Molecular moieties masking Ca$^{2+}$-dependent facilitation of voltage-gated Ca$_{2.2}$ Ca$^{2+}$ channels

Jessica R. Thomas,1,2 Jussara Hagen,1 Daniel Soh,5 and Amy Lee1,3,4

1Department of Molecular Physiology and Biophysics, 2Interdisciplinary Graduate Program in Neuroscience, 3Department of Otolaryngology Head-Neck Surgery, and 4Department of Neurology, University of Iowa, Iowa City, IA
5Medical Sciences Program, Boston University, Boston, MA

Voltage-gated Ca$_{2.1}$ (P/Q-type) Ca$^{2+}$ channels undergo Ca$^{2+}$-dependent inactivation (CDI) and facilitation (CDF), both of which contribute to short-term synaptic plasticity. Both CDI and CDF are mediated by calmodulin (CaM) binding to sites in the C-terminal domain of the Ca$_{2.1}$ α subunit, most notably to a consensus CaM-binding IQ-like (IQ) domain. Closely related Ca$_{2.2}$ (N-type) channels display CDI but not CDF, despite overall conservation of the IQ and additional sites (pre-IQ, EF-hand–like [EF] domain, and CaM-binding domain) that regulate CDF of Ca$_{2.1}$. Here we investigate the molecular determinants that prevent Ca$_{2.2}$ channels from undergoing CDF. Although alternative splicing of C-terminal exons regulates CDF of Ca$_{2.1}$, the splicing of analogous exons in Ca$_{2.2}$ does not reveal CDF. Transfer of sequences encoding the Ca$_{2.1}$ EF, pre-IQ, and IQ together (EF-pre-IQ-IQ), but not individually, are sufficient to support CDF in chimeric Ca$_{2.2}$ channels; Ca$_{2.1}$ chimeras containing the corresponding domains of Ca$_{2.2}$, either alone or together, fail to undergo CDF. In contrast to the weak binding of CaM to just the pre-IQ and IQ of Ca$_{2.2}$, CaM binds to the EF-pre-IQ-IQ of Ca$_{2.2}$ as well as to the corresponding domains of Ca$_{2.1}$. Therefore, the lack of CDF in Ca$_{2.2}$ likely arises from an inability of its EF-pre-IQ-IQ to transduce the effects of CaM rather than weak binding to CaM per se. Our results reveal a functional divergence in the CDF regulatory domains of Ca$_{2.2}$ channels, which may help to diversify the modes by which Ca$_{2.1}$ and Ca$_{2.2}$ can modify synaptic transmission.

INTRODUCTION

Voltage-gated Ca$_{v}$ Ca$^{2+}$ channels are multi-subunit complexes that regulate a variety of biological activities such as gene expression, muscle contraction, and neurotransmitter release. Ca$_{v}$ channels consist of an α subunit, which forms the pore, and two auxiliary subunits, β and αδ (Simms and Zamponi, 2014). Of the multiple Ca$_{v}$ channels that have been characterized (Ca$_{1.x}$–Ca$_{3.3}$), Ca$_{2.1}$ (P/Q-type) and Ca$_{2.2}$ (N-type) channels play prominent presynaptic roles in regulating neurotransmitter release (Dunlap et al., 1995). Ca$_{2.1}$ Ca$^{2+}$ signals promote exocytosis at most synapses, including CA3-CA1 hippocampal synapses (Wheeler et al., 1994), the calyx of Held auditory brainstem synapse (Forsythe et al., 1998; Inchauspe et al., 2004), and the parallel fiber–Purkinje cell synapse in the cerebellum (Mintz et al., 1995). Although Ca$_{2.2}$ plays a secondary role to Ca$_{2.1}$ at many central synapses, Ca$_{2.2}$ is the major Ca$_{v}$ channel regulating neurotransmitter release from terminals of spinal nociceptive neurons (Hatakeyama et al., 2001) and superior cervical ganglion neurons (Boland et al., 1994). Genetic inactivation of Ca$_{2.2}$ in mice causes no overt phenotypes except for higher pain thresholds (Hatakeyama et al., 2001). In contrast, knockout of Ca$_{2.1}$ causes ataxia, seizures, and premature death (Jun et al., 1999).

