CaBP1 Regulates Voltage-dependent Inactivation and Activation of CaV1.2 (L-type) Calcium Channels

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CaBP1 is a Ca²⁺-binding protein that regulates the gating of voltage-gated (Caᵥ) Ca²⁺ channels. In the Caᵥ1.2 channel α₁-subunit (α₁C), CaBP1 interacts with cytosolic N- and C-terminal domains and blunts Ca²⁺-dependent inactivation. To clarify the role of the α₁C N-terminal domain in CaBP1 regulation, we compared the effects of CaBP1 on two alternatively spliced variants of α₁C containing a long or short N-terminal domain. In both isoforms, CaBP1 inhibited Ca²⁺-dependent inactivation but also caused a depolarizing shift in voltage-dependent activation and enhanced voltage-dependent inactivation (VDI). In binding assays, CaBP1 interacted with the distal third of the N-terminal domain in a Ca²⁺-independent manner. This segment is distinct from the previously identified calmodulin-binding site in the N terminus. However, deletion of a segment in the proximal N-terminal domain of both α₁C isoforms, which spared the CaBP1-binding site, inhibited the effect of CaBP1 on VDI. This result suggests a modular organization of the α₁C N-terminal domain, with separate determinants for CaBP1 binding and transduction of the effect on VDI. Our findings expand the diversity and mechanisms of Caᵥ channel regulation by CaBP1 and define a novel modulatory function for the initial segment of the N terminus of α₁C.

EF-hand Ca²⁺-binding proteins (CaBPs)² regulate a wide variety of voltage- and ligand-gated ion channels (1–4). For voltage-dependent Ca²⁺ (Caᵥ) channels, Ca²⁺-binding proteins can differentially regulate inactivation, activation, open probability (Pₒ), Ca²⁺-dependent facilitation, and surface expression. These diverse forms of regulation may expand the functional repertoire of Caᵥ channels (5–13), tailoring Ca²⁺ signals to the specific needs of excitable cells.

Inactivation is a negative feedback process that prevents excessive Ca²⁺ entry via Caᵥ channels. For Caᵥ1 and Caᵥ2 channels, inactivation occurs by two routes: voltage-dependent inactivation (VDI) and Ca²⁺-dependent inactivation (CDI) (14). Both CDI and VDI involve multiple determinants in the cytoplasmic domains of the pore-forming subunit (α₁) (15–17). CDI depends on calmodulin (CaM) interactions with the C-terminal tail of the Caᵥ1 and Caᵥ2 α₁-subunits (18–21). CaBPs can interact with CaM-binding sites in the Caᵥ α₁-subunit (5, 6, 12, 22, 23) and may act by displacing or mimicking CaM (e.g. Refs. 6 and 11).

CaBP1 is a Ca²⁺-binding protein enriched in the brain and retina (2). It has emerged as a prominent regulator of Caᵥ channel CDI (4). The molecular determinants underlying CaBP1 regulation have been most well characterized for Caᵥ1.2 (α₁C) channels, which play crucial roles in Ca²⁺ signaling and excitability in the nervous and cardiovascular systems (14). In contrast to Ca²⁺/CaM, which stabilizes the inactivated state of Caᵥ1.2 (24–26), CaBP1 fully eliminates CDI and supports Ca²⁺-dependent facilitation (6, 27). CaBP1 binds to the C-terminal CaM-binding α₁C sites but also interacts with the α₁C N-terminal domain, the deletion of which blunts the effect of CaBP1 on CDI (28). The N terminus of α₁C regulates several aspects of channel gating as well as modulation by protein kinase C (29–32). Within the N terminus, two modular segments are involved in CaM binding (33, 34) and regulation of Pₒ (35). A “long N-terminal” alternatively spliced α₁C isoform possesses a 20-amino acid (aa) initial segment (N-terminal inhibitory (NTI) module), which controls the channel’s maximal Pₒ (30, 35). A second module in the central part of the N-terminal domain is a CaM-binding site, the N-terminal spatial Ca²⁺-transforming element (NCSaTE), which regulates CDI in Caᵥ1.3 (33), but its role in Caᵥ1.2 is less clear (34). The exact location of the CaBP1-binding site within the N terminus of α₁C has not been determined, and whether CaBP1 influences parameters of Caᵥ1.2 function other than CDI is unknown.

