autism (Singh et al., 2006), and cardiac action potential duration (Alseikhan et al., 2002; Dick et al., 2016; Mahajan et al., 2008; Morotti et al., 2012; Splawski et al., 2004, 2005) and is impacted by alternative splicing (Barbets et al., 2018; Shen et al., 2006; Tan et al., 2011), RNA editing (Huang et al., 2012), as well as mutations in CaM associated with long QT syndrome (Limptikul et al., 2014, 2017). Because CDI is central to both the biophysical and physiological functions of CaVs, its molecular origins have been extensively investigated, especially from the vantage point of the intracellular, CaM-based sensor and the role of the IQ domain (Ben-Johny and Yue, 2014; Minor and Findelen, 2010; Kim et al., 2008, 2010; Mori et al., 2008; Van Petegem et al., 2005).

Although the involvement of this CaM-based sensor in CDI is firmly established, how conformational changes of this cytoplasmic element result in the cessation of ion flow through the channel and the exact CDI endpoint have remained unresolved (Babich et al., 2007; Barrett and Tsien, 2008; Cens et al., 2006; Findelen and Minor, 2009; Kim et al., 2004; Tadross et al., 2010). Our observations provide the first evidence that the CaV SF is a central element of the CDI mechanism and constitutes the CDI gate.

Three CaV channel intracellular elements contribute to CDI: the N-terminal cytoplasmic domain (Ben-Johny et al., 2013; Dick et al., 2008; Ivanina et al., 2000; Tadross et al., 2008), the CaV/I-II loop complex (Almagor et al., 2012; Findelen and Minor, 2009), and the C-terminal tail:CaM complex (Ben-Johny and Yue, 2014; Minor and Findelen, 2010; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Van Petegem et al., 2005; Zühlke et al., 1999), with the C-terminal tail:CaM complex serving as the CaV2.1 sensor that initiates CDI. Given this functionally interconnected network of domains that influence CDI, there has been an ongoing search to identify the molecular endpoints of CaV CDI (Barrett and Tsien, 2008; Benmocha Guggenheimer et al., 2016; Tadross et al., 2010). There has been much focus on the S6 pore lining helices as a candidate for the CDI endpoint (Benmocha Guggenheimer et al., 2016; Raybaud et al., 2006; Tadross et al., 2010; Tadross and Yue, 2010), as these transmembrane helices form the channel intracellular gate (Wu et al., 2015, 2016) and are directly linked to two of the three domains that affect CDI. Given the involvement of many channel parts in CDI, an allosteric framework has been
Figure 5. CaV1.2 CDI Requires a Negative Charge at the DII, DIII, or DIV SF (+1) Position

(A) Exemplar normalized recordings at +20 mV in Ca\(^{2+}\) (black) or Ba\(^{2+}\) (gray) from Xenopus oocytes expressing the CaV1.2 or the indicated mutants.

(B) Fractional current remaining 300 ms post-depolarization (R\(_{300}\)) as a function of the membrane potential for channels in (A). Data in (A) and (B) are identical to Figures 1A and 1B.

(C) Exemplar normalized recordings at +20 mV in Ca\(^{2+}\) (blue) or Ba\(^{2+}\) (orange) from Xenopus oocytes expressing the indicated CaV1.2 mutants moving the negative charge at the (+1) position of the selectivity filter from domain II to domains I, III, or IV. Ca\(^{2+}\) (black) and Ba\(^{2+}\) (gray) currents from CaV1.2 are shown for comparison.

(D) Fractional current remaining 300 ms post-depolarization (R\(_{300}\)) as a function of the membrane potential for channels in (C).

(E) Average f values at 20 mV. **p < 0.001 compared to the corresponding single mutant.

