Dynamic switching of calmodulin interactions underlies Ca\(^{2+}\) regulation of Ca\(_V\)1.3 channels

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Calmodulin regulation of Ca\(_V\) channels is a prominent Ca\(^{2+}\) feedback mechanism orchestrating vital adjustments of Ca\(^{2+}\) entry. The long-held structural correlation of this regulation has been Ca\(^{2+}\)-bound calmodulin, complexed alone with an IQ domain on the channel carboxy terminus. Here, however, systematic alanine mutagenesis of the entire carboxyl tail of an L-type Ca\(_V\)1.3 channel casts doubt on this paradigm. To identify the actual molecular states underlying channel regulation, we develop a structure–function approach relating the strength of regulation to the affinity of underlying calmodulin/channel interactions, by a Langmuir relation (individually transformed Langmuir analysis). Accordingly, we uncover frank exchange of Ca\(^{2+}\)–calmodulin to interfaces beyond the IQ domain, initiating substantial rearrangements of the calmodulin/channel complex. The N-lobe of Ca\(^{2+}\)–calmodulin binds an N-terminal spatial Ca\(^{2+}\) transforming element module on the channel amino terminus, whereas the C-lobe binds an EF-hand region upstream of the IQ domain. This system of structural plasticity furnishes a next-generation blueprint for Ca\(_V\) channel modulation.
Calmodulin (CaM) regulation of the CaV1–1.2 family of Ca\(^{2+}\) channels ranks among the most consequential of biological Ca\(^{2+}\) decoding systems.\(^{12}\) In this regulation, the Ca\(^{2+}\)-free form of CaM (apoCaM) already pre-associates with channels\(^{3-5}\), ready for ensuing Ca\(^{2+}\)-driven modulation of channel opening. Upon elevation, intracellular Ca\(^{2+}\) binds to this indwelling CaM, driving conformational changes that enhance opening in some channels\(^{6-9}\) (positive-feedback ‘facilitation’) and inhibit opening in others\(^{9,10}\) (negative-feedback ‘inactivation’). Intriguingly, Ca\(^{2+}\) binding to the individual carboxy- and amino-terminal lobes of CaM can semiautomously induce distinct components of channel regulation\(^{7,11}\), where the C-lobe responds well to Ca\(^{2+}\) entering through the channel on which the corresponding CaM resides (‘local Ca\(^{2+}\) selectivity’), and the N-lobe may, in some channels, require the far weaker Ca\(^{2+}\) signal from distant Ca\(^{2+}\) sources\(^{6,7,12-14}\) (‘global Ca\(^{2+}\) selectivity’). Such Ca\(^{2+}\)-feedback regulation influences many biological functions\(^{1,15-17}\) and furnishes mechanistic lessons for Ca\(^{2+}\) decoding\(^{14}\). Indeed, CaM regulation of L-type (CaV1.2) channels strongly influences cardiac electrical stability\(^{15,18}\), and pharmacological manipulation of such regulation looms as a future antiarrhythmic strategy\(^{18,19}\).

Crucial for understanding and manipulating this CaM regulatory system is the identification of the conformations that underlie such Ca\(^{2+}\) modulation. Figure 1a summarizes the currently accepted conceptual framework, with specific reference to L-type CaV1.3 channels for concreteness. Configuration E (‘empty’ of CaM) represents channels lacking preassociated apoCaM. Such channels can open normally, but do not exhibit Ca\(^{2+}\)/CaM-dependent inactivation (CDI) over the typical ~300 ms duration of channel-activity measurements\(^{20}\). Over this period, Ca\(^{2+}\)/CaM from the bulk solution cannot appreciably access a channel in configuration E to produce CDI\(^{20-23}\). ApoCaM preassociation with configuration E yields channels in configuration A, where opening can also proceed normally, but subsequent CDI can now ensue. A thereby denotes channels that are ‘active’ and capable of CDI. Switching between configurations E and A occurs slowly (>10 s of seconds\(^{24}\)), and almost exclusively involves apoCaM, because typical experiments only briefly activate Ca\(^{2+}\) channels every 20–30 s. Thus, there is negligible exchange with configuration E during typical measurements of current. Regarding CDI, Ca\(^{2+}\) binding to both lobes of CaM yields configuration \(I_{CN}\) (both C- and N-lobes of CaM engaged towards CDI), corresponding to a fully inactivated channel with strongly reduced opening\(^{22,25}\). As for intermediate configurations\(^{20,21,25}\), Ca\(^{2+}\) binding only to the C-lobe induces configuration \(I_{C}\), representing a C-lobe inactivated channel with reduced opening; Ca\(^{2+}\) binding only to the N-lobe yields an analogous N-lobe-inactivated configuration \(I_{N}\), also with reduced opening. Subsequent entry into configuration \(I_{CN}\)...

**Figure 1 | General schema for CaM regulation of representative L-type CaV1.3 channels.** (a) Primary configurations of CaM/channel complex with respect to CaM-regulatory phenomena (E, A, \(I_{C}\), \(I_{N}\) and \(I_{CN}\)). Inset at far right, cartoon of main channel landmarks involved in CaM regulation, with only the pore-forming \(\lambda_{1}\) subunit of CaV1.3 diagrammed. Ca\(^{2+}\)-inactivation (CI) region, in the proximal channel C terminus (~160 amino acids (aa)), contains elements potentially involved in CaM regulation. IQ domain (IQ), comprising the C-terminal ~30 aa of the CI segment, long proposed as preeminent for CaM/channel binding. Dual vestigial EF-hand (EF) motifs span the proximal C-terminus of the CI region. \(I_{CN}\) (both C- and N-lobes of CaM engaged towards CDI), corresponding to a fully inactivated channel with strongly reduced opening\(^{22,25}\). As for intermediate configurations\(^{20,21,25}\), Ca\(^{2+}\) binding only to the C-lobe induces configuration \(I_{C}\), representing a C-lobe inactivated channel with reduced opening; Ca\(^{2+}\) binding only to the N-lobe yields an analogous N-lobe-inactivated configuration \(I_{N}\), also with reduced opening. Subsequent entry into configuration \(I_{CN}\)...

