Half-calciﬁed calmodulin promotes basal activity and
inactivation of the L-type calcium channel CaV1.2

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The L-type Ca$^{2+}$ channel CaV1.2 controls gene expression, cardiac contraction, and neuronal activity. Calmodulin (CaM) governs CaV1.2 open probability (Po) and Ca$^{2+}$-dependent inactivation (CDI) but the mechanisms remain unclear. Here, we present electrophysiological data that identify a half Ca$^{2+}$-saturated CaM species (Ca$_{2/3}$/CaM) with Ca$^{2+}$ bound solely at the third and fourth EF-hands (EF3 and EF4) under resting Ca$^{2+}$ concentrations (50–100 nM) that constitutively preassociates with CaV1.2 to promote Po and CDI. We also present an NMR structure of a complex between the CaV1.2 IQ motif (residues 1644–1665) and Ca$_{2/3}$/CaM$_{12}$, a calmodulin mutant in which Ca$^{2+}$ binding to EF1 and EF2 is completely disabled. We found that the CaM$_{12}$ N-lobe does not interact with the IQ motif. The CaM$_{12}$ C-lobe bound two Ca$^{2+}$ ions and formed close contacts with IQ residues I1654 and Y1657. I1654A and Y1657D mutations impaired CaM binding, CDI, and Po, as did disabling Ca$^{2+}$ binding to EF3 and EF4 in the CaM$_{12}$ mutant when compared to WT CaM. Accordingly, a previously unappreciated Ca$_{2/3}$/CaM species promotes CaV1.2 Po and CDI, identifying Ca$_{2/3}$/CaM as an important mediator of Ca signaling. CaV1.2 is the main L-type channel in heart, blood vessels, and brain (1, 2). Ca$^{2+}$ influx through CaV1.2 triggers cardiac contraction, regulates arterial tone (1), mediates synaptic long-term potentiation (3, 4), controls neuronal excitability (5), and mediates Ca$^{2+}$-dependent gene expression (6). Defects in inactivation of CaV1.2 cause Timothy syndrome, a rare congenital abnormality leading to lethal arrhythmias, autistic-like behaviors, and immune deficiency (7). Thus, defining mechanisms of CaV1.2 regulation is highly relevant for understanding its physiological and pathological functions. Ca$^{2+}$ inactivation (CDI) (8, 9). CDI is mediated by calmodulin (CaM) (8) that is preassociated with CaV1.2 under basal Ca$^{2+}$ conditions ([Ca$^{2+}$], = 100 nM) (10, 11). Ca$^{2+}$-free apoCaM has been suggested to be preassociated with CaV1.2 (12) and the closely related CaV1.3 (13). However, under physiological conditions, apoCaM binds to the isolated CaV1.2 IQ-motif with a dissociation constant ($K_D$) of ~10 μM (14, 15) and ~1 μM for full-length CaV1.2 (11). The concentration of free apoCaM is ~100 nM in neurons and cardiomyocytes (15, 16). Accordingly, the fractional binding of CaV1.2 to apoCaM is predicted to be less than 10% and may not be the prevalent CaM species bound to CaV1.2 or the closely related CaV1.3 under basal conditions as proposed previously (12, 13, 17).

To fill a critical gap in our understanding of how CaM governs CaV1.2 function, we used NMR structural analysis, protein biochemistry, and patch-clamp electrophysiology of WT and mutated CaV1.2 bound to CaM. Our studies uncovered a half-calciﬁed form of CaM (with Ca$^{2+}$ bound solely at EF3 and EF4, called Ca$_{2/3}$/CaM) that is functionally pre-associated with CaV1.2 under basal conditions. The NMR structure of Ca$_{2}$/CaM bound to the CaV1.2 IQ-motif (residues 1644–1664) suggests that the Ca$^{2+}$-bound CaM C-lobe (residues F93, M110, L113, M125) forms intermolecular interactions with the side chain atoms from CaV1.2 residues (Y1649, I1654, Y1657, and F1658), whereas the Ca$^{2+}$-free CaM N-lobe does not interact with the IQ motif. Electrophysiological data of key mutants of CaV1.2 (I1654A and Y1657E) contrasted with the earlier ﬁndings for the K1662E mutant along with the consequences of ectopic expression of CaM$_{12}$ all suggest that Ca$_{2/3}$/CaM, rather than apoCaM, preassociates with CaV1.2 under basal conditions to augment channel open probability (Po) and mediate rapid CDI.

Results

A CaM intermediate with two Ca$^{2+}$ bound

Isothermal titration calorimetry (ITC) studies have suggested that apoCaM binds to the IQ peptide with submicromolar affinity in the absence of salt (12). However, in the presence of physiological salt levels, apoCaM binds to the CaV1.2 IQ-motif with a dissociation constant ($K_D$) of 10 μM (14, 15). Earlier work suggests that binding of apoCaM to full-length CaV1.2 is ~10 times stronger than binding to the IQ segment (11). Collectively, these data suggest that apoCaM binds to full-length CaV1.2 with a $K_D$ of ~1 μM, which is outside the physiological concentration range of free CaM.

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(<100 nM) in neurons and cardiomyocytes (15, 16), implying low fractional binding. Furthermore, the recent NMR structure of apoCaM bound to the CaV1.2 IQ-motif revealed an intermolecular salt bridge involving CaV1.2 residue K1662, and the K1662E mutation signiﬁcantly and selectively weakened apoCaM binding to CaV1.2 (15). At the same time, the K1662E mutation does not affect single-channel Po (15). These previous results suggest that apoCaM may not be the main CaM species to support CaV1.2 activity under basal conditions as proposed previously (12, 13, 17). The current study tested the hypothesis that the CaV1.2 channel may preassociate mostly with apoCaM bound to its C-lobe under basal conditions ([Ca2+]i = 100 nM). Since the apoCaM N-lobe (CaMN) does not bind to IQ under physiological conditions (14, 18), we calculate that CaMC-IQ (Fig. 1) and CaMN-IQ (Fig. 2) have apparent Ca2+-binding afﬁnity of 100 nM and 1.0 μM, respectively:

\[
2\text{Ca}^{2+} + \text{CaMC} \xrightarrow{K_1 = 10^{12} M^{-2}} \text{Ca}_2/\text{CaMC}
\] (18)

\[
\text{Ca}_2/\text{CaMC} + IQ \xrightarrow{K_2 = 10^{17} M^{-1}} \text{Ca}_2/\text{CaMC} - IQ
\] (14)

\[
\text{CaMC} - IQ \xrightarrow{K_3 = 10^{-5} M} \text{CaMC} + IQ
\] (14)

Thus, the CaM C-lobe is calculated to have a 10-fold higher apparent Ca2+- afﬁnity compared to CaM N-lobe. This calculation implies that ~50% of CaM-IQ complex will have Ca2+ bound to its C-lobe under basal conditions ([Ca2+]i = 100 nM), whereas the N-lobe should be devoid of Ca2+. To test this prediction, we prepared a CaM mutant (D21A/D23A/D25A/E32Q/D57A/D59A/N61A/E68Q, called CaM12') that completely disabled Ca2+ binding to EF1 and EF2 but retained normal Ca2+ binding to EF3 and EF4. The apparent Ca2+- afﬁnity of CaM12' in the presence of saturating IQ peptide under physiological conditions (27 °C and 37 °C) was measured by ITC (Fig. 3, A and B). The ITC isotherm at 27 °C is biphasic, suggesting possible sample heterogeneity. The major binding component (N2 = 1.7 ±0.3 Ca2+ /protein; Table 1) represents binding of two Ca2+ to CaM12'-IQ as deﬁned by K2, ΔH2, and N2 (Table 1). The other isotherm component is non-stoichiometric (N1 = 0.2 ±0.1 Ca2+ /protein) and may be an artifact of IQ partial self-association or other sample heterogeneity. Fitting the ITC isotherm with a two-site model reveals a Ca2+-binding apparent K_D (K_D^app) of 60 ± 20 nM (Table 1), which agrees within experimental error with the predicted value in Figure 1 and with previously measured values of K_D^app obtained by UV ﬂuorescence (20). The Ca2+-binding ITC isotherm became monophasic at 37 °C, which more accurately demonstrates that two Ca2+ bind to CaM12' with a K_D^app of 72 ± 20 nM and ΔH = -7.7 ± 1 kcal/mol (Fig. 3B and Table 1). The relatively high apparent Ca2+- afﬁnity (K_D^app = 72 nM at 37°C) implies that at least 50% of the CaM/IQ complex will have Ca2+ bound to EF3 and EF4 (Y = [Ca2+] / [Ca2+]+[K_D]) at basal Ca2+ concentrations (~100 nM). This analysis predicts that slightly more than half of the CaV1.2 channels should be preassociated with the CaM intermediate, Ca2/CaM, under basal conditions.