(F) Dose-response curves for Ca\(^{2+}\) block of Li\(^{+}\) currents for CaV1.2, D\(_{\text{II}}\)Asp G364D/D707G (blue triangles), D\(_{\text{III}}\)Asp D707G/G1116D (inverted blue triangles), and D\(_{\text{IV}}\)Asp D707A/A1417D (lavender diamonds). Each data point at each Ca\(^{2+}\) concentration is normalized to the current at 3 nM Ca\(^{2+}\) and averaged for n = 7–9 oocytes.
used to try to understand the actions of disease mutants on CDI (Dick et al., 2016; Limpitikul et al., 2016; Tadross et al., 2010). An alternative proposal is that the SF may participate in CDI through an ion-blocking model in which Ca\textsuperscript{2+} affinity is increased in the inactivated state (Babich et al., 2007). Our observations that SF DII (+1) site mutations such as D707A eliminate CDI but spare biophysical parameters related to the activation process are inconsistent with the proposal that the activation gate formed by S6 is the CDI endpoint (Tadross et al., 2010). Nevertheless, S6 is likely to be critical for coupling to the various intracellular domains that contribute to CDI and may mediate direct coupling between the activation gate and the SF-based CDI gate, similar to its role in other VGIC superfamily members (Ader et al., 2009; Cuello et al., 2017; Imai et al., 2010; Panyi and Deutsch, 2006; Peters et al., 2013). Further, in contrast to the proposal that CDI occurs through an increased affinity of the SF for Ca\textsuperscript{2+} (Babich et al., 2007), we find that mutations that remove the aspartate at the SF DII (+1) site can have identical Ca\textsuperscript{2+} affinities but exhibit very different degrees of CDI (Figures 1 and 5) (Shaya et al., 2014). Together, our findings establish that the Ca\textsubscript{v} SF serves as the CDI gate and recast the paradigm for understanding the Ca\textsubscript{v} SF mechanism with a focus on the SF.

The pore domain of all VGIC superfamily members is made from four subunits arranged around the central ion-conducting pore (Yu et al., 2005) that share a common architectural fold comprising two transmembrane helices, the SF, and a short pore helix that supports the SF architecture (Catterall et al., 2017; Payandeh and Minor, 2015). Within the context of this shared architecture, studies of diverse VGIC superfamily members have begun to uncover a central role for SF in inactivation mechanisms, being best characterized in diverse potassium channel types including: KcsA (Cuello et al., 2010, 2017), Kv5 (Liu et al., 1996; López-Barneo et al., 1993; Ogielska and Aldrich, 1999; Peters et al., 2013), and K\textsubscript{v16} (Bagriantsev et al., 2011; Cohen et al., 2008; Lolicato et al., 2017; Piechotta et al., 2011; Schewe et al., 2016). Additionally, structural observations of asymmetric BacNa\textsubscript{v} SF conformations (Catterall et al., 2017; Payandeh et al., 2012) and functional studies of SF (+4) position mutants (Pavlov et al., 2005) have suggested a role for the SF in BacNa\textsubscript{v} VDI that may be shared with eukaryotic Na\textsubscript{v}s and Ca\textsubscript{v}s (Catterall et al., 2017). Studies of members of the TRP channel branch, TRPV1 (Cao et al., 2013; Steinberg et al., 2017) and TRPM4 (Autzen et al., 2018), also support the idea that the SF constitutes a gate. Hence, the discovery that Ca\textsubscript{v} CDI also relies on a SF-based mechanism sets Ca\textsubscript{v}s squarely within the growing list of VGICs in which SF-based gating is central to function. The apparent widespread role of the SF in the inactivation processes of VGIC superfamily members suggests that these SF-based inactivation mechanisms capitalize on a fundamental pore domain (PD) property that predates the evolutionary divergence in ion selectivity and gating cue responses among the VGIC superfamily branches. This prevalence of SF-based inactivation mechanisms seems likely to have its origins in the shared ancient structure that forms the PD.

