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Allostery in Ca²⁺ channel modulation by calcium binding proteins

Philemon S. Yang, Manu Ben Johny, and David T. Yue

Calcium Signals Laboratory, Departments of Biomedical Engineering and Neuroscience, The Johns Hopkins University School of Medicine, Ross Building, Room 713, 720 Rutland Avenue, Baltimore, MD 21205, voice: (410) 955-0078, fax: (410) 955-0549

David T. Yue: dyue@bme.jhu.edu

Abstract

Distinguishing between allostery and competition among modulating ligands is challenging for large target molecules. Of practical necessity, inferences are often drawn from *in vitro* assays on target fragments, but such inferences may belie actual mechanisms. One key example of such ambiguity concerns calcium-binding proteins (CaBPs) that tune signaling molecules regulated by calmodulin (CaM). Since CaBPs resemble CaM, CaBPs are believed to competitively replace CaM on targets. Yet, brain CaM expression far surpasses that of CaBPs, so how can CaBPs exert appreciable biological actions? Here, we devise a live-cell, holomolecule approach that reveals an allosteric mechanism for calcium channels, whose CaM-mediated inactivation is eliminated by CaBP4. Our strategy is to covalently link CaM and/or CaBP to holochannels, enabling live-cell FRET assays to resolve a cyclical allosteric binding scheme for CaM and CaBP4 to channels, thus explaining how trace CaBPs prevail. This approach may apply generally for discerning allostery in live cells.

Distinct modulatory ligands frequently act upon common target molecules¹⁻³, supporting molecular computations important to biological signaling networks^{3,4}. These ligands may act by competition wherein only one ligand can bind at a time, or by allostery allowing simultaneous interaction⁵. This contrast in classic biochemical mechanism holds crucial implications for signaling behavior, but can be notoriously difficult to establish⁶. This is particularly true for large target molecules resistant to reconstitution outside of live cells. If studied piecemeal as subdomain peptides, such targets often exhibit multiple potential ligand binding sites, but the relevance of these within the holomolecule often remains uncertain⁷.

One prime example of such mechanistic ambiguity concerns the interaction of voltage-gated Ca²⁺ channels (Ca_v) with two modulatory ligands: calmodulin (CaM) and calcium binding proteins (CaBPs). To start, CaM regulation of Ca_v channels plays a major role in the intracellular Ca²⁺ feedback orchestrating many biological processes⁸. This modulation

Correspondence to: David T. Yue, dyue@bme.jhu.edu.

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frequently manifests as a Ca^{2+} -dependent inactivation (CDI) of channel opening^{9,10}, important for Ca^{2+} homeostasis¹¹. Additionally, exciting recent discoveries identify Ca^{2+} -binding proteins (CaBPs), a family of CaM-like brain molecules¹²⁻¹⁷ that may also bind Ca_v channels and other targets^{18,19}. CaBPs are bi-lobed like CaM, with each lobe containing two EF-hand Ca^{2+} binding motifs¹². However, while all four EF hands bind Ca^{2+} in CaM, one of them is non-functional in CaBPs. Coexpressing CaBPs with Ca_v1 channels then eliminates their CDI^{14,20}. Indeed, modulation like this diversifies biological responses to Ca^{2+} signals^{13,14,18,21}.

Based on the similarity of CaM and CaBPs, the prevailing hypothesis is that CaBPs competitively replace CaM on channels^{14,22,23}. Indeed, these ligands exhibit competitive binding to an isolated channel IQ domain (Fig. 1a, blue oval), a principal element for CDI. The interaction of CaM and CaBPs may thus be formulated as in Fig. 1b. State 1 portrays an 'empty' channel without ligand and thus incapable of CaM-mediated Ca^{2+} regulation^{24,25}. This empty channel may bind CaM at the IQ domain to form a complex (state 2) that can then undergo Ca^{2+} regulation (e.g., inactivation). Alternatively, CaBP may bind at the IQ domain, yielding a state 3 with altered Ca^{2+} regulatory behavior (e.g., non-inactivatable). Importantly, both CaM and CaBP cannot simultaneously bind (state 4 excluded) under competition. Hypothetical dispositions of CaBP and CaM are cartooned in Fig. 1b.

As with many large molecules, studies of isolated channel peptides raise the possibility of more complex scenarios. Specifically, peptide assays hint at multiple potential binding sites for CaM and CaBPs. Thus, these two ligands need not compete at the IQ domain, and the possibility of an allosteric mechanism arises (i.e., Fig. 1b, state 4). For CaM regulation itself, interaction sites extend beyond the IQ domain (Fig. 1a, blue oval). In fact, CaM regulation starts with a single apoCaM (Ca^{2+} -free CaM) preassociated, with the C-lobe of apoCaM embracing the IQ domain²⁴⁻²⁷ (Fig. 1a, blue oval), and the N-lobe engaging upstream vestigial EF-hands²⁴ (Fig. 1a, squares), all within an encompassing CI region^{9,28,29}. Ca^{2+} binding to this 'resident' CaM alters channel opening by driving rearrangements that may include the binding of the N-lobe of Ca^{2+} -bound CaM (Ca^{2+} /CaM) to the channel amino terminus³⁰ (Fig. 1a, tan oval). Regarding CaBPs, peptide assays also hint at interactions beyond the IQ domain^{31,32}, but the relevance of these assays to holochannels remains unsettled. One study argues for the functional impact of such sites³², while another argues otherwise³¹. In all, the scheme of CaM and CaBP interaction remains ambiguous (Fig. 1b), and clear tests for concurrent ligand binding to holochannels (i.e., state 4) have proven infeasible. This state of impasse is common to many large molecules^{33,34}.