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likely involves cooperative interactions denoted by a $\lambda$ symbol. Overall, CDI reflects redistribution from configuration $A$ into $I_C$, $I_N$ and $I_{CN}$. Of note, we exclude cases where one Ca$^{2+}$ binds to a lobe of CaM, because binding within lobes is highly cooperative$^{27}$. Moreover, only one CaM is included, based on multiple lines of evidence$^{22,23}$.

The structural basis of this conceptual foundation is less certain, but has been dominated by an IQ-centric hypothesis, where an IQ domain, present on the C termini of all CaV1–2 channels$^2$ (Fig. 1a, far right, blue circle), serves as the dominant CaM-binding locus on the channel. By this hypothesis, not only does this element comprise much of the preassociation surface for apoCaM$^{4,5,20}$ (Fig. 1a, configuration A), it also constitutes the primary effector site$^{5,7,9,10,25,28}$. For Ca$^{2+}$/CaM rebinding to induce Ca$^{2+}$ regulation (for example, Fig. 1a, $I_{CN}$). The predominance of the IQ-centric paradigm$^{2}$ has prompted resolution of several crystal structures of Ca$^{2+}$/CaM complexed with IQ-domain peptides of various CaV1–2 channels$^{29–32}$.

Nonetheless, certain findings fit poorly with this viewpoint. First, crystal structures of Ca$^{2+}$/CaM complexed with wild-type (WT) and mutant IQ peptides of CaV1.2 indicate that a signature isoform line the IQ module is deeply buried within the C-lobe of Ca$^{2+}$/CaM, and that anilinium substitution at this site negligibly perturbs structure$^{30}$. Moreover, Ca$^{2+}$/CaM affinities for analogous WT and mutant IQ peptides are nearly identical$^{12}$.

How then does anilinium substitution at this well-encapsulated locus influence the rest of the channel to strongly disrupt functional regulation?$^{30}$ Second, in CaV1.2/1.3 channels we have demonstrated that the effector interface for the N-lobe of Ca$^{2+}$/CaM resides within an N-terminal spatial Ca$^{2+}$ transforming element (NScTE) of the channel N terminus$^{13,14,33}$ (Fig. 1a, far right), separate from the IQ element. Third, analysis of the atomic structure of Ca$^{2+}$/CaM bound to an IQ peptide of CaV2.1 channels hints that the C-lobe effector site also resides somewhere outside the IQ module$^1$. In all, the long disconnect between challenges like these and IQ-centric theory represents a critical impasse in the field.

A major concern with prior IQ-domain analyses is that function was mostly characterized with only endogenous CaM present$^{5,10,25,28,31}$. This regime is problematic, as IQ-domain mutations could alter CaM regulation via perturbations at multiple steps within Fig. 1a, whereas interpretations mainly ascribe effects to altered Ca$^{2+}$/CaM binding with an IQ effector site. Serious interpretive challenges thus include the following: (1) although the high apoCaM affinity of most WT channels$^{4,20}$ renders configuration $E$ unlikely (Fig. 1a), this may not hold true for mutant channels, just as observed for certain CaV1.3 splice variants$^{20}$. Mutations weakening apoCaM preassociation could thereby reduce CDI by favouring configuration $E$ (Fig. 1a, incapable of CDI), without affecting Ca$^{2+}$/CaM binding. (2) Mutations that do weaken interaction with one lobe of Ca$^{2+}$/CaM may have their functional effects masked by cooperative steps ($\lambda$ in Fig. 1a).

This study systematically investigates the IQ-centric hypothesis, minimizing the above challenges by focusing on CaV1.3 channels, a representative L-type channel whose CDI is particularly robust and separable into distinct C- and N-lobe components$^{11,13,14}$. These attributes simplify analysis as follows. For orientation, Fig. 1b illustrates the CDI of CaV1.3 channels expressed in HEK293 cells, with only endogenous CaM present. Strong CDI is evident from the rapid decay of whole-cell Ca$^{2+}$ current (black trace) compared with the nearly absent decline of Ba$^{2+}$ current (gray trace). As Ba$^{2+}$ binds negligibly to CaM$^{34}$, the fractional decline of Ca$^{2+}$ versus Ba$^{2+}$ current after 300 ms depolarization quantifies the steady-state extent of CDI (Fig. 1b, right, CDI parameter). The CDI here reflects the operation of the entire Fig. 1a system, as schematized at the bottom of Fig. 1b. We can formally isolate the diamond-shaped subsystem lacking configuration $E$ (Fig. 1c, bottom), by using mass action and strong overexpression of WT CaM (CaM$_{WT}$). The resulting CDI (Fig. 1c) is indistinguishable from that with only endogenous CaM present (Fig. 1b), owing to the high apoCaM affinity of WT CaV1.3 channels. Full deconstruction of CDI arises upon strong coexpression of channels with a mutant CaM that only allows Ca$^{2+}$ binding to its C-terminal lobe$^6$ (Fig. 1d, CaM$_{I2}$).

With respect to Fig. 1a, this manoeuvre depopulates configuration $E$ by mass action, and forbids access into configurations $I_N$ and $I_{CN}$. Thus, the isolated C-lobe component of CDI$^{11,14}$ is resolved (Fig. 1d), with its signature rapid timecourse of current decay. Importantly, this regime avoids interplay with cooperative $\lambda$ steps in Fig. 1a. Likewise, strongly coexpressing mutant CaM exhibiting Ca$^{2+}$ binding to its N-lobe alone$^6$ (CaM$_{E4}$) isolates the slower N-lobe form of CDI$^{11,14}$ (Fig. 1e), with attendant simplifications.

Thus armed, we here exploit selective monitoring of CaV1.3 subsystems (Fig. 1b–e), combined with alanine, scanning mutagenesis of the entire carboxyl tail of CaV1.3 channels. In doing so, we argue against the IQ-centric paradigm and propose a new framework for the CaM regulation of Ca$^{2+}$/CaM channels.