Elements from the cytoplasmic side of the channel drive conformational changes that stop ion permeation for both CDI and VDI. Whether Ca\textsubscript{v} CDI and VDI share a common mechanism has been unresolved (Barrett and Tsien, 2008; Cens et al., 2006; Findeisen and Minor, 2009; Kim et al., 2004; Tadross et al., 2010). Our studies support the idea that both processes involve the SF. Notably, we find that there are selective effects on CDI or VDI depending on which parts of the SF structure are altered (Figures 1 and S4), supporting the idea that CDI and VDI have different endpoints even though they are affected by common channel elements (Barrett and Tsien, 2008; Findeisen and Minor, 2009; Tadross et al., 2010). The VDI effects match those reported for BacNa\textsubscript{v}s (Pavlov et al., 2005) and support the idea that there is a common SF-based mechanism underlying VDI in BacNa\textsubscript{v}s, Ca\textsubscript{v}s, and Na\textsubscript{v}s (Catterall et al., 2017). Considering our new findings of the importance of the SF in CDI together with prior cytoplasmic domain studies (Almagor et al., 2012; Ben Johny et al., 2013; Dick et al., 2008; Findeisen and Minor, 2009; Ivanina et al., 2000; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Tadross et al., 2008; Van Petegem et al., 2005; Zühlke et al., 1999), we propose the following model in which SF conformational changes constitute the end stage of a CDI process that is initiated by CaM on the cytoplasmic side of the channel (Figure 6). Upon activation by voltage, Ca\textsuperscript{2+} influx through the channel is sensed by CaM and initiates a set of conformational rearrangements that end with a conformational change in the SF that stops ion flow. Although the exact conformational changes underlying Ca\textsubscript{v} SF inactivation and complete accounting of the residues involved remain to be elaborated and ultimately will require structural studies of the channel.

Figure 6. Ca\textsubscript{v} CDI Model
Closed channels (left) are activated by depolarization that includes voltage sensor domain (VSD) activation and pore opening. Flow of Ca\textsuperscript{2+} through the channel leads to Ca\textsuperscript{2+} (white circles) binding to CaM (green) that initiates CDI. CDI results in a selectivity filter (SF) conformational change that obstructs ion flow. Pore domain, PD (violet), voltage sensor domain, VSD (cyan), and SF (black) are labeled. Gray bars indicate membrane. Other intracellular elements that affect CDI such as the N-terminal cytoplasmic domain and Ca\textsubscript{v}I-I-II loop complex are not shown.
trapped in various states, our data indicate that this process exploits the previously identified functional asymmetry in the CaV SF (Ellinor et al., 1995; Parent and Gopalakrishnan, 1995; Yang et al., 1995). In this regard, it is interesting to note that the SF DII (+1) aspartate seems capable of making interactions that involve other SF domain residues (Cheng et al., 2010) and that we can restore CDI to DII (+1) SF mutants by supplying an aspartate at the equivalent site in two of the three other domains (Figure 5).

Our studies provide clear evidence that the CaV SF plays a central role in the CDP process by forming the CDI gate. The evidence for this CaV SF gate, together with the presence of an inner gate formed by the S6 helices (Benmocha Guggenheimer et al., 2016; Raybaud et al., 2006; Tadross et al., 2010; Tadross and Yue, 2010; Wu et al., 2015, 2016), establishes that CaVβs use two gates to control their activity, similar to other VGIC superfamily members (Autzen et al., 2018; Cao et al., 2013; Cuello et al., 2017; Steinberg et al., 2017). This finding creates a new framework for addressing how the two CaV gates interact, whether they are coupled in a manner similar to structurally related potassium channels (Cuello et al., 2017), whether there are commonalities in SF-based inactivation mechanisms between channels that have wide SFs such as CaVβs versus those that intimately contact the permeant ions such as potassium channels (Cuello et al., 2017), and how the intracellular components that contribute to CDI (Almagor et al., 2012; Ben Johny et al., 2013; Ben-Johny and Yue, 2014; Dick et al., 2008; Finsidein and Minor, 2009; Minor and Findelstein, 2010; Ivanina et al., 2000; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Tadross et al., 2008; Van Petegem et al., 2005; Zühlke et al., 1999) affect structural transitions in the SF gate. Such issues may be important for understanding how CaV disease mutations that impact CDI act (Dick et al., 2008; Finsidein and Minor, 2009; Minor and Findeisen, 2010; Ivanina et al., 2000; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Tadross et al., 2008; Van Petegem et al., 2005; Zühlke et al., 1999) affect structural transitions in the SF gate. Such issues may be important for understanding how CaV disease mutations that impact CDI act (Dick et al., 2008; Finsidein and Minor, 2009; Minor and Findeisen, 2010; Ivanina et al., 2000; Kim et al., 2008, 2010; Lee et al., 1999; Mori et al., 2008; Peterson et al., 1999; Tadross et al., 2008; Van Petegem et al., 2005; Zühlke et al., 1999). We thank M. Grabe and L. Jan for comments on the manuscript. This work was supported by grant NIH-NHLBI R01-HL080050 to D.L.M. and a Marcel Bleus-tein-Blanchet Foundation fellowship to F.A.-A.