Half-calciﬁed CaM represented by CaM12'

The concentration proﬁles in Fig. S1A show that half saturated CaM (Ca2/CaM) coexists in an equilibrium mixture with apoCaM and Ca2+-saturated CaM (Ca4/CaM). At a basal Ca2+ concentration of 100 nM, the fractional occupancy of Ca2/CaM is calculated to be 55% compared to 7% occupancy of Ca4/CaM and 38% occupancy of apoCaM. Therefore, under basal conditions, Ca2/CaM cannot be resolved from the other CaM species. To isolate the half Ca2+-saturated species,
we performed structural studies on the CaM mutant (D21A/D23A/D25A/E32Q/D57A/D59A/N61A/E68Q, called CaM12) that completely disables Ca2+ binding to EF1 and EF2 but retains Ca2+ binding to EF3 and EF4. The NMR assignments of Ca2+-bound CaM12 bound to the IQ peptide (Ca2+/CaM12-IQ) reveal two downfield NMR peaks assigned to G99 (EF3) and G135 (EF4) that indicate Ca2+ is bound to EF3 and EF4 (21). The corresponding Gly residues in EF1 (G26) and EF2 (G62) do not exhibit downfield amide resonances, indicating that EF1 and EF2 in Ca2/CaM12-IQ are both devoid of Ca2+.

The NMR spectrum of Ca2/CaM12-IQ is a hybrid of the spectra of Ca2+-bound and Ca2+-free CaM (Fig. 4, A and B). The chemical shifts assigned to the CaM12 C-lobe (residues 80–149) of Ca2/CaM12-IQ (peaks labeled red in Fig. 4A) are nearly identical to those of the isolated Ca2+-bound CaM C-lobe bound to IQ (blue peaks in Fig. 4A). NMR peaks assigned to CaM12 N-lobe (residues 1–79) of Ca2/CaM12-IQ are similar to those of apoCaM12 in the absence of IQ (black peaks in Fig. 4B), indicating that the CaM12 N-lobe is Ca2+ free and does not interact with the IQ peptide. Thus, only the C-lobe, but not N-lobe, residues in Ca2/CaM12 exhibit IQ-induced spectral shifts.

**Table 1**

| Temp (°C) | N1 | K1 (x10^6 M^-1) | ΔH1 (kcal/mol) | N2 | K2 (x10^7 M^-1) | ΔH2 (kcal/mol) | KappD (nM) |
|-----------|----|----------------|----------------|----|----------------|----------------|------------|
| 27        | 0.2 ± 0.1 | 6 ± 4 | -10 ± 1 | 1.7 ± 0.3 | 1.7 ± 0.4 | -7.5 ± 1 | 60 ± 20 |
| 37        | - | - | - | 1.8 ± 0.3 | 1.4 ± 0.3 | -7.7 ± 1 | 72 ± 20 |

**Figure 3. Isothermal titration calorimetry (ITC) binding assays.** A and B, ITC measurement of Ca2+ binding to CaM12-IQ at 27 °C (A) and 37 °C (B). The Ca2+ binding isotherms at 27 °C and 37 °C were fit to a two-site and one-site model, respectively. The apparent Ca2+ affinity (KappD) and enthalpy difference (ΔH1 and ΔH2) are given in Table 1. The CaM12-IQ complex in the sample cell (10 μM at 27 °C or 8.0 μM at 37 °C, 1.5 ml) was titrated with aqueous CaCl2 (0.23 mM at 27 °C or 0.30 mM at 37 °C) using 35 injections of 10 μl each. C–D, ITC measurement of Ca2+/CaM12 binding to IQ at 27 °C (C) and 37 °C (D). The dissociation constant (Kd) and enthalpy difference (ΔH) for Ca2/CaM12 binding to IQ mutants (IQWT, IQY1649A, IQI1654A, IQY1657D, IQF1658D, and IQF1658A) are given in Table 3. The binding of Ca2/CaM12 to IQK1662E could not be accurately measured by ITC because IQK1662E formed aggregated species under the conditions required for ITC. E–H, ITC measurement at 27 °C of Ca2/CaM12 binding to IQWT (E), IQY1649A (F), IQI1654A (G), and IQF1658A (H). The IQ peptide concentrations for WT, Y1649A, and I1654A were each 10 μM (27 °C) or 7.0 μM (37 °C) in 1.5 ml in the sample cell for titration with 0.1 mM Ca2/CaM12, and Y1657D and F1658D concentrations were each 50 μM in 1.5 ml for titration with 0.5 mM Ca2/CaM12 using 35 injections of 10 μl each. CaM, calmodulin.
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Figure 4. NMR-derived structures of Ca2+/CaM12–IQ. A, 15N–1H HSQC NMR spectrum of 15N-labeled Ca2+/CaM12 bound to unlabeled IQ (red) is overlaid with the spectrum of Ca2+-bound CaMwt C-lobe/IQ complex (blue). B, NMR spectrum of 15N-labeled Ca2+/CaM12 bound to unlabeled IQ (black) is overlaid with the spectrum of Ca2+-free CaM12 (red). C, ensemble of 10 lowest energy NMR structures of Ca2+/CaM12 (PDB ID: 7L8V). Main chain structures are depicted by a ribbon diagram. Structures of the C-lobe (residues 85–149) are overlaid and highlighted in red; N-lobe structures (residues 1–84) are highlighted in pink. Bound Ca2+ ions are yellow. Structural statistics are given in Table 2. D, the lowest energy structure of Ca2+/CaM12–IQ complex is shown as a ribbon diagram of Ca2+/CaM12 bound to the IQ peptide (cyan). The CaM N-lobe and C-lobe are highlighted pink and red, respectively. Side-chain atoms of key residues are depicted by sticks and are colored yellow and blue. E, overlay of the NMR structure of Ca2+/CaM12–IQ (C-lobe in red) with the crystal structure of Ca4/CaM (cyan, 2B6E). The C-lobe structures overlay with an RMSD of 1.8 Å. F, ﬂuorescence polarization assay showing the binding of half Ca2+-saturated CaM mutant (Ca2+/CaM12) with ﬂuorescently labeled IQ peptides (WT: black; K1662E: red; both: K0 < 100 nM), and of apoCaM binding to Y1657D (blue, K0 = 60 μM). CaM, calmodulin; PDB, Protein Data Bank.

Table 2

| NMR structural statistics for Ca2+/CaM12–IQ | Value (restraint violation) |
|-------------------------------------------|----------------------------|
| Short-range NOEs                          | 327 (0.0 ± 0.0)            |
| Long-range NOEs                           | 172 (0.0 ± 0.0)            |
| Hydrogen bonds                            | 81 (not used in water reﬁnement) |
| Dihedrals angles                          | 187 (0.1 ± 0.3)            |
| 1DHN RDC                                  | 24 (0.0 ± 0.0)             |
| RDC Q-Factor                              | 0.292                      |
| Coordinate precision (Å)                   |                            |
| RMSD backbone atoms                       | 0.83 ± 0.09                |
| RMSD all heavy atoms                      | 1.56 ± 0.1                 |
| Deviation from idealized geometry Bonds (Å) | 0.007 ± 0.000             |
| Angles (°)                                 | 0.753 ± 0.012              |
| Improper (°)                               | 0.927 ± 0.029              |
| Ramachandran Plot (%)                      | 75.0                       |
| Favored region                            | 19.0                       |
| Allowed region                            | 7.0                        |
| Outlier region                            |                            |
| Structure qualityb                         | 24                         |
| Clash score                               | 6.6%                       |
| Ramachandran outliers                     | 16.3%                      |

* Coordinate precision was calculated for C-lobe residues 85 to 149.

** Structure quality metrics assessed by MolProbity [51].

(22) and long-range orientational restraints derived from RDC data (23) as described in the Experimental procedures. The final NMR-derived structures of Ca2+/CaM12 are overlaid in Figure 4C and structural statistics summarized in Table 2. The two domains of Ca2+/CaM12 (N-lobe in pink and C-lobe in red, Fig. 4C) are separately folded and noninteracting, as was seen previously for the NMR structures of apoCaM (24–26). The overall precision of the NMR ensemble is expressed by a RMSD of 0.83 ± 0.09 Å calculated from the coordinates of the main chain atoms in the C-lobe (Fig. 4C) and 0.9 ± 0.1 Å from the main chain atoms in the N-lobe. The lowest energy NMR structure of Ca2+/CaM12 bound to the IQ peptide is shown in Figure 4D. The quality of the NMR structures of Ca2+/CaM12–IQ was assessed using PROCHECK-NMR (27), which shows that 93% of the residues occur in the allowed or favorable regions from the Ramachandran plot. The NMR structure of the Ca2+-bound CaM C-lobe (residues 80–149) of Ca2+/CaM12–IQ (dark red in Fig. 4, D and E) looks similar to that observed in the crystal structure of Ca2+-saturated CaM bound to the IQ (cyan in Fig. 4E) (28). The structure of the Ca2+-free CaM N-lobe (residues 1–78) of Ca2+/CaM12–IQ (light red in Fig. 4D) adopts a closed conformation and looks similar to that of apoCaM (26). The IQ peptide was veriﬁed by NMR to have a helical conformation (cyan in Fig. 4D). In the Ca2+/CaM12–IQ structure (Fig. 4D), the IQ residues (Y1649, I1654, Y1657, and F1658) point toward CaM and make extensive contacts with CaM C-lobe residues (E85, A89, F93, M110, L113, M125). The IQ peptide in the Ca2+/CaM12–IQ structure does not make any contacts with the Ca2+-free N-lobe, in contrast to the crystal structure of Ca4/CaM12–IQ (28–30) where IQ aromatic residues (F1648, Y1649, and F1652) make extensive contacts with N-lobe residues (F13, F69, M73).