**Results**

**Individually transformed Langmuir analysis of CaM/channel regulation.** Identifying channel effector interfaces for Ca$^{2+}$/CaM is challenging. The main subunit of CaV channels alone spans about 2,000 amino acids or more; and peptide assays indicate that Ca$^{2+}$/CaM can bind to multiple segments of uncertain function$^{25,35–39}$. Even if mutating these segments alters CaM regulation, the observed functional effects could reflect perturbations of apoCaM preassociation, Ca$^{2+}$/CaM binding or transduction. To address these challenges, we initially consider an expanded conceptual layout believed valid for either isolated N- or C-lobe CDI$^{14}$ (Fig. 2a), then deduce from this arrangement a simple quantitative analysis to identify bona fide effector interfaces. An apoCaM lobe begins prebound to a channel preassociation surface (state 1), Ca$^{2+}$/CaM binding to CaM in this prebound state is considered rare$^{14,40}$. However, after apoCaM releases (state 2), it may bind Ca$^{2+}$ to produce Ca$^{2+}$/CaM (state 3), or return to state 1. The transiently dissociated lobe of CaM (state 2 or 3) remains within a channel above the usual timescale of CaM regulation ($<\text{seconds}$). Finally, Ca$^{2+}$/CaM binds a channel effector site (state 4, square pocket), ultimately inducing regulation via transduction to state 5. Emergent behaviours of this scheme rationalize local and global Ca$^{2+}$ selectivities, as argued previously$^{14}$.

Despite the multiple transitions present even for this reduced CDI subsystem (Fig. 2a, left schematics), a straightforward relationship emerges that will aid detection of Ca$^{2+}$/CaM interfaces on the channel, as follows. Suppose we can introduce point alanine mutations into the channel that selectively perturb the Ca$^{2+}$/CaM-binding equilibrium association constant $K_{\text{eff}}$ (Fig. 2a). Moreover, suppose we can measure Ca$^{2+}$/CaM binding to a corresponding channel peptide, and the associated association constant $K'_{\text{eff}}$ is proportional to $K_{\text{eff}}$. It then turns out that our metric of inactivation (CDI in Fig. 1b) will always be given by the Langmuir function $C$.

\[
C(t) = C_{\text{max}} - C_{\text{max}} K_{\text{eff}} / (K_{\text{eff}} + t)\]

where $C_{\text{max}}$ is the value of CDI if $K_{\text{eff}}$ becomes exceedingly large, and $\Lambda$ is a constant comprised of other association constants in the layout (Supplementary Note S1). Figure 2b plots this function, where the green symbol marks a hypothetical WT
channel position, and mutations should create data symbols that decorate the remainder of the curve. Importantly, the requirement that peptide $K_{a,EFF}$ need only be proportional to (not equal to) holochannel $\gamma_1$ increases the chances that tagged peptides may suffice to correlate with holochannel function. In addition, equation 1 will hold true only if these two suppositions are satisfied (Supplementary Note S2). For example, if mutations alter two transitions within the holochannel, a function with different shape will result. Alternatively, if mutations change the peptide interaction with $Ca^{2+}/CaM (K_{a,EFF})$, but not any of the actual association constants within the channel, the outcome in Fig. 2c will emerge. In this case, the channel peptide can bind $Ca^{2+}/CaM$ in isolation, this reaction has no bearing on transitions within the intact holochannel (Fig. 2a). By contrast, Fig. 2d diagrams a scenario where mutations actually do affect transition(s) governing $Ca^{2+}/CaM$ binding CDI (defined Fig. 1b, right) as a function of $K_{a,EFF}$. If $K_{a,EFF}$ is proportional to one of the actual association constants in the scheme as in a. Black symbols, hypothetical results for various channel/peptide mutations; green symbol, hypothetical WT. (b) Unique Langmuir relation (equation 1) that will emerge upon plotting channel CDI (defined Fig. 1b, right) as a function of $K_{a,EFF}$ (association constant measured for isolated channel peptide), if $K_{a,EFF}$ is proportional to one of the actual association constants in the scheme as in a. Black symbols, hypothetical results for various channel/peptide mutations; green symbol, hypothetical WT. (c) Predicted outcome if peptide association constant $K_{a,EFF}$ has no bearing on association constants within holochannels. (d) Outcome if mutations affect holochannel association constants, but not peptide association constants. (e) Outcome if mutations affect holochannel association constant(s) and peptide association constant, but in ways that are poorly correlated.

**Figure 2** Probing functionally relevant CaM regulatory interactions via iTL analysis. (a) Isolated C- or N-lobe regulatory system (denoted by stick-figure diagrams on left) can be coarsely represented by a five-state scheme on right. A single lobe of apoCaM begins preasssociated to channel (state 1). Following disassociation (state 2), CaM may bind two $Ca^{2+}$ ions (state 3, black dots). $Ca^{2+}/CaM$ may subsequently bind to channel effector site (state 4). From here, transduction step leads to state 5, equivalent to CDI. Association constant for lobe of apoCaM binding to preassociation site is $\varepsilon$; whereas $\gamma_1$ and $\gamma_2$ are association constants for respective transitions from states 3 to 4, and states 4 to 5. (b) Unique Langmuir relation (equation 1) that will emerge upon plotting channel CDI (defined Fig. 1b, right) as a function of $K_{a,EFF}$ (association constant measured for isolated channel peptide), if $K_{a,EFF}$ is proportional to one of the actual association constants in the scheme as in a. Black symbols, hypothetical results for various channel/peptide mutations; green symbol, hypothetical WT. (c) Predicted outcome if peptide association constant $K_{a,EFF}$ has no bearing on association constants within holochannels. (d) Outcome if mutations affect holochannel association constants, but not peptide association constants. (e) Outcome if mutations affect holochannel association constant(s) and peptide association constant, but in ways that are poorly correlated.