Importantly, the distinctions between competitive versus allosteric mechanism concern more than academic interest; they appear to be critical to the biological activity of CaBPs. In particular, the affinity of Ca_v channels for apoCaM may be greater than that for CaBPs^{14,25,26}, as the present study will substantiate. Moreover, this study will highlight a predominance of CaM over CaBP expression throughout the brain. Under competition, then, how could CaBPs exert functional effects? By contrast, an allosteric mechanism with the right mix of parameters would readily explain how naturally occurring CaBPs could still exert modulation.

Here, we establish an allosteric mechanism for $\text{Ca}_v1.3$ Ca^{2+} channels, a robust prototype system where strong CaM-mediated CDI can nonetheless be completely suppressed by CaBPs. Our approach is to exploit covalent linkage of holochannels to CaM and/or CaBP, along with live-cell FRET binding assays with intact holochannel complexes. In so doing, we fully resolve a cyclical allosteric binding scheme for CaM and CaBP4 to channels (i.e., Fig. 1b). The parameters we obtain explain how trace CaBP can functionally prevail over excess CaM. This approach may furnish a general means for discriminating allosteric mechanisms in the live-cell context.

Results

Predominance of CaM over CaBPs in the CNS

To motivate exploration of competitive versus allosteric mechanisms, we first considered the relative expression of CaM and CaBPs in the CNS. Given a competitive mechanism of CaBP modulation of Ca^{2+} channels, CaBP must at least approach the abundance of CaM to be functionally relevant, particularly if channels were to exhibit a greater affinity for apoCaM over CaBPs. In native systems, however, CaBP expression pales in comparison to that for CaM³⁵, even for the more prominent CaBP1 and CaBP4 isoforms. Deep sequencing in mouse neocortex³⁶ reveals CaBP1 transcripts to be ~5-fold rarer than those for CaM; and CaBP4 transcripts 10,000-fold rarer (Fig. 1c). Similarly, SAGE analysis of whole mouse retina³⁷ estimates a 1:15 ratio for CaBP4 to CaM transcripts, and an even smaller ratio for CaBP1 (Fig. 1d). Moreover, deep sequencing of human prefrontal cortex³⁸ indicates a 1:8 ratio for CaBP1 to CaM transcripts, and a 1:6,000 ratio for CaBP4 (Fig. 1e). Although transcript abundance is not equivalent to protein expression, the enormous degree of transcript imbalance here would certainly yield protein expression mismatch³⁹. Normalized *in-situ* hybridization profiles of mouse brain (Figs. 1f-h) underscore the predominance of CaM³⁵, raising a challenge for competitive mechanisms, and prompting a search for potential allostery.

As a critical prelude to this search, we sought to resolve the functional contribution of ancillary CaBP binding sites outside the IQ domain, especially within the relevant configuration of the holochannel complex. This step would be a necessary ante for considering an allosteric scenario. Thus, we undertook a two-step sequence: first, to consolidate the baseline functional effects and binding properties of CaBP to $\text{Ca}_v1.3$ channels; and second, to validate the functional relevance of potential ancillary CaBP binding sites in chimeric Ca^{2+} channels.

CaBP modulation and binding of $\text{Ca}_v1.3$ channels

Figure 2a displays the baseline effects of CaBPs on $\text{Ca}_v1.3$ channels. We mainly focused on the rarer CaBP4 isoform, because it faces the larger competitive challenge. Absent CaBP, CaM-mediated CDI triggers strong decay of Ca^{2+} current¹⁴ when compared to Ba^{2+} (left subpanel). Ba^{2+} binds CaM poorly⁴⁰ and thus furnishes a baseline reference without CDI. Upon strong overexpression of CaBP4, CDI is eliminated in exemplar traces¹⁴ (right subpanel). Population data in Fig. 2b fully confirms these trends, where the fraction of peak current remaining after 300-ms depolarization (r_{300}) gauges steady-state inactivation. When

plotted versus step potential, the r_{300} relation obtained with Ba^{2+} (black) is nearly flat, corroborating weak voltage-dependent inactivation. In control, the corresponding Ca^{2+} r_{300} relation exhibits a U shape (left subpanel, red), indicating genuine CDI⁹. The difference between these relations (f_{300} , at 10-mV step depolarization) then quantifies CDI in isolation. Upon coexpressing CaBP4, the Ba^{2+} and Ca^{2+} relations coalesce (right subpanel), substantiating elimination of CDI. Figure 2c portrays this effect in f_{300} bar-graph format, used for compactness in subsequent figures.