Here, we report that CaBP1 expression in Xenopus oocytes regulates activation gating and accelerates VDI of Caᵥ1.2. Although CaBP1 binds to a site in the distal N-terminal domain that is distinct from the CaM-binding site, CaBP1 regulation of VDI requires an intact proximal domain in the N terminus of α₁C. Deletion of the entire N-terminal domain inhibits but does not fully abolish effects of CaBP1 on VDI, which indicates the presence of additional relevant molecular determinants. These results suggest a new modulatory role for the α₁C N terminus, in which CaBP1 binding and transduction of the modulation of VDI are encoded by distinct N-terminal domains.
EXPERIMENTAL PROCEDURES

Expression Systems, DNAs, and RNAs—Maintenance of female frogs (*Xenopus laevis*), preparation of oocytes, and *in vitro* RNA synthesis were carried out as described previously (30). Oocytes were injected 3–5 days before the experiment with equal amounts (by weight, 0.8–5 ng of RNA) of cRNAs from the three channel subunits, α1C, non-palmitoylated CaVβ2b (GenBank accession no. X64297), and skeletal muscle αδ-1 (P13806) (30), with or without 10 ng of cRNA from CaBP1-S (AF169148), unless mentioned otherwise. We used rabbit long N-terminal α1C (LNTα1C, X15539) or SNTα1C, based on rabbit LNTα1C, with the initial segment (46 aa long) replaced with that encoded by exon 1 (16 aa long) as in the human short isoform (Z34815) (35). RNAs of the N-terminal clamp. 25–30 nl of 50 mM BAPTA (Ca2+ chelator) was injected by a P1000 microinjector (GenSonic) into the cytoplasm of oocytes. Electrophysiological studies of HEK293T cells involved transient transfection of HEK293T cells with CaV1.2 with or without CaBP1. In some experiments, we utilized T-REx-293 cells (Invitrogen) stably transfected with GFP-tagged CaBP1 under tetracycline-inducible expression. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO2. Cells plated in 35-mm tissue culture dishes were grown to 65–80% confluency and transfected with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Cells were transfected with 2 μg of DNA coding for the rabbit LNTα1C or rat rbcIα1C variant or their truncated forms, 1 μg of β2a, 1 μg of αδ, and 0.05 μg of enhanced GFP with or without 0.05–0.1 μg of CaBP1, all in the pcDNA3 vector. For stably transfected T-REx-293 cells, cells were transfected only with CaV1.2 subunit cDNAs and 0.05 μg of mCherry plasmid to mark transfected cells. Tetracycline (1 μg/ml) was added to the culture medium 6–8 h before recording to induce CaBP1 expression. Patch-clamp recordings of all cells were made at least 24 h after transfection. There were no significant differences between channel properties or regulation by CaBP1 in the transiently transfected and stably transfected cells, so the data from both systems were combined for analysis.

Electrophysiology and Data Analysis—In *Xenopus* oocytes, the procedures were essentially as described (35). In brief, whole cell currents were recorded with the GeneClamp 500 amplifier Molecular Devices using a two-electrode voltage clamp. 25–30 nl of 50 mM BAPTA (Ca2+ chelator) was routinely injected into the oocytes 0.5–3 h before the measurement of currents. This procedure usually blocked the endogenous Ca2+-dependent Cl− currents; cells with residual Cl− currents (distinguished by long-lasting inward tails at −80 mV) were excluded from analysis. The extracellular solutions contained 40 mM Ba(OH)2 or Ca(OH)2, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid. In each oocyte, the net current was obtained by subtraction of the residual currents recorded with the same protocols after applying 200 μM CdCl2 to the same solution (supplemental Fig. S1A) (35). Stimulation, data acquisition, and analysis were performed using pCLAMP 10.2 software (Molecular Devices). All experiments were performed at 20–22 °C. Currents were measured by 400-ms, 1000-ms, or 10-s pulses from a resting potential of −80 mV to different potentials, with 60-s intervals for the 10-s pulses and with 10-s intervals for shorter pulses. A current-voltage (I-V) curve was fitted to the Boltzmann equation in the form I = Gmax(Vm − Vrev)/(1 + exp(-(Vm − Vrev)/Kf)), where Gmax is the maximal conductance, Vrev is the reversal potential of the current. The parameters obtained for Gmax and Vrev were then used to calculate fractional conductance at each Vm using the equation G/Gmax = I/(Gmax(Vm − Vrev)).