IQ residue K1662 interacts with apoCaM more strongly than Ca2+/CaM12:

The NMR structure of Ca2+/CaM12–IQ (Fig. 4D) looks quite different from the recent NMR structure of apoCaM bound to IQ (15). In the apoCaM–IQ structure, K1662 forms intermolecular salt bridges with CaM residues, E85 and E88. By contrast, K1662 is mostly solvent exposed in the Ca2+/CaM12–IQ structure and does not contact either E85 or E88 (Fig. 4D). This analysis predicts that the CaV1.2 mutation K1662E K1662E was not soluble enough for ITC with Ca2+/CaM12, we used ﬂuorescence polarization (FP) to measure binding afﬁnity in the nanomolar
range. As predicted, titration of the IQ peptides with Ca$_2$/Ca$_{M12}^*$ reached full saturation at 100 nM Ca$_2$/Ca$_{M12}^*$, indicating a $K_D < 100$ nM for both, IQ$^{WT}$ and IQ$^{K1662E}$ (Fig. 4F). It was not possible to more accurately determine the actual $K_D$ because the IQ peptide concentration in Figure 4F had to be 100 nM due to limited detection sensitivity. This concentration is much larger than the $K_D$ for IQ$^{WT}$ (16 nM in Table 3) and apparently also for IQ$^{K1662E}$, as binding was clearly saturated at 100 nM for both peptides. The free concentrations of Ca$_2$/Ca$_{M12}^*$ ($[\text{Ca}_2/\text{Ca}_{M12}]_{\text{free}} = [\text{Ca}_2/\text{Ca}_{M12}]_{\text{total}} - [\text{IQ}]$ (fractional saturation)) are within the sample noise level during the first half of the titration when $[\text{Ca}_2/\text{Ca}_{M12}]_{\text{free}} < 100$ nM (see SD bars in Fig. 4F). During the second half of the titration, $[\text{Ca}_2/\text{Ca}_{M12}]_{\text{free}}$ was above the noise level and the titration curves show clear saturation at 100 nM providing an upper limit of 100 nM for the $K_D$ of both, IQ$^{WT}$ and IQ$^{K1662E}$, consistent with the 16 nM $K_D$ for IQ$^{WT}$ as seen by ITC (Table 3). As a result, Ca$_2$/Ca$_{M12}$ can bind to IQ$^{K1662E}$ in the nanomolar range in contrast to apoCaM, which binds to IQ$_{WT}$ with a $K_D$ of 100 nM (see SD bars in Fig. 4F). The $K_D$ for IQ$^{WT}$ is 16 ± 5 nM (at 27 °C), in contrast to the nanomolar binding of IQ$^{K1662E}$ with Ca$_2$/Ca$_{M12}^*$ (Fig. 4F). Accordingly, the K1662E mutation can be used to selectively disable apoCaM binding to CaV1.2, while retaining CaV1.2 binding to Ca$_2$/CaM.

**IQ residues Y1649, I1654, Y1657, and F1658 interact with Ca$_2$/Ca$_{M12}^*$**

The NMR structure of Ca$_2$/Ca$_{M12}^*$-IQ reveals intermolecular contacts with IQ residues, Y1649, I1654, Y1657, and F1658, that are each located on the same side of the IQ helix pointing toward the Ca$_2$-$^{2+}$-occupied C-lobe of Ca$_2$/Ca$_{M12}^*$ (Fig. 4D). As predicted by this analysis, the IQ peptide mutants IQ$^{Y1649A}$, IQ$^{I1654A}$, IQ$^{Y1657D}$, and IQ$^{F1658D}$ each exhibited weaker binding to Ca$_2$/Ca$_{M12}^*$ compared to IQ$^{WT}$. The $K_D$ was 16 ± 5 nM for IQ$^{WT}$, 26 ± 5 nM for IQ$^{Y1649A}$, 60 ± 10 nM for IQ$^{I1654A}$, 8000 ± 10 nM for IQ$^{Y1657D}$, 4000 ± 10 nM for IQ$^{F1658D}$, and 32 ± 5 nM for IQ$^{F1658A}$ (Fig. 3, C and E–H and Table 3). These findings validate our structural analysis and verify that Y1657 makes the strongest contact with CaM.

The highly exothermic binding of the IQ peptide to Ca$_2$/ Ca$_{M12}^*$ ($\Delta H = -15$ kcal/mol in Figure 3D and Table 3) predicts the $K_D$ to increase by 2.3-fold when the temperature is increased from 27 °C to 37 °C. As predicted, the $K_D$ for IQ binding to Ca$_2$/Ca$_{M12}^*$ increased from 16 ± 5 nM (at 27 °C) to 37 ± 10 nM at 37 °C. Also, the temperature dependence of $\Delta H$ (-10 kcal/mol at 27 °C versus −15 kcal/mol at 37 °C) indicates a negative $\Delta C_p$ value, which is consistent with the relatively large change in solvent accessible hydrophobic surface area that occurs when Ca$_2$/Ca$_{M12}^*$ binds to the IQ peptide.

**The K1662E mutation affects binding of apoCaM but not CDI of CaV1.2**

The aforementioned analysis suggests that K1662E retains binding to Ca$_2$/Ca$_{M12}^*$ but not apoCaM under physiological conditions (i.e., with free CaM < 100 nM (16)) (Fig. 4F). This differential effect informs interpretation of recently published data that showed that the K1662E mutation has no effect on Po (15), while the I1654A mutation, which affects binding of both apoCaM and Ca/CaM, decreased Po by 6-fold (15). A similar effect has been seen for an analogous Ile to Ala mutation in the closely related CaV1.3 (17). Collectively, these findings suggest that CaM promotes Po when it forms a complex with CaV1.2 with Ca$^{2+}$ bound to EF3 and EF4 to give rise to a half-saturated Ca$_2$/CaM state in this complex. To further test the idea of preassociation of half Ca$^{2+}$-saturated Ca$_2$/CaM with CaV1.2 at basal Ca$^{2+}$ concentrations, we wanted to compare CDI of CaV1.2$^{K1662E}$ with WT and also CaV1.2$^{K1664A}$, which served as a well-established reference point for loss of CDI (8, 13, 17). For that purpose, we measured whole-cell current density for I$_{ba}$ and I$_{ca}$. Consistent with the earlier Po analysis, I$_{ba}$ and I$_{ca}$ were reduced by the I1654A but not K1662E mutation (Fig. 5, A–D and Table S1A). Strikingly, the K1662E mutation had no significant effect on CDI (nor on voltage-dependent inactivation), in contrast to the I1654A mutation, which reduced CDI by ~75% (Fig. 5, B, E, and F and Table S1B). The small, remaining CDI seen for the I1654A mutant channel may be due to N-lobe effects such as its binding to the N terminus of the CaV1.2 a1 subunit (31). The differential effect on I$_{bar}$, I$_{car}$, and CDI by the K1662E versus I1654A mutation is consistent with the differential effect of the K1662E versus I1654A mutation on Po (15) and suggests that formation of a complex of CaV1.2 with half Ca$^{2+}$-saturated Ca$_2$/CaM is important for Po and for predisposing CaV1.2 to CDI.

**The Y1657D mutation strongly affects binding of half-saturated Ca$_2$/CaM as well as I$_{bar}$, I$_{car}$, Po, and CDI of CaV1.2**

Our new Ca$_2$/Ca$_{M12}^*$-IQ structure indicates that Y1657 makes the most and closest contacts among all IQ residues with Ca$_2$/Ca$_{M12}^*$ (Fig. 4). In support of its central role in mediating this interaction, binding studies indicate that the Y1657D mutation has the strongest negative effect on the affinity of the Ca$_2$/Ca$_{M12}^*$-IQ interaction of all tested IQ peptides ($K_D$ for IQ$^{WT}$ is 16 nM and for IQ$^{Y1657D}$ 8 μM; Table 3). The Y1657D mutation decreased whole-cell currents, I$_{ba}$ and I$_{ca}$, as well as CDI with no apparent effect on voltage dependent inactivation (Fig. 6, A–F). Single-channel recordings show a remarkably strong decrease in Po for Y1657D versus WT.

### Table 3

| Temp (°C) | IQ peptide | $K_D$ (nM) | $\Delta H$ (kcal/mol) | n-value |
|-----------|------------|------------|----------------------|---------|
| 37        | WT         | 37 ± 10    | -15 ± 0.2            | 0.76 ± 0.25 |
| 27        | WT         | 16 ± 5     | -10 ± 0.2            | 0.77 ± 0.25 |
| 37        | Y1649A     | 26 ± 5     | -9.7 ± 0.2           | 0.88 ± 0.25 |
| 27        | I1654A     | 60 ± 10    | -9.2 ± 0.2           | 0.89 ± 0.25 |
| 27        | Y1657D     | 8000 ± 900 | -5.6 ± 0.7           | 0.72 ± 0.5 |
| 27        | Y1658A     | 32 ± 5     | -9.5 ± 0.2           | 1.0 ± 0.25 |
| 27        | F1658D     | 4000 ± 700 | -5.9 ± 0.7           | 0.8 ± 0.5 |

The errors are the SD calculated from three independent trials.