Perhaps to support their distinct physiological roles, Ca$_{2.1}$ and Ca$_{2.2}$ channels are differentially modulated by a variety of factors, including the Ca$^{2+}$ ions that pass through the pore. Like other high voltage–activated Ca$_{v}$ channels (Liang et al., 2003), Ca$_{2.1}$ and Ca$_{2.2}$ undergo Ca$^{2+}$-dependent inactivation (CDI) mediated by calmodulin (CaM) binding to sites in the intracellular C-terminal domain (CTD) of the α subunit (Lee et al., 1999; DeMaria et al., 2001). These include a consensus IQ-like domain for binding CaM (IQ) as well as a CaM-binding domain (CBD; Fig. 1). During a train of depolarizations, the amplitude of Ca$_{2.1}$ Ca$^{2+}$ currents increases and then declines because of the onset of CDI. The initial increase is caused by Ca$^{2+}$-dependent facilitation (CDF), which also requires CaM (Lee et al., 1999; DeMaria et al., 2001) and potentially other Ca$^{2+}$ sensor proteins in neurons (Tsujimoto et al., 2002). CDF and CDI of Ca$_{2.1}$ currents contribute to the facilitation and depression, respectively, of synaptic transmission at the calyx of Held (Cuttle et al., 1998; Forsythe et al., 1998; Tsujimoto et al., 2002) and other brain synapses (reviewed in Catterall et al., 2013). Despite the physiological importance of CDF of Ca$_{2.1}$ in short-term synaptic plasticity (Nanou et al., 2016),
there is little evidence that Ca\textsubscript{v}2.2 channels are similarly regulated. In a heterologous expression system, CDF is not observed for Ca\textsubscript{v}2.2 under conditions that evoke robust CDF of Ca\textsubscript{v}2.1 (Liang et al., 2003). At the calyx of Held of mice lacking Ca\textsubscript{v}2.1, Cav2.2 channels compensate for the loss of Cav2.1, but the resulting Ca\textsuperscript{2+} currents do not facilitate or support short-term plasticity (Inchauspe et al., 2004). Although a form of CDF has been reported for Cav2.2 channels in dorsal root ganglion neurons, the mechanism relies on CaM-dependent protein kinase II and is distinct from CaM-dependent CDF of Cav2.1 channels (Tang et al., 2012).

What prevents Ca\textsubscript{v}2.2 from undergoing CDF is unknown but may involve unique sequence elements in the CTD of the \(\alpha\) subunit based on analyses of Ca\textsubscript{v}2.1 splice variants. Alternative splicing of exons in the proximal or distal CTD of the Ca\textsubscript{v}2.1 \(\alpha\) subunit (exons 37 and 47, respectively; Fig. 1) gives rise to channels with altered CDF (Chaudhuri et al., 2004). Notably, the corresponding exons of Ca\textsubscript{v}2.2 also undergo alternative splicing with effects on Ca\textsubscript{v}2.2 current density, modulation by G-proteins, and synaptic trafficking in neurons (Maximov and Bezprozvanny, 2002; Bell et al., 2004; Lipscombe et al., 2013). The potential of these alternatively spliced exons to regulate CDF of Ca\textsubscript{v}2.2 has not been investigated.

In this study, we tested whether sequences encoded by exons 37 and 46, as well as other regions of the CTD, underlie the absence of CDF in Cav2.2. We find that although splice variation of exons 37 and 46 was inconsequential, the transfer of the key CDF regulatory sites in Cav2.1 to Cav2.2 unmasked strong CDF in the chimeric channels. However, transfer of any of these sites alone was ineffective. Our results reveal an unexpected variance in the molecular determinants controlling CaM regulation of Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2, which may shape the
distinct coupling of these channels to vesicle release at the synapse.

MATERIALS AND METHODS

cDNAs and molecular biology

The following cDNAs were used: Cav2.1 (NM_001127221), Cav2.2 e37a (AF055477), Cav2.2 e37b (NM_147141), β2A (NM_053851), and α2δ-1 (NM_000722.3). The plasmid for β2A-CaM was a gift from I. Dick (University of Maryland, Baltimore, MD). Chimeras were constructed using NEBuilder HiFi DNA Assembly Cloning System (New England Biolabs) and Cav2.1 and Cav2.2 e37a as templates. The following constructs were generated by swapping the amino acids indicated in parentheses: Cav2.2-CT2.1, Cav2.1-CT2.2 (1,681–2,334 of Cav2.2, 1,786–2,261 of Cav2.1); Cav2.2-EF2.1, Cav2.1-EF2.2 (1,681–1,788 of Cav2.2, 1,786–1,892 of Cav2.1); Cav2.2-pre-IQ-IQ2.1, Cav2.1-pre-IQ-IQ2.2 (1,789–1,875 of Cav2.2, 1,893–1,985 of Cav2.1); and Cav2.2-CBD2.1, Cav2.1-CBD2.2 (1,912–1,990 of Cav2.2, 2,009–2,084 of Cav2.1). Additional chimeric channels containing subsets of the EF-hand, pre-IQ, IQ, and CBD were generated using the residues indicated above. For Cav2.2 Δe46, the sequence encoding exons 42–45 of Cav2.2 (1,927–2,162) followed by a stop codon was amplified by PCR and cloned into the corresponding site of Cav2.2 as an XbaI fragment. All chimeras and Cav2.2 Δe46 constructs were cloned into the pcDNA6V5His vector. For generating glutathione S-transferase (GST) fusion proteins, sequences corresponding to the aforementioned Cav2 domains were amplified by PCR and cloned into BamHI and XhoI sites of the pGEX-4T-1 vector.

Cell culture and transfection

Human embryonic kidney 293 cells transformed with the SV40 T-antigen (HEK 293T, CRL-3216, RRID:CVCL_0063; ATCC) were maintained in Dulbecco’s modified Eagle’s medium with 10% FBS at 37°C in a humidified atmosphere with 5% CO2. Cells were grown to 80% confluence and transfected using FuGene 6 (Promega) according to the manufacturer’s protocol. Cells were plated in 35-mm dishes and transfected with cDNAs encoding Cav channel subunits (for Cav2.1 and chimeras with Cav2.2 CTDs: 1.0 µg α1, 0.5 µg β2A, and 0.5 µg α2δ1; for Cav2.2 and chimeras with Cav2.1 CTDs: 1.8 µg α1, 0.6 µg β2A, and 0.6 µg α2δ1). Cotransfection with cDNA encoding enhanced green fluorescent protein (pEGFP, 50 ng) allowed visualization of transfected cells.

