Calmodulin (CaM) regulation of voltage-gated calcium (CaV,1-2) channels is a powerful Ca^{2+}-feedback mechanism to adjust channel activity in response to Ca^{2+} influx. Despite progress in resolving mechanisms of CaM-CaV feedback, the stoichiometry of CaM interaction with CaV channels remains ambiguous. Functional studies that tethered CaM to CaV,1.2 suggested that a single CaM sufficed for Ca^{2+} feedback, yet biochemical, FRET, and structural studies showed that multiple CaM molecules interact with distinct interfaces within channel cytosolic segments, suggesting that functional Ca^{2+} regulation may be more nuanced. Resolving this ambiguity is critical as CaM is enriched in subcellular domains where CaV channels reside, such as the cardiac dyad. We here localized multiple CaMs to the CaV nanodomain by tethering either WT or mutant CaM that lack Ca^{2+}-binding capacity to the pore-forming α-subunit of CaV,1.2, CaV,1.3, and CaV,2.1 and/or the auxiliary β_{2A} subunit. We observed that a single CaM tethered to either the α or β_{2A} subunit tunes Ca^{2+} regulation of CaV,1 or CaV,2 channels. However, when multiple CaMs are localized concurrently, CaV channels preferentially respond to signaling from the α-subunit–tethered CaM. Mechanistically, the introduction of a second IQ domain to the CaV,1.3 carboxyl tail switched the apparent functional stoichiometry, permitting two CaMs to mediate functional regulation. In all, Ca^{2+} feedback of CaV channels depends exquisitely on a single CaM preassociated with the α-subunit carboxyl tail. Additional CaMs that colocalize with the channel complex are unable to trigger Ca^{2+}-dependent feedback of channel gating but may support alternate regulatory functions.

Calmodulin (CaM) regulation of high-voltage activated calcium (CaV,1-2) is a dynamic feedback modulation that sculpts calcium entry into neurons and cardiac myocytes (1–4). This regulatory process is mechanistically rich, affording insights into powerful Ca^{2+}-decoding schemes (1, 3), and biologically consequential in determining the cardiac action potential (5, 6) and in furnishing stable Ca^{2+} influx at presynaptic terminals (7). Indeed, alterations in CaM regulation of CaV channels have emerged as an important contributor to cardiac arrhythmogenic disorders (6), including Timothy syndrome (8) and calmodulinopathies (9–11), and for neuropsychiatric disorders (12, 13). Therefore, elucidating underlying molecular mechanisms is critical for in-depth physiological understanding and to delineate pathophysiological alterations. More broadly, CaM is increasingly recognized as a pervasive regulatory partner (14) for multiple ion channel families, including NaV channels (15, 16), KV channels (17–21), SK channels (22), ryanodine receptors (23, 24), and transient receptor potential (25, 26) channels, hinting that insights from CaV modulation may shed light on common regulatory mechanisms.

Indeed, progress over the past three decades has revealed core mechanistic details regarding CaV calmodulation (2–4). First, CaM tunes multiple aspects of CaV,1 and CaV,2 function, including channel gating (27–29), surface-membrane trafficking (30), and transduction of local Ca^{2+} fluctuations to downstream signaling pathways (31–34). Second, to elicit gating changes, Ca^{2+}-free CaM pore-forming α1 subunit with the pore-forming channel subunit (35–38). This interaction itself up-regulates channel openings (39). Following channel activation, permeant Ca^{2+} ions bind CaM, and ensuing conformational rearrangements (40, 41) trigger Ca^{2+}-dependent feedback modulation. For many CaV,1 and CaV,2 channels, this conformational change diminishes channel activity, a process termed Ca^{2+}-dependent inactivation (CDI) (27–29, 37, 42, 43). For CaV,2.1, however, this modulatory process can also enhance channel activity, a positive feedback known as Ca^{2+}-dependent facilitation (CDF) (42–44). Third, multiple channel domains that interact with CaM have been identified. Specifically, Ca^{2+}-free CaM preassociates with a canonical CaM-binding IQ motif localized to the channel carboxyl tail (CT) and the closely juxtaposed EF-hand segment (35, 41, 45). Ca^{2+}/CaM, on the other hand, interacts with multiple domains, including the IQ domain (27, 35, 43, 46–49) and the pre-IQ segment (50–53) of CaV,1-2 family CT, the I-II intracellular loop of CaV,1.2 (53, 54), the N-terminal spatial Ca^{2+}-transforming element (NSCaTE) on the CaV,1.2 and CaV,1.3 N termini (55–57), and a CaM-binding domain (CBD) distal to the CaV,2.1 IQ domain (49). Fourth, CaM signaling to the CaV channel is ultimately conveyed to a selectivity filter gate (58).

Despite these advances, one uncertainty pertains to the stoichiometry of CaM interaction with the CaV complex. Early functional studies that tethered CaM onto the CT of the pore-forming α1 subunit suggested that a single CaM is both necessary and sufficient for CaV,1 regulation (59). However, biochemical and structural analysis point to the binding of multiple CaM molecules within the CaV complex. Briefly, atomic structures of the CaV,1.2 and CaV,2.1 CT show Ca^{2+}/CaM interaction with the IQ domain (47, 60, 61), as well as two pre-IQ domains cross-bridged by two additional Ca^{2+}/CaM molecules (46, 61).
For CaV1.2, NMR structures show the binding conformation of Ca\(^{2+}\)/CaM to the CaV1.2 NSCaTE domain (56). It remains unknown whether a single CaM molecule switches between conformations (62) or whether multiple CaMs molecules engage distinct sites (63) to orchestrate channel regulation. This mechanistic ambiguity is biologically important as CaM is enriched in subcellular regions, such as the cardiac dyad where CaV1 channels also reside (64). In vitro analysis suggests that Ca\(^{2+}\)/CaM is not capable of bridging the aforementioned channel domains (63, 65). Furthermore, previous FRET-based analysis of CaM stoichiometry showed that whereas a single apo-CaM preassociates with the holo-CaV1.2 channel, in the presence of Ca\(^{2+}\), up to two CaMs can bind to the holo-channel complex (66). Given this ambiguity, we here sought to dissect the potential role of multiple CaMs in eliciting Ca\(^{2+}\)-dependent modulation of CaV channel gating by tethering mutant or WT CaM to distinct locations within the channel complex. We found that CaM linked to the channel CT is privileged in eliciting Ca\(^{2+}\) regulation of CaV channels. Furthermore, when additional Ca\(^{2+}\)/CaMs are present in the channel complex, signaling by these molecules is rejected by the channel pore domain with regard to dynamic Ca\(^{2+}\)-feedback modulation.

