Calmodulin (CaM) regulation of voltage-gated calcium (CaV) channels is a powerful Ca2+-feedback mechanism to adjust channel activity in response to Ca2+ 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 Ca2+ feedback, yet biochemical, FRET, and structural studies showed that multiple CaM molecules interact with distinct interfaces within channel cytosolic segments, suggesting that functional Ca2+ 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,1.2 nanodomain by tethering either WT or mutant CaM that lack Ca2+-binding capacity to the pore-forming α-subunit of CaV,1.2, CaV,1.3, and CaV,2.1 and/or the auxiliary β2A subunit. We observed that a single CaM tethered to either the α or β2A subunit tunes Ca2+ regulation of CaV,1 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, Ca2+ 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 Ca2+-dependent feedback of channel gating but may support alternate regulatory functions.

Calmodulin (CaM) regulation of high-voltage activated calcium (CaV,1-2) channels 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 Ca2+-decoding schemes (1, 3), and biologically consequential in determining the cardiac action potential (5, 6) and in furnishing stable Ca2+ 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), K+ 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 Ca2+ fluctuations to downstream signaling pathways (31–34). Second, to elicit gating changes, Ca2+-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, permeal Ca2+ ions bind CaM, and ensuing conformational rearrangements (40, 41) trigger Ca2+-dependent feedback modulation. For many CaV,1 and CaV,2 channels, this conformational change diminishes channel activity, a process termed Ca2+-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 Ca2+-dependent facilitation (CDF) (42–44). Third, multiple channel domains that interact with CaM have been identified. Specifically, Ca2+-free CaM associates with a canonical CaM-binding IQ motif localized to the channel carboxyl tail (CT) and the closely juxtaposed EF-hand segment (35, 41, 45). Ca2+/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 Ca2+-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 Ca2+/CaM interaction with the IQ domain (47, 60, 61), as well as two pre-IQ domains cross-bridged by two additional Ca2+/CaM molecules (46, 61).
Stoichiometry of CaM in tuning CaV feedback

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 CaM 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 (CaMWT) or mutant CaM that lacks Ca\(^{2+}\) binding (CaM1234) with known stoichiometries through genetic fusion to either the pore-forming \(\alpha_{1C}\) subunit\(^{3}\) or the auxiliary \(\beta_{2A}\) subunit (59, 67) (Fig. 1A). Here, CaM1234 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 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 – \(r_{300}/r_{300,Ba}\)) (Table 1). Further representative currents and current-voltage relationships are provided in Fig. S1A. Previous studies have

\(^{3}\)\(\alpha_{1C}, \alpha_{1D}, \text{and } \alpha_{1A} \text{ denote CaV1.2, CaV1.3, and CaV2.1, respectively.}

does not alter CDI (\(r_{1671}\)). The fusion of CaMWT to the \(\alpha_{1C}\) subunit (\(\alpha_{1C}-\text{CaMWT}\)) supports normal CDI (F). Fusion of CaM1234 to the \(\alpha_{1C}\) subunit (\(\alpha_{1C}-\text{CaM1234}\)) abolishes CDI (F). CaM fusion to the \(\beta_{2A}\) subunit (i.e., \(\beta_{2A}-\text{CaMWT}\)) supports strong CDI (G). CDI is absent when the \(\beta_{2A}\) subunit is fused to CaM1234 (\(\beta_{2A}-\text{CaM1234}\)). The format is as described for B.
demonstrated that fusion of CaMWT to the carboxyl terminus of the truncated αICΔ1671 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, F, and Table 1). We further validated that αICΔ1671-CaMWT supported strong CDI (Fig. 1D and Fig. S1C), whereas αICΔ1671-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 αIC with either β2A-CaMWT or β2A-CaM1234-β2A-CaMWT co-expression with αIC 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 αIC 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 Ca$^{2+}$ 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 αICΔ1671-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 Ca$^{2+}$/CaM independently orchestrate channel modulation, then this maneuver would result in a partial disruption of CDI regardless of whether CaM1234 is tethered to αIC CT or β2A (Scenario I); if two Ca$^{2+}$/CaM cooperatively modulate CAV1.2 regulation, then the presence of one CaM1234 in the channel complex tethered to either αIC or β2A would exert a dominant negative effect and fully inhibit CDI (Scenario II); if instead, functional channel modulation relied on a single Ca$^{2+}$-bound CaM, then