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## STAR METHODS

### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniel L. Minor, Jr. (daniel.minor@ucsf.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Oocytes collection
Oocytes were harvested from female *Xenopus laevis* frogs purchased from Nasco and housed in the UCSF Laboratory Animal Resource Center (LARC) facilities. The use of these *Xenopus* oocytes was approved by IACUC (protocol approval # AN178461-01) and experiments were performed in accordance with University of California guidelines and regulations.

Cell Culture
Human embryonic kidney cells (HEK293) were purchased from ATCC (CRL-1573) and were grown at 37°C under 5% CO₂, in a Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% L-glutamine, and antibiotics (100 IU mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin) (University of California, San Francisco Cell Culture Facility). The sex of cell line is not determined.

METHOD DETAILS

Molecular Biology
Human CaV1.2 (*α₁C77*, GenBank: Z34815), human CaV2.1 (*α₁A*, GenBank: NM_001127221.1), rat Cav1.3 (*α₁D*, GenBank: AF370009.1), rat CaV₂β₂α (GenBank: NM_053851), rabbit CaV₂β₃ (GenBank: NM_001101715.2), and rabbit CaV₂β₅-1 (GenBank: NM_001082276.1) were used for both patch clamp and two-electrode voltage clamp experiments. CaV1.2 mutations were introduced by two separate PCR reactions. First the region of interest was PCR amplified using pcDNA3.1 Cav1.2 as template. The PCR product was then subcloned into pcDNA3.1 by restriction-ligation. The new plasmid containing the region of interest was then used as template to introduce the desired mutation using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The region of interest containing the desired mutation was then subcloned back into the pcDNA3.1 Cav1.2 to form the mutant full-length channel using the following restriction sites: NheI-HpaI, HpaI-PpuMI, KpnI-AgeI, and AgeI-FseI, for DI, DII, DIII, and DIV mutants, respectively. CaV₂β₃ and CaV₁.3 mutants were made using the QuickChange Site-Directed Mutagenesis Kit (Agilent). All mutants were validated by complete sequencing of the genes encoding for the proteins of interest.

Two-electrode voltage clamp electrophysiology
Linearized cDNA was translated into capped mRNA using the T7 messenger kit (Ambion). We injected 50 nL of CaV₁.2*α₁*, CaV₂β₂α or CaV₂β₅ and CaV₂β₅-1 mRNA at a 1:1:1 molar ratio into *Xenopus* oocytes. Two-electrode voltage clamp experiments were performed 2–3 days post-injection.

Oocytes were injected with 50 nL of 100 mM BAPTA four minutes before recording, to minimize calcium-activated chloride currents as previously described (Findeisen and Minor, 2009). For recording of Ca²⁺ or Ba²⁺ currents, bath solutions contained 40 mM CaCl₂ or 40 mM BaCl₂, respectively, 50 mM NMDG-Cl, 1 mM KOH, 10 mM HEPES, adjusted to pH 7.4 with HNO₃. Measurements of Ca²⁺ block of Li⁺ currents followed previously described protocols (Shaya et al., 2014). The bath solution contained 100 mM LiOH, Ca(NO₃)₂ at test concentrations between 3 nM and 100 μM, and 10 mM HEPES, adjusted to pH 7.4 with HNO₃. Ca²⁺ concentrations were verified using a Ca²⁺ electrode. A solution having a nominal 3 nM free Ca²⁺ concentration was used as control condition and contained 170 μM Ca(NO₃)₂ and 15 mM ethylene glycol-bis(2-aminoethyl ether)-N, N’,N”-tetraacetic acid (EGTA). Electrodes were filled with 3 M KCl and had resistances of 0.3–1.0 MΩ. Recordings were conducted at room temperature from a holding potential of −90 mV. Leak currents were subtracted using a P/4 protocol.
Whole-cell Patch-clamp electrophysiology

HEK293 cells were transfected (in 35-mm diameter wells) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and plated onto coverslips coated with Matrigel (BD Biosciences, San Diego, CA, USA).