Electrophysiological recordings

Whole-cell patch recordings were performed 24–72 h after transfection with a EPC-8 patch clamp amplifier and PatchMaster software (HEKA Elektronik). External recoding solution contained (mM) 150 Tris, 1 MgCl2, and 5 CaCl2 or BaCl2. Intracellular solution contained (mM) 140 N-methyl-d-glucamine, 10 Hepes, 10 or 0.5 EGTA, 2 MgCl2, and 2 Mg-ATP. The pH of both solutions was adjusted to 7.3 using methanesulfonic acid. Electrode resistances were 4–6 MΩ in the bath solution. Series resistance was compensated 60–70%. Leak currents were subtracted using a P/−4 protocol. Data were analyzed using Igor Pro software (WaveMetrics). Averaged data represent mean ± SEM and results from at least three independent transfections.

Figure 2. The absence of CDF in Cav2.2 is not affected by alternative splicing of exon 37. (A–C) Left, representative ICa and IBa evoked before (P1, gray trace) and after (P2, red trace) a prepulse to 20 mV for Cav2.1 (A) and Cav2.2 variants with exon 37b (B) or exon 37a (C). Current traces were overlaid for comparison. Voltage protocol is shown above. P1 and P2 pulses were 10-ms steps from −80 mV to −5 mV (for ICa) or −10 mV (for IBa) 1 s before and 5 ms after, respectively, a 50-ms prepulse to various voltages. For P2 and P1, tail currents were resolved by repolarization to −60 mV for 2 ms before stepping to −80 mV. Right, the ratio of P2 and P1 tail currents is plotted against prepulse voltages for ICa and IBa. Numbers of cells for ICa and IBa are indicated in Table 1. Data represent mean ± SEM.
Pull-down binding assays
The cDNA encoding full-length rat CaM (rCaM1–148 [Pedigo and Shea, 1995], provided by M. Shea) was expressed in BL21 DE3 Escherichia coli bacteria and purified as described previously (Theoharis et al., 2008). Purified CaM (1–10 µg) was added to GST or GST-tagged Ca v2.1 or Ca v2.2 proteins (5 µg) immobilized on glutathione Sepharose beads (GE Healthcare Life Sciences). The reaction was brought to a total volume of 750 µl with binding buffer (20 mM Tris-HCl, pH 7.3, 2 mM CaCl2, ± 150 mM NaCl; results were similar with or without the added NaCl and so were combined). Binding reactions were incubated at 4°C, rotating for 1 h. The beads were washed three times with 1 ml ice-cold binding buffer, and bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose. To detect the GST-proteins, the nitrocellulose was first stained with Ponceau S. Bound CaM was then detected by Western blot with rabbit polyclonal antibodies against CaM (1:1,000, 301 003, RRID:AB_2620046; Synaptic Systems). Blots were processed with HRP-conjugated secondary antibodies (anti–rabbit IgG, 1:4,000, I5006, RRID: AB_1163659; Sigma-Aldrich) and reagents for enhanced chemiluminescent detection (Thermo Fisher Scientific) before autoradiography.

For quantitative analysis, densitometry was performed using a Canon LIDE 200 scanner and ImageJ (NIH) software. The Western blot signal for CaM was normalized to the signal corresponding to the Ponceau-stained GST fusion proteins. Results from at least three independent experiments were pooled for statistical analysis.

Data presentation and statistical analysis
Data were incorporated into figures using SigmaPlot (Systat Software) and Adobe Illustrator software. Statistical analysis was performed with SigmaPlot or GraphPad Prism software. The data were first analyzed for Ca2+-dependent facilitation of Ca v2.2 | Thomas et al.

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Table 1. F_CDF and P2/P1 for I_{Ca} and I_{Ba} from double-pulse protocol (20-mV prepulse)

| Construct     | P2/P1 for I_{Ca} | P2/P1 for I_{Ba} | P-value, I_{Ca} vs. I_{Ba} | F_CDF  | P-value vs. Cav2.1 | P-value vs. Cav2.2 |
|---------------|------------------|------------------|---------------------------|--------|-------------------|-------------------|
| Cav2.1        | 1.40 ± 0.07 (5)  | 1.11 ± 0.02 (7)  | 0.002                     | 0.28 ± 0.07 (5) | 0.015 | 1.000 |
| Cav2.2 e37b   | 1.28 ± 0.06 (10) | 1.35 ± 0.01 (5)  | 0.437                     | −0.07 ± 0.06 (10) | 0.032 | 0.019 |
| Cav2.2 e37a   | 1.39 ± 0.06 (8)  | 1.38 ± 0.04 (6)  | 0.232                     | 0.01 ± 0.06 (7)  | 0.032 | 0.019 |
| Cav2.2-CT12   | 1.65 ± 0.10 (13) | 1.32 ± 0.04 (11) | 0.099                     | 0.33 ± 0.10 (15) | 1.000 | 0.019 |
| Cav2.1-CT12   | 1.16 ± 0.10 (4)  | 1.16 ± 0.02 (5)  | 0.717                     | 0.00 ± 0.10 (4)  | 0.035 | 1.000 |

F_CDF and P2/P1 (mean ± SEM) were determined as indicated in the text. Number of cells in parentheses.