Regarding binding, CaBP4 might outcompete excess CaM, if CaBP4 were to exhibit a higher affinity for channels than CaM. Prior peptide assays conflict on this point^{14,27}, and quantification of CaM and CaBP4 binding to functional holochannel complexes has seldom been achieved. We previously developed a holochannel FRET 2-hybrid assay^{26,41} enabling characterization of CaM binding to functional $\text{Ca}_v1.2$ in live cells. Here, we utilize this approach to quantify interaction between YFP-tagged $\text{Ca}_v1.3$ channels and CFP-tagged CaM or CaBP4 (Figs. 2d, e). Within the scheme in Fig. 1b, these assays measure the association constants between states 1 and 2 (Fig. 2d, diamond schematic), and between states 1 and 3 (Fig. 2e). The rightmost subpanel in Fig. 2d plots FRET efficiency (E_A) versus the relative free concentration of apoCaM (D_{free}), yielding a binding curve with a large association constant of $K_{12} = 44 \pm 9 \mu\text{M}^{-1}$ ($K_{a,\text{eff}} = 143 \times 10^{-5}$ microscope-specific units). By contrast, though CaBP4 does bind well to the channel (Fig. 2e), the affinity is ten-fold lower with a $K_a = 4.4 \pm 0.7 \mu\text{M}^{-1}$. Likewise, CaBP1 binding was $2.6 \pm 0.4 \mu\text{M}^{-1}$ (Supplementary Results, Supplementary Fig. 1) Of note, interaction affinity (i.e., $K_{a,\text{EFF}}$) is the reciprocal of the free concentration of donor-tagged molecules (D_{free}) at half-maximal E_A , whereas the distance/orientation of donor and acceptor fluorophores in the bound complex specifies maximal E_A ($E_{A/\text{max}}$) at large D_{free} values^{26,41}.

To identify candidate segments for CaBP4 binding with holochannels (Fig. 2e), we scanned the intracellular loops of $\text{Ca}_v1.3$ for CaBP4 interaction (Fig. 2e, left subpanel, dark blue segments), using a peptide version of FRET 2-hybrid assays^{26,41}. CaBP4-ECFP was the donor, and EYFP fused to various channel segments the acceptor. Though peptide affinities may not translate quantitatively to the intact context, they serve to identify regions meriting full analysis via integrative holochannel assays. Figure 2f confirms CaBP4 binding to the isolated IQ domain of $\text{Ca}_v1.3$ (black), as observed previously^{14,27}. Notably, the interaction affinity is lower than for the holochannel (gray fit), suggesting that other segments contribute. Indeed, CaBP4 also interacts with the upstream PCI region shown in Fig. 2g (black data), and further results identify other candidate interaction sites at the channel amino terminus (NT) and the C-terminal third of the loop between domains III and IV (III-IV loop) (Fig. 2h, red regions; Supplementary Fig. 2). These findings largely agree with those in homologous $\text{Ca}_v1.2$ channels^{20,22}.

Donating CaBP4 modulation to chimeric channels

To test for functional relevance of candidate CaBP4 binding segments (Fig. 2h, red) at the holochannel level, we sought to confer CaBP4 sensitivity to CaBP-insensitive channels via donation of essential $\text{Ca}_v1.3$ modules. We thus turned to the $\text{Ca}_v2.3$ isoform (Fig. 3a, left; Supplementary Fig. 3a), which readily forms functional chimeras with Ca_v1 channels^{28,42}.

FRET between CaBP4-CFP and Cav2.3 fused to YFP revealed little binding (Fig. 3a, right). Moreover, CaBP4 also did not influence CDI (Figs. 3b, c). Thus, Cav2.3 channels served as a 'blank slate' lacking both binding and modulation by CaBP4. First, we replaced the CI region of Cav2.3 with the corresponding segment of Cav1.3 (Fig. 3d, left, eeee). CaBP4 now bound appreciably to this chimeric channel (Fig. 3d, right). More importantly, CDI in these chimeric channels could now also be attenuated, though not eliminated by CaBP4 (Figs. 3e, f; Supplementary Fig. 3b). Further chimeric-channel experiments indicated that adding both the NT and III-IV loops of Cav1.3 were required for full CaBP4 sensitivity (Supplementary Fig. 3-4). The final outcome is demonstrated by the chimeric channel (Fig. 3g, left, deedd) containing all three candidate binding sites (NT, III-IV loop, and CI region) of Cav1.3. CaBP4 binding to this chimera shows the highest affinity (Fig. 3g, right; Supplementary Fig. 3e), and CDI is now completely eliminated by CaBP4 (Figs. 3h, i; Supplementary Fig. 3d). Direct deletions and mutagenesis performed on the Cav1.3 channel backbone further confirmed the functional contribution of all these segments to CaBP4 sensitivity (Supplementary Figs. 5-7). Overall, the Cav1.3 CI region appeared necessary, and all three segments (NT, III-IV, PCI, and IQ) together were required for full functional sensitivity to CaBP4.