**Results**

**Strategy for probing effects of multiple CaM in tuning CaV1 CDI**

To dissect the potential functional contribution of multiple CaM in evoking CDI of CaV1.2, we localized either WT (CaM\(_{WT}\)) or mutant CaM that lacks Ca\(^{2+}\) binding (CaM\(_{1234}\)) with known stoichiometries through genetic fusion to either the pore-forming \(\alpha_{1C}\) subunit\(^a\) or the auxiliary \(\beta_{2A}\) subunit (59, 67) (Fig. 1A). Here, CaM\(_{1234}\) mutant is generated by alanine substitution of key Ca\(^{2+}\)-coordinating aspartate residues in all four EF-hand domains of CaM. This overall strategy allows us to localize either one or two CaM molecules to the channel complex and assess changes in CDI. Fig. 1B shows baseline extent of CDI for full-length CaV1.2 in the absence of CaM fusion to either the \(\alpha_{1C}\) or \(\beta_{2A}\) subunits. In response to a step-voltage depolarization to +10 mV, Ca\(^{2+}\) current decay (red) is accelerated compared with Ba\(^{2+}\) current (black). Population data shows the fraction of peak current remaining after a 300-ms depolarization (\(r_{300}\)) with either Ca\(^{2+}\)/(red) or Ba\(^{2+}\)/(black) as the charge carrier. As Ba\(^{2+}\) binds poorly to CaM (68), the Ba\(^{2+}\) relation provides a baseline measure of voltage-dependent inactivation (VDI). The magnitude of CDI is quantified as the fractional difference between \(r_{300}\) relations obtained with Ca\(^{2+}\) and Ba\(^{2+}\) as permeant ions (i.e. CDI\(_{300} = 1 - \frac{r_{300, \text{Ca}}}{r_{300, \text{Ba}}}\)) (Table 1). Further representative currents and current-voltage relationships are provided in Fig. S1A. Previous studies have shown that channels does not alter CDI (\(r_{300,\alpha_{1C}}\Delta1671 + \beta_{2A} - \text{CaM}\)). D. fusion of CaM\(_{WT}\) to the carboxyl terminus of \(\alpha_{1C}\) subunit (\(\alpha_{1C}\Delta1671 - \text{CaM}_{\text{WT}}\)) supports normal CDI. E. fusion of CaM\(_{1234}\) to the \(\alpha_{1C}\) subunit (\(\alpha_{1C}\Delta1671 - \text{CaM}_{1234}\)) abolishes CDI. F. CaM fusion to the \(\beta_{2A}\) subunit (\(\beta_{2A} - \text{CaM}_{1234}\)) supports strong CDI. G, CDI is absent when the \(\beta_{2A}\) subunit is fused to CaM\(_{1234}\) (\(\beta_{2A} - \text{CaM}_{1234}\)). The format is as described for B.

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\(^a\)\(\alpha_{1C}\), \(\alpha_{1D}\), and \(\alpha_{1A}\) denote CaV1.2, CaV1.3, and CaV2.1, respectively.
demonstrated that fusion of CaMWT to the carboxyl terminus of the truncated α1CΔ1671 subunit preserves strong CDI, whereas tethering CaM1234 to the same location abolishes CDI, suggesting that CaM fusion to the channel carboxyl terminus preserves modulatory function and permits interaction with key effector interface on the channel (59, 69). As such, we confirmed that truncation of the distal carboxyl tail does not appreciably alter CDI (Fig. 1C, Fig. S1B, and Table 1). We further validated that α1CΔ1671-CaMWT supported strong CDI (Fig. 1D and S1C), whereas α1CΔ1671-CaM1234 abolished CDI (Fig. 1E and Fig. S1D). To determine whether genetic fusion of CaM to the β2A subunit similarly supports CaV1.2 regulation, we tethered CaMWT and CaM1234 onto the β2A subunit, yielding β2A-CaMWT or β2A-CaM1234, respectively. Notably, the β2A subunit binds to the α1 subunit with a high affinity and 1:1 stoichiometry (70, 71), and this subunit is obligatory for channel function in HEK293 cells. As such, we co-expressed α1C with either β2A-CaMWT or β2A-CaM1234; β2A-CaMWT co-expression with α1C subunit preserved strong CDI (Fig. 1F, Fig. S1E, and Table 1) similar to control conditions. By contrast, co-expression of β2A-CaM1234 abolished CDI (Fig. 1G, Fig. S1F, and Table 1). Thus, CaM tethered to the β2A Subunit is also capable of binding to critical channel effector motifs and eliciting functional regulation.

**CaV1 preferentially responds to CaM tethered to the channel carboxyl terminus**

Having verified the functionality of tethered CaM, we sought to determine channel regulation when multiple CaM molecules are localized within the channel nanodomain. As both the α1C CT and the β2A subunit are within close proximity of the channel pore (<10 nm) based on cryo-EM structure (71), CaM tethered to either domain is exposed to similar local Ca2+ fluctuations (72, 73). Thus, if two WT CaM molecules are attached to the channel complex, we anticipate strong CDI akin to channels that lack tethered CaM, because either one or both CaM molecules can interact with respective channel effector interfaces. Indeed, co-expression of β2A-CaMWT with α1CΔ1671-CaMWT resulted in appreciable CDI (Fig. 2A, Fig. S1G, and Table 1), albeit modestly reduced compared with channels lacking tethered CaM (~25% reduction). However, if the channel complex comprises of one mutant and one WT CaM, then four distinct functional outcomes emerge, depending on the underlying mechanism of channel modulation: If two Ca2+/CaM independently orchestrate channel modulation, then this maneuver would result in a partial disruption of CDI regardless of whether CaM1234 is tethered to α1C CT or β2A (Scenario I); if two Ca2+/CaM cooperatively modulate CaV1.2 regulation, then the presence of one CaM1234 in the channel complex tethered to either α1C or β2A would exert a dominant negative effect and fully inhibit CDI (Scenario II); if instead, functional channel modulation relied on a single Ca2+-bound CaM, then