* Determined by Student’s t test.

† Determined by one-way ANOVA test and post-hoc Dunnett’s test.

Table 2. F_CDF calculated from F_{96–100} for I_{Ca} and I_{Ba} from 100-Hz protocol

| Construct     | F_{96–100} for I_{Ca} | F_{96–100} for I_{Ba} | P-value, I_{Ca} vs. I_{Ba} | F_CDF  | P-value vs. Cav2.1 | P-value vs. Cav2.2 |
|---------------|------------------------|------------------------|---------------------------|--------|-------------------|-------------------|
| Cav2.1        | 1.33 ± 0.03 (10)       | 1.06 ± 0.02 (12)       | <0.001                    | 0.27 ± 0.03 (10) | <0.001 | 0.960 |
| Cav2.2 e37b   | 0.91 ± 0.03 (10)       | 0.83 ± 0.02 (11)       | 0.067                     | 0.07 ± 0.05 (10) | <0.001 | 0.960 |
| Cav2.2 e37a   | 0.89 ± 0.02 (10)       | 0.88 ± 0.04 (10)       | 0.970                     | −0.01 ± 0.02 (10) | <0.001 | 0.960 |
| Cav2.2 e37a +46 (10 mM) | 0.95 ± 0.03 (12) | 0.83 ± 0.03 (10) | 0.005 | 0.12 ± 0.03 (12) | 0.002 | 0.926 |
| Cav2.2 e37a +46 (0.5 mM) | 0.82 ± 0.04 (10) | 0.82 ± 0.03 (10) | 0.968 | 0.00 ± 0.04 (10) | <0.001 | 1.000 |
| Cav2.2 e37b +46 (10 mM) | 0.94 ± 0.03 (8) | 0.94 ± 0.02 (4) | 0.985 | 0.00 ± 0.03 (8) | <0.001 | 1.000 |
| Cav2.2 e37b +46 (0.5 mM) | 0.86 ± 0.04 (5) | 0.88 ± 0.02 (5) | 0.646 | −0.02 ± 0.04 (5) | <0.001 | 0.985 |

F_CDF and F_{96–100} (mean ± SEM) were determined as indicated in the text. Number of cells in parentheses.

* Determined by Student’s t test.

† Determined by one-way ANOVA test and post-hoc Dunnett’s test.

Figure 3. Repetitive depolarizations cause CDF for Cav2.1 but not Cav2.2. (A–C) I_{Ca} or I_{Ba} were evoked by 2-ms steps from −80 mV to 0 mV for I_{Ca} or −10 mV for I_{Ba} at 100 Hz in cells transfected with Cav2.1 (A) or Cav2.2 containing exon 37b (B) or exon 37a (C). The amplitude of each current was normalized to the first current of the train and plotted against pulse number. For clarity, every fifth point is plotted. Numbers of cells for I_{Ca} and I_{Ba} are indicated in Table 2. Data represent mean ± SEM.
normality using the Shapiro–Wilk test. For parametric
data, significant differences were determined by Student’s t test or ANOVA with post hoc Dunnett or Tukey
test. For nonparametric data, Kruskal–Wallis and post
hoc Dunn’s tests were used.

Online supplemental material
Effects of varying EGTA concentration in the intracellular
recording solution are presented in Fig. S1. Fig. S2
shows that enrichment of local CaM does not produce
CDF of Cav2.1-EF-pre-IQ-IQ2.2 or Cav2.2 e37a.

RESULTS

Effects of alternative splicing on CDF of Ca,2.2
In both Ca,2.1 and Ca,2.2, exon 37 encodes a portion
of an EF-hand-like (EF) domain similar to those found
in a variety of Ca\(^{2+}\) binding proteins (Kawasaki and
Kretsinger, 1995). Conserved in the proximal CTD of
all Ca,1 and Ca,2 channels, the EF domain has been im-
plicated in the regulation of CDI and Mg\(^{2+}\)-dependent
inhibition of Cav1,2 channels (Peterson et al., 2000; Kim
et al., 2004; Brunet et al., 2005). Alternative splicing of
exon 37 gives rise to two Ca,2.1 variants with distinct