Cells were transfected using a total of 4.4 μg DNA having a ratio by weight of 2:1.6:0.4:0.4 of CaVα1:CaVβ2a:CaVα2d-1:sv40 T-antigen plasmids. The SV40 T-antigen plasmid was used to increase channel expression. Transfected cells were identified visually using an enhanced green fluorescent protein (EGFP) expression in the second cassette of the plasmid expressing the β2a subunit. Whole cell patch clamp (Hamill et al., 1981) was used to record Ca2+ and Ba2+ currents at room temperature (23 ± 2°C) 48–72 h post-transfection. Data acquisition was performed using pCLAMP 9 (Molecular Devices, Sunnyvale, CA, USA) and an Axopatch 200B amplifier (Molecular Devices). Pipettes were pulled from borosilicate glass capillaries (TW150F-3; World Precision instruments, Sarasota, FL, USA) and polished (MF-900 microforge; Narishige, Tokyo, Japan) to obtain 2–3 MΩ resistances. Sixty to eighty percent of the voltage error due to the series resistance was compensated, and leak currents were subtracted using a P/4 protocol. For CaV1.2 and CaV1.3 experiments, the pipette solution contained 120 mM NMDG-Cl, 1 mM MgCl2, 5 mM EGTA, 4 mM Mg-ATP, 42 mM HEPES (pH 7.3 adjusted with Methane sulfonic acid). Bath solution contained 40 mM CaCl2 or 40 mM BaCl2, 1 mM MgCl2, 105 mM Tris (pH 7.3 adjusted with Methane sulfonic acid). For CaV2.1 experiments, the pipette solution contained 120 mM NMDG-Cl, 1 mM MgCl2, 0.5 mM EGTA, 2 mM Mg-ATP, 60 mM HEPES (pH 7.3 adjusted with Methane sulfonic acid). Bath solution contained 10 mM CaCl2 or 10 mM BaCl2, 1 mM MgCl2, 150 mM Tris (pH 7.3 adjusted with Methane sulfonic acid).

Single-channel recordings

HEK cells were maintained and transfected as described above. Cell-attached configuration of the patch clamp technique (Hamill et al., 1981) was used to record Ba2+ currents from single or multiple CaV1.2 channels at room temperature (23 ± 2°C) 48–72 h post-transfection. Data acquisition was performed using pCLAMP 9 (Molecular Devices, Sunnyvale, CA, USA) and an Axopatch 200B amplifier (Molecular Devices). Pipettes were pulled from quartz glass capillaries (QF100-70-7.5; Sutter Instrument, Novato, CA, USA) using a Laser-Based Micropipette puller (P-2000, Sutter Instrument, Novato, CA, USA), and filled with 140 mM TEA-Cl, 40 mM BaCl2, 10 mM HEPES (pH 7.4 adjusted with TEA-OH). To zero membrane potential, the bath solution contained 132 mM K glutamate, 5 mM KCl, 5 mM NaCl, 3 mM MgCl2, 2 mM EGTA, 10 mM glucose, 20 mM HEPES (pH 7.4 adjusted with KOH) (Dick et al., 2016). Recordings were low pass filtered with a cutoff frequency of 2 kHz and digitized at 50 ms. Patches were stimulated by a voltage ramp from −80 mV to 50 mV over the duration of 200 ms. The leak for each trace was subtracted using a linear fit added to an exponential fit.