CaM and CaBP4 concurrently bind to holochannels

With ample evidence for the functional contribution of CaBP binding beyond the IQ module, we pursued definitive tests for competitive versus allosteric interaction between CaM and CaBP4 (Fig. 1b). A crucial discriminator would be the presence or absence of simultaneous ligand binding and modulation of holochannels. Such a test would establish or exclude the existence of state 4 in Fig. 1b, thus furnishing a decisive mechanistic distinction.

To this end, we covalently fused CaM or CaBP4 to Cav1.3 channels, a strategy we used previously with functional assays alone to probe CaM interactions with Cav1.2 channels⁴³. Figures 4a, b introduce the approach, here applied anew to Cav1.3 channels. To start, we considered Cav1.3 channels without covalently linked CaM, which again demonstrate robust CDI (Fig. 4a, control), just as observed earlier. Upon strong overexpression of a dominant-negative mutant CaM^{9,14} (CaM₁₂₃₄), which is Ca²⁺ insensitive, CDI is completely eliminated¹⁴ (Fig. 4a, traces labeled +CaM₁₂₃₄ and bargraph). This outcome occurs because CaM₁₂₃₄ has competitively replaced endogenous functional CaM on the channel IQ domain²⁴. By contrast, when the analogous experiment is performed with Cav1.3 channels fused to a single CaM (Fig. 4b, far left, Cav1.3–CaM construct), a completely different outcome results. Before coexpressing CaM₁₂₃₄ (Fig. 4b, control), these channels undergo robust CDI indicating unfettered functionality of the covalently attached CaM. Importantly, however, the covalent linkage of a single CaM would enrich enormously the local concentration of CaM to levels extending into the millimolar range⁴³. This local concentration would far surpass the concentrations of CaM₁₂₃₄ achievable by expression as a separate molecule²⁴ (~10 μM). One would thereby predict that separately coexpressing CaM₁₂₃₄ in this context would have little or no effect on CDI. Indeed, we observed exactly this preservation of CDI (Fig. 4b, +CaM₁₂₃₄ traces, and bar graph; Supplementary Figs. 8a, b). This outcome demonstrates that covalently attached CaM effectively occludes the IQ module in Cav1.3.

Thus armed, we could perform a pivotal experiment—to test the effect of coexpressing CaBP4 with the Ca_v1.3–CaM construct. If CaBP4 and CaM compete at the IQ module, then CaBP4 should hardly influence CDI of Ca_v1.3–CaM, just as observed for CaM₁₂₃₄ (Fig. 4b). In striking contrast, we observed complete elimination of CDI (Fig. 4c, control versus +CaBP4 traces), as confirmed by population data (Fig. 4c, bar graph; Supplementary Fig. 8c). We also observed similar outcomes with CaBP1 acting on Ca_v1.3–CaM (Supplementary Fig. 9), as well as with CaBP modulation of analogous Ca_v1.2–CaM channels (Supplementary Fig. 10). These remarkable results strongly argue that CaBP and CaM can bind to distinct sites on the channel, in contradiction to a competitive mechanism (Fig. 1b).

We advanced the approach of tethered CaM even further, and directly corroborated the functional result above with holochannel binding assays involving Ca_v1.3–CaM. Holochannel FRET 2-hybrid analysis demonstrated that CaBP4 still binds to Ca_v1.3–CaM (Fig. 4d, black data and curve), with only moderately reduced affinity compared to channels without covalently linked CaM (Fig. 4d, unlinked gray fit reproduced for reference). Interestingly, owing to occlusion of the CaM binding site by tethered CaM, the binding of CaBP4 to Ca_v1.3–CaM directly probes transitions between states 2 and 4 (Fig. 4d, schematic). This yields an association constant $K_{24} = 1.3 \pm 0.2 \mu\text{M}^{-1}$. Thus, we would argue that state 4 in Fig. 1b exists.

A key remaining issue was the possibility of more than one CaBP4 molecule working together to modulate channels. Accordingly, we extended our linkage strategy still further, and fused a single CaBP4 to the amino terminus of a channel that already had a carboxy-tail linkage to CaM. This created the dual-linkage construct in Fig. 5a (left subpanel, CaBP4–Ca_v1.3–CaM). Remarkably, this single CaBP4 entirely eliminated CDI, demonstrating that only one CaBP4 molecule suffices for full modulation (Fig. 5a, other subpanels; Supplementary Fig. 8d). Furthermore, FRET 2-hybrid assays between CaBP4–Ca_v1.3–YFP holochannels and CaBP4–CFP resulted in negligible binding (Fig. 5b), demonstrating explicitly that only a single CaBP4 can bind to the channel. In this dual-linkage construct, the fused CaBP4 must certainly be interacting with the channel, otherwise CDI would not have been eliminated. Yet, the tethered CaM might conceivably be dislodged, retaining affiliation only via its linker. This possibility was excluded, however, because holochannel FRET assays confirmed that CaM, as a separate molecule, can indeed bind holochannels fused to CaBP4 (Fig. 5c, black data and fit). Because of occlusion of the CaBP4 binding pocket by linked CaBP4, binding of CaM to CaBP4–Ca_v1.3 directly probes transitions between states 3 and 4 (Fig. 5c, schematic), with an association constant $K_{34} = 10 \pm 2 \mu\text{M}^{-1}$. Moreover, the maximal FRET efficiency at large D_{free} values ($E_{\text{A}/\text{max}}$) is indistinguishable from that for the corresponding experiment with Ca_v1.3 channels without fusion (as reference, gray trace for unlinked channel experiment in Fig. 3d). Thus, apoCaM appears to bind to holochannels in largely the same configuration, whether CaBP is present or not.