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**Stoichiometry of CaM in tuning CaV feedback**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Ca2+ r300 at +10 mV</th>
<th>Ba2+ r300 at +10 mV</th>
<th>CDI300 at +10 mV</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1C + β2A</td>
<td>0.46 ± 0.07</td>
<td>1.08 ± 0.09</td>
<td>0.57 ± 0.05 (n = 5)</td>
<td>NA</td>
</tr>
<tr>
<td>α1CΔ1671-CaMWT + β2A</td>
<td>0.42 ± 0.04</td>
<td>0.81 ± 0.08</td>
<td>0.48 ± 0.02 (n = 5)</td>
<td>0.910 Δ</td>
</tr>
<tr>
<td>α1CΔ1671-CaM1234 + β2A</td>
<td>0.42 ± 0.03</td>
<td>0.85 ± 0.07</td>
<td>0.49 ± 0.04 (n = 5)</td>
<td>&gt;0.9777, &gt;0.9999 Δ</td>
</tr>
<tr>
<td>β2A-CaMWT</td>
<td>0.86 ± 0.05</td>
<td>0.91 ± 0.01</td>
<td>0.05 ± 0.05 (n = 6)</td>
<td>&lt;0.0001 Δ, &lt;0.0001 Δ</td>
</tr>
<tr>
<td>α1C + β2A-CaMWT</td>
<td>0.35 ± 0.02</td>
<td>0.96 ± 0.02</td>
<td>0.64 ± 0.02 (n = 8)</td>
<td>0.9688 Δ, 0.1326 Δ, 0.2591 Δ</td>
</tr>
<tr>
<td>α1C + β2A-CaM1234</td>
<td>0.89 ± 0.05</td>
<td>0.98 ± 0.04</td>
<td>0.09 ± 0.05 (n = 7)</td>
<td>&lt;0.0001 Δ, &lt;0.0001 Δ Δ</td>
</tr>
<tr>
<td>α1CΔ1671-CaMWT + β2A-CaMWT</td>
<td>0.63 ± 0.03</td>
<td>1.00 ± 0.02</td>
<td>0.03 ± 0.03 (n = 7)</td>
<td>0.0271 Δ, 0.6852 Δ Δ</td>
</tr>
<tr>
<td>α1CΔ1671-CaMWT + β2A-CaM1234</td>
<td>0.47 ± 0.04</td>
<td>0.92 ± 0.05</td>
<td>0.49 ± 0.05 (n = 8)</td>
<td>0.9095 Δ, &lt;0.9999 Δ</td>
</tr>
<tr>
<td>α1CΔ1671-CaM1234 + β2A-CaMWT</td>
<td>0.80 ± 0.03</td>
<td>0.86 ± 0.03</td>
<td>0.06 ± 0.04 (n = 6)</td>
<td>&lt;0.0001 Δ, &lt;0.0001 Δ</td>
</tr>
<tr>
<td>α1CΔ1671-CaM1234 + β2A-CaM</td>
<td>0.86 ± 0.04</td>
<td>0.89 ± 0.02</td>
<td>0.00 ± 0.03 (n = 5)</td>
<td>&lt;0.0001 Δ, &lt;0.0001 Δ Δ</td>
</tr>
<tr>
<td>α1C + β2A-CaMWT + CaM</td>
<td>0.79 ± 0.07</td>
<td>0.90 ± 0.07</td>
<td>0.13 ± 0.03 (n = 5)</td>
<td>&lt;0.0001 Δ, &lt;0.0001 Δ</td>
</tr>
</tbody>
</table>

*Tukey’s multiple-comparison test shows p values in comparison with α1C + β2A.

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**Figure 2. CaV1.2 is preferentially regulated by CaM tethered to the channel carboxyl terminus.** A, localizing two WT CaM to the channel complex via fusion to both the α1C and β2A subunits (α1CΔ1671-CaMWT + β2A-CaMWT) supports CDI, albeit the extent of CDI is modestly diminished compared with channels with a single tethered CaMWT. B, co-expression of α1CΔ1671-CaM1234 with β2A-CaMWT sufficed to abolish CDI, suggesting that CT-tethered CaM is critical for CDI. C, co-expression of α1CΔ1671-CaMWT with β2A-CaM1234 resulted in strong CDI, suggesting that CaV1.2 is preferentially regulated by a single CaM associated with the channel carboxyl tail. Data are presented as mean ± S.E. obtained from a specified number of cells (n).
the presence of one CaMWT tethered to either α1C CT or β2A would elicit full CDI (Scenario III); and a final nuanced possibility is that CDI depends only on a single CaM, but one that is prebound to a particular interface (Scenario IV). In this last scenario, the modulatory effect will be binary, depending on whether CaMWT or CaM1234 occupies the interface responsible for triggering CDI. To dissect between these possibilities, we first co-expressed α1CΔ1671-CaM1234 with β2A-CaMWT. Comparison of Ca2+ versus Ba2+ current decay demonstrates a strong reduction of CDI (Fig. 2B, Fig. S1H, and Table 1). This result eliminates both Scenarios I and III. To distinguish between Scenarios II and IV, we co-expressed α1CΔ1671-CaMWT with β2A-CaM1234 (Fig. 2C and Fig. S1I). This maneuver resulted in strong CDI indistinguishable from that observed upon co-expression of either α1C or α1CΔ1671 with β2A (Table 1). This result confirmed Scenario IV with a single CaM pre-bound to the channel carboxyl tail being privileged in triggering CDI. To further ensure that the glycine linkage of CaM to either the α1CΔ1671 or the β2A subunit did not occlude accessibility of CaM to effector interfaces, we measured CDI of α1CΔ1671-CaM1234 with β2A and α1C in the presence of freely diffusible CaM. As with α1CΔ1671-CaM1234, we observed no CDI even upon CaM overexpression (Fig. S2 (A and B) and Table 1). As a further control, we also co-expressed α1C with β2A-CaM1234 and freely diffusible CaM. In this case, we again found no CDI, consistent with β2A-localized CaM occupying the carboxyl tail site (Fig. S2 (C and D) and Table 1). These findings further confirm that glycine linkage does not prevent CaM from reaching critical sites. In all, these results demonstrate that CaV1.2 is preferentially regulated by a single CaM associated with the channel CT, in effect rejecting Ca2+/CaM signaling from the β2A-tethered CaM.

To assess generality, we considered the stoichiometric basis for CaM regulation of CaV1.3. Accordingly, CaV1.3 exhibits strong CDI at baseline as shown in Fig. 3A (Fig. S3A and Table 2), consistent with previous studies. As with CaV1.2, we have previously demonstrated that fusion of CaMWT to the CaV1.3 CT (α1D-CaMWT) supports strong CDI, whereas attaching CaM1234 at the same locus (α1D-CaM1234) abolishes CDI (69, 74). To confirm functionality of CaM linkage to the β2A subunit, we co-expressed β2A-CaMWT or β2A-CaM1234 with the α1D pore-forming subunit. We observed robust CDI for CaV1.3 in the presence of β2A-CaMWT similar to that observed with the β2A subunit alone (Fig. 3B, Fig. S3B, and Table 2). By contrast, co-expression of β2A-CaM1234 abolished CDI (Fig. 3C, Fig. S3C, and Table 2), suggesting that CaM linked to the β2A subunit is capable of eliciting functional regulation. Thus assured, we sought to deduce the effect of localizing two CaMWT to the CaV1.3 complex. As anticipated, strong CDI was observed when α1D-CaMWT was co-expressed with β2A-CaMWT (Fig. 3D, Fig. S3D, and Table 2). Subsequently, we co-expressed α1D-CaM1234 with β2A-CaMWT and measured CDI (Fig. 3E, Fig. S3E, and Table 2). As with CaV1.2, this combination sufficed to strongly attenuate CDI. In contrast, co-expression of α1D-CaMWT with β2A-CaM1234 fully spared CDI (Fig. 3F, Fig. S3F, Table 2). To ensure that these findings did not result from a steric limitation imposed by tethered CaM, we considered whether overexpression of freely diffusible recombinant CaMWT could reverse CDI deficits of either α1D-CaM1234 with β2A (Fig. S4 (A and B) and Table 2) or α1D with β2A-CaM1234 (Fig. S4 (C and D) and Table 2). In both cases, we observed no CDI, confirming that CaM localized to the channel preferentially regulated channel function (Fig. S4). Taken together, the binary switching of channel regulatory behavior observed with localizing one CaMWT and one CaM1234 suggests that functional CaV1.3 regulation is preferentially triggered by CaM in close vicinity of the channel CT.
Stoichiometry of CaM in tuning CaV feedback