**iTL analysis of IQ domain as $Ca^{2+}/CaM$ effector site.** We first addressed whether the $Ca_{1.3}$ IQ domain serves as a $Ca^{2+}/CaM$ effector site for CDI, as IQ-centric theory postulates. Single alanines were substituted at each position of the entire IQ domain of $Ca_{1.3}$ channels, whose sequence appears atop Fig. 3a, with the signature isoleucine bolded at position '0'. Naturally occurring alanines were changed to threonine. CDI of these mutants was then characterized for the isolated N- and C-lobe CDI subsystems described above (Fig. 3a,b, left schematics), thus minimizing potential complications from diminished preassociation with apoCaM (Fig. 1a, configuration $E$), or masking of CDI effects by cooperative $k$ steps (Fig. 1a). Whereas little deficit in N-lobe CDI was observed (Fig. 3a), the C-lobe CDI was strongly attenuated by alanine substitutions at I[0]A (Fig. 3b, red bar, exemplar traces) and nearby positions (rose). To test for correspondence between reductions in C-lobe CDI and altered $Ca^{2+}/CaM$ binding, we performed FRET two-hybrid assays of $Ca^{2+}/CaM$ binding to alanine-substituted IQ peptides, with substitutions encompassing sites associated with the strongest CDI effects (Fig. 3b, red and rose bars). Hatched bars denote additional sites chosen at random. The left aspect of Fig. 3c cartoons the FRET interaction partners, and the right portion displays the resulting binding curve for the WT IQ peptide (Fig. 3c, right, black). FR-1 is proportional to FRET efficiency, as indicated by the efficiency $E_A$ scale bar on the right. $D_{free}$ is the free concentration of donor-tagged molecules (cyan fluorescent protein (CFP)–CaM), where 200 nM is approximately 6,100 $D_{free}$ units. At odds with a $Ca^{2+}/CaM$ effector role of the IQ domain, the binding curve for the I[0]A substitution (Fig. 3c, right, red) resembled that for the WT peptide (black), whereas C-lobe CDI was strongly decreased (Fig. 3b). Figure 3c (middle) displays a bar-graph summary of the

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resulting association constants ($K_{a,EFF}$); the WT value is shown as a dashed green line, and that for I[0]A as a red bar (Supplementary Note S5). If the IQ domain were the effector site for the C-lobe of Ca$^{2+}$/CaM, C-lobe CDI over various substitutions should correlate with association constants according to equation 1 (Supplementary Notes S1 and S6). However, our data markedly deviate from such a relation (Fig. 3e), much as in Fig. 2e. The green symbol denotes the WT IQ case. Likewise, plots of N-lobe CDI versus $K_{a,EFF}$ deviate from a Langmuir (blue symbols, Fig. 3d), much as in Fig. 2c. These outcomes fail to support the IQ domain as an effector site for either lobe of Ca$^{2+}$/CaM. The actual role of the IQ domain in CDI will be explored later in Fig. 6.

To undertake a still more stringent test, we investigated a Y[3]D construct, based on a prior analogous mutation in Ca$\text{v}$2.1 that intensely diminished Ca$^{2+}$/CaM affinity$^{31}$. Indeed, the Y[3]D substitution in Ca$\text{v}$1.3 resulted in a large 13.5-fold decrement in $K_{a,EFF}$ (Fig. 3c, blue symbol). However, there was no change in C-
or N-lobe CDI (Fig. 3a,b, blue symbols; Supplementary Note S7). These data deviated yet more strongly from a Langmuir (blue symbols, Fig. 3d,e), arguing further against the IQ domain as a Ca$^{2+}$/CaM effector site.

**NSCaTE element upheld as effector site for N-lobe of Ca$^{2+}$/CaM.** Given the absence of a positive outcome for iTL analysis of the IQ domain (that is, Fig. 2b), we turned to the N-terminal NSCaTE module (Fig. 4a, oval), previously proposed as an effector site for N-lobe CDI$^{13,14}$. For reference, Fig. 4b displays the WT Ca$\text{v}$1.3 profile for N-lobe CDI. Single alanines were substituted across the NSCaTE module (Fig. 4d, top), at residues that were not originally alanine. The bar-graph summary below (Fig. 4d) indicates strongly diminished N-lobe CDI upon alanine substitution at three residues, previously identified as critical$^{13,14}$ (W[44]A, I[48]A and R[52]A). For comparison, the