In all, these results argue that CaBP4 and CaM can bind concurrently to Ca_v1.3 channels. While apoCaM likely interacts with the IQ domain²⁴, CaBP4 modulation may rely on binding to elements outside the IQ domain.

Modulation by trace CaBP levels explained

That shown, how could trace concentrations of CaBPs modulate channels in excess CaM (Figs. 1c-h)? Accordingly, we elaborated the four-state scheme of Fig. 1b, by including relevant association constants obtained above (Figs. 2, e, 4d, and 5c). Figure 6a displays the result, which comprises a thermodynamic cycle of CaM and CaBP interaction with channels. Thus viewed, it was reassuring that $(K_{12} \cdot K_{24}) / (K_{13} \cdot K_{34}) \sim 1$, in accord with thermodynamic constraints⁴⁴. We thus proceeded to in-depth analysis of this scheme. If CaBP4 were to interact in a purely competitive manner, state 4 would not exist. Given a rough estimate of free endogenous CaM at 10 μM ²⁵, the remaining 3-state system would predict that a CaBP concentration of 107 μM would be required for half block of CDI (Fig. 6b, Competitive Model, black). This concentration of CaBP4 far exceeds that generally present in the brain (Figs. 1c-h). If state 4 is included, so that an allosteric mechanism pertains, a very different outcome arises. In particular, if the binding of CaM and CaBP4 were completely independent, then transitions into state 4 would be governed by association constants K_{34} and K_{24} , which would be respectively identical to K_{12} and K_{13} . Alternatively, if the transitions were to interact cooperatively, then K_{34} and K_{24} would be equal to K_{12} and K_{23} multiplied by a common factor λ that derives from thermodynamic mandates⁴⁴. Here, $\lambda > 1$ would signify positive cooperativity in the binding of CaBP4 and CaM to the channel, $\lambda < 1$ would denote negative cooperativity, and $\lambda = 0$ would correspond to purely competitive binding of CaM and CaBP4. Intriguingly, our experiments permitted direct determination of the λ factor. K_{34} is lower than K_{12} by about 4 fold, as is K_{24} compared to K_{13} . Thus, we estimate $\lambda \sim 0.23$, confirming a modest degree of negative cooperativity in the binding of both CaBP4 and apoCaM to channels. Importantly, given this precise diamond-shaped system, the projected effects of CaBP4 on CDI corresponds to the red-dashed curve in Fig. 6b, with a half CDI block at a CaBP4 concentration of $\sim 0.8 \mu\text{M}$. This concentration is plausibly consistent with levels in the brain. Moreover, accounting for the effects of endogenous CaM on our binding analysis produced little change in this prediction (Supplementary Fig. 11; Supplementary Notes 1-2) Thus, physiological CaBP4 may be able to exert modulation as a direct consequence of the allosteric mechanism in Fig. 6a.

This notable prediction nonetheless relies on the accuracy of holochannel FRET assays. We thus utilized patch fluorometry to directly determine the sensitivity of $\text{Ca}_v1.3$ CDI to CaBP4 (Fig. 6c). Specifically, whole-cell electrophysiology measured CDI, and concurrent whole-cell fluorescence measurements estimated the concentration of fluorescently labeled CaBP4 molecules. CaM was fused to $\text{Ca}_v1.3$ channels to ensure a stable and high local concentration of CaM. Data thus obtained should decorate the predicted dashed-red curve in Fig. 6b. Exemplar calcium traces (Fig. 6d) obtained under various CaBP4 concentrations illustrate variable CDI attenuation. Absent CaBP4 (point a in Figs. 6b, d), the full measure of wild-type CDI is apparent. Notably, at estimated CaBP concentrations of less than 2.5 μM , CDI already exhibits substantial attenuation (points b and c in Figs. 6b, d). Indeed, CDI becomes virtually absent at [CaBP4] of 4 μM and higher (point d in Figs. 6b, d). Population data corroborate these trends (Fig. 6b, symbols, solid-red curve) and essentially overlays the predicted outcome from holochannel FRET assays (Fig. 6b, dashed-red curve). This correspondence substantiates our allosteric model (Fig. 6a), and its explanation for how trace amounts of CaBPs may exert their action on channels despite an abundance of CaM.