Table 2
Comparison of CaV1.3 CDI_{apo} values when one or two CaM molecules are localized to the channel complex

Values are mean ± S.E. with number of samples designated in the CDI_{apo} column. One-way ANOVA test shows significant differences with p < 0.0001, and Dunnett’s multiple-comparison test shows p values in comparison with the untreated channels as reference. NA, not applicable.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Ca^{2+} _r_{500}</th>
<th>Ba^{2+} _r_{500}</th>
<th>CDI_{100} at ± 10 mV (mean ± S.E.)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_{1D} + β_{2A}</td>
<td>0.23 ± 0.02</td>
<td>0.97 ± 0.01</td>
<td>0.765 ± 0.025 (n = 8)</td>
<td>NA</td>
</tr>
<tr>
<td>α_{1D} + β_{2A}-CaM_{WT}</td>
<td>0.17 ± 0.06</td>
<td>0.94 ± 0.03</td>
<td>0.820 ± 0.057 (n = 6)</td>
<td>0.9231</td>
</tr>
<tr>
<td>α_{1D} + β_{2A}-CaM_{1234}</td>
<td>0.06 ± 0.02</td>
<td>1.00 ± 0.01</td>
<td>0.033 ± 0.029 (n = 7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α_{1D}-CaM_{WT} + β_{2A}-CaM_{WT}</td>
<td>0.32 ± 0.03</td>
<td>0.98 ± 0.01</td>
<td>0.673 ± 0.036 (n = 8)</td>
<td>0.3858</td>
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<tr>
<td>α_{1D}-CaM_{WT} + β_{2A}-CaM_{1234}</td>
<td>0.23 ± 0.03</td>
<td>0.90 ± 0.03</td>
<td>0.746 ± 0.025 (n = 6)</td>
<td>0.9996</td>
</tr>
<tr>
<td>α_{1D}-CaM_{1234} + β_{2A}-CaM_{WT}</td>
<td>0.97 ± 0.09</td>
<td>0.99 ± 0.01</td>
<td>0.025 ± 0.084 (n = 5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α_{1D}-CaM_{1234} + β_{2A}-CaM_{1234}</td>
<td>0.91 ± 0.05</td>
<td>0.95 ± 0.05</td>
<td>0.041 ± 0.047 (n = 5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α_{1D} + β_{2A}-CaM_{1234} + CaM</td>
<td>0.83 ± 0.05</td>
<td>0.86 ± 0.06</td>
<td>0.035 ± 0.025 (n = 5)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Functional CaM stoichiometry for CaV1 is limited by the number of CaV1 IQ domains

To delineate mechanisms that govern CaM CaM stoichiometry for channel regulation, we considered whether CaV1 could be engineered to be responsive to multiple CaM molecules. Accordingly, we constructed CaV1.3 channels containing two IQ domains in tandem in the carboxyl tail (CaV1.3-2IQ), fused to either CaM_{WT} (termed α_{1D/2IQ}-CaM_{WT} to denote CaM_{WT} fusion to the pore-forming α-subunit) or CaM_{1234} (α_{1D/2IQ}-CaM_{1234}) and co-expressed with β_{2A}, β_{2A}-CaM_{WT}, or β_{2A}-CaM_{1234}. We observed strong CDI for α_{1D/2IQ}-CaM_{WT} similar to CaV1.3 (Fig. 4A, Fig. S5A, and Table 3). By comparison, CDI of α_{1D/2IQ}-CaM_{1234} was sharply diminished similarly to α_{1D}-CaM_{1234}, although not fully eliminated (Fig. 4B, Fig. S5B, and Table 3). These findings suggest that CT-linked CaM remains vital for CDI of CaV1.3 containing tandem IQ domains. Furthermore, co-expression of α_{1D/2IQ}-CaM_{WT} with β_{2A}-CaM_{1234} also revealed strong CDI similarly to α_{1D/2IQ}-CaM_{WT} co-expressed with β_{2A} alone (Fig. 4C, Fig. S5C, and Table 3). However, when α_{1D/2IQ}-CaM_{WT} is co-expressed with β_{2A}-CaM_{1234}, we observed a partial reduction in CDI (Fig. 4D, Fig. S5D, and Table 3). In like manner, co-expression of α_{1D/2IQ}-CaM_{1234} with β_{2A}-CaM_{WT} also showed a partial reduction in CDI (Fig. 4E, Fig. S5E, and Table 3). By contrast, localizing two-mutant CaM_{1234} to the channel complex by co-expressing α_{1D/2IQ}-CaM_{1234} with β_{2A}-CaM_{1234} revealed a complete disruption of CDI (Fig. 4F, Fig. S5F, and Table 3). This behavior is distinctly different from a single IQ domain—containing CaV1.3 (Fig. 3), where a binary change in CDI is observed, depending on the Ca^{2+}-binding ability of carboxyl-terminally linked CaM. Instead, the stepwise change in CDI with one- versus two-mutant CaM_{1234} is consistent with Scenario I considered above. This outcome suggests a 2:1 functional CaM stoichiometry for mutant CaV1.3-2IQ.