The waveform of decay of the Ba2+ current (I_Ba) was fitted, using a Levenberg-Marquardt algorithm, to a two-exponential equation in the form f(t) = A_faste−t/τfast + A_slowe−t/τslow + C, where A is the contribution of each kinetic component (τfast or τslow) of the decay of I_Ba and C is the non-inactivating component (supplemental Fig. S1B and Table S2).

In transfected HEK293T cells, Ba2+ currents were recorded in whole cell patch–clamp configuration at room temperature. Extracellular recording solutions contained 150 mM Tris, 2 mM MgCl2, 10 mM BaCl2. Intracellular recording solutions contained 130 mM N-methyl-D-glucamine, 60 mM HEPES, 1 mM MgCl2, 2 mM MgATP, and 5 mM EGTA. The pH of the extracellular and intracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Reagents used for electrophysiological recordings were obtained from Sigma. Currents were recorded with an EPC 9 patch-clamp amplifier driven by PULSE software (HEKA Electronics, Lambrecht/Pfalz, Germany). Leak and capacitive transients were subtracted using a P4 protocol. To measure VDI, I_Ba was evoked by 1-s depolarizations to +10 mV from −80 mV.

r_3000, r_10000, and r_20000 are defined as the residual current (I_res) left after 400, 1000, and 2000 ms, respectively, divided by the maximal current, r = I_res/I_peak. Data were analyzed using pCLAMP (Molecular Devices) or routines written in IGOR Pro software (WaveMetrics, Lake Oswego, OR). Averaged data are presented as means ± S.E. Statistical differences between two groups were determined by Student’s t test, and multiple group comparisons were done by one-way analysis of variance followed by Dunnett’s or Bonferroni tests. Graphs and statistical analysis were done with SigmaPlot (SPSS, Inc., Chicago, IL).

Pulldown Assays with GST-Fused Proteins—Procedures were essentially as described (29). In brief, [35S] Met/Cys-labeled CaBP1 or CaM was translated on the template of an *in vitro* synthesized RNA using a rabbit reticulocyte translation kit (Promega). DNAs of α1C segments designed to create GST fusion proteins were cloned into the pGEX-4T-1 vector. GST-fused proteins were produced in *Escherichia coli* (strain BL21-RIL) transfected with the cDNAs (see Fig. 3A) and grown in standard medium at 37 or 18 °C after induction with isopropyl β-D-thiogalactopyranoside. The GST fusion proteins were extracted from *E. coli* using an Amersham Biosciences kit. The protein concentration was estimated using a Bio-Rad protein assay kit. Purified GST fusion proteins (5–10 μg) or purified GST (10 μg) was incubated with 5 μl of the lysate containing the 35S-labeled proteins in PBS containing 0.05% Tween 20
with 1 mM CaCl2 or EGTA. The final reaction volume was 300 μl. GST fusion protein was immobilized on glutathione-Sepharose beads (Amersham Biosciences) for 30 min at 4 °C and washed. Following washing, GST fusion proteins were eluted with 20 mM reduced glutathione in 120 mM NaCl and 100 mM Tris-HCl (pH 8) and analyzed by SDS-PAGE. Gels were stained to detect proportional protein concentration with Coomassie Brilliant Blue R-250 (Bio-Rad). The labeled products were identified by autoradiography using a PhosphorImager (Molecular Dynamics).