As these experiments employed Ca_v1.3 fused covalently to CaM, the CaBP sensitivity here is a lower-limit estimate, making the outcome still more compelling.

Discussion

Calcium-binding proteins (CaBPs) tune the Ca²⁺ responsiveness of numerous Ca²⁺ signaling molecules regulated by CaM, thereby projecting influence over diverse biological processes^{13,18,19,21}. Channel peptide studies and the homology between CaBPs and CaM support the view that CaBPs act by competitively replacing CaM on target molecules^{14,22,27}. However, CaM expression far exceeds that of CaBPs in the brain, raising questions about the ability of CaBPs to exert appreciable effects. To overcome the interpretive limitations of peptide studies, we devised functional and live-cell FRET interaction assays on Ca_v1.3 holochannels covalently linked to CaM and/or CaBP4. Notably, Ca_v1.3 channels fused to CaM are still strongly modulated by CaBP4, and holochannel FRET assays directly confirm CaBP4 binding to such channels. These results firmly establish that apoCaM and CaBP4 can bind simultaneously, implicating an allosteric scheme that predicts and explains how trace CaBP4 can modulate channels in excess CaM. We directly confirm this prediction using patch fluorometry.

These results project a new view of CaBP modulation of Ca_v1.3 channels that incorporates our recent molecular model of CDI²⁴ (Supplementary Fig. 12). The channel/apoCaM complex in Supplementary Fig. 12a corresponds to configuration 2 in Fig. 6a. The C-terminal lobe of apoCaM interacts strongly with the IQ domain, and the N-lobe engages more weakly with EF-hand regions. CDI occurs upon Ca²⁺/CaM switching interactions to its effector sites (Supplementary Fig. 12b), with the N-lobe of Ca²⁺/CaM embracing an NSCaTE module in the channel amino terminus, and the C-lobe forming a tripartite complex with the channel IQ module and upstream EF-hand region. To overlay CaBP4, we positioned CaBP4 with major contacts on the amino terminus, III-IV loop, and EF-hand regions (Supplementary Fig. S12c, corresponding to Fig 6a, state 4). During rest, CaBP and apoCaM may bind simultaneously because principal interaction sites appear distinct for the two ligands. This proposed arrangement retains strong interaction of the IQ domain with the C-lobe of apoCaM, based on interpreting the equivalent $E_{A/\max}$ values in Figs. 2d and 5c as evidence that apoCaM adopts a similar bound configuration, whether CaBP is absent or also bound. As this equivalence could conceivably accommodate different apoCaM arrangements, the suggested configuration is provisional. That said, the proposed layout (Supplementary Fig. 12c) does project a potential steric clash between the N-lobe of apoCaM and CaBP4 at the EF-hand region, which nicely rationalizes the modest negative cooperativity factor of $\lambda \sim 0.23$. On Ca²⁺ elevation, access of Ca²⁺/CaM to its effector sites may be inhibited by CaBP4 preoccupying nearby or partially overlapping sites, thus prohibiting CDI. In this regard, the mechanism of CDI inhibition may be a nuanced combination of allosteric and competitive mechanisms. Under resting Ca²⁺, CaBP and apoCaM posture for baseline position via an allosteric mechanism (i.e., Fig. 6a); however, upon Ca²⁺ elevation, CDI fails to occur because CaBP occludes Ca²⁺/CaM effector sites via a potentially competitive regime. This class of mechanism may generalize to other CaBP target molecules that share distinctions between sites for apoCaM preassociation and Ca²⁺/CaM effectuation. Ca_v1.2 channels are homologous to Ca_v1.3⁴⁵, and likely to subscribe to an analogous mechanism.

Ryanodine receptors are CaM regulated and exhibit movement of CaM between distinct apoCaM and Ca²⁺/CaM sites⁴⁶. Closely related IP3 receptors host both apoCaM⁴⁷ and Ca²⁺/CaM^{48,49} binding sites, and are modulated by CaBP^{18,19}.

More broadly, measuring binding and function in holomolecules fused to modulatory ligands may apply to many systems. Particular advantages include the ability to readily isolate individual transitions within cyclical binding schemes, and the interpretive relevance of results obtained on holomolecules in live cells. Indeed, the approaches developed here enrich a burgeoning toolkit for pursuing quantitative biochemistry in functional assemblies within live cells. This endeavor promises rapid progress in understanding native molecular mechanisms⁵⁰.