### Table 1

| Construct                          | Ca$^{2+}$ r$_{300}$ at +10 mV | Ba$^{2+}$ r$_{300}$ at +10 mV | CDI$_{300}$ at +10 mV | p value |
|------------------------------------|-------------------------------|-------------------------------|-----------------------|---------|
| αIC + β2A                          | 0.46 ± 0.07                   | 1.08 ± 0.09                   | 0.57 ± 0.05 (n = 5)   | NA      |
| αICΔ1671-CaMWT + β2A               | 0.42 ± 0.04                   | 0.81 ± 0.08                   | 0.48 ± 0.02 (n = 5)   | 0.91$^a$|
| αICΔ1671-CaM1234 + β2A              | 0.42 ± 0.03                   | 0.85 ± 0.07                   | 0.49 ± 0.04 (n = 5)   | >0.9777$^a$, >0.9999$^a$ |
| αICΔ1671-CaM1234 + β2A              | 0.86 ± 0.05                   | 0.91 ± 0.01                   | 0.05 ± 0.05 (n = 6)   | <0.0001$^a$, <0.0001$^a$ |
| αIC + β2A-CaMWT                    | 0.35 ± 0.02                   | 0.96 ± 0.02                   | 0.64 ± 0.02 (n = 8)   | 0.9688$^a$, 0.1326$^a$, 0.2591$^a$ |
| αIC + β2A-CaM1234                   | 0.89 ± 0.05                   | 0.98 ± 0.04                   | 0.09 ± 0.05 (n = 7)   | <0.0001$, <0.0001$ |
| αICΔ1671-CaMWT + β2A-CaMWT         | 0.63 ± 0.03                   | 1.00 ± 0.02                   | 0.03 ± 0.03 (n = 7)   | 0.0271$^a$, 0.6832$^a$ |
| αICΔ1671-CaM1234 + β2A-CaMWT       | 0.47 ± 0.04                   | 0.92 ± 0.05                   | 0.49 ± 0.05 (n = 8)   | 0.9995$^a$, >0.9999$^a$ |
| αICΔ1671-CaM1234 + β2A + CaM        | 0.80 ± 0.03                   | 0.86 ± 0.03                   | 0.06 ± 0.04 (n = 6)   | <0.0001$, <0.0001$ |
| αICΔ1671-CaM1234 + β2A              | 0.86 ± 0.04                   | 0.89 ± 0.02                   | 0.03 ± 0.03 (n = 5)   | >0.9999$^a$, <0.0001$^a$ |
| αICΔ1671-CaMWT + CaM                | 0.79 ± 0.07                   | 0.90 ± 0.07                   | 0.13 ± 0.03 (n = 5)   | >0.9999$^a$, <0.0001$^a$ |

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$^a$Tukey’s multiple-comparison test shows p values in comparison with αIC + β2A.
$^b$Tukey’s multiple-comparison test shows p values in comparison with αICΔ1671 + β2A.
$^c$Tukey’s multiple-comparison test shows p values in comparison with αICΔCaMWT + 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 αIC and β2A subunits (αICΔ1671-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 αICΔ1671-CaM1234 with β2A-CaMWT sufficed to abolish CDI, suggesting that CT-tethered CaM is critical for CDI. C, co-expression of αICΔ1671-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 CaM\textsubscript{WT} tethered to either $\alpha_{1C}$ CT or $\beta_{2A}$ 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 CaM\textsubscript{WT} or CaM\textsubscript{1234} occupies the interface responsible for triggering CDI. To dissect between these possibilities, we first co-expressed $\alpha_{1C}\Delta1671$-CaM\textsubscript{1234} with $\beta_{2A}$-CaM\textsubscript{WT}. Comparison of Ca\textsuperscript{2+} versus Ba\textsuperscript{2+} 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 $\alpha_{1C}\Delta1671$-CaM\textsubscript{WT} with $\beta_{2A}$-CaM\textsubscript{1234} (Fig. 2C and Fig. S1I). This maneuver resulted in strong CDI indistinguishable from that observed upon co-expression of either $\alpha_{1C}$ or $\alpha_{1C}\Delta1671$ with $\beta_{2A}$ (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 $\alpha_{1C}\Delta1671$ or the $\beta_{2A}$ subunit did not occlude accessibility of CaM to effector interfaces, we measured CDI of $\alpha_{1C}\Delta1671$-CaM\textsubscript{1234} with $\beta_{2A}$ and $\alpha_{1C}$ in the presence of freely diffusible CaM. As with $\alpha_{1C}\Delta1671$-CaM\textsubscript{1234}, we observed no CDI even upon CaM overexpression (Fig. S2 (A and B) and Table 1). As a further control, we also co-expressed $\alpha_{1C}$ with $\beta_{2A}$-CaM\textsubscript{1234} and freely diffusible CaM. In this case, we again found no CDI, consistent with $\beta_{2A}$-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 CaV\textsubscript{1.2} is preferentially regulated by a single CaM associated with the channel CT, in effect rejecting Ca\textsuperscript{2+}/CaM signaling from the $\beta_{2A}$-tethered CaM.