Figure 4. Deletion of exon 46 does not influence the ab-



Figure 5. The CTDs of Ca,2.1 and Ca,2.2 distinguish their
abilities to undergo CDF. (A–E) As in Fig. 2 (double-pulse
protocol) and Fig. 3 (100-Hz protocol) except cells transfected
with Cav2.2 channels with the CTD of Ca,2.1 (A–C) or Ca,2.1
channels with the CTD of Ca,2.2 (D and E). In C, interpulse
voltage was −140 mV. Gray line representing strong CDF of
Ca,2.1 ICa (from Fig. 3 A) is overlaid for comparison. Data rep-
resent mean ± SEM.
We tested this possibility in whole-cell patch clamp recordings of transfected HEK 293T cells. To analyze CDF, we used a classic voltage protocol in which the amplitudes of currents evoked before (P1) and after (P2) a conditioning prepulse are compared (Thomas and Lee, 2016). The extracellular solution contained either Ca2+ or Ba2+, and the intracellular recording solution contained a high concentration of EGTA (10 mM), which blocks CDI while sparing CDF of Cav2 channels (Lee et al., 2000; Liang et al., 2003). With this protocol, Cav2.1 (containing exon 37a) exhibited the hallmarks of CDF: the ratio of P2 to P1 was greater for ICa than for IBa (Fig. 2 A). Consistent with a role for Ca2+ influx during the prepulse in promoting CDF (Lee et al., 2000), the difference between P2/P1 for ICa and IBa was greatest at prepulse voltages evoking the peak inward ICa (20 mV) and was used as a metric for CDF (FCDF). Similar to previous findings (Liang et al., 2003), Cav2.2 e37b did not undergo CDF, in that P2/P1 was similar for ICa and IBa across all prepulse voltages (Fig. 2 B) and FCDF was nominal (Table 1). The P2/P1 ratio for both ICa and IBa increased monotonically with prepulse voltage (Fig. 2 B), likely because of voltage-dependent removal of basal G-protein inhibition (Li et al., 2004). FCDF was not significantly different for Cav2.2 containing exons 37a or 37b (Fig. 2 C; and Table 1), which argued against this exon being permissive for CDF.

To determine whether CDF of the Cav2.2 splice variants might be revealed with more physiological stimuli, we analyzed ICa and IBa evoked by trains of depolarizations at 100 Hz. The amplitude of each current was normalized to that of the first pulse (Fractional I) and plotted against pulse number. As shown previously (Lee et al., 2000), ICa mediated by Cav2.1 undergoes a robust and sustained increase, whereas IBa undergoes relatively modest voltage-dependent facilitation during the train (Fig. 3 A). The mean of the last five pulses (F96–100) was significantly greater for ICa than for IBa (∼25%; Table 2), indicative of CDF. In contrast, there was no difference in F96–100 for ICa and IBa mediated by Cav2.2 e37a or e37b (Fig. 3, B and C; and Table 2). These results confirm that inclusion of exon 37a is insufficient to confer Cav2.2 channels with an ability to undergo CDF, in contrast to the role of the analogous exon in Cav2.1 (Chaudhuri et al., 2004).

For Cav2.1 channels, the insensitivity of CDF to high intracellular Ca2+ buffering arises from its dependence on local Ca2+ signals detected by the C-terminal lobe of CaM (Chaudhuri et al., 2007). In the context of exon 37b, deletion of exon 47 from Cav2.1 (Ca2.1 Δe47) converts CDF to a reliance on global elevations in Ca2+, which are sensed by the N-terminal lobe of CaM and can be blunted by a high intracellular concentration of Ca2+ chelator (Chaudhuri et al., 2004). Therefore, we tested whether deletion of the analogous exon 46 of Cav2.2 (Δex46) might reveal CDF under conditions of limited Ca2+ buffering (0.5 mM EGTA). With this approach, there was no significant difference in ICa and IBa evoked by the 100-Hz protocol in cells transfected with Cav2.2 e37b Δex46 with either 10 or 0.5 mM EGTA (Fig. 4, A and B; and Table 2). With 10 mM EGTA, deletion of exon 46 from Cav2.2e37a led to a small increase in the F96–100 for ICa at the end of the train compared with IBa, but FCDF was nominal and significantly weaker than...
that for Cav2.1 (Fig. 4, C and D; and Table 2). Our strategy of manipulating the Ca²⁺-dependent effects of CaM was effective in that strong inactivation of I_Ca caused by CDI with 0.5 mM EGTA was significantly reduced with 10 mM EGTA in the intracellular recording solution (Fig. S1). Collectively, our results show that alternative splicing of exons in the proximal and distal CTD do not account for the lack of CDF of Cav2.2.

Role of CaM-regulatory regions in CDF of Cav2 channels

Mutations of the IQ-like domain that inhibit CaM binding abolish CDF (DeMaria et al., 2001; Lee et al., 2003), whereas deletion of the CBD diminishes CDF and CDI (Lee et al., 1999, 2000, 2003). Although its role in CDF of Cav2.1 is not established, the pre-IQ domain upstream of the IQ domain also interacts with CaM and regulates CDI and CDF of Ca,1.2 channels (Pitt et al., 2001; Kim et al., 2004, 2010). Each of these domains is conserved in Cav2.1 and Cav2.2 (Fig. 1), but key differences in their amino acid sequences may allow CDF of Cav2.1 but not Cav2.2. If so, then CDF should be conferred to Cav2.2 upon transfer of the corresponding domains from Cav2.1. Consistent with this prediction, chimeric Cav2.2 channels containing the CTD of Cav2.1 (Cav2.2-CT2.1; Cav2.2a variant was used for all Cav2.2 chimeras) exhibited robust CDF with the double-pulse protocol, with F_CDF not significantly different from that of Cav2.1 channels (Fig. 5 A and Table 1).