**CaV1.2 channel regulation by half-calciﬁed CaM**
CaV1.2 channel regulation by half-calciﬁed CaM

Figure 5. Effects of IQ mutants I1654A and K1662E on CaV1.2 activity and inactivation. A, topology of the hypothetical CaV1.2 Ca2+-channel pore and localization of the IQ domain and its mutations in the α1.2 subunit. At rest with [Ca2+]i ≤ 100 nM the C-lobe (green) of half-calciﬁed Ca2+/CaM is predicted to bind to the C-terminal portion of the IQ motif, making hydrophobic contacts with I1654 and Y1657 but not with K1662.

A. For the green patches we omit the leucine residues (EAAAR; see text). A prominent 45 kDa band and a weaker signal for the endogenous 17 kDa band. Comparison of the 17 kDa band in mock-transfected (no CaM vectors) cell lysate to the same MR indicates no endogenous CaM is expressed in 293T cells transfected with CaV1.2 expression constructs ± WT CaM or CaM34 plasmids (Fig. S2). We found that overexpression of WT CaM compared to endogenous CaM is about ~10 fold, while CaM34 is ~20 fold (Fig. S2, A–D). To test whether ectopic expression of CaM affects levels of endogenous CaM, we expressed YFP-tagged WT CaM or CaM34, which migrate as a prominent 45 kDa band and a weaker signal for the endogenous 17 kDa band. Comparison of the 17 kDa band in mock-transfected (no CaM vectors) cell lysate to the same MR immunoreactive band in the CaM plasmid-transfected samples did not indicate a significant effect of ectopic CaM on endogenous CaM levels (Fig. S2, E and F).

Consistent with earlier work on CaV1.3 by Adams et al. (17), we ﬁnd that overexpression of WT CaM strongly increases Po by ~300% as compared to expression of CaV1.2 alone (Fig. 7, A and B and Table S3). This effect could be due to increased binding of apoCaM, half Ca2+-saturated Ca2+/CaM, or both. Because earlier work did not differentiate between these possibilities (17), we tested the effect of ectopic expression of CaM34 and found no increase at all in Po as compared to

CaV1.2 (Fig. 6, F and G). This loss in Po and CDI is comparable to similarly strong effects for the I1654A mutation on Po (15) and CDI (9) but the K1662E mutation, which speciﬁcally affects apoCaM but not Ca/CaM binding, did not affect Po (15) or CDI (Fig. 5). The decrease in Po is also well reﬂected when calculating the ensemble averages of unitary single-channel currents (Fig. 6F and Table S2). To test whether there is also a change in channel surface expression in addition to a decrease in Po of individual channels, we conducted surface biotinylation experiments. We determined that CaV1.2 surface expression was reduced by almost 50% (Fig. 6, H and I), which can explain some, but not all, of the 80% loss in Po.

CaM intermediate (Ca2+/CaM) increases Po of CaV1.2

To further analyze the role of CaM in Po, we ectopically expressed CaM in HEK 293T cells. Although this approach has been used before to deﬁne the role of CaM in CDI, the level to which exogenous CaM was expressed in these CDI studies had not been thoroughly assessed (32). Thus, we investigated whether the expression of CaM34 (described by (8)) was sufﬁcient to allow detection of an effect (i.e., many fold greater than endogenous CaM) by immunoblotting extracts of 293T cells transfected with CaV1.2 expression constructs ± WT CaM or CaM34 plasmids (Fig. S2). We found that overexpression of WT CaM compared to endogenous CaM is about ~10 fold, while CaM34 is ~20 fold (Fig. S2, A–D). To test whether ectopic expression of CaM affects levels of endogenous CaM, we expressed YFP-tagged WT CaM or CaM34, which migrate as a prominent 45 kDa band and a weaker signal for the endogenous 17 kDa band. Comparison of the 17 kDa band in mock-transfected (no CaM vectors) cell lysate to the same MR immunoreactive band in the CaM plasmid-transfected samples did not indicate a significant effect of ectopic CaM on endogenous CaM levels (Fig. S2, E and F).

Consistent with earlier work on CaV1.3 by Adams et al. (17), we ﬁnd that overexpression of WT CaM strongly increases Po by ~300% as compared to expression of CaV1.2 alone (Fig. 7, A and B and Table S3). This effect could be due to increased binding of apoCaM, half Ca2+-saturated Ca2+/CaM, or both. Because earlier work did not differentiate between these possibilities (17), we tested the effect of ectopic expression of CaM34 and found no increase at all in Po as compared to
expression of CaV1.2 alone. This result demonstrates that Ca2+ binding to EF3 and EF4 in CaM is essential for promoting the increased Po. There was no detectable effect on surface expression of CaV1.2 by either WT CaM or CaM34 (Fig. 7, C and D and Table S3). Given the ~20-fold higher expression levels of CaM34 versus endogenous CaM, it seems especially remarkable that this overexpression had no effect at all on Po when a lesser degree of overexpression of WT CaM induced a ~3-fold increase in Po (Fig. 7). Collectively, these data indicate that binding of Ca2+/CaM and not apoCaM to CaV1.2 facilitates rapid CDI (8, 9). We provide multiple lines of evidence that CaV1.2 preassociates with half-calciﬁed Ca2/CaM that contains two Ca2+ bound to the CaM C-lobe. The fact that the CaM34 mutant abolished the 300% increase in channel open probability of CaV1.2 caused by WT CaM (Fig. 7, A and B) implies that Ca2+ binding to EF3 and EF4 (hence half-calciﬁed CaM) is essential for CaV1.2 channel function. Also, our binding analysis reveals that IQ binding to CaM increases the apparent Ca2+ afﬁnity by at least 10-fold (see Fig. 1 and Table 3), consistent with observations from previous binding studies (14, 20). Hence, the IQ-bound CaM C-lobe is more than 50% saturated with Ca2+ at basal Ca2+ concentrations when CaM is saturated with the IQ peptide (Fig. S1). The concentration of free endogenous CaM inside a cell is estimated to be between 50 to 100 nM (16). As Ca2+/CaM binds to the IQ motif with a KD of 16 nM, we estimate

Discussion

Preassociation of CaM with CaV1.2 and the highly homologous CaV1.3 under basal conditions has been suggested to both augment channel activity at low Ca2+ levels (17) and
that ~50% of CaV1.2 is bound to Ca2+/CaM under basal conditions, which would put the channel regulation by CaM in the middle of its dynamic range.

The NMR structure of Ca2+/CaM12-IQ reveals that half Ca2+-saturated CaM (Ca2+/CaM) has a closed conformation in the Ca2+-free N-lobe and a Ca2+-bound open conformation in the C-lobe (Fig. 4). The N- and C-lobe structures of Ca2+/CaM12-IQ are separately folded and do not exhibit interdomain contacts (Fig. 4C). The two separate lobes in Ca2+/CaM12-IQ are dynamically independent, similar to apoCaM (26, 33, 34). The Ca2+-free N-lobe structure in Ca2+/CaM12-IQ does not interact with the IQ peptide, in contrast to the IQ contacts with the N-lobe observed in the crystal structure of Ca2+-saturated CaM (28–30). The IQ peptide binds exclusively to the Ca2+-bound C-lobe of Ca2+/CaM (Fig. 4D), whose structure is similar to the C-lobe of Ca3/CaM bound to the IQ (Fig. 4E) (28–30). The IQ peptide bound to Ca3/CaM12 is rotated 180° compared to the orientation of the IQ bound to apoCaM (15). The opposite binding orientation may explain in part why the IQ binds to Ca3/CaM with at least 100-fold higher affinity (Fig. 4F) compared to that of apoCaM (14, 15). The contrasting binding orientation also suggests why the preassociation of CaV1.2 with Ca2+/CaM (rather than with apoCaM) predisposes CaV1.2 for CDI. Since Ca2+/CaM and Ca4/CaM both bind to CaV1.2 with the same orientation, CaM can remain bound to CaV1.2 upon Ca2+ influx to facilitate rapid CDI. By contrast, preassociated apoCaM would first need to dissociate from CaV1.2 upon Ca2+ influx and then subsequently rebind in the conformation adopted by Ca2+-saturated Ca4/CaM to engage CDI (28–30). This unbinding of apoCaM and rebinding of Ca4/CaM would likely prevent rapid CDI and defeat the purpose of the CaM preassociation.