Methods

Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Potential mechanisms of CaM and CaBP interaction with calcium channels
(a) Calcium channel landmarks. Ca²⁺-inactivating (CI) region (~160 aa of proximal carboxy terminus), containing elements involved in CaM regulation. IQ domain (blue oval), harbors apoCaM pre-association and proposed site of CaBP interaction. Vestigial EF hands (squares). NSCaTE element (brown oval), N-lobe Ca²⁺/CaM effector site. **(b)** Potential mechanisms of CaM and CaBP interaction. Prevailing competitive hypothesis restricts model to states 1-3, and excludes state 4. State 1, channel lacking both CaM and CaBP. State 2, channel with single apoCaM bound (black circle), primarily to IQ domain. State 3, channel with single CaBP bound (orange square). Under competition, CaBP would occupy same site as CaM (i.e., IQ domain), allowing channel binding to either ligand, but not both. Under allostery, CaBP binds to alternate site (state 3, dashed outline square), allowing simultaneous binding of CaM and CaBP (state 4). Existence of state 4 is unknown. Far right and left, hypothetical interaction of CaBP (left) and CaM (right) with IQ element under competition. **(c)** Relative transcript count (RPKM, Reads Per Kilobase Million) of CaM, CaBP4, and CaBP1, obtained by deep sequencing of transcriptomes from adult mouse neocortex. **(d)** Serial analysis of gene expression (SAGE) reveals transcript levels from adult mouse retina. CaM expression ~15× CaBP4, and ~70× CaBP1. **(e)** Deep sequencing of transcriptomes from human prefrontal cortex. CaM transcripts ~8× CaBP1, and ~6000× CaBP4. **(f-h)** *In-situ* hybridization mouse brain images from Allen Brain Atlas, normalized by scaling neocortical region to match RPKM signals in **c**.

Figure 2. CaBP4 modulation and binding of Ca_v1.3 channels

(a) Functional CaBP4 effects on whole-cell Ca_v1.3 current traces. Left, CaM-mediated CDI without CaBP4. Scale bar pertains to Ca²⁺ currents (red). Ba²⁺ currents (black) scaled down $\sim 3\times$ for comparison of decay kinetics. Format utilized throughout. Right, coexpressing CaBP4 eliminates CDI. (b) Population data confirming CaBP4 elimination of CDI. Left, fraction of peak current after 300-msec depolarization (r_{300}), versus step voltage for control cells. Black relation for Ba²⁺ currents; red for Ca²⁺ (mean $\pm$ SEM, $n = 7$ cells). Difference between relations at +10 mV (f_{300}) reports steady-state CDI¹⁴. Right, elimination of CDI by

CaBP4 ($n = 7$). (c) Bar-graph summary for relations in b ($n = 7$). (d) Left, cartoon of FRET 2-hybrid pairs: Ca_v1.3 fused with YFP on carboxy terminus (Ca_v1.3–YFP), and CFP–CaM_{WT}. Middle, diamond schematic (cf., Fig. 1b) showing predominant transition (black segment) probed by FRET experiment. Right, FRET efficiency (E_A) for binned groups of cells (~ 4 –5 cells per symbol) plotted versus D_{free} , relative free donor concentration^{26,41} (CFP–CaM_{WT}), forming a binding isotherm^{26,41}. D_{free} , microscopic-specific units are calibrated to μM by horizontal green scale bar²⁴ (here and in e–g). Half-maximal E_A reached at $K_{d,\text{EFF}} = 700 D_{\text{free}}$ units yielding $K_{a,\text{EFF}} = (1 / K_{d,\text{EFF}}) = 143 \times 10^{-5} (D_{\text{free}}^{-1} \text{ units})$, equivalent to $K_a \sim 44 \mu\text{M}^{-1}$. (e) FRET analysis for CaBP4–CFP versus Ca_v1.3–YFP. Format as in d. Black symbols bin ~ 7 –8 cells. $K_{a,\text{EFF}} \sim 14 \times 10^{-5} (D_{\text{free}}^{-1} \text{ units})$, or $K_a \sim 4.4 \mu\text{M}^{-1}$. (f) FRET 2-hybrid analysis for CaBP4–CFP versus YFP–IQ. Left, cartoon of FRET partners. Right, corresponding binding curve (black); symbols average ~ 5 –6 cells. Gray, fit to holochannel relation from e. (g) FRET 2-hybrid analysis for CaBP4–CFP versus YFP–PCI peptide. Format as in e. Black symbols average ~ 7 –8 cells. (h) Cartoon of main α_{1D} subunit of Ca_v1.3, comprising four homologous domains, with intracellular loops (dark blue) facing downwards. Red, potential CaBP binding regions, based on peptide FRET 2-hybrid analysis.