To assess generality, we considered the stoichiometric basis for CaM regulation of CaV\textsubscript{1.3}. Accordingly, CaV\textsubscript{1.3} exhibits strong CDI at baseline as shown in Fig. 3A (Fig. S3A and Table 2), consistent with previous studies. As with CaV\textsubscript{1.2}, we have previously demonstrated that fusion of CaM\textsubscript{WT} to the CaV\textsubscript{1.3} CT ($\alpha_{1D}$-CaM\textsubscript{WT}) supports strong CDI, whereas attaching CaM\textsubscript{1234} at the same locus ($\alpha_{1D}$-CaM\textsubscript{1234}) abolishes CDI (69, 74). To confirm functionality of CaM linkage to the $\beta_{2A}$ subunit, we co-expressed $\beta_{2A}$-CaM\textsubscript{WT} or $\beta_{2A}$-CaM\textsubscript{1234} with the $\alpha_{1D}$ pore-forming subunit. We observed robust CDI for CaV\textsubscript{1.3} in the presence of $\beta_{2A}$-CaM\textsubscript{WT} similar to that observed with the $\beta_{2A}$ subunit alone (Fig. 3B, Fig. S3B, and Table 2). By contrast, co-expression of $\beta_{2A}$-CaM\textsubscript{1234} abolished CDI (Fig. 3C, Fig. S3C, and Table 2), suggesting that CaM linked to the $\beta_{2A}$ subunit is capable of eliciting functional regulation. Thus assured, we sought to deduce the effect of localizing two CaM\textsubscript{WT} to the CaV\textsubscript{1.3} complex. As anticipated, strong CDI was observed when $\alpha_{1D}$-CaM\textsubscript{WT} was co-expressed with $\beta_{2A}$-CaM\textsubscript{WT} (Fig. 3D, Fig. S3D, and Table 2). Subsequently, we co-expressed $\alpha_{1D}$-CaM\textsubscript{1234} with $\beta_{2A}$-CaM\textsubscript{WT} and measured CDI (Fig. 3E, Fig. S3E, and Table 2). As with CaV\textsubscript{1.2}, this combination sufficed to strongly attenuate CDI. In contrast, co-expression of $\alpha_{1D}$-CaM\textsubscript{WT} with $\beta_{2A}$-CaM\textsubscript{1234} 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 CaM\textsubscript{WT} could reverse CDI deficits of either $\alpha_{1D}$-CaM\textsubscript{1234} with $\beta_{2A}$ (Fig. S4 (A and B) and Table 2) or $\alpha_{1D}$ with $\beta_{2A}$-CaM\textsubscript{1234} (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 CaM\textsubscript{WT} and one CaM\textsubscript{1234} suggests that functional CaV\textsubscript{1.3} 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 CDI300 values when one or two CaM molecules are localized to the channel complex

| Construct | Ca2⁰⁺ r300 | Ba2⁺ r300 | CDI300 at +10 mV (mean ± S.E.) | p value |
|-----------|------------|-----------|-------------------------------|---------|
| α1D + β2A | 0.23 ± 0.02 | 0.97 ± 0.01 | 0.765 ± 0.025 (n = 8) | NA |
| α1D + β2A-CaMWT | 0.17 ± 0.06 | 0.94 ± 0.03 | 0.820 ± 0.057 (n = 6) | 0.9231 |
| α1D + β2A-CaM1234 | 0.96 ± 0.02 | 1.00 ± 0.01 | 0.033 ± 0.029 (n = 7) | <0.0001 |
| α1D-CaMWT + β2A-CaMWT | 0.32 ± 0.03 | 0.98 ± 0.01 | 0.673 ± 0.036 (n = 8) | 0.3858 |
| α1D-CaMWT + β2A-CaM1234 | 0.23 ± 0.05 | 0.90 ± 0.03 | 0.746 ± 0.025 (n = 6) | 0.9996 |
| α1D-CaM1234 + β2A-CaMWT | 0.97 ± 0.09 | 0.99 ± 0.01 | 0.025 ± 0.084 (n = 5) | <0.0001 |
| α1D-CaM1234 + β2A + CaM | 0.91 ± 0.05 | 0.95 ± 0.05 | 0.041 ± 0.047 (n = 5) | <0.0001 |
| α1D + β2A-CaM1234 + CaM | 0.83 ± 0.05 | 0.86 ± 0.06 | 0.035 ± 0.025 (n = 5) | <0.0001 |