Figure 3 | Inconsistencies with IQ domain role as Ca$^{2+}$/CaM effector site. (a) No appreciable deficit in isolated N-lobe CDI upon point alanine substitutions across the IQ domain (sequence at top with bolded isoleucine at ‘0’ position). Left, corresponding subsystem schematic. Middle, bar-graph summary of CDI metric, as defined in Fig. 1b. Bars, mean ± s.e.m. for ~6 cells each. Green dashed line, WT profile; red bar, I[0]A; blue symbol in all panels, Y[3]D. Right, exemplar currents, demonstrating no change in N-lobe CDI upon I[0]A substitution. Horizontal scale bar, 100 ms; vertical scale bar, 0.2 nA Ca$^{2+}$ current. Red, Ca$^{2+}$ current; gray, Ba$^{2+}$ current. (b) Isolated C-lobe CDI (corresponding subsystem schematized on left) exhibits significant attenuation by mutations surrounding the central isoleucine (coloured bars). Format as in a. I[0]A shows the strongest attenuation (red bar and exemplar currents at right). Bars average ~5 cells ± s.e.m. Dashed green line, WT profile. Timebase as in b, vertical scale bar, 0.2 nA Ca$^{2+}$ current. (c) Bar-graph summary of association constants ($K_{a,EFF}$) for Ca$^{2+}$/CaM binding to IQ, evaluated for constructs exhibiting significant effects in b (coloured bars, with I[0]A in red), or chosen at random (hashed in b). Error bars, non-linear s.d. estimates. FRET partners schematized on the left, and exemplar binding curves on the right for I[0]A (red) and WT (black). Symbols average ~7 cells. Smooth curve fits, 1:1 binding model. Calibration to efficiency $E_a=0.1$, far right vertical scale bar. Horizontal scale bar corresponds to 100 nM. (d) Plots of N-lobe CDI versus $K_{a,EFF}$ deviate from equation 1, much as in Fig. 2c. Green, WT; red, I[0]A; blue, Y[3]D. (e) Plots of C-lobe CDI versus $K_{a,EFF}$ also diverge from Langmuir, as in Fig. 1e. This result further argues against the IQ per se acting as an effector site for the C-lobe of Ca$^{2+}$/CaM. Symbols as in d. (d,e) Y[3]D (blue symbol, CDI mean of four cells) yields poor Ca$^{2+}$/CaM binding, but unchanged CDI. Supplementary Note S7, further FRET data.
WT level of CDI is represented by the green dashed line and affiliated error bars. W[44]A featured the strongest CDI decrement, as shown by the Ca\(^{2+}\) current (Fig. 4b, red trace) and population data (Fig. 4d, red bar). To pursue iTL analysis, we characterized corresponding binding curves between NSCaTE and Ca\(^{2+}\)/CaM\(_{34}\) FRET pairs (Fig. 4c, left; Supplementary Note S8). The WT pairing exhibited a well-resolved binding curve with \(K_{\text{a,EFF}} = 4 \times 10^{-4}\) units (Fig. 4c, right, black), whereas the W[44]A variant yielded a far lower affinity with \(K_{\text{a,EFF}} \approx 0\) (red). A summary of binding affinities is shown for this and additional mutations within NSCaTE in Fig. 4e (Supplementary Note S9), where the dashed green line signifies the WT profile. The crucial test arises by plotting N-lobe CDI as a function of \(K_{\text{a,EFF}}\), which resolves the Langmuir relation in Fig. 4f. For reference, WT is shown in green and W[44]A in red. The particular formulation of equation 1 for this arrangement is given in Supplementary Note S1. Hence, iTL analysis does uphold NSCaTE as a predominate effector site for N-lobe CDI, as argued before by other means\(^{13,14}\). By contrast, analysis of C-lobe CDI (Fig. 4g–k and Supplementary Note S10) reveals deviation from equation 1 (Fig. 4l), much as in Fig. 2c. Thus, NSCaTE mutations have little bearing on C-lobe CDI of the holochannel, though such mutations affect Ca\(^{2+}\)/CaM\(_{12}\) binding to an isolated NSCaTE peptide.

Identification of the C-lobe Ca\(^{2+}\)/CaM effector interface. Satisfied by proof-of-principle tests of the iTL approach, we turned to identification of the as-yet-unknown effector site for the C-lobe form of CDI. Our screen focused upon the entire carboxy tail of CaV1.3 channels upstream of the IQ domain (Fig. 5a,
Figure 5 | iTL analysis of PCI segment as C-lobe Ca\(^{2+}\)/CaM effector interface. (a) Channel cartoon depicting PCI segment as putative effector site for C-lobe of Ca\(^{2+}\)/CaM. (b) Isolated N-lobe CDI for WT and LGF → AAA (LGF) mutant channels. Ca\(^{2+}\) current for WT in red, and for LGF in red. Gray, averaged Ba\(^{2+}\) trace. Horizontal scale bar, 100 ms; vertical scale bar, 0.2 nA Ca\(^{2+}\) current (red, LGF; black, WT). (c) Isolated C-lobe CDI for WT and LGF mutant channels, indicating strong CDI attenuation by LGF mutation. Format as in b. (d) FRET two-hybrid binding curves for Ca\(^{2+}\)/CaM pitted against PCI segments, for WT (black) and LGF (red). Each symbol, mean ± s.e.m. from ~9 cells. (e) Bar-graph summary confirming no appreciable reduction of isolated N-lobe CDI, over all alanine scanning mutants across the PCI region (sequence at the top). Schematic of corresponding system under investigation at the left. Green dashed line, WT; red, LGF mutant; gaps, non-expressing configurations. Bars, mean ± s.e.m. of ~5 cells. (f) Bar-graph summary, C-lobe CDI for alanine scan of PCI. Red bar, LGF → AAA mutant showing strong CDI reduction. Rose bar, other loci showing substantial CDI reduction. Hashed, randomly chosen loci for subsequent FRET analysis below. Bars, mean ± s.e.m. of ~5 cells. (e,f) CDI decrease for YLT cluster (Fig. 5e,f) reflects reduced Ca\(^{2+}\) entry from 30 mV depolarizing shift in activation, not CDI attenuation per se. Shifts for all other loci were at most ± 10 mV (not shown). (g) Association constants for Ca\(^{2+}\)/CaM binding to PCI region, with FRET partners as diagrammed on the left. Green dashed line, WT profile. PCI mutations yielding large C-lobe CDI deficits were chosen for FRET analysis (red and rose in i), as well as those chosen at random (hashed in i). Error bars, nonlinear estimates of standard deviation. (h) Plots of N-lobe CDI versus \(K_{\text{eff}}\) for Ca\(^{2+}\)/CaM binding to PCI deviated from Langmuir. Red, LGF; green, WT. (i) Alternatively, plotting C-lobe CDI revealed Langmuir relation, supporting PCI as C-lobe Ca\(^{2+}\)/CaM effector site. Symbols as in h.
C-lobe CDI also requires IQ domain interaction with the PCI element. Though the IQ domain alone does not appear to be an effector site for Ca\(^{2+}\)/CaM (Fig. 3), alanine substitutions in this element nonetheless attenuated the C-lobe CDI\(^{7,10,11,28,31}\), a result reproduced for reference in Fig. 6a,b. Might the departure of Ca\(^{2+}\)/CaM to NSCaTE (Fig. 4) and PCI elements (Fig. 5) then allow the IQ domain to rebind elsewhere, in a manner also required for C-lobe CDI? Thus viewed, IQ-domain mutations could diminish C-lobe CDI by weakening this rebinding, but in a way that correlates poorly with IQ-peptide binding to Ca\(^{2+}\)/CaM in isolation. As C-lobe CDI can be conferred to Ca\(_{V2}\) channels by substituting PCI and IQ elements from Ca\(_{V1}\)\(^{31,41}\), will the requisite rebinding involve association between these very elements?