Data Analysis

All results are from at least two independent oocyte batches or at least two independent transfections. Data were analyzed with Clampfit 10.6 (Axon Instruments). Activation curves were obtained by fitting the data with the following Boltzmann equation: \( \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp \left( \frac{V_{0.5} - V_{m}}{k} \right)} \), where \( V_{0.5} \) is the half-activation potential, \( V_{m} \) is the membrane potential, and \( k \) is the slope factor. Dose–response curves were calculated as follows: \( \frac{I_x}{I_{3nM Ca^{2+}}} = \frac{1}{1 + \frac{x}{IC_{50}}} \), where \( I_x \) is the current amplitude at the Ca2+ concentration \( x \), \( I_{3nM Ca^{2+}} \) is the current amplitude at 3 nM and \( IC_{50} \) is the half-maximal inhibitory concentration. VDI time constant (\( \tau_{\text{TDI}} \)) was determined at a test potential of +20 mV using the formula \( I = A \exp \left( -t/\tau_{\text{TDI}} \right) + C \), where \( I \) is the recorded current, \( A \) is the peak current, \( C \) is the residual current at steady state, and \( t \) is the time. Current density was determined as the ratio between current amplitude (pA) and the membrane capacitance (pF).

QUANTIFICATION AND STATISTICAL ANALYSIS

All the details of data analysis and statistical analysis can be found in the Method Details and figure/table legends. All data values are presented as mean ± SEM ‘n’ represents the number of cells. Statistical significance of the observed effects was assessed by Student’s t test, using SigmaStat 3.1 software. \( p < 0.01 \) was considered significant, unless otherwise stated.
Supplemental Information

A Selectivity Filter Gate Controls
Voltage-Gated Calcium Channel
Calcium-Dependent Inactivation

Supplementary material for

A selectivity filter gate controls voltage-gated calcium channel calcium-dependent inactivation

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Figure S1 Exemplar Ca\textsuperscript{2+} currents for Ca\textsubscript{\text{V}1.2}, Ca\textsubscript{\text{V}1.3}, Ca\textsubscript{\text{V}2.1}, and mutants co-expressed with Ca\textsubscript{\text{V}2a}. Related to Figures 1, 2, and 4. Exemplar traces recorded in A, Xenopus oocytes expressing Ca\textsubscript{\text{V}1.2} or the indicated mutants, B, HEK 293 cells expressing Cav1.2 or the indicated mutants, C, HEK 293 cells expressing Ca\textsubscript{\text{V}1.3} or the indicated mutants, and D, HEK 293 cells expressing Ca\textsubscript{\text{V}2.1} or the indicated mutants. Currents were evoked using a multi-step activation protocol (insets).
Figure S2 SFII (+1) mutants do not dramatically reduce CaV selectivity properties. Related to Figures 1, 2, and 4. A, Exemplar recordings at +20 mV in Ca²⁺ (black) or Ba²⁺ (grey) from *Xenopus* oocytes expressing CaV1.2 or indicated mutants, B, from HEK293 cells expressing CaV1.2 or indicated mutants, C, from HEK293 cells expressing CaV1.3 or indicated mutants, and D, HEK293 cells expressing CaV2.1 or indicated mutants. Right panels represent averaged values of ratios between Ba²⁺ and Ca²⁺ peak current amplitude at +20 mV or CaV1.2 and CaV1.3 or at +10 mV for CaV2.1. ‘*’ indicates p<0.01, ‘**’ indicates p<0.001, and ‘N.S.’ indicates ‘not statistically significant’ compared to wild-type channels. 

n values for all bar graphs are in Table 1. All recordings were made with CaVβ2a.
Figure S3 Ca\textsubscript{v}1.2 selectivity filter mutations affect CDI independently of Ca\textsubscript{v}\textbeta. Related to Figure 1. 

A, Exemplar raw traces recorded at +20 mV in Ca\textsuperscript{2+} (black) or Ba\textsuperscript{2+} (grey) from Xenopus oocytes expressing Ca\textsubscript{v}1.2 or the indicated mutants with Ca\textsubscript{v}\textbeta.

B, Normalized traces from ‘A’. 

C, Ratio of normalized \(I_{Ca}/I_{Ba}\) currents (net CDI, (Barrett and Tsien, 2008; Findeisen and Minor, 2009)) showing average plots ± s.e.m.