RESULTS

**CaBP1 Accelerates VDI of CaV1.2—**CaBP1 inhibition of CDI in CaV,1.2 has been described for a SNTα1C isoform from rat brain, rbcII (6, 28). However, a LNTα1C isoform is also expressed in the brain (37). The LNTα1C isoform includes a 46-aa initial segment encoded by exon 1a (38), which contains the NTI module (aa 1–20) (see Fig. 3A and supplemental Fig. S4 for sequence) that regulates the channel's Po (35). The SNTα1C isoform (39–41) has an initial N-terminal segment encoded by the alternative exon 1 (42, 43). This 16-aa segment is partly homologous to aa 6–20 of the long N terminus (see Fig. 3A and supplemental Fig. S4) but does not retain functionality of the NTI module in terms of modulation of Po of CaV,1.2 (35). Considering the importance of the rbcII N terminus in CaBP1 regulation of CDI (28), we examined whether CaBP1 might differentially regulate the LNTα1C and SNTα1C isoforms. We employed two-electrode voltage-clamp recordings in *Xenopus* oocytes injected with RNAs encoding LNTα1C (left panel) or SNTα1C (right panel) without (control, black) or with CaBP1 (gray). Currents were recorded in 40 mM Ca2+ solution (I(Ca)) or Ba2+ solution (I(Ba)). α, β-1 and Caβ2-subunits were coexpressed in all experiments done in *Xenopus* oocytes. B, summary of changes in τ400 at different voltages in the presence (○) or absence (●) of CaBP1. Data are presented as means ± S.E. (n = 14–39). C, summary of the effects of CaBP1 on τ400 in the LNTα1C and SNTα1C isoforms in +20 mV. Bars represent means ± S.E.; the numbers within the bars indicate n in B and C, the statistical significance of differences between the control and CaBP1 was determined by t test. *p < 0.05,** p < 0.01,***, p < 0.001.

**CaBP1 Regulates Voltage-dependent Inactivation of CaV1.2**

CaBP1 enhances VDI mainly by changing the fraction of fast and slow components of inactivation—Given the relatively slow time course of CaV,1.2 VDI, we next used 10-s depolarizing pulses to characterize the effects of CaBP1 on the kinetics of VDI (Fig. 2). For LNTα1C and SNTα1C alone, I(Ba) showed faster decay kinetics at more positive membrane voltages, a hallmark of VDI (Fig. 2, A–C, and supplemental Fig. S3). The decay of I(Ba) was best fitted with a double-exponential function (Fig. 2A and supplemental Fig. S1B, B, and C), giving time constants of τfast ~ 0.6 s and τslow ~ 3 s at +10 mV (Fig. 2C, Table 1, and supplemental Fig. S1B and Table S1), supporting the involvement of at least two processes in VDI of CaV,1.2 (21). CaBP1 accelerated I(Ba) decay kinetics at all voltages in both LNTα1C and SNTα1C isoforms (Fig. 2B and supplemental Fig. S3A). Acceleration of VDI by CaBP1 was dose-dependent and maximal with 5–10 ng of CaBP1 RNA/oocyte in both LNTα1C and SNTα1C isoforms (supplemental Fig. S2A). The kinetic analysis of current decay showed that, at less depolarized voltages, CaBP1 decreased both τfast and τslow. At more depolarized voltages, CaBP1 significantly increased the fast inactivating fraction (Afast) with a concomitant decrease in the slowly inactivating fraction (Aslow) (Fig. 2C). Together, these changes account for the acceleration of VDI by CaBP1 at all voltages.