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

To delineate mechanisms that govern 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 CaMWT (termed α1D/2×IQ-CaMWT to denote CaMWT fusion to the pore-forming α-subunit) or CaM1234 (α1D/2×IQ-CaM1234) and co-expressed with β2A, β2A-CaMWT, or β2A-CaM1234. We observed strong CDI for α1D/2×IQ-CaMWT similar to CaV1.3 (Fig. 4A, Fig. S5A, and Table 3). By comparison, CDI of α1D/2×IQ-CaM1234 was sharply diminished similarly to α1D-CaM1234, 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 α1D/2×IQ-CaMWT with β2A-CaMWT also revealed strong CDI similarly to α1D/2×IQ-CaMWT co-expressed with β2A alone (Fig. 4C, Fig. S5C, and Table 3). However, when α1D/2×IQ-CaMWT is co-expressed with β2A-CaM1234, we observed a partial reduction in CDI (Fig. 4D, Fig. S5D, and Table 3). In like manner, co-expression of α1D/2×IQ-CaM1234 with β2A-CaMWT also showed a partial reduction in CDI (Fig. 4E, Fig. S5E, and Table 3). By contrast, localizing two-mutant CaM1234 to the channel complex by co-expressing α1D/2×IQ-CaM1234 with β2A-CaM1234 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 Ca²⁺-binding ability of carboxyl-terminally linked CaM. Instead, the stepwise change in CDI with one versus two-mutant CaM1234 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 Ca²⁺/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 Ca²⁺-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-NaV1.4IQ). 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 Ca²⁺-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 Ca²⁺/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 Ca²⁺/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 with similar underpinnings as CaV1.3 (15). Intriguing, whole-cell recordings of CaV1.3-NaV1.4IQ revealed recognizable CDI although with reduced magnitude compared with WT CaV1.3 (Fig. 5C, Fig. S6C, and Table S1). Taken together, these findings suggest that the CaV and NaV IQ domains are privileged in CaV/NaV channel modulation, and this domain likely plays an important role in orchestrating downstream structural rearrangements of channel cytosolic domains. Overall, these results are consistent with the possibility that the functional CaM stoichiometry for CaV1 is dictated by the number of IQ domains in the channel carboxyl terminus.
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 \(\alpha_{1A}\) and the \(\beta_{2A}\) subunits. For these experiments, the whole-cell dialyzate contained low Ca\(^{2+}\) buffering (1 mM EGTA) to permit global Ca\(^{2+}\) elevations necessary to trigger CDI. 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 CaMWT to the CT of \(\alpha_{1A}\) subunit (\(\alpha_{1A}\)-CaMWT) and co-expression with the \(\beta_{2A}\) subunit yields CDI comparable with baseline conditions (Fig. 6B, Fig. S7B, and Table S2). By contrast, fusion of CaM1234 to the CT of \(\alpha_{1A}\) subunit results in a reduction in CDI (Fig. 6C, Fig. S7C, and Table S2). Subsequently, we tested whether CaM fusion to the \(\beta_{2A}\) subunit also evoked similar regulatory effects. Accordingly, co-expression of \(\alpha_{1A}\) subunit with \(\beta_{2A}\)-CaMWT showed robust CDI comparable with baseline conditions (Fig. 6D, Fig. S7D, and Table S2), whereas \(\beta_{2A}\)-CaM1234 strongly diminished CDI (Fig. 6E, Fig. S7E, and Table S2). Thus, fusion of CaM to either the \(\alpha_{1A}\) or the \(\beta_{2A}\) 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 \(\alpha_{1A}\)-CaMWT with \(\beta_{2A}\)-CaMWT. As expected, this maneuver elicited strong CDI (Fig. 7A, Fig. S7F, and Table S2). To determine stoichiometric requirements, we measured CDI of \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\)-CaMWT. Comparison of Ca\(^{2+}\) versus Ba\(^{2+}\) currents revealed markedly blunted CDI (Fig. 7B, Fig. S7G, and Table S2). In comparison, co-expression of \(\alpha_{1A}\)-CaMWT with \(\beta_{2A}\)-CaM1234 revealed strong CDI (Fig. 7C, Fig. S7H, and Table S2) similar to untagged channels. As a control, we co-expressed of \(\alpha_{1A}\)-CaM1234 with \(\beta_{2A}\)-CaM1234 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 CaMWT could reverse CDI deficits of \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\) (Fig. S8, A, B, and D and Table S2). Indeed, CDI remained blunted for \(\alpha_{1A}\)-CaM1234 despite CaM overexpression. Similarly, overexpression of CaMWT failed to reverse the reduction in CDI of \(\alpha_{1A}\) in the presence of \(\beta_{2A}\)-CaM1234 (Fig. S8, E and F, Fig. 8H, 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 \(\alpha_{1A}\) 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.** A, \(\alpha_{1D}/2\times IQ\)-CaMWT with \(\beta_{2A}\) exhibits strong CDF. The format is as in Fig. 18.B, \(\alpha_{1D}/2\times IQ\)-CaM1234 with \(\beta_{2A}\) exhibit strongly reduced CDI. C, co-expression of \(\alpha_{1D}/2\times IQ\)-CaMWT with \(\beta_{2A}\)-CaMWT also demonstrates strong CDF. D, localizing one CaMWT and one CaM1234 to the CaV1.3 IQ channel complex by co-expressing \(\alpha_{1D}/2\times IQ\)-CaMWT with \(\beta_{2A}\)-CaM1234 results in a partial reduction in CDI. E, similarly, co-expression of \(\alpha_{1D}/2\times IQ\)-CaM1234 with \(\beta_{2A}\)-CaMWT also revealed a partial reduction in CDI. F, by contrast, localizing two CaM1234 molecules by expressing \(\alpha_{1D}/2\times IQ\)-CaM1234 with \(\beta_{2A}\)-CaM1234 results in a complete absence of CDI.
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 \(\alpha_{1A}\) and \(\beta_{2A}\) subunits supports CDF. Briefly, co-expression of \(\alpha_{1A}\)-CaMWT with the \(\beta_{2A}\) subunit elicits strong CDF, whereas \(\alpha_{1A}\)-CaM1234 exhibits strongly diminished CDF (Fig. 8 (B and C) and Table S3). In like manner, expressing \(\beta_{2A}\)-CaMWT with \(\alpha_{1A}\) subunit supports strong CDF, whereas \(\beta_{2A}\)-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 \(\alpha_{1A}\) and \(\beta_{2A}\) 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 \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\)-CaMWT. CDF was nearly absent for this pair (Fig. 9B and Table S3). By contrast, \(\alpha_{1A}\)-CaMWT in the presence of \(\beta_{2A}\)-CaM1234 revealed no appreciable change in CDF (Fig. 9D and Table S3). Taken together, these findings suggest that CaM linked to the \(\alpha_{1A}\) CT is privileged in triggering CDF. Of note, co-expression of \(\alpha_{1A}\)-CaM1234 with \(\beta_{2A}\)-CaM1234 exhibited minimal CDF (Fig. 9D and Table S3). As a further test, we probed whether freely diffusible recombinant CaMWT could reverse reduced CDF observed for \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\) or for \(\alpha_{1A}\) in the presence of \(\beta_{2A}\)-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 3