Initially disappointing was the existence of only low-affinity binding between IQ and PCI modules (Fig. 6c, left cartoon; Fig. 6d, gray) under conditions of resting intracellular calcium\(^{5}\). By contrast, under elevated Ca\(^{2+}\), robust interaction between the same IQ/PCI FRET pair was observed, with \(K_{\text{eff}} = 4.35 \times 10^{-5} \text{ D}_{\text{free}} \text{ units}^{-1}\) (Fig. 6d, black). In fact, this Ca\(^{2+}\)-dependent domain interaction accords well with a role in triggering CDI, and likely arises from a requirement for Ca\(^{2+}\)/CaM to bind the PCI domain before appreciable IQ association occurs (Supplementary Note S13). Beyond mere binding, however, functionally relevant interaction would be decreased by the same IQ-domain mutations that reduced C-lobe CDI. In this regard, IQ peptides bearing I[0]A or Q[1]A substitutions actually demonstrated strong and graded reductions in affinity (Fig. 6e, respective red and blue symbols), coarsely matching observed deficits in C-lobe CDI (Fig. 6b). Figure 6c summarizes the results.
of these and other FRET-binding assays (Supplementary Note S14) performed for loci with the strongest effects on C-lobe CDI (Fig. 6b, coloured bars under dashed black threshold). With these data, quantitative iTL analysis could be undertaken, where the presumed CDI transition in question would be the $\gamma_2$ transduction step in Fig. 2a, and the relevant form of equation 1 is specified in Supplementary Note S15. Remarkably, plotting C-lobe CDI (Fig. 6b) versus IQ/PCI-

![Diagram](image-url)
binding affinity (Fig. 6c) indeed resolves a Langmuir (Fig. 6f). Thus, C-lobe CDI likely requires a tripartite complex of IQ, PCI, and C-lobe Ca\(^{2+}\)/CaM (Fig. 6a).

ApocaM preassociation within the PCI domain. Having explored Ca\(^{2+}\)/CaM, we turned to apocaM interactions. Elsewhere\(^\text{22}\) we have shown that apocaM preassociates with a surface that at least includes\(^\text{24,25}\) the IQ element. Furthermore, homology modelling\(^\text{42}\) of a related apocaM/IQ structure for Na\(_{\text{V}}\) channels\(^\text{44,45}\) suggests that the Ca\(_{\text{A}}\)-I IQ module interacts with the C-lobe of apoCaM. Will the N-lobe of apoCaM then bind the PCI domain (Fig. 7a)? If so, then our earlier PCI mutations could have weakened N-lobe apoCaM interaction, and potentially diminished CDI by favouring configuration E channels (Fig. 1a, incapable of inactivation). This effect would not have been apparent thus far, as we invariably overexpressed CaM. However, with only endogenous CaM present in Fig. 7e, CDI reflects the operation of a system that includes configuration I, corresponding to the surface of the upstream EF-hand region of an alternative C-lobe complexed with the IQ domain\(^\text{42}\) (blue), based on an analogous atomic structure from Na\(_{\text{V}}\) channel\(^\text{44,45}\). Key IQ-domain hotspots for apoCaM preassociation (red) are rationalized by this model\(^\text{42}\). To portray the N-lobe as shown in Fig. 8b, we utilized \textit{ab initio} structural prediction of the CI domain with the Rosetta package\(^\text{19}\) (Supplementary Note S19), yielding a PCI domain (green) with two vesigial EF hands, and a protruding helix (‘preIQ’ subelement). The EF-hand module (EF) resembles the structure of a homologous segment of Na\(_{\text{V}}\) channels\(^\text{36,37}\), and a helical segment has been resolved in atomic structures of analogous Ca\(_{\text{V}}\)_1.2 segments\(^\text{36,37}\). Reassuringly, N-lobe apoCaM hotspots adorn the surface of this PCI model (red coloration), within the more C-terminal of the two EF hands. Accordingly, we appose the atomic structure of the N-lobe (1 CFD) to this segment of the PCI model, initially using a shape-complementarity docking algorithm\(^\text{52}\) (PatchDock), followed by refinement with docking protocols of Rosetta (Supplementary Note S20). Of note, the configuration of the N-lobe explains the outright enhancement of N-lobe CDI by PCI mutations in the region of putative N-lobe contact (compare Figs 5e and 7e, GKL through TLF). Weakening channel binding to the N-lobe (Fig. 2a, state 1) would, through connection to other states, increase state 5 occupancy, thereby boosting N-lobe CDI (Supplementary Note S21). By contrast, no N-lobe CDI enhancement was observed for IQ substitutions at the central isoleucine (I(0)) and downstream\(^\text{42}\), consistent with IQ binding the C-lobe of apoCaM.

Figure 8c displays a model of Ca\(^{2+}\)/CaM complexed with the channel. The N-lobe bound to NSCaTE is an NMR structure\(^\text{33}\), and functional N-lobe CDI hotspots correspond well with intimate contact points (red). C-lobe CDI hotspots also adorn the surface of the upstream EF-hand region of an alternative \textit{ab initio} model of the PCI (Fig. 8c, red; Supplementary Note S19). The IQ domain and atomic structure of the C-lobe of Ca\(^{2+}\)/CaM (3BXL) were then computationally docked (Supplementary Note S22), yielding a rather canonical CaM/peptide complex where the channel contributes a surrogate lobe of CaM. Overall, this framework promises to set the table for future structural biology and structure–function work.