Two mechanistic possibilities may engender this switch in functional CaM stoichiometry. First, the number of apo-CaM molecules within the CaV1 complex may be the determining parameter for functional CaM stoichiometry. Our previous work using a holo-channel FRET two-hybrid assay showed that although two Ca^{2+}/CaM molecules bind the holo-CaV1 channels, only a single apo-CaM preassociates with the full-length channel (66). Furthermore, with two IQ domains, up to two apo-CaM molecules may interact with the channel complex. Second, functional CaM stoichiometry may be fundamentally limited by the number of IQ domains, a critical segment for initiating CDI. We previously showed that Ca^{2+}-binding to pre-bound CaM triggers a conformational rearrangement of the channel CT, resulting in the formation of a tripartite complex involving CaM, the channel dual vestigial EF-hand domains, and the IQ domain (41). If so, the functional CaM stoichiometry may be limited by the number of IQ domains available to initiate formation of the tripartite EF/CaM/IQ complex. To test these possibilities, we replaced the CaV1.3 IQ domain with three different CaM-binding segments (75): 1) M13 peptide from the myosin light-chain kinase (CaV1.3-M13), 2) the IQ domain of unconventional myosin Va (CaV1.3-MyoIQ), and 3) the IQ domain of related NaV1.4 channels (CaV1.3-NaV14IQ). Thus probed, the CaV1.3-M13 channels revealed minimal CDI (Fig. 5A, Fig. S6A, and Table S1) compared with WT CaV1.3. As M13 interacts only with the Ca^{2+}-bound form of CaM, this result suggests that apo-CaM preassociation is obligatory for CDI. Notably, CaV1.3-M13 channels also exhibited increased VDI, reminiscent of previous observations of increased VDI upon disrupting apo-CaM binding. Unlike the M13 peptide, the IQ domain of unconventional myosin Va interacts with both apo-CaM and Ca^{2+}/CaM with a high affinity comparable with CaV1 channel IQ domain. If the number of apo-CaM molecules in the channel complex sufficed to determine functional regulation and stoichiometry, then substitution of the CaV1.3 IQ domain with the IQ domain from the unconventional myosin Va would preserve CDI triggered by a single CaM. However, CaV1.3-MyoIQ channels failed to trigger appreciable CDI, suggesting that high-affinity apo-CaM and Ca^{2+}/CaM interaction with the channel alone are insufficient for CDI (Fig. 5B, Fig. S6B, and Table S1). As a further test, we considered whether substitution of the CaV1.3 IQ domain with the IQ domain from the related NaV1.4 channels might support functional channel regulation. Of note, NaV1.4 undergoes CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5C, Fig. S6C, and Table S1). Taken together, these findings suggest that high-affinity apo-CaM and Ca^{2+}/CaM interaction with the channel alone are insufficient for CDI. Whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI (Fig. 5D, Fig. S6D, and Table S1). As a further test, we considered whether substitution of the CaV1.3 IQ domain with the IQ domain from the related NaV1.4 channels might support functional channel regulation. Note, NaV1.4 undergoes CDI with similar underlying mechanisms as CaV1.3 (15). Intriguingly, whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5E, Fig. S6E, and Table S1). As a further test, we considered whether substitution of the CaV1.3 IQ domain with the IQ domain from the related NaV1.4 channels might support functional channel regulation. Of note, NaV1.4 undergoes CDI with similar underlying mechanisms as CaV1.3 (15). Intriguingly, whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5E, Fig. S6E, and Table S1). As a further test, we considered whether substitution of the CaV1.3 IQ domain with the IQ domain from the related NaV1.4 channels might support functional channel regulation. Note, NaV1.4 undergoes CDI with similar underlying mechanisms as CaV1.3 (15). Intriguingly, whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5E, Fig. S6E, and Table S1). As a further test, we considered whether substitution of the CaV1.3 IQ domain with the IQ domain from the related NaV1.4 channels might support functional channel regulation. Of note, NaV1.4 undergoes CDI with similar underlying mechanisms as CaV1.3 (15). Intriguingly, whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5E, Fig. S6E, and Table S1).
Stoichiometry of CaM in tuning CaV feedback

Distinct modes of CaV2.1 regulation are preferentially evoked by carboxyl-terminally linked CaM

CaM regulation of CaV2.1 is bifurcated resulting in two mechanistically distinct forms of regulation: 1) rapid CDF that evolves over ~1–10 ms and is sensitive to local Ca\(^{2+}\) fluctuations, and 2) kinetically slower CDI that evolves over ~300-800 ms and is sensitive to global Ca\(^{2+}\) elevations (43, 76). The two modes of channel regulation rely on Ca\(^{2+}\)/CaM interaction with distinct channel domains (48, 61). CDF is triggered primarily by CaM C-lobe interaction with the canonical IQ domain (48), whereas CDI relies on Ca\(^{2+}\)/CaM N-lobe interacting with binding sites elsewhere on the channel (76). To determine whether both modes of channel regulation are triggered by a single CaM, we again applied our strategy of localizing multiple CaM molecules to the CaV2 complex through linkage to the pore-forming α1A and the β2A subunits. For these experiments, the whole-cell dialyze contained low Ca\(^{2+}\) buffering (1 mM EGTA) to permit global Ca\(^{2+}\) elevations necessary to trigger CDF. A family of depolarizing voltage pulses of 800-ms duration were utilized to elicit Ca\(^{2+}\) and Ba\(^{2+}\) currents for CDI measurements. Thus probed, Fig. 6A shows baseline CDI of CaV2.1 (Fig. S7A and Table S2). Tethering CaM\(_{WT}\) to the CT of α1A subunit (α1A-CaM\(_{WT}\)) and co-expression with the β2A subunit yields CDI comparable with baseline conditions (Fig. 6B, Fig. S7B, and Table S2). By contrast, fusion of CaM\(_{1234}\) to the CT of α1A subunit results in a reduction in CDI (Fig. 6C, Fig. S7C, and Table S2). Subsequently, we tested whether CaM fusion to the β2A subunit also evoked similar regulatory effects. Accordingly, co-expression of α1A subunit with β2A-CaM\(_{WT}\) showed robust CDI comparable with baseline conditions (Fig. 6D, Fig. S7D, and Table S2), whereas β2A-CaM\(_{1234}\) strongly diminished CDI (Fig. 6E, Fig. S7E, and Table S2). Therefore, fusion of CaM to either the α1A or the β2A subunit permits interaction with key effector interfaces and preserves functional modulation.

Thus informed, we considered changes in channel regulatory behavior in the presence of multiple CaM molecules. Accordingly, we co-expressed α1A-CaM\(_{WT}\) with β2A-CaM\(_{WT}\). As expected, this maneuver elicited strong CDI (Fig. 7A, Fig. S7F, and Table S2). To determine stoichiometric requirements, we measured CDI of α1A-CaM\(_{1234}\) in the presence of β2A-CaM\(_{WT}\). Comparison of Ca\(^{2+}\) versus Ba\(^{2+}\) currents revealed markedly blunted CDI (Fig. 7B, Fig. S7G, and Table S2). In comparison, co-expression of α1A-CaM\(_{WT}\) with β2A-CaM\(_{1234}\) revealed strong CDI (Fig. 7C, Fig. S7H, and Table S2) similar to untagged channels. As a control, we co-expressed of α1A-CaM\(_{1234}\) with β2A-CaM\(_{1234}\) and found nearly complete inhibition of CDI (Fig. 7D, Fig. S7I, and Table S2). Once again, to further corroborate these findings, we considered whether overexpression of freely diffusible recombinant CaM\(_{WT}\) could reverse CDI deficits of α1A-CaM\(_{1234}\) in the presence of β2A-CaM\(_{WT}\) (Fig. S8 A, B, and D and Table S2). Indeed, CDI remained blunted for α1A-CaM\(_{1234}\) despite CaM overexpression. Similarly, overexpression of CaM\(_{WT}\) failed to reverse the reduction in CDI of α1A in the presence of β2A-CaM\(_{1234}\) (Fig. S8 E and F, Fig. S8H, and Table S2). These findings suggest that a single CaM bound to the CaV2.1 CT is primarily responsible for signaling CDI.

To determine whether CaM localized to the α1A CT is also responsible for eliciting CDF, we first established baseline CDF of CaV2.1 using a paired-pulse facilitation protocol (Fig. 8A and Table S3). Briefly, in the absence of a prepulse, CaV2.1 current displays biphasic kinetics corresponding to rapid activation of the channel and a subsequent slower interconversion into a facilitated gating configuration following Ca\(^{2+}\) binding to CaM.