With the 100-Hz protocol, F_96–100 for Cav2.2-CT2.1 I_Ca was not as great as that for Cav2.1 (Fig. 5 B and Tables 2 and 3) perhaps because of closed-state inactivation, which is prominent for Cav2.2 during repetitive depolarizations and relieved by hyperpolarized interpulse voltages (Patil et al., 1998). Cav2.2 inactivation (I_Ca and I_Ba) was stronger than that for Cav2.1 during 100-Hz trains (Fig. 3) and could partially occlude facilitation of Cav2.2-CT2.1 I_Ca. Changing the interpulse voltage from −80 to −140 mV increased F_96–100 for I_Ca (1.19 ± 0.03 for −140 mV, n = 10, vs. 1.09 ± 0.03 for −80 mV, n = 15, P = 0.013 by t test) to a similar extent as for I_Ba (F_96–100 = 0.99 ± 0.02 for −140 mV, n = 10, vs. 0.89 ± 0.03 for −80 mV, n = 15, P = 0.014 by t test; Fig. 5 C). Thus, although closed-state inactivation does indeed underlie the smaller F_96–100 for Cav2.2-CT2.1 I_Ca compared with Cav2.1 I_Ca, it does not affect the magnitude of CDF. In fact, F_CDF of Cav2.2-CT2.1 was not significantly different from that for Cav2.1 (Table 3). Collectively, our results indicate that molecular determinants within the CTD of Cav2.1 are sufficient to enable Cav2.2-CT2.1 to undergo CDF.

We next tested the converse prediction that transfer of the Cav2.2 CTD to Cav2.1 should blunt CDF. In contrast to the wild-type Cav2.1, I_Ca and I_Ba behaved similarly in double-pulse and 100-Hz protocols in cells transfected with the chimeric Cav2.1-CT2.1 channels (Fig. 5, D and E; and Tables 1 and 3). To further refine the molecular determinants in the CTD responsible for “turning off” Cav2.2 CDF, we analyzed additional chimeric channels. For these studies, data are shown only for the 100-Hz protocol because similar results were obtained with double-pulse protocols. If the CDF-regulatory domains of Cav2.1 and Cav2.2 distinguish their abilities to undergo CDF, Cav2.2 channels containing the proximal CTD (Cav2.2-pCT2.1) but not the distal CTD (Cav2.2-dCT2.1) should exhibit CDF. As expected, Cav2.2-pCT2.1 underwent CDF (Fig. 6 A and Table 3). In contrast, Cav2.2-dCT2.1 was similar to wild-type Cav2.2 in that there was no difference in F_96–100 for I_Ca and I_Ba (Fig. 6 B and Table 3). Consistent with these findings, transfer of the proximal CTD but not the distal CTD...
of CaV2.2 to CaV2.1 resulted in chimeric channels that did not undergo CDF (Fig. 6, C and D; and Table 3). Therefore, the proximal CTD contains the sequence elements that distinguish the ability of CaV2.1 and CaV2.2 to undergo CDF.

We next determined the relative contributions of the EF, pre-IQ, IQ, and CBDs in disabling CDF in CaV2.2 channels. In these experiments, the pre-IQ and IQ sequences were transferred together because they work in concert to transduce effects of CaM in CaV1.2 (Pitt et al., 2001; Kim et al., 2004). None of the CaV2.2 chimeras containing these domains from CaV2.1 exhibited CDF (Fig. 7, A–C; and Table 3), indicating that the individual CDF-regulatory sites in CaV2.1 are dysfunctional within the context of the CaV2.2 proximal CTD. At the same time, substitution of the pre-IQ-IQ or EF-hand domain, but not the CBD, of CaV2.2 into CaV2.1 abolished CDF normally observed for the wild-type CaV2.1 (Fig. 7, D–F; and Table 3). Collectively, these results suggested that functional differences primarily in the EF-hand and pre-IQ-IQ domain of CaV2.2 prevent CDF (Fig. 8, A–C; and Table 3). Conversely, CDF was abolished in CaV2.1 channels containing the CaV2.2 EF-pre-IQ-IQ domain (Fig. 8 D and Table 3).

The inability of EF-pre-IQ-IQ to support CDF in CaV2.2 could be caused by weaker interactions with CaM compared with this region in CaV2.1. Indeed, past work suggests that CaM binds with lower affinity to the pre-IQ and IQ regions of CaV2.2 than of CaV2.1 (Peterson et al., 1999; Liang et al., 2003). To test whether this is the case in the context of EF-pre-IQ-IQ, we compared binding to GST-tagged CaV2.1 or CaV2.2 fusion proteins in pull-down assays. Consistent with previous results (Liang et al., 2003), CaM binding was significantly stronger to the pre-IQ-IQ of CaV2.1 than to this region of CaV2.2 or the GST control (Fig. 9, A and B). Remarkably, addition of the EF-hand to the pre-IQ-IQ of CaV2.2 greatly enhanced the interaction with CaM such that there was no significant difference in CaM binding to the EF-pre-IQ-IQ domain of CaV2.2 and CaV2.1 (Fig. 9, A and B). In contrast, CaM bound equally well to the pre-IQ-IQ and EF-pre-IQ-IQ domains of CaV2.1 (Fig. 9, A and B). The impact of the EF-hand on CaM binding to the CaV2.2 pre-IQ-IQ was particularly apparent with increasing amounts of CaM added to the binding reactions. For all concentrations of CaM tested, the amount of CaM
bound to the Ca\textsubscript{v}\textsubscript{2.2} pre-IQ-IQ was only \sim 20\% of that to the Ca\textsubscript{v}\textsubscript{2.1} pre-IQ-IQ (Fig. 9, C and D), whereas there was no difference in CaM binding to the EF-pre-IQ-IQ of the two channels (Fig. 9, E and F).