**Discussion**

These experiments fundamentally transform the prevailing molecular view of CaM regulation of Ca\(^{2+}\) channels. The field has long been dominated by an IQ-centric scheme\(^\text{2,5,7,8,10,25,28}\), wherein indwelling apoCaM begins preassociated with a C-terminal IQ domain, and remains bound to this element upon CaM interaction with Ca\(^{2+}\). Here our new proposal establishes substantial exchange of CaM to alternate effector loci (Fig. 8a). ApocaM preassociates with an interface that includes, but is not limited to, the IQ domain (configuration I): the C-lobe binds the IQ (cyan circle) and the N-lobe binds the central midsection of the PCI (green box). Ca\(^{2+}\) binding to the N-lobe yields configuration I\(_\text{CS}\), wherein this lobe binds the NSCaTE module on the channel N terminus (pink oval) to trigger the N-lobe CDI. Ensuing Ca\(^{2+}\) binding to the C-lobe induces configuration I\(_\text{CS}\), with C-lobe binding the proximal PCI midsection (green square) and IQ engagement\(^\text{25}\). If Ca\(^{2+}\) only binds the C-lobe, the system adopts configuration I\(_\text{C}\), corresponding to C-lobe CDI. Ultimately, Ca\(^{2+}\)/CaM exchange to effector loci diminishes opening, perhaps via allosteric coupling of carboxytail conformation to a contiguous IVS6 segment implicated in activation\(^\text{46,47}\) and inactivation\(^\text{48}\). Only a single CaM is present\(^\text{22,23}\) (Supplementary Note S17).

The structures of many of these configurations are presently unknown, but \textit{ab initio} and homology modelling here confirms the plausibility of these configurations. Concerning the apocaM/IQ-channel complex, Fig. 8b displays a homology model of the C-lobe complexed with the IQ domain\(^\text{42}\) (blue), based on an analogous atomic structure from Na\(_{\text{V}}\) channel\(^\text{44,45}\). Key IQ-domain hotspots for apoCaM preassociation (red) are rationalized by this model\(^\text{42}\). To portray the N-lobe as shown in Fig. 8b, we utilized \textit{ab initio} structural prediction of the CI domain with the Rosetta package\(^\text{19}\) (Supplementary Note S19), yielding a PCI domain (green) with two vesigial EF hands, and a protruding helix (‘preIQ’ subelement). The EF-hand module (EF) resembles the structure of a homologous segment of Na\(_{\text{V}}\) channels\(^\text{36,37}\), and a helical segment has been resolved in atomic structures of analogous Ca\(_{\text{V}}\)_1.2 segments\(^\text{36,37}\). Reassuringly, N-lobe apoCaM hotspots adorn the surface of this PCI model (red coloration), within the more C-terminal of the two EF hands. Accordingly, we appose the atomic structure of the N-lobe (1 CFD) to this segment of the PCI model, initially using a shape-complementarity docking algorithm\(^\text{52}\) (PatchDock), followed by refinement with docking protocols of Rosetta (Supplementary Note S20). Of note, the configuration of the N-lobe explains the outright enhancement of N-lobe CDI by PCI mutations in the region of putative N-lobe contact (compare Figs 5e and 7e, GKL through TLF). Weakening channel binding to the N-lobe (Fig. 2a, state 1) would, through connection to other states, increase state 5 occupancy, thereby boosting N-lobe CDI (Supplementary Note S21). By contrast, no N-lobe CDI enhancement was observed for IQ substitutions at the central isoleucine (I(0)) and downstream\(^\text{42}\), consistent with IQ binding the C-lobe of apoCaM.

Figure 8c displays a model of Ca\(^{2+}\)/CaM complexed with the channel. The N-lobe bound to NSCaTE is an NMR structure\(^\text{33}\), and functional N-lobe CDI hotspots correspond well with intimate contact points (red). C-lobe CDI hotspots also adorn the surface of the upstream EF-hand region of an alternative \textit{ab initio} model of the PCI (Fig. 8c, red; Supplementary Note S19). The IQ domain and atomic structure of the C-lobe of Ca\(^{2+}\)/CaM (3BXL) were then computationally docked (Supplementary Note S22), yielding a rather canonical CaM/peptide complex where the channel contributes a surrogate lobe of CaM. Overall, this framework promises to set the table for future structural biology and structure–function work.
Figure 8 | New view of CaM regulatory configurations of CaV1.3 channels. (a) Molecular layout of configurations A, I$_C$, I$_N$ and I$_{CN}$ for conceptual scheme in Fig. 1a. ApoCaM preassociates with CI region; C-lobe articulates IQ domain, and N-lobe engages the PCI segment. Once Ca$^{2+}$/CaM CI region docked to apoCaM (PCI region: green; IQ domain: blue). ApoCaM hotspots (Fig. 6e–g) in red. C-lobe of apoCaM contacts IQ, whereas N-lobe binds EF-hand region. (b) Left, atomic structure of NSCaTE bound to N-lobe of Ca$^{2+}$/CaM (2LQC$^{33}$). NSCaTE peptide in tan; and N-lobe Ca$^{2+}$/CaM in cyan. Ca$^{2+}$, yellow. N-lobe CDI hotspots on NSCaTE in red. Right, de novo model of tripartite IQ-PCI-Ca$^{2+}$/CaM complex (PCI region, green; IQ domain, blue). C-lobe CDI hotspots in red for both PCI and IQ domains.