![Figure 4. Engineering CaV1.3 with tandem IQ domains switches functional CaM stoichiometry.](image-url)
With a prepulse, CaV2.1 current is monophasic with enhanced activation, Ca$^{2+}$ entry during the prepulse having already triggered facilitation. RF is quantified as the excess charge entry following prepulse, and CDF is quantified as the difference in RF with Ca$^{2+}$ versus Ba$^{2+}$ as charge carriers. Once again, we validated that CaM fusion to the a$_{1A}$ and b$_{2A}$ supports CDF. Briefly, co-expression of a$_{1A}$-CaMWT with the b$_{2A}$ subunit elicits strong CDF, whereas a$_{1A}$-CaM1234 exhibits strongly diminished CDF (Fig. 8 (B and C) and Table S3). In like manner, expressing b$_{2A}$-CaMWT with a$_{1A}$ subunit supports strong CDF, whereas b$_{2A}$-CaM1234 diminishes CDF (Fig. 8 (D and E) and Table S3), thus confirming functionality of tethered CaM. Furthermore, robust CDF was also observed when both a$_{1A}$ and b$_{2A}$ subunits were both fused with CaMWT (Fig. 9A and Table S3), albeit the magnitude of CDF was modestly reduced compared with WT channels. To determine whether CDF of CaV2.1 is also dependent on carboxyl-terminally linked CaM, we probed CDF of a$_{1A}$-CaM1234 in the presence of b$_{2A}$-CaMWT. CDF was nearly absent for this pair (Fig. 9B and Table S3). By contrast, a$_{1A}$-CaMWT in the presence of b$_{2A}$-CaM1234 revealed no appreciable change in CDF (Fig. 9C and Table S3). As a further test, we probed whether freely diffusible recombinant CaMWT could reverse reduced CDF observed for a$_{1A}$-CaM1234 in the presence of b$_{2A}$ or for a$_{1A}$ in the presence of b$_{2A}$-CaM1234. Indeed, CDF was strongly diminished in both cases (Fig. S8, C and G). Thus, localized CaM is privileged in initiating CDF. Taken together, these

<table>
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<tr>
<th>Construct</th>
<th>Ca$^{2+}$ r$_{300}$</th>
<th>Ba$^{2+}$ r$_{300}$</th>
<th>CDF$_{100}$ at $+10$ mV (mean ± S.E.)</th>
<th>p value</th>
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<tbody>
<tr>
<td>a$<em>{1D}$/2-M13 + b$</em>{2A}$</td>
<td>0.13 ± 0.02</td>
<td>0.81 ± 0.07</td>
<td>0.836 ± 0.020 ($n=5$)</td>
<td>0.3953</td>
</tr>
<tr>
<td>a$<em>{1D}$/2-M13 + b$</em>{2A}$</td>
<td>0.89 ± 0.01</td>
<td>1.01 ± 0.04</td>
<td>0.124 ± 0.017 ($n=6$)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>a$<em>{1D}$/2-IQ-CA1234 + b$</em>{2A}$</td>
<td>0.26 ± 0.01</td>
<td>0.97 ± 0.03</td>
<td>0.724 ± 0.017 ($n=6$)</td>
<td>0.7614</td>
</tr>
<tr>
<td>a$<em>{1D}$/2-IQ-CA1234 + b$</em>{2A}$</td>
<td>0.60 ± 0.05</td>
<td>0.94 ± 0.03</td>
<td>0.354 ± 0.070 ($n=5$)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>a$<em>{1D}$/2-IQ-CA1234 + b$</em>{2A}$</td>
<td>0.48 ± 0.05</td>
<td>0.98 ± 0.01</td>
<td>0.478 ± 0.061 ($n=5$)</td>
<td>&lt;0.0001</td>
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<tr>
<td>a$<em>{1D}$/2-IQ-CA1234 + b$</em>{2A}$</td>
<td>0.99 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>0.011 ± 0.006 ($n=5$)</td>
<td>&lt;0.0001</td>
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Table 3

Comparison of Ca$_{1.3}$ tandem IQ CDI$_{300}$ values when one or two CaM molecules are localized to the channel complex

Values are mean ± S.E., with the number of samples designated in the CDI$_{300}$ column. One-way ANOVA test shows significant differences with $p < 0.0001$, and Dunnett’s multiple-comparison test shows p values in comparison with the WT Ca$_{1.3}$ channels as reference.

Figure 5. The IQ domain is essential for functional CaM regulation of CaV$_{1.3}$. A, replacing the Ca$_{1.3}$ IQ domain with M13 peptide from myosin light-chain kinase that only interacts with Ca$^{2+}$/CaM strongly reduces CDI. The format is as in Fig. 1B. Data are presented as mean ± S.E. obtained from a specified number of cells ($n$). B, substitution of Ca$_{1.3}$ IQ domain with IQ domain 1 from unconventional myosin Va also abolished CDI. C, by contrast, replacement of Ca$_{1.3}$ IQ domain with IQ domain of Na$_{v}1.4$ supports CDI. The magnitude of CDI is diminished compared with WT Ca$_{1.3}$. 
FRET analysis suggests a stoichiometry of up to two Ca$^{2+}$/CaM.

The carboxyl terminus of peak current remaining following 800-ms depolarization (Right panel) serves functional channel regulation.

Discussion

The stoichiometry of CaM interaction with the CaV channel complex and the functional requirements for channel regulation have long been debated (3, 4). Biochemical and structural studies demonstrate the interaction of multiple CaM with distinct channel peptide segments (38, 46, 48, 52, 55, 57, 60, 65). FRET analysis suggests a stoichiometry of up to two Ca$^{2+}$/CaM molecules associating with the holo-CaV channel complex (66). Functional studies, however, indicate that a single CaM suffices for Ca$^{2+}$-dependent feedback regulation (59). To reconcile these differences, we dissected the potential role of multiple CaM in orchestrating CaV feedback modulation. We localized up to two WT CaM or mutant CaM$_{1234}$ to the CaV$_{1-2}$ channel complex through linkage to the $\alpha$ and $\beta$ subunits. Consistent with prior studies, we found that a single CaM tethered to the CaV$_{1}$ complex through either the $\alpha$ or $\beta$ subunits is fully capable of replacing endogenous CaM (59, 67, 74). Nonetheless, when multiple CaMs are localized to the CaV$_{1-2}$ channel complex, functional Ca$^{2+}$-regulation of channel gating depends primarily on CaM tethered to the CT of the $\alpha$-subunit. More specifically, when CaM$_{WT}$ is attached to the CaV$_{1/2}$ CT locus, both CDI (for CaV$_{1.2/1.3/2.1}$) and CDF (for CaV$_{2.1}$) are fully intact; however, when CaM$_{1234}$ is attached at this locus, Ca$^{2+}$ regulation is absent. Furthermore, we found that introduction of a second IQ domain in the channel carboxyl tail switches the functional CaM stoichiometry for CaV$_{1.3}$ channels such that channel regulation is responsive to two CaM molecules. These results are consistent with a model whereby a single CaM preassociated with the channel CT serves as a dedicated sensor for Ca$^{2+}$-dependent modulation of CaV$_{1/2}$ gating.