**CaBP1-binding Site in the N Terminus, and No Overlap with the NTI or NSCaTE Module—**The N-terminal domain of α1C binds both CaM and CaBP1 (28, 29). The binding of CaM is Ca2+-dependent and has been mapped to aa 60–100, with crucial binding determinants between aa 80 and 92 (33, 34) conserved in all isoforms of α1C (supplemental Fig. S4A), as well as in CaV,1.3α1 (s112). We tested if CaBP1 and CaM share similar molecular determinants for binding in the α1C N-terminal domain in GST pulldown assays. GST fusion proteins that cover the complete long N terminus of α1C, as shown schematically in Fig. 3A, were used to pull down in vitro synthesized radiolabeled CaBP1. CaBP1 bound to aa 95–154 and to a shorter segment (aa 95–140) but not to segments located N-terminally to aa 94 (Fig. 3B). Importantly, CaBP1 did not bind to the segment at aa 60–100, which has been shown to strongly bind CaM in the same assay (supplemental Fig. S4B) (34). These results locate the CaBP1-binding site to aa 95–140, a segment fully conserved in all α1C isoforms; the crucial binding determinants are probably between aa 120 and 140. Thus, unlike the C terminus (6), in the N terminus, there is no overlap between CaM- and CaBP1-binding sites (Fig. 3A).
CaBP1 binding to the N-terminal fragments was Ca\(^{2+}\)/H11001-independent in that binding was similar in the presence of either Ca\(^{2+}\) or EGTA (Fig. 3B). We estimate that the Ca\(^{2+}\)-dependent binding of CaBP1 to the N terminus is weaker than that of CaM because the ratio of bound to loaded protein (‘input,’ i.e. initial amount of protein in the binding reaction) was always smaller for CaBP1 than for CaM (compare Fig. 3B and supplemental Fig. S4B). These results further underscore differences in the physical interactions of CaBP1 and CaM with Ca\(_{\text{V}1.2}\) that could contribute to their distinct modulation of channel function.

**FIGURE 2.** **Kinetic analysis of CaBP1-induced changes in VDI in LNT\(_{\alpha_{1C}}\).** A, records of \(I_{\text{Ba}}\) from a representative cell expressing LNT\(_{\alpha_{1C}}\) evoked by 10-s depolarizing voltage steps from \(-80\) mV to different voltages. Net \(I_{\text{Ba}}\), obtained by subtraction of Cd\(^{2+}\)-insensitive currents, is shown in gray. The decay time course was fitted to a two-exponential function (see supplemental Table S1 for fitted parameters), and the fitted curve (black line) is shown superimposed on the original trace. B, CaBP1 accelerates the kinetics of decay of \(I_{\text{Ba}}\) of LNT\(_{\alpha_{1C}}\). Currents were elicited by 10-s pulses from \(-80\) mV to various voltages. Traces, averaged from 10–13 oocytes, show the decay phase of \(I_{\text{Ba}}\), starting from the peak and to the end of the voltage step. For clarity, S.E. is shown only for every 20th acquisition point. Student’s t test was used to determine statistical significance at the \(t = 2000\) ms time point. C, summary of the two-exponential fit analysis of \(I_{\text{Ba}}\) decay in LNT\(_{\alpha_{1C}}\). Averaged values of \(A_{\text{slow}}\) and \(A_{\text{fast}}\) (the contribution of each kinetic component), \(C\) (the non-inactivating current), and \(\tau_{\text{slow}}\) and \(\tau_{\text{fast}}\) (the time constants) at different voltages are shown with (●) or without (○) CaBP1. Statistical significance between values was calculated at each voltage using a non-paired t test. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).

**TABLE 1**

Parameters of VDI (\(r_{400}\) and \(r_{2000}\)) at +10 and +20 mV in the different channel constructs

| RNA constructs injected | \(r_{400} \pm \text{S.E.}\) | \(\Delta r_{400}\) (effect of CaBP1) | \(n\) | \(r_{2000} \pm \text{S.E.}\) | \(\Delta r_{2000}\) (effect of CaBP1) | \(n\) |
|------------------------|-----------------|-----------------|-----|-----------------|-----------------|-----|
| At 10 mV               |                 |                 |     |                 |                 |     |
| LNT\(_{\alpha_{1C}}\)  | 0.77 ± 0.02     | 0.20a           | 39  | 0.49 ± 0.05     | 0.22b           | 10  |
| +CaBP1                | 0.57 ± 0.03     | 0.23a           | 29  | 0.27 ± 0.04     | 0.19b           | 13  |
| SNT\(_{\alpha_{1C}}\)  | 0.77 ± 0.03     | 0.23a           | 16  | 0.38 ± 0.05     | 0.19b           | 7   |
| +CaBP1                | 0.55 ± 0.05     |                 | 14  | 0.19 ± 0.04     | 0.01 (NS)       | 7   |
| Δ46-LNT\(_{\alpha_{1C}}\) | 0.73 ± 0.05   | 0.01 (NS)       | 10  | 0.40 ± 0.04     | 0.01 (NS)       | 10  |
| +CaBP1                | 0.72 ± 0.04     |                 | 14  | 0.39 ± 0.04     |                 | 14  |
| Δ139-LNT\(_{\alpha_{1C}}\) | 0.62 ± 0.04   | 0.12a           | 6   | 0.23 ± 0.05     | 0.07 (NS)       | 6   |
| +CaBP1                | 0.50 ± 0.03     |                 | 11  | 0.16 ± 0.03     |                 | 11  |