Comparison of CaV1.3 tandem IQ CDI300 values when one or two CaM molecules are localized to the channel complex

| Construct                        | \(\text{Ca}^{2+} r_{300}\) | \(\text{Ba}^{2+} r_{300}\) | \(\text{CDI}_{300}\) at +10 mV (mean ± S.E.) | \(p\) value |
|----------------------------------|----------------------------|----------------------------|-----------------------------------------------|-------------|
| \(\alpha_{1D/2}\)-M13-CaMWT + \(\beta_{2A}\) | 0.13 ± 0.02               | 0.81 ± 0.07               | 0.836 ± 0.020 (\(n=5\))                      | 0.3953      |
| \(\alpha_{1D/2}\)-M13-CaM1234 + \(\beta_{2A}\) | 0.89 ± 0.04               | 1.01 ± 0.04               | 1.24 ± 0.017 (\(n=6\))                      | <0.0001     |
| \(\alpha_{1D/2}\)-IQ-CaMWT + \(\beta_{2A}\)-CaMWT | 0.26 ± 0.01               | 0.97 ± 0.03               | 0.724 ± 0.017 (\(n=6\))                      | 0.7614      |
| \(\alpha_{1D/2}\)-IQ-CaMWT + \(\beta_{2A}\)-CaM1234 | 0.60 ± 0.05               | 0.94 ± 0.03               | 0.554 ± 0.070 (\(n=5\))                      | <0.0001     |
| \(\alpha_{1D/2}\)-IQ-CaM1234 + \(\beta_{2A}\)-CaMWT | 0.48 ± 0.05               | 0.98 ± 0.01               | 0.478 ± 0.061 (\(n=5\))                      | <0.0001     |
| \(\alpha_{1D/2}\)-IQ-CaM1234 + \(\beta_{2A}\)-CaM1234 | 0.99 ± 0.01               | 1.00 ± 0.01               | 0.011 ± 0.006 (\(n=5\))                      | <0.0001     |