Figure 6. CaM to CaV$_{2.1}$ via fusion to either the $\alpha$ or $\beta$ subunits preserves functional channel regulation. A, baseline CDI of CaV$_{2.1}$ is assessed at low Ca$^{2+}$ buffering. Representative traces correspond to Ca$^{2+}$ and Ba$^{2+}$ currents evoked in response to a $\pm$ 20 mV voltage step. Ba$^{2+}$ traces are scaled to about one-third actual magnitude to match Ca$^{2+}$ traces (at scale with bar). Right, levels of inactivation at different voltages are assessed as the fraction of peak current remaining following 800-ms depolarization ($i_{\text{fmax}}$) and averaged from n cells. Symbols and error bars, mean $\pm$ S.E.; B, fusion of CaM$_{WT}$ to the carboxyl terminus of $\alpha_{1A}$-subunit ($\alpha_{1A}$-CaM$_{WT}$) supports normal CDI. C, fusion of mutant CaM$_{1234}$ ($\alpha_{1A}$-CaM$_{1234}$) abolishes CDI. D, localizing a single CaM to the CaV$_{2.1}$ complex by co-expression of $\beta_{2A}$-CaM$_{WT}$ supports robust CDI. E, CDI of CaV$_{2.1}$ is strongly diminished when $\beta_{2A}$-CaM$_{1234}$ is co-expressed.

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A few mechanistic implications merit further attention. First, in vitro measurements of CaM affinity have demonstrated that apo-CaM interaction with the channel CT peptides is much weaker (~1 $\mu$M) compared with Ca$^{2+}$/CaM interaction (<1 nM) (51, 77). Thus, if both a mutant CaM incapable of binding Ca$^{2+}$ and a WT CaM are within the same channel complex, one would expect the WT CaM to competitively displace the mutant CaM on the CT, owing to the 3-order of magnitude affinity advantage. However, we found that this was not the case for CaV$_{1/2}$ channels; $\beta$-subunit–tethered CaM$_{WT}$ was unable to displace CaM$_{1234}$ to trigger CDI. One possibility is that the apo-CaM affinity for the CaV channel complex may be stronger than estimated in vitro, presumably reflecting unconventional interactions with the channel complex, as has been observed in cryo-EM structures of holo-K$_{V}$7 channels (17) and ryanodine receptors (24). Indeed, previous studies have shown that reducing free apo-CaM levels to nanomolar concentrations was insufficient to appreciably deplete apo-CaM preassociation from the CaV$_{1.3}$ channel, suggesting a higher apo-CaM affinity (78). An alternative possibility is that additional channel regulatory proteins, such as $\alpha$-actinin, may fine-tune CaM interactions with the CaV channel, thereby imparting distinct effects on channel gating (79). Second, our findings also point toward potential mechanisms that underlie the singular CaM stoichiometry observed for Ca$^{2+}$ regulation of CaV gating. For CaV$_{1.3}$ channels, we previously found that Ca$^{2+}$-binding to CaM elicits a conformational rearrangement of the channel CT, resulting in the formation of a tripartite complex involving the channel IQ domain, Ca$^{2+}$/CaM, and the channel dual vestigial EF-hand segments (41). Thus, one possibility is that functional stoichiometry for CaM regulation of channel gating may be ultimately limited by the number of IQ domains available to initiate formation of the tripartite complex. Consistent with this possibility, when CaV$_{1.3}$ channels contained two IQ domains, functional Ca$^{2+}$ regulation appeared to depend on both the channel CT-tethered CaM and the $\beta$-subunit–tethered CaM.
Furthermore, we found that replacement of the CaV1.3 IQ domain with either M13 peptide or an IQ domain from the unconventional myosin Va resulted in a nearly complete inhibition of CDI. By contrast, substitution of the NaV1.4 IQ domain still permits functional Ca\textsuperscript{2+} regulation. Importantly, NaV1.4 channels are homologous to CaV channels, and they undergo CDI in a similar manner as CaV1 channels. In this scenario, it is possible that specific residues unique to the CaV/NaV IQ domain and not in myosin Va IQ may be critical in triggering tripartite complex formation. It is also possible that the precise orientation or arrangement of CaM may also be relevant in this process (48). Furthermore, although M13 and myosin Va IQ domain are widely recognized as CaM-binding peptides, it is possible that attachment to the CaV channel carboxyl tail may perturb the ability of these peptides to interact with CaM. Third, the traditional model of Ca\textsuperscript{2+}-dependent regulation is that Ca\textsuperscript{2+}/CaM interaction with effector sites is sufficient to signal to the pore domain. For most CaV channels, the carboxyl tail IQ domain is thought to harbor key effector sites for triggering Ca\textsuperscript{2+}/CaM regulation (4); however, for CaV1.2 and CaV1.3, one critical interface for N-lobe-mediated CDI is the NSCaTE motif located on the channel N terminus (55). Importantly, NSCaTE only interacts with CaM in the presence of Ca\textsuperscript{2+} (55, 57). As such, if mutant CaM\textsubscript{1234} were prebound to the channel CT, then the NSCaTE motif remains unoccupied at basal conditions (55, 57, 62). Following Ca\textsuperscript{2+} influx, the second CaM\textsubscript{WT} within the channel complex localized via the \(\beta\) subunit would be able to interact with NSCaTE segment and trigger CDI. However, the complete absence of Ca\textsuperscript{2+}/CaM regulation when CaV1.2 and CaV1.3 \(\alpha\) subunit is linked to mutant CaM\textsubscript{1234} and \(\beta\textsubscript{2A}-\)CaM suggests that the simple interaction of the N-lobe of Ca\textsuperscript{2+}/CaM with the NSCaTE domain is insufficient. Instead, these results suggest that a Ca\textsuperscript{2+}-dependent conformational rearrangement of the CT-bound CaM is obligatory for CaM regulation. Fourth, for CaV2 channels, CaM regulation
Figure 8. CaM tethered to both the Ca$_{\text{v}}$2.1 a and b subunits is able to trigger CDF. A, top left, schematic of WT Ca$_{\text{v}}$2.1 co-expressed with a$_2$β and b$_{2A}$ subunits. Top right, a two-pulse protocol is used to quantify the extent of CDF. Ba$^{2+}$ current kinetics are similar in the presence (black) or absence (gray) of a depolarizing prepulse. Bottom right, without prepulse, a 0 mV step depolarization elicits a biphasic inward Ca$^{2+}$ current with an initial rapid phase followed by a slow phase corresponding to Ca$^{2+}$-dependent facilitation. With a +20-mV prepulse, the channels are already facilitated, and as such, the ensuing test pulse elicits currents that exhibit enhanced channel activation. The area between the two current traces ($\Delta Q$) approximates CDF triggered by the prepulse. Bottom left, population data (mean ± S.E.) shows RF (relative facilitation) at different prepulse potentials averaged from n cells and assessed as $\Delta Q$ divided by the time constant ($t$) of facilitation. CDF is determined as the difference in RF with Ba$^{2+}$ versus Ca$^{2+}$ as charge carrier. B, fusion of CaM$_{\text{WT}}$ to the carboxyl terminus of a$_1$A subunit (a$_1$A-CaM$_{\text{WT}}$) supports strong CDF. C, fusion of CaM$_{1234}$ to the a$_1$A carboxyl terminus (a$_1$A-CaM$_{1234}$) abolishes CDF. D, co-expression of b$_2$A-CaM$_{\text{WT}}$ with a$_1$A elicits strong CDF. E, CDF is abolished in the presence of b$_2$A-CaM$_{1234}$. 