Our functional analysis fully supports the relevance of prebinding of Ca3/CaM to the CaV1.2 IQ motif. The K166E mutation, which impaired binding of apoCaM (15) but retained binding to Ca2+/CaM at physiological CaM concentrations of ~100 nM (16) (Fig. 4F), did not affect Po (15), CDI,
I_{Ca} or I_{Ba} (Fig. 5). Furthermore, the Y1657D mutation impaired binding of apoCaM ($K_D = 60 \, \mu M$, Fig. 4f), as well as Ca_{2+}/CaM ($K_D = 8 \, \mu M$, Table 3), and reduced Po, CDI, I_{Ba} and I_{Ca} (Fig. 6). We also tested the effect of ectopic expression of CaM_{34} and CaM_{1234}. Consistent with the earlier work on the closely related CaV1.3 (17), overexpression of WT CaM strongly augmented Po (Fig. 7 and Table S3). The main finding of these authors (17) was that substitution of the eponymous Ile in the IQ motif by Met reduced Po and overexpression of WT CaM rescued this loss. Because mutating this Ile reduces binding of apoCaM, these authors concluded that it is apoCaM that binds to the IQ motif under resting Ca_{2+} concentrations to augment Po. However, they did not test the effect of overexpression of CaM_{1234} or CaM_{34} on single channel activity as is required for measuring Po and thus did not rule out that Po is driven by the binding of Ca_{2+}/CaM, whose binding to the IQ motif is also strongly impaired by mutating this Ile. Importantly, we found that neither CaM_{34} (Fig. 7 and Table S3) nor CaM_{1234} (Fig. S3 and Table S4) increased Po, despite the fact that the exogenous CaM levels were much higher (by 20-fold) than that of endogenous CaM. In addition, the differential effects of (1) the K1662E mutation on CaV1.2 binding to apoCaM versus Ca_{2+}/CaM; (2) K1662E versus Y1657D on Po and CDI; and (3) WT CaM versus CaM_{34} or CaM_{1234} on Po collectively indicate that preassociated Ca_{2+}/CaM is an important factor in determining channel Po.

As discussed previously, we estimate that ~50% of CaV1.2 is occupied by Ca_{2+}/CaM with little occupancy by apoCaM due to its low concentration in the cytosol ($50–100 \, nM$ (16)) and low affinity binding to the IQ ($K_D = 10 \, \mu M$ (15)) and full-length CaV1.2 ($K_D = 1 \, \mu M$ (11)). How then can the remainder of the CaV1.2 population possess a reasonable level of activity? We previously found that binding of α-actinin to the IQ motif also strongly augments Po (15). Thus, we propose a model in which CaV1.2 is either occupied by α-actinin, which at the same time anchors CaV1.2 at the cell surface and especially in dendritic spines where α-actinin is concentrated (35) or by Ca_{2+}/CaM. Accordingly, in addition to strongly promoting Po, α-actinin also augments the Ca_{2+}/CaM surface expression (15), perhaps by connecting to F-actin (36). On the other hand, Ca_{2+}/CaM augments Po with apparently little if any effect on surface expression. Channel occupancy by Ca_{2+}/CaM could be increased upon modest increases of basal Ca_{2+} influx potentially in a positive feedback loop at low Ca_{2+} levels and low channel activity. However, prolonged displacement of α-actinin by Ca_{2+}/CaM also triggers endocytosis of CaV1.2 as a negative feedback mechanism (35). At this point, we cannot be certain about how α-actinin and CaM intersect at the IQ motif to govern CaV1.2 activity, and much needs to be learned with respect to the exact function of these interactions.

In conclusion, our analysis provides novel mechanistic insight into preassociation of CaM with CaV1.2 and its role in controlling channel activity and CDI. These findings are not only of functional relevance for understanding the physiological effects of CaV1.2 but also inform the current understanding of pathological events such as arrhythmias due to impaired CDI (37, 38).

### Experimental procedures

**CaM_{12} mutagenesis and purification and IQ peptide for NMR**

The CaM_{12} mutation ((D21A/D23A/D25A/E32Q/D57A/D59A/N61A/E68Q) was introduced into Xenopus CaM complementary DNA by PCR QuickChange procedure (39). The mutated complementary DNA was inserted into the Neol/BamHI sites of a pET11d vector and verified by automated Sanger sequencing. The recombinant CaM_{12} protein was expressed from a pET11d vector in a BL21(DE3) Codon Plus Escherichia coli strain (Stratagene) and purified as described previously (40). The CaV1.2 IQ peptide (residues 1644–1664) was purchased from ChinaPeptides. The peptide was dissolved in d_{6}-dimethyl sulfoxide to give a peptide concentration of 7.8 mM. The peptide concentration was determined by measuring absorbance at 280 nm with $ε_{280} = 2980 \, M^{-1} \, cm^{-1}$. An aliquot of peptide (1.5 equivalents) was added to a dilute solution of CaM_{12} (50 μM protein dissolved in 20 mM 2-amino–2–hydroxymethyl-propane-1,3-diol-d11 (Tris-d_{11}) with 95% H_{2}O/5% D_{2}O). The complex was then concentrated to a final concentration of 500 μM in a final volume of 500 μl for NMR experiments. The 1.5-fold excess of IQ peptide in the NMR sample of Ca_{2+}/CaM_{12}–IQ was necessary to minimize the occupancy of a 2:1 complex, in which two molecules of CaM_{12} were bound to one IQ. The HSQC spectrum of a sample that contained an equal concentration of CaM_{12} and IQ revealed two distinct peaks for each C-lobe residue of CaM_{12} (Fig. S4D). The most intense peak represented a 1:1 complex (~90% occupancy) and a weaker second peak (marked by arrows in Fig. S4D) represented a second CaM_{12} molecule bound to IQ in a 2:1 complex (~10% occupancy). The relative occupancy of the 2:1 complex could approach nearly 100% when the CaM_{12} concentration is more than 10-fold higher than that of CaV1.2, like what exists inside HEK293 cells used in the CaV1.2 electrophysiological experiments (Fig. S2). The 2:1 complex likely consists of a single IQ peptide that binds tightly to a Ca_{2+}-bound C-lobe on one side of the IQ helix (CaM_{12} C-lobe contacting I1654 and Y1657) as well as a second CaM_{12} C-lobe that binds with lower affinity to the opposite side of the IQ helix (CaM_{12} C-lobe contacting F1648 and F1652). The binding of a second C-lobe from CaM_{12} mimics the binding of the Ca_{2+}-bound N-lobe from WT CaM. Therefore, we suggest that the CDI observed for CaV1.2 in the presence of CaM_{12} (13) is likely an artifact of the formation of a 2:1 complex in HEK293 cells involving two of the overexpressed CaM_{12} molecules bound to a single CaV1.2.

**ITC**

ITC experiments were performed using a VP-ITC calorimeter (Micro-Cal) at 27 °C and 37 °C. The data were acquired and processed with MicroCal software (https://www.originlab.com) as described previously (41). The first data point from each ITC isotherm was deleted because the amount...
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of injectant delivered during the first injection has significant error caused by a dead volume void in the injection syringe. For ITC experiments in Figure 3, A and B, samples of Ca\textsuperscript{2+} (injectant) and CaM\textsubscript{2-}–IQ complex (tirrent) were prepared by exchanging each into buffer containing 20 mM Tris, pH 7.4, and 100 mM KCl. The CaM\textsubscript{2-}–IQ complex in the sample cell (10 μM at 27 °C or 8.0 μM at 37 °C in 1.5 ml) was titrated with aqueous CaCl\textsubscript{2} (0.23 mM at 27 °C or 0.3 mM at 37 °C) using 35 injections of 10 μl each. For the ITC experiments in Fig. 3, C, E–H, samples of Ca\textsubscript{2}/CaM\textsubscript{2+} (injectant) and IQ peptide (tirrent) were prepared by exchanging each into buffer containing 20 mM Tris, pH 7.4, 100 mM KCl, and 1 mM CaCl\textsubscript{2}. The concentrations of the IQ peptides (WT, Y1649A, I1654A, or F1658A) were each 10 μM in 1.5 ml in the sample cell for titration with 0.1 mM Ca\textsubscript{2}/CaM\textsubscript{2+} and the concentrations of Y1657D and F1658D were each 50 μM in 1.5 ml for titration with 0.5 mM Ca\textsubscript{2}/CaM\textsubscript{2+} using 35 injections of 10 μl each.