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 \(\alpha_{1A}\) and \(\beta_{2A}\) supports CDF. Briefly, co-expression of \(\alpha_{1A}\)-CaMWT with the \(\beta_{2A}\) subunit elicits strong CDF, whereas \(\alpha_{1A}\)-CaM1234 exhibits strongly diminished CDF (Fig. 8 (B and C) and Table S3). In like manner, expressing \(\beta_{2A}\)-CaMWT with \(\alpha_{1A}\) subunit supports strong CDF, whereas \(\beta_{2A}\)-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 \(\alpha_{1A}\) and \(\beta_{2A}\) 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 \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\)-CaMWT. CDF was nearly absent for this pair (Fig. 9B and Table S3). By contrast, \(\alpha_{1A}\)-CaMWT in the presence of \(\beta_{2A}\)-CaM1234 revealed no appreciable change in CDF (Fig. 9C and Table S3). Taken together, these findings suggest that CaM linked to the \(\alpha_{1A}\) CT is privileged in triggering CDF. Of note, co-expression of \(\alpha_{1A}\)-CaM1234 with \(\beta_{2A}\)-CaM1234 exhibited minimal CDF (Fig. 9D and Table S3). As a further test, we probed whether freely diffusible recombinant CaMWT could reverse reduced CDF observed for \(\alpha_{1A}\)-CaM1234 in the presence of \(\beta_{2A}\) or for \(\alpha_{1A}\) in the presence of \(\beta_{2A}\)-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
FRET analysis suggests a stoichiometry of up to two Ca\(^{2+}\) bound to the carboxyl terminus of the CaM molecule following 800-ms depolarization. Studies demonstrate the interaction of multiple CaMs with disparate IQ domains, levels of inactivation at different voltages are assessed as the fraction of peak current remaining following depolarization. Figure 6. CaM to CaV2.1 via fusion to either the \(\alpha\) or \(\beta\) subunits preserves functional channel regulation. A, baseline CDI of CaV2.1 is assessed at low Ca\(^{2+}\) buffering. Representative traces correspond to Ca\(^{2+}\)- and Ba\(^{2+}\)-evoked currents. B, traces are scaled to about one-third actual magnitude to match Ca\(^{2+}\) traces. C, functional Ca\(^{2+}\)-regulation of channel gating depends primarily on CaM tethered to the CT of the \(\alpha\)-subunit. D, localization of a single CaM to the CaV2.1 complex by co-expression of \(\beta\) subunit supports robust CDI. E, CDI of CaV2.1 is strongly diminished when \(\beta\) subunit is co-expressed.

Results also indicate that the same CaM molecule prebound to the CaV channel CT is responsible for mediating both CDI and CDF.

Discussion

The stoichiometry of CaM interaction with the CaV channel complex and the functional requirements for channel regulation have long been debated. Biochemical and structural studies demonstrate the interaction of multiple CaMs with distinct channel peptide segments. FRET analysis suggests a stoichiometry of up to two Ca\(^{2+}\)/CaM molecules associating with the holo-CaV channel complex. Functional studies, however, indicate that a single CaM suffices for Ca\(^{2+}\)-dependent feedback regulation. To reconcile these differences, we dissected the potential role of multiple CaMs in orchestrating CaV feedback modulation. We localized up to two WT CaM or mutant CaM1234 to the CaV1-2 channel complex through linkage to the \(\alpha\) and \(\beta\) subunits. Consistent with prior studies, we found that a single CaM tethered to the CT is responsible for mediating both CDI and CDF (for CaV2.1) are fully intact; however, when CaM1234 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 CaV1.3 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 CaV1/2 gating.

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). 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 CaV1/2 channels; \(\beta\)-subunit–tethered CaMWT was unable to displace CaM1234 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-Kv7 channels and ryanodine receptors. Indeed, previous studies have shown that reducing free apo-CaM levels to nanomolar concentrations was insufficient to appreciably deplete apo-CaM preassociation from the CaV1.3 channel, suggesting a higher apo-CaM affinity. 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.