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manifests as both CDF and CDI with distinct spatial Ca\(^{2+}\) selectivity and kinetics (43, 80). These findings support the possibility that both modes of channel regulation are mediated by the same CaM that is initially preassociated with the channel CT.

Our findings also bear important biological implications. In cardiac myocytes, a vast majority of Ca\(^{2+}\)-free CaM is enriched in the dyad with a large fraction bound to the RyR (64, 81). Following Ca\(^{2+}\) binding, however, CaM is mobilized and is available to interact with targets including Ca\(^{2+}\)/CaM-dependent kinases and phosphatases that are also localized at the dyad (81). Our findings suggest that Ca\(^{2+}\)-mobilized CaM would be unable to inhibit the L-type Ca\(^{2+}\)-channels. Instead, only CaM initially preassociated with the channel CT would be able to trigger Ca\(^{2+}\) regulation. Physiologically, this scheme is advantageous in cardiomyocytes as additional CaMs in the dyad are free to signal to other regulatory processes, including activation of kinases and phosphatases (31, 32, 82, 83), channel coupling (84), or translocation to nucleus (85), all without disrupting Ca\(_V\) channel inactivation, an essential factor for normal cardiac repolarization (5). In the disease context, this arrangement, however, makes L-type channels particularly vulnerable to misregulation in cardiac arrhythmias associated with calmodulinopathies (10). The singular functional stoichiometry implies that the preassociation of even a small fraction of mutant CaM with weakened Ca\(^{2+}\) binding could appreciably disrupt L-type channel inactivation and increase risk of arrhythmogenesis. In like manner, in neurons, CaM localized to L-type channels serve multiple functions (44), including modulation of channel gating, trafficking (30, 86), and a key role in excitation-transcription coupling, where local Ca\(^{2+}\) signaling near L-type channels results in rapid shuttling of Ca\(^{2+}\)/CaM to the nucleus through γCaMKII (34, 87, 88). Having a resident CaM dedicated for Ca\(_V\) channel feedback modulation ensures that local Ca\(^{2+}\)/CaM signaling can be multiplexed without detrimental effects on cellular electrical excitability.

Figure 9. CDF of Ca\(_{V}2.1\) channel is preferentially evoked by carboxyl terminus linked CaM. A, localization of two CaMWT molecules via co-expression of a\(_{1A}\)-CaMWT with b\(_{2A}\)-CaMWT supports CDF, although its magnitude is blunted compared with WT channels that lack tethered CaM. B, localizing a mutant CaM\(_{1234}\) via fusion to the a\(_{1A}\) subunit CT (a\(_{1A}\)-CaM\(_{1234}\)) and a CaMWT via linkage to the b\(_{2A}\) subunit (b\(_{2A}\)-CaMWT) resulted in a partial reduction of CDF. C, fusion of a\(_{1A}\)-CaMWT with b\(_{2A}\)-CaM\(_{1234}\) demonstrates strong CDF comparable with channels that lack a tethered CaM. D, co-expression of a\(_{1A}\)-CaM\(_{1234}\) with b\(_{2A}\)-CaM\(_{1234}\) completely abolishes CDF.
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Whole-cell electrophysiology recordings

Whole-cell voltage-clamp recordings for HEK293 were collected at room temperature using an Axopatch 200A amplifier (Axon Instruments). Glass pipettes (World Precision Instruments, MTW-150-F4) were pulled with a horizontal puller (P-97; Sutter Instruments Co.) and fire-polished (Microforge, Narishige, Tokyo, Japan), resulting in 1–3-megohm resistances, before series resistance compensation of 70%. For CaV1.2 and CaV1.3 recordings, the internal solutions contained 135 mM CsMeSO\(_3\), 5 mM CsCl\(_2\), 1 mM MgCl\(_2\), 4 mM MgATP, 10 mM HEPES, 10 mM BAPTA, adjusted to 295 mosm with CsMeSO\(_3\) and pH 7.4 with CsOH. The external solution contained 140 mM TEA-MeSO\(_3\), 10 mM HEPES, 40 mM CaCl\(_2\) or BaCl\(_2\), adjusted to 300 mosm with TEA-MeSO\(_3\) and pH 7.4 with TEA-OH. This external solution composition was chosen based on previous studies to ensure that local Ca\(^{2+}\) signals are saturating to drive maximal local CDI (72, 93). For CaV2.1 recording, the internal solutions contained 135 mM CsMeSO\(_3\), 5 mM CsCl\(_2\), 1 mM MgCl\(_2\), 4 mM MgATP, 10 mM HEPES, 1 mM EGTA, adjusted to 295 mosm with CsMeSO\(_3\) and pH 7.4 with CsOH. The external solution contained 140 mM TEA-MeSO\(_3\), 10 mM HEPES, 5 mM CaCl\(_2\) or BaCl\(_2\), adjusted to 300 mosm with TEA-MeSO\(_3\) and pH 7.4 with TEA-OH. For CDI measurements, we used a family of test pulses from −50 to +50 mV with repetition intervals of 20 s, at a holding potential of −80 mV. Custom MATLAB (Mathworks) software was used to determine peak current and fraction of peak current remaining after either 300 ms (TP300) of depolarization for CaV1 or 800 ms (TP800) of depolarization for CaV2. Ca\(^{2+}\)-dependent facilitation was quantified using the normalized charge difference $\Delta Q$, obtained by integrating the difference between normalized traces $\Delta t$ prepulse is directly proportional to $Q_{\text{Ca}}$ divided by the slow time constant ($t$) of facilitation, yielding relative facilitation ($RF = \Delta Q/\Delta t$). For knockouts of Ca\(^{2+}\)-dependent facilitation, $t$ was set to 12 ms (matching the average time constant for facilitation in channels lacking tethered CaM). $RF_{\text{Ca}}$ corresponds to relative facilitation with Ca\(^{2+}\) as charge carrier, whereas $RF_{\text{Ba}}$ corresponds to that obtained with Ba\(^{2+}\) as charge carrier representing voltage-dependent facilitation. CDF is measured as the difference $RF_{\text{Ca}} - RF_{\text{Ba}}$ (43).

Data availability

All original data are fully available upon request from Manu Ben-Johny (mbj2124@cumc.columbia.edu).

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Abbreviations—The abbreviations used are: CaM, calmodulin; CDI, Ca2+-dependent inactivation; CT, carboxyl tail; NSCaTe, N-terminal spatial Ca2+-transforming element; CDF, Ca2+-dependent facilitation; ANOVA, analysis of variance; TEA, tetraethylammonium; VDI, voltage-dependent inactivation.

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