D, net CDI 300-ms post-depolarization. ‘*’ indicates \(p<0.01\) and ‘**’ indicates \(p<0.001\) compared to Cav1.2. \(n = 5-10\).
Figure S4 DII (+1) aspartate mutations affecting CDI spare VDI. Related to Figure 1. A, Exemplar two electrode voltage clamp recordings at +20 mV from *Xenopus* oocytes expressing Ca\(\nu\)1.2 (black), Ca\(\nu\)1.2 D707A (grey), Cav1.2 D707E (light blue), or Cav1.2 D707N (orange) with Ca\(\nu\)\(\beta\)3. B, \(\tau\) inactivation \((\tau_{\text{inact}})\) for ‘A’. \(n=6-15\). C, Exemplar two electrode voltage clamp recordings at +20 mV from *Xenopus* oocytes expressing Ca\(\nu\)1.2 (black), Ca\(\nu\)1.2 D707A (grey), Cav1.2 D367A (green), Cav1.2 E1119A (blue), or Cav1.2 D1420A (pink) with Ca\(\nu\)\(\beta\)3. D, \(\tau\) inactivation \((\tau_{\text{inact}})\) for ‘C’. \(n=9-19\). ‘*’ indicates \(p<0.001\), and ‘N.S.’ indicates ‘not statistically significant’ compared to Ca\(\nu\)1.2. E, Voltage-dependent inactivation curves representing channel availability after a steady-state inactivation at the indicated potentials.
Figure S5 SF (+4) mutations affecting VDI spare CDI. Related to Figure 1. A, Exemplar traces recorded in *Xenopus* oocytes co-expressing Ca$_{v}$1.2, Ca$_{v}$1.2 E1119A, or Cav1.2 D1420A with Cav$\beta_{2a}$ in response to the indicated protocol. B, Exemplar normalized recordings at +20 mV in Ca$^{2+}$ (black) or Ba$^{2+}$ (grey) from *Xenopus* oocytes expressing Ca$_{v}$1.2, Ca$_{v}$1.2 E1119A, or Cav1.2 D1420A. C, Fractional current remaining 300 ms post-depolarization ($R_{300}$) as a function of the membrane potential for channels in ‘B’. D, Average fraction CDI (f) at +20 mV. ‘N.S.’ indicates ‘not statistically significant’. n values are found in Table 1. E, Voltage-dependent activation curves for Ca$_{v}$1.2 (black circles), Ca$_{v}$1.2 E1119A (blue triangles), and Cav1.2 D1420A (red inverted triangles). F, I-V relationships for the indicated channels. Symbols are the same as ‘E’.
Figure S6 Ca\textsubscript{v}1.2 CDI requires a negative charge at +1 position on DII, DIII, or DIV, not DI, regardless of the amount of Ca\textsuperscript{2+} influx. Related to Figures 3 and 5. A, Exemplar Ca\textsuperscript{2+} currents in response to a +20 mV depolarization from \textit{Xenopus} oocytes, co-expressing Ca\textsubscript{v}1.2 or the indicated mutants with Cav\beta\textsubscript{2a} in the absence (black) or presence of 5 \textmu M Bay K8644 (blue). Raw traces (upper panels) and normalized traces (lower panels) are shown to illustrate Bay K8644 effects on both the peak current and inactivation, respectively. B, Exemplar normalized Ba\textsuperscript{2+} currents from \textit{Xenopus} oocytes expressing Ca\textsubscript{v}1.2 or the indicated mutants, in response to a +20 mV depolarization in absence (black) or in presence of 5 \textmu M Bay K8644 (blue). C, Ratio of Ca\textsuperscript{2+} current amplitudes from \textit{Xenopus} oocytes expressing Ca\textsubscript{v}1.2 or the indicated mutants, in response to a +20 mV depolarization in absence (black) or in presence of 5 \textmu M Bay K8644 (blue). ‘N.S.’ indicates not statistically different. n = 4-7. D and E, Percentage of inactivation 300 ms post-depolarization (t\textsubscript{300}) in the absence (dark bars) and presence of Bay K8644 (light bars) on recordings performed using D, Ca\textsuperscript{2+}, or E, Ba\textsuperscript{2+} as the charge carrier. ‘*’ indicates p<0.01 and ‘**’ indicates p<0.001. n = 6-11. F, Difference in t\textsubscript{300} induced by Bay K8644 in Ca\textsuperscript{2+} (dark bars) and Ba\textsuperscript{2+} (light bars).