Second, our findings also point toward potential mechanisms that underlie the singular CaM stoichiometry observed for Ca\(^{2+}\)-dependent regulation of CaV gating. For CaV1.3 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. 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 CaV1.3 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 Ca²⁺ regulation. Importantly, NaV1.4 channels are homologous to CaV channels, and they undergo CDI in a similar manner as CaV 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 Ca²⁺-dependent regulation is that Ca²⁺/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 Ca²⁺/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 Ca²⁺ (55, 57). As such, if mutant CaM1234 were prebound to the channel CT, then the NSCaTE motif remains unoccupied at basal conditions (55, 57, 62). Following Ca²⁺ influx, the second CaMWT within the channel complex localized via the β subunit would be able to interact with NSCaTE segment and trigger CDI. However, the complete absence of Ca²⁺/CaM regulation when CaV1.2 and CaV1.3 α subunit is linked to mutant CaM1234 and β2A-CaM suggests that the simple interaction of the N-lobe of Ca²⁺/CaM with the NSCaTE domain is insufficient. Instead, these results suggest that a Ca²⁺-dependent conformational rearrangement of the CT-bound CaM is obligatory for CaM regulation. Fourth, for CaV2 channels, CaM regulation

**Figure 7. CaM fused to the CaV2.1 carboxyl terminus is privileged in triggering CDI.** A, co-expression of α1A-CaMWT with β2A-CaMWT results in robust CDI. The format is as in Fig. 18. B, by contrast, α1A-CaM1234 co-expressed with β2A-CaMWT results in strong reduction in CDI, suggesting that CT-fused CaM is privileged in modulating CDI. C, strong CDI is evident for α1A-CaMWT in the presence of β2A-CaM1234. D, CDI is strongly diminished when mutant CaM1234 is tethered to both the α1A and β2A subunits.
Figure 8. CaM tethered to both the CaV2.1 α and β subunits is able to trigger CDF. A, top left, schematic of WT CaV2.1 co-expressed with α2δ and β2A subunits. Top right, a two-pulse protocol is used to quantify the extent of CDF. Ba2+ 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 Ca2+ current with an initial rapid phase followed by a slow phase corresponding to Ca2+-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 (ΔQ) approximates CDF triggered by the prepulse. Bottom left, population data (mean ± S.E.) shows RF (relative facilitation) at different prepulse potentials averaged form n cells and assessed as ΔQ divided by the time constant (t) of facilitation. CDF is determined as the difference in RF with Ba2+ versus Ca2+ as charge carrier. B, fusion of CaMWT to the carboxyl terminus of α1A subunit (α1A-CaMWT) supports strong CDF. C, fusion of CaM1234 to the α1A carboxyl terminus (α1A-CaM1234) abolishes CDF. D, co-expression of β2A-CaMWT with α1A elicits strong CDF. E, CDF is abolished in the presence of β2A-CaM1234.
manifests as both CDF and CDI with distinct spatial Ca²⁺ 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 Ca²⁺-free CaM is enriched in the dyad with a large fraction bound to the RyR (64, 81). Following Ca²⁺ binding, however, CaM is mobilized and is available to interact with targets including Ca²⁺/CaM-dependent kinases and phosphatases that are also localized at the dyad (81). Our findings suggest that Ca²⁺-mobilized CaM would be unable to inhibit the L-type Ca²⁺-channels. Instead, only CaM initially preassociated with the channel CT would be able to trigger Ca²⁺ 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 Cav channel inactivation, an essential factor for normal cardiac repolarization (5). In the disease context, this arrangement, however, makes L-type channels particularly vulnerable to mis-regulation in cardiac arrhythmias associated with calmodulino-pathies (10). The singular functional stoichiometry implies that the preassociation of even a small fraction of mutant CaM with weakened Ca²⁺ binding could appreciably disrupt L-type channel inactivation and increase risk of arrhythmogenesis. In like manner, in neurons, CaM localized to Ca²⁺ channels serve multiple functions (44), including modulation of channel gating, trafficking (30, 86), and a key role in excitation-transcription coupling, where local Ca²⁺ signaling near L-type channels results in rapid shuttling of Ca²⁺/CaM to the nucleus through γCaMKII (34, 87, 88). Having a resident CaM dedicated for Cav channel feedback modulation ensures that local Ca²⁺/CaM signaling can be multiplexed without detrimental effects on cellular electrical excitability.
Experimental procedures