**NMR spectroscopy**

All NMR measurements were performed at 303 K using a Bruker Avance III 600 MHz spectrometer equipped with a four-channel interface and triple-resonance cryoprobe. NMR sample preparation of Ca\textsubscript{2}/CaM\textsubscript{12–IQ} was described previously (21). Two-dimensional NMR experiments (heteronuclear single quantum coherence [HSQC] and HSQC-IPAP) were recorded on samples of \textsuperscript{15}N-labeled Ca\textsubscript{2}/CaM\textsubscript{12} (0.5 mM) bound to unlabeled IQ (0.75 mM). Each sample was dissolved in 20 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol-d\textsubscript{11} (Tris-d\textsubscript{11} at pH 7.5), 1.0 mM CaCl\textsubscript{2}, and 95% H\textsubscript{2}O/5% D\textsubscript{2}O. Three-dimensional NMR experiments for assigning backbone and side-chain resonances, and NOESY distance restraints were analyzed as described previously (42). NMR data were processed using NMRPipe (43) and analyzed with SPARKY (Goddard T.D. and Kneller D.G., University of California at San Francisco). To measure RDCs (23) of Ca\textsubscript{2}/CaM\textsubscript{12} bound to the IQ peptide, the filamentous bacteriophage Pf1 (Asla Biotech Ltd) was used as an orienting medium. Pf1 (12 mg/ml) was added to an NMR sample that contained either \textsuperscript{15}N-labeled Ca\textsubscript{2}/CaM\textsubscript{12} bound to unlabeled IQ, \textsuperscript{3}H,\textsuperscript{15}N residual dipolar coupling constants (DNH) were measured using a 2D IPAP (inphase/antiphase) \textsuperscript{1}H-\textsuperscript{15}N HSQC experiment as described by (44). Representative IPAP-HSQC spectra of \textsuperscript{13}C-labeled Ca\textsuperscript{2}/CaM\textsuperscript{12} bound to the IQ peptide are shown in Fig. S4A. Briefly, the backbone N-H RDCs were calculated by measuring the difference in \textsuperscript{15}N splitting for each amide resonance, both in the presence and absence of the orienting medium. The RDC Q-factor and analysis of RDC data were calculated by PALES (45). The Q-factor is calculated as Q = RMS(D\textsubscript{meas}−D\textsubscript{calc})/RMS(D\textsubscript{meas}), where D\textsubscript{meas} is the measured RDC, D\textsubscript{calc} is the calculated RDC, and RMS is the root mean square difference. A Q-factor of 30% corresponds to 2 Å resolution.

**NMR structure calculation**

NMR-derived structures of Ca\textsubscript{2}/CaM\textsubscript{12} bound to the IQ peptide were calculated using restrained molecular dynamics simulations within Xplor-NIH (46). RDCs, NOE distances, dihedral angles from TALOS+ (47), and backbone hydrogen bonds were used as structural restraints. NOEs were obtained from \textsuperscript{1}H-\textsuperscript{15}N-edited NOE-SY-HSQC, \textsuperscript{13}C-edited NOE-SY-HSQC (aliphatic), and \textsuperscript{13}C-filtered NOE-SY-HSQC as described by (48). Representative \textsuperscript{13}C-edited NOE-SY-HSQC and \textsuperscript{13}C-filtered NOE-SY-HSQC spectra of \textsuperscript{13}C-labeled Ca\textsubscript{2}/CaM\textsubscript{12} bound to unlabeled IQ peptide are shown in Fig. S4, B and C, respectively. Backbone dihedral angles were calculated by TALOS+ (47) using backbone chemical shifts (H\textalpha, C\textalpha, C\textbeta, CO, \textsuperscript{13}N, and HN) as input. Hydrogen bond restraints in helices and \textbeta-sheets were verified by measuring amide hydrogen-deuterium exchange rates as described by (49). The Xplor-NIH structure calculation was performed in three stages: annealing, refinement, and water refinement (50). Annealing started from an extended random structure. A total of 200 structures were calculated and the one with lowest energy was used as a starting structure during the refinement. The lowest energy structure was refined in an explicit water environment. A Ramachandran plot was generated by PROCHECK-NMR (27) and structure quality was assessed by MolProbity (51).

**FP assays**

Fluorescein-labeled peptides (100 nM; ChinaPeptides) were titrated with increasing concentrations of purified Ca\textsubscript{2}/CaM\textsubscript{12} in FP buffer (20 mM Tris, pH 7.4, 100 mM KCl, 1 mM MgCl\textsubscript{2}, 1.0 mM CaCl\textsubscript{2}) or apoCaM in Ca\textsuperscript{2+}-free buffer (20 mM Tris, pH 7.4, 100 mM KCl, 1 mM MgCl\textsubscript{2}, 2.0 mM EGTA). FP was measured with a Synergy 2 plate reader (BioTek) as described (52). FP was calculated as P = (I\textsubscript{u} − g\textsubscript{b}I\textsubscript{b})/(I\textsubscript{u} + g\textsubscript{h}I\textsubscript{h}); I\textsubscript{u} and I\textsubscript{b} are vertical and horizontal fluorescence intensity, respectively, and g is the correction factor for fluorescein. To obtain binding curves and K\textsubscript{P} values, data were fitted in GraphPad Prism 5 (GraphPad Software Inc) to the equation Y = B\textsuperscript{X}/(K\textsubscript{P} + X); B is maximal FP value that would be reached at saturation as determined by extrapolation of the fitted curve.

**Concentration profiles of CaM species versus [Ca\textsuperscript{2+}]**

The concentration profiles of apoCaM-IQ, Ca\textsubscript{2}/CaM-IQ, and Ca\textsubscript{4}/CaM-IQ as a function of the free Ca\textsuperscript{2+} concentration were calculated according to the following scheme in Figure 8.

**Expression of CaV1.2 IQ domain mutants and CaM species in HEK 293T/17 cells**

HEK 293T/17 cells (ATCC) were maintained as previously described (15, 53). For electrophysiology, Lipofectamine 2000 (Invitrogen) or JetPrime (Polyplus Transfection) was used to transiently transfect cells with indicated plasmid DNAs in 35 mm dishes. For biochemistry experiments, transient transfection of HEK 293T/17 cells in 100 mM dishes was
achieved using either JetPrime or, as previously described (15, 53), the calcium phosphate method. Cells were cotransfected with plasmids encoding the pore-forming α1.2 subunit N-terminally tagged with eCFP (15, 53) or mCherry (54) plus pGWIH-based plasmids encoding the auxiliary subunits rat β2A (55) and rabbit αδ-1 (56) as previously described (15, 53). For all transfections, equimolar ratio of 1:1:1 was used for CaV1.2 channel subunits and later further optimized (JetPrime) for CaM (at ratio of 1:1:0.5 for α1.2:β2A:αδ-1:CaM). Rat brain α1.2 (GenBank ID: M67515.1) N-terminally fused to eCFP was utilized as previously described (15). The point mutations in plasmids encoding single-residue I1654A, Y1657D (this report), and K1662E exchanges in α1.2 were generated via QuickChange II as previously described (15, 53) using N-terminally tagged eCFP (15, 53) or mCherry tagged (54) rat brain α1.2 plasmid template DNAs. We studied CDI using mCherry-tagged α1.2 subunit coexpressed with the other, untagged CaV1.2 subunits and WT CaM or the calmodulin 34 mutant CaM34 (kindly provided by JP Adelman, (8)). For some biochemical experiments shown in Fig. S2, YFP-tagged CaM was used (32).

Whole-cell patch clamp recording

Macroscopic Ba²⁺- (Iba) and Ca²⁺ currents (Ica) of CaV1.2 L-type Ca²⁺ channels were obtained in the whole-cell configuration using external bath solution containing (in mM) 134 N-methyl-D-glucamine, 10 BaCl₂ (for CDI, 10 CaCl₂), 1 MgCl₂, 10 Heps, and 10 glucose with an adjusted pH of 7.4 (Cs-OH) and an osmolarity of 300 to 310 mOsm (sucrose). Intracellular pipette solution contained (in mM) 125 Cs-MeSO₄, 5 CsCl, 10 EGTA, 10 Heps, 1 MgCl₂, 4 Mg-ATP, and pH 7.3 (CsOH), mOsm 290 to 300 (sucrose). Cells were clamped at a holding potential of ~80 mV and depolarized for 900 ms to a series of activating potentials, from ~60 mV to ~50 mV (or ~80 mV for Ca²⁺ currents), in increments of 10 mV at an interval of 0.033 Hz. The series resistance and the cell capacitance were directly taken from the Amplifier (Axopatch 200B, Molecular Device) and compensated to ~40%. Data were sampled at 10 kHz and lowpass filtered at 2 kHz. Leak subtracted raw data were analyzed with Pclamp10 and GraphPad Prism IX software. All recordings were performed at room temperature (RT).

Cell-attached patch clamp recording

Single-channel recordings were performed as described previously (15, 31). In brief, low noise raw data were recorded with an Axopatch 200B amplifier and data were sampled at 10 kHz with a low-pass filter at 2 kHz (3 dB, four pole Bessel) and digitalized with a Digidata 1440 digitizer. Recording electrodes were pulled from borosilicate capillary glass (0.86 OD/1.25 ID) with a Flaming/Brown micropipette puller (Model P-97, Sutter Instruments), heat polished, and coated with Sylgard (Sylgard 289) until close to the electrode tip. Electrode resistance in solution was usually 5 to 10 MΩ. To keep the membrane potential close to 0 mV the extracellular bath solution contained (in mM) 120 K-Glutamate, 25 KCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 Heps, and 2 Na₂-ATP pH 7.4 (KOH). The intracellular pipette solution contained (in mM) 110 BaCl₂ and 10 Heps, adjusted to pH 7.4 (TEA-OH). Cells were depolarized for 2 s from a holding potential of ~80 mV to 0 mV every 7 s. Event lists were created from raw Ba²⁺ currents after leak and capacity transients were digitally subtracted by pClamp 10. Unitary current events were then analyzed based on the half-height criterium (57) using the single-channel software provided by pClamp 10.