Methods

Molecular biology. To simplify mutagenesis, the WT construct in this study was an engineered Ca$_{v1.3}$ construct $\alpha_{1D}$N$^{1626}$, nearly identical to and derived from the native rat brain variant ($\alpha_{1D}$, AF3070009). Briefly, the $\alpha_{1D}$N$^{1626}$ construct, as contained with mammalian expression plasmid pCDNA6 (Invitrogen), features introduction of a silent and unique KpnI site at a position corresponding to $\sim$50 amino acid residues upstream of the C-terminal IQ domain ($G_{1538}T_{1539}$). As well, a unique BglII restriction site is present at a locus corresponding to $\sim$450 amino acid residues upstream of the IQ domain. Finally, a unique Xhel and stop codon have been engineered to occur immediately after the IQ domain. These attributes accellerated construction of cDNAs encoding triple alanine mutations of $\alpha_{1D}$N$^{1626}$. Point mutations of channel segments were made via QuickChange mutagenesis (Agilent) before PCR amplification and insertion into the full-length $\alpha_{1D}$N$^{1626}$ channel construct via restriction sites BglII/KpnI, KpnI/Xhel or BglII/Xhel. Some triple alanine mutation constructs included a seven amino acid extension (SRGPAVRR) after residue 1626. For FRET two-hybrid constructs, fluorophore-tagged (all based on enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP)) CaM constructs were made as described.$^4$ Other FRET constructs were made by replacing CaM with appropriate PCR-amplified segments, via unique NolI and Xhel sites flanking CaM.$^4$. YFP–CaM–. (Supplementary Fig. S10) was YFP fused to the C-lobe of CaM (residues 78–149). To aid cloning, the YFP-tagged CI region was made with a 12 residue extension (SRGPYSIVSPKC) via NotI/Xhel sites as above. This linker did not alter apoCaM binding affinity versus WT YFP-tagged CI region (not shown). Throughout, all segments subject to PCR or QuickChange mutagenesis were verified in their entirety by sequencing.

More broadly, this regulatory scheme may explain paradoxes and open horizons. First, it has been asked how Ca$^{2+}$/CaM could ever leave the IQ domain, when the binding affinity between these elements is so high.$^{4,5,29,39,53}$ (for example, $K_a$/CaM–IQ $\approx 5.88 \times 10^{-4}$ $D_{\text{free}}$ units$^{-1}$ in Fig. 3c). The answer may arise from the competing binding affinity for the tripartite complex (Fig. 8a, I$_{CN}$), which multivalent ligand binding theory$^{54}$ would approximate as $K_a$/CaM–PCI–IQ $\sim K_a$/CaM–PCI $\times K_a$/PCI–IQ $\times$ (local concentration of IQ) $= (4.35 \times 10^{-5} D_{\text{free}}$ units$^{-1}) \times (3.45 \times 10^{-5} D_{\text{free}}$ units$^{-1}) \times (1.36 \times 10^9 D_{\text{free}}$ units$^{-1}) \sim 0.2$ $D_{\text{free}}$ units$^{-1}$, a value far larger than $K_a$/CaM–IQ (Supplementary Note S23). Second, our scheme offers new interfaces targetable by native modulators and drug discovery. As L-type channel CDI influences cardiac arrhythmogenic potential$^{15,18}$ and Ca$^{2+}$ load in substantia nigral neurons prone to degeneration in Parkinson’s,$^{55}$ one could envisage a screen for selective modulators of N- or C-lobe CDI.$^{19}$ Third, our results offer a fine-grained roadmap for Ca$_{v1.2}$ splice/editing variants and channelopathies.$^{56}$ Indeed, we suspect that the design principles revealed here may generalize widely to other molecules modulated by CaM.$^{45,50,51,57}$
**Transfection of HEK293 cells.** For whole-cell patch clamp experiments, HEK293 cells were cultured on 10-cm plates, and channels transiently transfected by a calcium phosphate method. We applied 8 µg of rat brain β2a (M80545) and 8 µg of rat brain γ2δ (NM012919.2) subunits. We utilized the β2a auxiliary subunit to minimize voltage-dependent inactivation. For experiments involving CaM overexpression, we coexpressed 8 µg of rat CaMWT, CaMv, or CaM34, as described. All of the above cDNA constructs were included within mammalian expression plasmids with a cytomegalovirus promoter. To boost expression, cDNA encoding mRNAs was transfected using the calcium phosphate method. We applied 8 µg of cDNA and obtained high transfection efficiencies. All of the above cDNA constructs were included within mammalian expression plasmids with a cytomegalovirus promoter. To boost expression, cDNA encoding mRNAs was transfected using the calcium phosphate method. We applied 8 µg of cDNA and obtained high transfection efficiencies.

**Whole-cell recording.** Whole-cell recordings were obtained using an Axopatch 200A amplifier (Axon Instruments). Electrodes were made from borosilicate glass capillaries (World Precision Instruments, MTW 150-F4) yielding 1-3 MΩ resistances, which were in turn compensated for series resistance by >70%. Currents were low-pass filtered at 2 kHz before digital acquisition at several times that frequency. A P/2 leak-subtraction protocol was used. The internal solution contained (in mM): CaMeSO₄, 114; CaCl₂, 5; MgCl₂, 1; MgATP, 4; HEPES (pH 7.4); and BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid); 10; at 290 mOsM adjusted with glycerol. The bath solution was (in mM): TEA-MeSO₄, 102; HEPES (pH 7.4); 10; CaCl₂ or BaCl₂, 40; at 300 mOsM, adjusted with TEA-MeSO₄.

**FRET imaging.** We conducted FRET two-hybrid experiments in HEK293 cells cultured on glass-bottom dishes, using an inverted fluorescence microscope as extensively described by our laboratory. Experiments utilized a bath Tyrode’s solution containing (in mM): NaCl, 130; KCl, 5; MgCl₂, 1; MgATP, 4; HEPES (pH 7.4); and BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid); 10; at 290 mOsM adjusted with glycerol. The bath solution was (in mM): TEA-MeSO₄, 102; HEPES (pH 7.4); 10; CaCl₂ or BaCl₂, 40; at 300 mOsM, adjusted with TEA-MeSO₄.

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**Author contributions**

M.B.J., P.S.Y. and H.B. created mutant channels, performed electrophysiology and FRET experiments, and undertook extensive data analysis. M.B.J. pioneered and conducted many of the FRET binding assays, performed molecular modelling and undertook extensive software development. D.Y. conceived and supervised the project; and helped formalize iTL theory and translation of 33-FRET to E-FRET methodologies. All authors refined hypotheses, wrote the paper and created figures.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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