Figure 3. Chimeric channels reveal importance of CaBP sites beyond the IQ domain
(a-c) No CaBP4 binding or functional modulation of wild-type $\text{Ca}_v2.3$. **(a)** FRET experiment for $\text{Ca}_v2.3$ fused to YFP, versus CaBP4-CFP. Little binding. Each symbol bins ~ 4 -5 cells. **(b)** Exemplar whole-cell current traces illustrating absence of CaBP4 effect on CDI. **(c)** Population data confirm lack of CaBP4 effects on CDI. Bars average $n = 5$ cells each. **(d)** Left, cartoon of holochannel FRET experiment, pitting CaBP4-CFP versus YFP-tagged chimeric $\text{Ca}_v2.3$ with $\text{Ca}_v1.3$ CI substitution (eeeeed). Right, significantly increased binding affinity of eeeeeed with CaBP4 (symbols bin ~ 3 -4 cells each) compared to the control in gray (from a). **(e)** Exemplar whole-cell currents now exhibit partial CDI suppression by CaBP4. Control on left, with strong CaBP4 coexpression on right. **(f)** Population data confirms partial CDI suppression by CaBP4 in eeeeeed channels. Control bar averages $n = 5$ cells. Light gray bar for CaBP4 condition averages $n = 7$ cells. **(g, h, i)** Chimeric $\text{Ca}_v2.3$ channels that include $\text{Ca}_v1.3$ amino terminus, III-IV loop, and CI module (deedd) show strongest CaBP4 binding (g, symbols average ~ 5 cells each), and complete CDI elimination by CaBP4 (h, i). **(i)** Control bar graph averages $n = 8$ cells, and light gray bar for CaBP4 condition averages $n = 5$ cells.

Figure 4. CaBP4 and CaM simultaneously bind Ca_v1.3 channels

(a) Effect of Ca²⁺-insensitive mutant CaM (CaM₁₂₃₄) to completely eliminate CDI. Left, schematic of wild-type Ca_v1.3 channel. Control exemplar traces illustrating baseline CDI for reference. +CaM₁₂₃₄ traces illustrating complete loss of CDI upon CaM₁₂₃₄ coexpression. Bar graphs, confirming complete elimination of CDI by CaM₁₂₃₄ in population, where bars average $n = 5$ cells each. (b) Complete occlusion of CaM₁₂₃₄ effects in Ca_v1.3 channels attached at carboxy terminus to wild-type CaM via 12-glycine linker (Ca_v1.3–CaM in left schematic). Format as in a. Control traces show fullbore CDI, indistinguishable from that in Ca_v1.3 channels lacking covalent linkage. By contrast, +CaM₁₂₃₄ exemplar traces illustrate complete sparing of CDI. Bar-graph summaries confirm this trend, where each bar averages $n = 6$ cells each. (c) CaBP4 effects on Ca_v1.3–CaM. Format as in b. Control whole-cell current traces reproduced from b for reference. Remarkably, coexpressing CaBP4 with Ca_v1.3–CaM here completely eliminates CDI in both exemplar traces (+CaBP4), and population bar-graph data, where the dark control bar averages $n = 6$ cells, and the light gray bar for the CaBP4 condition averages $n = 7$ cells. (d) CaBP4 can still bind with Ca_v1.3–CaM, where black symbols bin ~ 5 -6 cells each. As reference, gray curve plots amplitude-normalized fit obtained without CaM fusion from Fig. 3e.

Figure 5. Only one CaBP4 can bind per CaV1.3 channel

(a) Fusion of both CaBP4 (by 8-glycine linker) and CaM (by 12-glycine linker) to CaV1.3 (CaBP4-CaV1.3-CaM, left cartoon) completely eliminates CDI, as seen from exemplar traces (middle left), overlay of Ba^{2+} (black) and Ca^{2+} (red) r_{300} relations (middle right, $n = 5$ cells per condition), and bar-graph f_{300} , averaging $n = 5$ cells for each bar. (b) CaBP4-CFP does not bind CaBP4-CaV1.3-YFP (left schematic showing CaV1.3 channel with CaBP4 fused to its amino terminus, and YFP to its carboxy terminus), suggesting only one CaBP4 can bind to CaV1.3. Middle diamond schematic indicates that CaBP4-CaV1.3-YFP cannot be perturbed from state 3 by overexpressing CaBP4-CFP as a separate molecule. Black symbols average ~ 3 -4 cells each. Reference gray fit, CaM binding CaV1.3 channels without fusion to CaBP4 from Fig. 3e. (c) Holochannel FRET indicates that CaM can still bind to CaBP4-CaV1.3-YFP. Right, corresponding CFP-CaM binding curve (black symbols bin ~ 3 -4 cells each, black fit). Amplitude-normalized reference gray fit, CaM binding CaV1.3 channels without fusion to CaBP4, from Fig. 3e.

Figure 6. Mechanism of Ca_v1.3 channel modulation by modest levels of CaBP4

(a) Allosteric model of CaM and CaBP4 binding to Ca_v1.3 channels, based on Fig. 1b, but now with experimentally determined association constants as listed and drawn from Figs. 2d, e, 4d, and 5c. (b) Model predictions for CaBP4 dependence of CDI. If dual-bound state 4 does not exist (i.e., pure competition), then black relation results under estimated free CaM of 10 μM . Entire 4-state allosteric scheme, with experimentally determined association constants predicts dashed curve for CDI dependence upon free CaBP concentration. Solid red curve fits experimental data from approach outlined in c, where symbols average $n = 5$ cells each. (c) Schematic of patch fluorometry experiments. (d) Top, schematic for increasing CaBP4 concentration. Bottom, Ca^{2+} traces corresponding to various symbols in b, as labeled.