At 20 mV

| RNA constructs injected | \(r_{400} \pm \text{S.E.}\) | \(\Delta r_{400}\) (effect of CaBP1) | \(n\) | \(r_{2000} \pm \text{S.E.}\) | \(\Delta r_{2000}\) (effect of CaBP1) | \(n\) |
|------------------------|-----------------|-----------------|-----|-----------------|-----------------|-----|
| LNT\(_{\alpha_{1C}}\)  | 0.73 ± 0.04     | 0.23a           | 39  | 0.41 ± 0.05     | 0.21b           | 10  |
| +CaBP1                | 0.50 ± 0.04     |                 | 29  | 0.20 ± 0.04     | 0.19b           | 13  |
| SNT\(_{\alpha_{1C}}\)  | 0.73 ± 0.02     | 0.22a           | 16  | 0.36 ± 0.03     | 0.19a           | 7   |
| +CaBP1                | 0.50 ± 0.03     |                 | 14  | 0.17 ± 0.03     | 0.03 (NS)       | 7   |
| Δ46-LNT\(_{\alpha_{1C}}\) | 0.66 ± 0.05    | 0.03 (NS)       | 10  | 0.33 ± 0.04     | 0.03 (NS)       | 10  |
| +CaBP1                | 0.63 ± 0.03     |                 | 14  | 0.30 ± 0.03     |                 | 14  |
| Δ139-LNT\(_{\alpha_{1C}}\) | 0.58 ± 0.03   | 0.13a           | 6   | 0.18 ± 0.04     | 0.05 (NS)       | 6   |
| +CaBP1                | 0.45 ± 0.02     |                 | 11  | 0.13 ± 0.03     |                 | 11  |

\(a \ p < 0.001.\)

\(b \ p < 0.01.\)

\(c \ p < 0.05.\)
CaBP1 Regulates Voltage-dependent Inactivation of CaV1.2

Because CaBP1 reduces the voltage sensitivity of activation in P/Q-type (CaV2.1) channels (5, 11), we also examined the possibility of such regulation in CaV1.2 channels expressed in Xenopus oocytes. CaBP1 caused a 10–12.5-mV depolarizing shift in the I-V curves and the corresponding conductance-voltage (G-V) curves of the LNTα1C and SNTα1C isoforms (Fig. 4; see Table 2 for Boltzmann fit parameters). CaBP1 inhibited CaV1.2 activation in a dose-dependent manner with the maximal effect achieved with 5–10 ng of CaBP1 RNA/oocyte (supplemental Fig. S2B). CaBP1 specifically altered the midpoint value of the G-V curve, the $V_a$, of all other parameters of the Boltzmann equation did not change significantly (Table 2).