The similar CaM binding abilities of the EF-hand and pre-IQ-IQ domains of Ca\textsubscript{v}\textsubscript{2.1} suggested that the lack of CDF in Ca\textsubscript{v}\textsubscript{2.2} (Chaudhuri et al., 2004) did not result from reduced affinity for CaM. If so, then increasing the concentration of CaM to overcome any such differences in CaM binding affinity between Ca\textsubscript{v}\textsubscript{2.1} and Ca\textsubscript{v}\textsubscript{2.2} should not uncover CDF. To test this prediction, we used a strategy to enrich the local concentration of CaM near Ca\textsubscript{v} channels in which CaM is tethered to the auxiliary Ca\textsubscript{v}\textsubscript{\beta\textsubscript{2a}} subunit (\beta\textsubscript{2a}-CaM; Sang et al., 2016). Using \beta\textsubscript{2a} as a control, we analyzed the effects of \beta\textsubscript{2a}-CaM on the amplitude of I\textsubscript{Ca} evoked by 100-Hz stimuli in cells cotransfected with Ca\textsubscript{v}\textsubscript{2.2} or Ca\textsubscript{v}\textsubscript{2.1} chimeras containing the EF-pre-IQ-IQ domain of Ca\textsubscript{v}\textsubscript{2.2} (Ca\textsubscript{v}\textsubscript{2.1}-EF-pre-IQ-IQ\textsubscript{2.2}). Coexpression of \beta\textsubscript{2a}-CaM (verified by Western blots) had no effect on I\textsubscript{Ca}; CDF was not rescued in Ca\textsubscript{v}\textsubscript{2.1}-EF-pre-IQ-IQ\textsubscript{2.2}, nor was it uncovered in Ca\textsubscript{v}\textsubscript{2.2} (Fig. S2). We conclude that the lack of CDF shown by Ca\textsubscript{v}\textsubscript{2.2} channels does not arise from weaker binding of CaM, but likely through an inability of the EF-pre-IQ-IQ domain to convert CaM binding into channel conformations that support CDF.

**DISCUSSION**

In this study, we uncovered new insights into the molecular determinants regulating CDF of Ca\textsubscript{v} channels. First, we discounted a role for alternatively spliced C-terminal exons 37 and 46. Inclusion of exon 37a, which is permissive for CDF in Ca\textsubscript{v}\textsubscript{2.1} (Chaudhuri et al., 2004), did not reveal CDF in Ca\textsubscript{v}\textsubscript{2.2} (Figs. 2 and 3), nor did deletion of exon 46 (Fig. 4), which influences the Ca\textsuperscript{2+} dependence of Ca\textsubscript{v}\textsubscript{2.1} CDF (Chaudhuri et al., 2004). Second, we identified the EF-hand and pre-IQ-IQ domains as the critical determinants distinguishing the abilities of Ca\textsubscript{v}\textsubscript{2.1} and Ca\textsubscript{v}\textsubscript{2.2} to undergo CDF. These domains in the proximal CTD of Ca\textsubscript{v}\textsubscript{2.2} functionally diverge from those in Ca\textsubscript{v}\textsubscript{2.1} because their transfer to Ca\textsubscript{v}\textsubscript{2.1} prevented CDF (Fig. 6 C). Third, we discovered an unexpected role for the EF-hand domain in strengthening the ability of the pre-IQ-IQ of Ca\textsubscript{v}\textsubscript{2.2} to bind CaM. Our results support a model in which CaM binds to the EF-pre-IQ-IQ of Ca\textsubscript{v}\textsubscript{2.2} in a way that is functionally uncoupled from CDF.