For statistical analysis, single-channel parameters were corrected by the channel number (k), respectively, the maximum of simultaneously open channels (P_MAX). The number of channels in the patch was estimated based on the observed simultaneous openings and is a precise parameter for k < 4, as included in this article and originally described by R. Horn (58). On average, 100 to 200 Ba²⁺ current traces were recorded for each cell for each experimental condition for an appropriate statistical analysis.

Surface biotinylation, NeutrAvidin pull downs, and immunoblotting

Surface biotinylation and analysis of CaV1.2 surface expression was carried out essentially as described (15, 53) with the following modifications. Twenty-two to twenty-four hours post transfection, HEK 293T/17 cells plated in 100 mm diameter dishes were rinsed with RT PBS-CM (PBS supplemented with 1 mM Ca²⁺ and 0.5 mM Mg²⁺) and placed on ice. Cell were incubated with freshly prepared 0.4 mg/ml of EZ-Link-Sulfo-NHS-LC-biotin (Thermo Fisher Scientific) in PBS-CM for 30 min, followed by quenching of remaining NHS reactive groups with ice-cold 100 mM glycine in PBS-CM, four separate washes with quenching buffer, and a final rinse with PBS alone. Labeled and quenched cells were dislodged by scraping and directly lysed into ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 1% NP-40, 0.05% SDS, 0.4% DOC, and 10% glycerol) supplemented with protease inhibitors: 1 μg/ml leupeptin (Merck Millipore), 2 μg/ml aprotinin (Merck Millipore), 1 μg/ml pepstatin A (Merck Millipore), and 34 μg/ml PMSF (Sigma). Lysates were cleared of insoluble material via centrifugation at 200,000g for 30 min at 4 °C. The protein concentration of the solubilized material in the cleared lysate was determined by a standard bicinechonic acid assay (Thermo Fisher Scientific). Biotinylated constituents in equal amount protein lysates (∼400 μg/sample) were affinity purified by incubation with 30 μl of NeutrAvidin-conjugated Sepharose beads (Thermo Fisher Scientific) for 2 h at 4 °C. Bead-bound material was sedimented by centrifugation, washed several times with ice-cold buffer, and bound proteins extracted in SDS sample buffer (with shaking at 65 °C for 15 min). Proteins from pull downs as well as directly loaded lysates were fractionated by 7.5% acrylamide SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF; Bio-Rad) membranes. For experiments used for analysis of CaM expression levels in directly loaded lysates (Fig. 6), 12% acrylamide gels were used. PVDF membranes were stained with

CaV1.2 channel regulation by half-calciﬁed CaM

Surface biotinylation and analysis of CaV1.2 surface expression was carried out essentially as described (15, 53) with the following modifications. Twenty-two to twenty-four hours post transfection, HEK 293T/17 cells plated in 100 mm diameter dishes were rinsed with RT PBS-CM (PBS supplemented with 1 mM Ca²⁺ and 0.5 mM Mg²⁺) and placed on ice. Cell were incubated with freshly prepared 0.4 mg/ml of EZ-Link-Sulfo-NHS-LC-biotin (Thermo Fisher Scientific) in PBS-CM for 30 min, followed by quenching of remaining NHS reactive groups with ice-cold 100 mM glycine in PBS-CM, four separate washes with quenching buffer, and a final rinse with PBS alone. Labeled and quenched cells were dislodged by scraping and directly lysed into ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 1% NP-40, 0.05% SDS, 0.4% DOC, and 10% glycerol) supplemented with protease inhibitors: 1 μg/ml leupeptin (Merck Millipore), 2 μg/ml aprotinin (Merck Millipore), 1 μg/ml pepstatin A (Merck Millipore), and 34 μg/ml PMSF (Sigma). Lysates were cleared of insoluble material via centrifugation at 200,000g for 30 min at 4 °C. The protein concentration of the solubilized material in the cleared lysate was determined by a standard bicinchonic acid assay (Thermo Fisher Scientific). Biotinylated constituents in equal amount protein lysates (∼400 μg/sample) were affinity purified by incubation with 30 μl of NeutrAvidin-conjugated Sepharose beads (Thermo Fisher Scientific) for 2 h at 4 °C. Bead-bound material was sedimented by centrifugation, washed several times with ice-cold buffer, and bound proteins extracted in SDS sample buffer (with shaking at 65 °C for 15 min). Proteins from pull downs as well as directly loaded lysates were fractionated by 7.5% acrylamide SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF; Bio-Rad) membranes. For experiments used for analysis of CaM expression levels in directly loaded lysates (Fig. 6), 12% acrylamide gels were used. PVDF membranes were stained with
Ca\textsubscript{v}1.2 channel regulation by half-calcified CaM

Ponceau S, imaged, washed, and then incubated in blocking buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4 (TBS) with 0.1% Tween (TBST) and 2% bovine serum albumin (RPI Corp.)) for 1 h at RT and then incubated with primary antibodies in blocking buffer for 3 h at RT. For analysis of surface expressed Ca\textsubscript{v}1.2, \( \alpha_{1.2} \) was detected using rabbit antibodies against epitopes in the intracellular loop II/III (FP1 or CNC1) and the CNC2 epitope near the C terminus of \( \alpha_{1.2} \) (59). When CaM expression in directly loaded lysates was assessed, the membranes were probed with a mouse anti-CaM monoclonal primary antibody (made against a synthetic peptide corresponding to the 21 carboxy terminal amino acids (128–148) of bovine calmodulin) obtained from from Sigma Millipore (catalog no.: # 05-173, Lot # 2717626). YFP-tagged CaM signals were further verified by the NeuroMab mouse anti-GFP monoclonal antibody N86/8 (UC Davis). Signals obtained from probing with antibodies against the cytosolic proteins GAPDH (mouse monoclonal, Sigma/Millipore 214592) and \( \alpha \)-tubulin (DM1A mouse monoclonal, Santa Cruz Biotechnology SC32293) were used (along with Ponceau S-stained bands) as loading controls for correction of variation in protein content between lysate samples. The absence of GAPDH and \( \alpha \)-tubulin antibody signals in NeutrAvidin-pull down samples also served as intracellular protein controls for assurance of plasma membrane integrity during the biotinylation of plated cells. PVDF membranes were washed for 40 min with at least five exchanges of TBST, incubated with horseradish peroxidase–conjugated secondary goat antimouse antibodies (Jackson) or mouse anti-rabbit antibodies (Jackson) for 1 h at RT, and washed again with TBST with at least five exchanges for 1.5 h. Immunosignals were detected using the horseradish peroxidase substrates Luminata Classic or Cre-scendo (Merck Millipore) or Femto (Thermo Fisher Scientific) by X-ray film (Denville Scientific Inc). Multiple exposures over increasing time periods were taken to ensure that all signals were in the linear range (60, 61).

Analysis of immunoblots

Signal intensity for each band in scanned film images of immunoblots were assessed using ImageJ (https://imagej.nih.gov). Background signals in individual lanes were subtracted from the band signal prior to quantitative analysis. Differences in immunosignal strengths were corrected for potential immunoblotted and film exposures differences between experiments, as described (15, 53). Loading control (e.g., GAPDH, \( \alpha \)-tubulin) lystate immunosignals were used to correct for minor differences in protein amounts loaded in individual sample lanes. To correct for variation in test immunosignals (e.g., \( \alpha_{1.2}, \) CaM) between experimental replicates, normalization was done according to the ‘sum of the replicates’ method as described (62). Each immunosignal for a protein (e.g., \( \alpha_{1.2}, \) CaM) on one blot was divided by the sum of all immunosignals from the same immunoblot exposure for that experimental run to obtain the relative signal fraction for each band (62). The means of these signal intensity fractions were calculated for each condition (e.g., \( \alpha_{1.2} \) WT, Y1657D) from all experiments (e.g., \( \alpha_{1.2} \) WT, Y1657D) and these means then divided by the mean value of the test control (e.g., \( \alpha_{1.2} \) WT, which is now equal to 1% or 100%). All data were statistically analyzed (GraphPad Prism IX software) applying either a Student’s \( t \) test (two-sample comparison) or ANOVA with Tukey post hoc test.

Data availability

Atomic coordinates were deposited in the Protein Databank (accession no. 7L8V), and all other data are contained within the article.

Supporting information—This article contains supporting information (13, 19, 28, 63).

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Abbreviations—The abbreviations used are: CaM, calmodulin; CDon, Ca\textsuperscript{2+}-dependent inactivation; FP, fluorescence polarization; ITC, isothermal titration calorimetry; PVDF, polyvinylidene difluoride; RDC, residual dipolar coupling.