**Molecular biology**

CaV1.2, CaV1.3, and CaV2.1 variant were unmodified from previously published constructs: rabbit CaV1.2 (NM001136522) (89), CaV1.2-glycine-(12)-CaMWT and CaV1.2-glycine-(12)-CaM1234 (59); rat CaV1.3 (AF370009.1) (78), CaV1.3-glycine-(12)-CaMWT and CaV1.3-glycine-(12)-CaM1234 (69). Human CaV1.2.1 clones (NP_001120693.1) were as previously published (80) with the exact splice background being Δ10A (+ G); 16+/17+; Δ17A (-VEA); -31* (−NP); 37a (EFa); 43+/44−; 47− (90). CaV1.2-glycine-(12)-CaMWT and CaV1.2-glycine-(12)-CaM1234 were obtained by attaching a polyglycine linker of length 8 fused to CaMWT/CaM1234 immediately downstream of the channel IQ domain (following the protein sequence MREEQ, residue 181) using PCR amplification and ligation using restriction enzyme sites XbaI and SalI. The channel IQ domain was constructed using gene synthesis. To do so, we utilized a CaV1.3 channel with a silent mutation at 1538GT1539 that introduces a KpnI restriction site (by replacing GGGACA with GGTACC). We synthesized (GENEWIZ) a tandem IQ domain using the normalized charge difference \( RF \) divided by the slow time constant \( t \) of facilitation, yielding relative facilitation \( RF = \Delta Q/t \). 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). \( RF_{Ca} \) corresponds to relative facilitation with \( Ca^{2+} \) as charge carrier, whereas \( RF_{Ba} \) corresponds to that obtained with \( Ba^{2+} \) as charge carrier representing voltage-dependent facilitation. CDF is measured as the difference \( RF_{Ca} - RF_{Ba} \).

**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 CsMeSO3, 5 mM CsCl2, 1 mM MgCl2, 4 mM MgATP, 10 mM HEPES, 10 mM BAPTA, adjusted to 295 mosm with CsMeSO3 and pH 7.4 with CsOH. The external solution contained 140 mM TEA-MeSO3, 10 mM HEPES, 40 mM CaCl2 or BaCl2, adjusted to 330 mosm with TEA-MeSO3 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 CsMeSO3, 5 mM CsCl2, 1 mM MgCl2, 4 mM MgATP, 10 mM HEPES, 1 mM EGTA, adjusted to 295 mosm with CsMeSO3 and pH 7.4 with CsOH. The external solution contained 140 mM TEA-MeSO3, 10 mM HEPES, 5 mM CaCl2 or BaCl2, adjusted to 330 mosm with TEA-MeSO3 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 (\( t_{300} \)) of depolarization for CaV1 or 800 ms (\( t_{800} \)) 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 \( z \) prepulse as reported previously (43). The fraction of channels facilitated by prepulse is directly proportional to \( \Delta Q \) divided by the slow time constant \( t \) of facilitation, yielding relative facilitation \( RF = \Delta Q/t \). 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). \( RF_{Ca} \) corresponds to relative facilitation with \( Ca^{2+} \) as charge carrier, whereas \( RF_{Ba} \) corresponds to that obtained with \( Ba^{2+} \) as charge carrier representing voltage-dependent facilitation. CDF is measured as the difference \( RF_{Ca} - RF_{Ba} \).

**Cell culture and transfection of HEK293 cells**

For whole-cell electrophysiology, HEK293 cells were cultured on glass coverslips in 60-mm dishes and transfected using a calcium phosphate method (28). We applied 2–4 μg of cDNA encoding the desired channel α1 subunit (WT or CaM-linked variant), along with 4 μg of rat brain β2A (WT or CaM-linked) and 4 μg of rat brain α2δ subunits (NM012919.2) (92). To enhance expression, cDNA for simian virus 40 T antigen (1 μg) was co-transfected. Electrophysiology recordings were done at room temperature 1–2 days after transfection.

**Stoichiometry of CaM in tuning CaV feedback**

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

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extraordinary mentor and teacher, and his unbridled passion for science continues to inspire us.

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Abbreviations—The abbreviations used are: CaM, calmodulin; CDI, Ca\(^{2+}\)-dependent inactivation; CT, carboxyl tail; NSCaTE, N-terminal spatial Ca\(^{2+}\)-transforming element; CDF, Ca\(^{2+}\)-dependent facilitation; ANOVA, analysis of variance; TEA, tetraethylammonium; VDI, voltage—dependent inactivation.

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