The importance of the IQ domain for CDF is demonstrated by findings that mutation of the initial isoleucine and glutamine in the Ca\textsubscript{v}\textsubscript{2.1} IQ domain diminishes CaM binding and blunts CDF (DeMaria et al., 2001; Lee et al., 2003). Although the IQ domain is highly conserved in Ca\textsubscript{v} and Ca\textsubscript{v}\textsubscript{2} channels, sequence alterations between Ca\textsubscript{v} subtypes could underlie functional differences in channel regulation by CaM. By x-ray crystallography, Kim et al. (2008) found subtle differences in how CaM interacts with peptides corresponding to the IQ domain of Ca\textsubscript{v}\textsubscript{2.1} and Ca\textsubscript{v}\textsubscript{2.2}. These differences include less contact with the methionine at position −1 and greater interaction with phenylalanine at position 1 relative to the central isoleucine (position 0; Kim et al., 2008). These alterations may account for weaker CaM binding to the IQ and pre-IQ-IQ of Ca\textsubscript{v}\textsubscript{2.2} compared with Ca\textsubscript{v}\textsubscript{2.1} (Fig. 9; DeMaria et al., 2001; Liang et al., 2003). However, they are not sufficient to explain the absence of CDF in Ca\textsubscript{v}\textsubscript{2.2} channels because transfer of the Ca\textsubscript{v}\textsubscript{2.1} pre-IQ-IQ region alone to Ca\textsubscript{v}\textsubscript{2.2} did not reverse the inability of Ca\textsubscript{v}\textsubscript{2.2} to undergo CDF (Fig. 7 B). Moreover, Ca\textsubscript{v}\textsubscript{2.3} does not undergo CDF, and yet the crystal structures of CaM bound to the Ca\textsubscript{v}\textsubscript{2.1} and Ca\textsubscript{v}\textsubscript{2.3} IQ domains are nearly identical (Kim et al., 2008; Mori et al., 2008).
In this context, the crystal structure presented by Kim et al. (2010) of CaM in complex with the Ca_{1.2} pre-IQ-IQ may be informative. The structure indicates a 2:1 stoichiometry with one Ca^{2+}/CaM bound to the IQ domain and a second to a lower-affinity site in the pre-IQ region. A key tryptophan residue in the Ca_{1.2} pre-IQ region was identified as an anchoring site for the C-terminal lobe of CaM, and the mutation of this residue disrupted CDF when the initial isoleucine in the IQ-domain was also mutated so as to disrupt CDI (Kim et al., 2010). This tryptophan is conserved among all Cav1 and Cav2 channels and therefore may serve as an analogous region for binding Ca^{2+}/CaM in Cav2 channels. Differences between the pre-IQ region of Ca_{2.1} and Ca_{2.2} include residues at positions -3, -4, and -12 from this tryptophan, which are all methionines in Ca_{2.1}. Such differences could prevent the ability of CaM bound to the pre-IQ to produce CDF in Ca_{2.2}, which would explain the absence of CDF in any of the Ca_{2.1} chimeras containing the Ca_{2.2} pre-IQ-IQ (Fig. 5, D and E; Fig. 6 C; and Fig. 7 E).

Considering the weak binding of CaM to the pre-IQ-IQ of Ca_{2.2} (Fig. 9; DeMaria et al., 2001; Liang et al., 2003), the equivalence of CaM binding of the EF-pre-IQ-IQ of Ca_{2.1} and Ca_{2.2} (Fig. 9) suggests that the EF-hand domain differentially regulates interactions with CaM in the two channels. This is surprising given the strong sequence conservation in the EF-hand domains of Ca_{2.1} and Ca_{2.2} (Fig. 1). The divergent residues in the Ca_{2.2} EF-hand may be significant enough to facilitate interaction of CaM with the pre-IQ-IQ in ways that are unnecessary for Ca_{2.1}. The Ca_{2.2} EF-hand might reposition CaM bound to the pre-IQ-IQ so as to prevent CDF, which could explain the absence of CDF in the Ca_{2.1} chimera containing the Ca_{2.2} EF-hand (Fig. 7 D). Alternatively, interactions of the EF-pre-IQ-IQ with other parts of the channel such as the cytoplasmic loops linking domains I and II (Kim et al., 2004) and III and IV (Wu et al., 2016) may be unfavorable for entry of Ca_{2.2} into the facilitated state that is normally triggered by Ca^{2+}/CaM in Ca_{2.1}.

Although it binds CaM and regulates CDI of Ca_{2.1} (Lee et al., 1999, 2000), the CBD plays a more modulatory role and works with the IQ domain to promote CDF (Lee et al., 2003). This is supported by our findings that Ca_{2.2} channels containing only the Ca_{2.1}
CBD were unable to undergo CDF (Fig. 7 C). Only when cotransfected with the Ca2.1 EF-hand and pre-IQ-IQ domain was the Ca2.1 CBD effective in producing CDF in Ca2.2 (Figs. 5 A and 6 D). The CBD may be functionally redundant in Ca2.1 and Ca2.2, because CDF in Ca2.1 channels containing the Ca2.2 CBD was comparable to that in WT Ca2.1 channels (Fig. 7 F). Considering that CDF was slightly weaker in Ca2.2- pCT2.1 than Ca2.2-CT2.1 (Table 3), it may be that the CBD requires the distal CTD of Ca2.1 to fully promote CDF. An understanding of how the EF-hand, pre-IQ-IQ, and CBD domains coordinate to regulate CDF is an important challenge for future studies.

The neurophysiological importance of disabling CDF in Ca2.2 channels is not entirely clear but may relate to the major roles of these channels in the peripheral nervous system (Hirning et al., 1988). Localized in the presynaptic terminals of small-diameter nociceptive neurons, Ca2.2 channels mediate the release of neuro-peptides into the superficial layers of the spinal dorsal horn in response to painful stimuli (Holz et al., 1988; Maggi et al., 1990). Because the amount of neurotransmitter released is proportional to the third or fourth power of the presynaptic Ca2+ concentration (Dodge and Rahaminoff, 1967; Sakaba and Neher, 2001), the inability of Ca2.2 to undergo Ca2+/CaM-dependent CDF may have evolved to limit additive effects with other forms of Ca2.2 modulation that could collectively exacerbate transmission of painful stimuli. For example, Ca2.2 channel spinal nociceptive neurons undergo a CaMKII-dependent longer-term CDF that is eliminated with peripheral nerve injury (Tang et al., 2012). In sympathetic neurons, Ca2.2 channels are inhibited by a wide range of hormones and neurotransmitters acting via G protein-coupled receptors (Hille, 1994). If present in Ca2.2, CDF would oppose this inhibition, leading to improper neurohumoral control of sympathetic outflow.

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