References

1. Ghosh, D., Syed, A. U., Prada, M. P., Nystoriak, M. A., Santana, L. F., Nieves-Cintron, M., et al. (2017) Calcium channels in vascular smooth muscle. Adv. Pharmacol. 78, 49–87
2. Hell, J. W., Westenbroek, R. E., Warner, C., Ahlijanian, M. K., Prystay, W., Gilbert, M. M., et al. (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J. Cell. Biol. 123, 949–962
3. Moosmang, S., Haider, N., Klugbauer, N., Adelsberger, H., Langwieser, N., Muller, I., et al. (2005) Role of hippocampal Cav1.2 Ca2+ channels in NMDA-receptor-independent synaptic plasticity and spatial memory. J. Neurosci. 25, 9883–9892
4. Qian, H., Patriarchi, T., Price, J. L., Matt, L., Lee, B., Nieves-Cintron, M., et al. (2017) Phosphorylation of Ser1928 mediates the enhanced activity of the L-type Ca2+ channel Cav1.2 by the beta2-adrenergic receptor in neurons. Sci. Signal. 10, eaaf9659
5. Berkefeld, H., Sailer, C., Bildl, W., Rohde, V., Thumfart, J., Eble, S., et al. (2006) BKCa-Cav channel complexes mediate rapid and localized Ca2+-activated K+ signaling. Science 314, 615–620
21. Salveson, I., Anderson, D. E., Hell, J. W., and Ames, J. B. (2019) Chemical
22. Saplonski, L., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., et al. (2004) Ca(V)1.2 calcium channel dysfunction causes a multi-system disorder including arthrythmia and autism. Cell 119, 19–31
23. Peterson, B., DeMaria, C., Adelman, J., and Yue, D. (1999) Calmodulin is the Ca2+ sensor for Ca2+-dependent inactivation of L-type calcium channels. Nature 22, 549–558
24. Zuhlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature 399, 159–162
25. Erickson, M., Abeikhan, B., Peterson, B., and Yue, D. (2001) Preassociation of calmodulin with voltage-gated Ca2+(+) channels revealed by FRET in single living cells. Neuron 31, 973–985
26. Finn, B. E., Evenas, J., Drakenberg, T., Waltho, J. P., Thulin, E., and Iacobucci, G. J., and Popescu, G. K. (2019) Spatial coupling tunes the Ca2+ sensitivity of bound calmodulin. Proc. Natl. Acad. Sci. U. S. A. 106, 5135–5140
27. Gilli, R., La, Wu, X., and Bers, D. M. (2007) Free and bound intracellular calmodulin between calmodulin domains binding calcium and contiguous sites in the C-terminal tail of Ca(V)1.2. Biophys. Chem. 159, 172–187
28. Hall, D. D., Sai, S., Tseng, P. Y., Malik, Z., Nguyen, M., Matt, L., et al. (2013) Apo states of calmodulin and CaBP1 control CaV1 voltage-gated calcium channel function through direct competition for the IQ domain. J. Mol. Biol. 425, 3217–3234
29. Halling, D. B., Georgiou, D. K., Black, D. J., Yang, G., Fallon, J. L., Quiocho, F. A., et al. (2009) Determinants in CaV1 channels that regulate the Ca2+ sensitivity of bound calmodulin. J. Biol. Chem. 284, 20041–20051
30. Halls, I., Anderson, D. E., Hell, J. W., and Ames, J. B. (2019) Chemical shift assignments of retinal degeneration 3 protein (RD3). J. Biomol. NMR Assign. 13, 233–237
31. Clore, G. M., and Gronenborn, A. M. (1998) Determining the structures of large proteins and protein complexes by NMR. Curr. Opin. Chem. Biol. 2, 564–570
32. Tjandra, N., and Bax, A. (1997) Direct measurement of disances and angles in biomolecules by NMR in a dilute liquid crystalline medium. Science 278, 1111–1114
33. Zischka, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C. B., and Bax, A. (1995) Structure of calcium-free calmodulin. Nat. Struct. Biol. 2, 768–776
34. Zhang, M., Tanaka, T., and Ikura, M. (1995) Calcium-induced conformational transition revealed by the solution structures of apo calmodulin. Nat. Struct. Biol. 2, 758–767
35. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J. Biomol. NMR 8, 477–486
36. Johnson, B. D., and Byerly, L. (1993) A cytoskeletal mechanism for Ca2+ channel metabolic dependence and inactivation by intracellular Ca2+. Neuron 10, 797–804
37. Jensen, H. H., Brohus, M., Nyegaard, M., and Overgaard, M. T. (2018) Human calmodulin mutations. Front. Mol. Neurosci. 11, 396
38. Lim, S., Cudia, D., Yu, Q., Peshenko, I., Dizhoor, A., and Ames, J. (2018) Chemical shift assignments of retinal degeneration 3 protein (RD3). J. Biomol. NMR Assign. 12, 167–170
39. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeiffer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293
40. Ottiger, M., Delaglio, F., Marquardt, J. L., Tjandra, N., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293
41. Schwiers, C. D., Kuszewski, J. I., Tjandra, N., and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. J. Magn. Reson. 160, 65–73
42. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. J. Biomol. NMR 44, 213–223
43. Ottiger, M., Delaglio, F., Marquardt, J. L., Tjandra, N., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293
44. Ottiger, M., Delaglio, F., Marquardt, J. L., Tjandra, N., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293
45. Schreiber, F., Kuszewski, J. I., Tjandra, N., and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. J. Magn. Reson. 160, 65–73
46. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. J. Biomol. NMR 44, 213–223
Ca\textsubscript{v}1.2 channel regulation by half-calci
ded CaM

48. Tanaka, T., Ames, J. B., Kainosho, M., Stryer, L., and Ikura, M. (1998) Differential isotype labeling strategy for determining the structure of myristoylated recoverin by NMR spectroscopy. *J. Biomol. NMR* 11, 135–152

49. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1994) Secondary structure of myristoylated recoverin determined by three-dimensional heteronuclear NMR: implications for the calcium-myristoyl switch. *Biochemistry* 33, 10743–10753

50. Nilges, M., Gronenborn, A. M., Brunger, A. T., and Clore, G. M. (1988) Determination of three-dimensional structures of proteins by simulated annealing with interproton distance restraints. Application to crambin, potato carboxypeptidase inhibitor and barley serine proteinase inhibitor 2. *Protein Eng.* 2, 27–38

51. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., et al. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. Sect. D, Biol. Crystallogr.* 66, 12–21

52. Zhang, Y., Matt, L., Patriarchi, T., Malik, Z. A., Chowdhury, D., Park, D. K., et al. (2014) Capping of the N-terminus of PSD-95 by calmodulin triggers its postsynaptic release. *EMBO J.* 33, 1341–1353

53. Tseng, P. Y., Henderson, P. B., Hergarden, A. C., Patriarchi, T., Coleman, A. M., Lilly, M. W., et al. (2017) Alpha-actinin promotes surface localization and current density of the Ca\textsuperscript{2+} channel Ca\textsubscript{v}1.2 by binding to the IQ region of the alpha1 subunit. *Biochemistry* 56, 3669–3681

54. Shen, A., Nieves-Cintron, M., Deng, Y., Shi, Q., Chowdhury, D., Qi, L., et al. (2018) Functionally distinct and selectively phosphorylated GPCR subpopulations co-exist in a single cell. *Nat. Commun.* 9, 1050

55. Perez-Reyes, E., Castellano, A., Kim, H. S., Bertrand, P., Baggstrom, E., Lacerda, A. E., et al. (1992) Cloning and expression of a cardiac/brain beta subunit of the L-type calcium channel. *J. Biol. Chem.* 267, 1792–1797

56. Ellis, S. B., Williams, N. R., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., et al. (1988) Sequence and expression of mRNAs encoding the alpha 1 and alpha 2 subunits of a DHP-sensitive calcium channel. *Science* 241, 1661–1664

57. Sachs, F., Neil, J., and Barkakati, N. (1982) The automated analysis of data from single ionic channels. *Pflugers Archiv.* 395, 331–340

58. Horn, R. (1991) Estimating the number of channels in patch recordings. *Biophys. J.* 60, 433–439

59. Buonarati, O. R., Henderson, P. B., Murphy, G. G., Horne, M. C., and Hell, J. W. (2017) Proteolytic processing of the L-type Ca\textsuperscript{2+} channel alpha 1.2 subunit in neurons. *F1000 Res.* 6, 1166

60. Davare, M. A., and Hell, J. W. (2003) Increased phosphorylation of the neuronal L-type Ca\textsuperscript{2+} channel Ca\textsubscript{v}1.2 during aging. *Proc. Natl. Acad. Sci. U. S. A.* 100, 16018–16023

61. Hall, D. D., Feekes, J. A., Arachchige, A. S., Shi, M., Hamid, I., Chen, L., et al. (2006) Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation. *Biochemistry* 45, 3448–3459

62. Degasperi, A., Birtwistle, M. R., Volinsky, N., Rauch, J., Kolch, W., and Khodolenko, B. N. (2014) Evaluating strategies to normalise biological replicates of Western blot data. *PLoS One* 9, e87293

63. Wu, J., Yan, Z., Li, Z., Qian, X., Lu, S., Dong, M., et al. (2016) Structure of the voltage-gated calcium channel Ca\textsubscript{v}1.1 at 3.6 Å resolution. *Nature* 537, 191–196