To assess whether the N terminus of $\alpha_{1C}$ is involved in CaBP1-induced changes in activation, we used several mutants. $\Delta 139$-LNT$\alpha_{1C}$ lacks most of the cytosolic N terminus of $\alpha_{1C}$, including both the NSCaTE module and the CaBP1-binding site. $\Delta 46$-LNT$\alpha_{1C}$ lacks the initial segment of the N terminus (46 aa encoded by exon 1a in LNT$\alpha_{1C}$ or 16 aa encoded by exon 1 in SNT$\alpha_{1C}$) but retains the CaM-binding site (NSCaTE module) and the CaBP1-binding segment. The shift in the G-V curve was preserved in both $\Delta 46$-LNT$\alpha_{1C}$ and $\Delta 139$-LNT$\alpha_{1C}$ (Fig. 4). CaBP1 similarly affected the voltage dependence of channels with other N-terminal deletions and mutations: $\Delta 20$-LNT$\alpha_{1C}$, $\Delta 20$-LNT$\alpha_{1C}$, the WIR mutant (a triple mutation that abolishes CaM binding to the N terminus (33, 34)), and channels in which the $\beta_{2a}$ subunit was replaced by $\beta_{2a}$ or $\beta_{2b}$. In all cases, a 6–10-mV shift in $V_a$ was observed upon coexpression of CaBP1 (Table 2). In all, the effect of CaBP1 on voltage-dependent activation appears to be independent of the N terminus of $\alpha_{1C}$.
CaBP1 Regulates Voltage-dependent Inactivation of CaV1.2

TABLE 2
Parameters of Boltzmann fits of I-V curves in the different α1C constructs expressed in oocytes and the effect of CaBP1

Values are means ± S.E.

|          | Without CaBP1 | With CaBP1 | ΔV_{ii} (t test) |
|----------|---------------|------------|------------------|
| LNTα1Cβ2n |               |            |                  |
| \(G_{\text{max}}\) | 23.75 ± 2.54  | 19.39 ± 2.38 |                  |
| \(V_{\text{rev}}\) | 68.67 ± 1.47  | 69.47 ± 0.78 |                  |
| \(V_{p}\) | −0.16 ± 1.04  | 10.01 ± 0.80 | 10.17*           |
| \(K_p\) | 7.35 ± 0.14   | 7.18 ± 0.20  |                  |
| \(n\) | 62            | 51          |                  |
| SNTα1Cβ2n |               |            |                  |
| \(G_{\text{max}}\) | 16.68 ± 4.3   | 26.37 ± 5.68 |                  |
| \(V_{\text{rev}}\) | 69 ± 0.65     | 71.25 ± 0.79 |                  |
| \(V_{p}\) | 0.5 ± 1.41    | 9.12 ± 0.92  | 12.48*           |
| \(K_p\) | 6.7 ± 0.35    | 7.73 ± 0.17  |                  |
| \(n\) | 19            | 16          |                  |
| Δ46-LNTα1Cβ2n |            |            |                  |
| \(G_{\text{max}}\) | 44.77 ± 8.08  | 31.06 ± 4.85 |                  |
| \(V_{\text{rev}}\) | 62.58 ± 0.59  | 68.65 ± 1.21 |                  |
| \(V_{p}\) | −2.34 ± 1.52  | 9.68 ± 0.92  | 12.02*           |
| \(K_p\) | 6.86 ± 0.21   | 7.02 ± 0.14  |                  |
| \(n\) | 19            | 25          |                  |

| Delta-LNTα1Cβ2n (20 molar Ba²⁺) | | |
| \(G_{\text{max}}\) | 38.16 ± 7.68 | 20.00 ± 5.64 |                  |
| \(V_{\text{rev}}\) | 62.05 ± 1.08 | 64.74 ± 1.14 |                  |
| \(V_{p}\) | 1.97 ± 1.15  | 8.77 ± 1.29  | 7.20*            |
| \(K_p\) | 7.21 ± 0.24  | 7.15 ± 0.33  |                  |
| \(n\) | 14            | 18          |                  |

| WIRα1Cβ2n | | |
| \(G_{\text{max}}\) | 22.44 ± 2.47 | 22.21 ± 2.35 |                  |
| \(V_{\text{rev}}\) | 63.99 ± 0.90 | 67.10 ± 0.89 |                  |
| \(V_{p}\) | −2.44 ± 0.98 | 5.51 ± 0.96  | 7.94*            |
| \(K_p\) | 6.41 ± 0.26  | 6.84 ± 0.19  |                  |
| \(n\) | 22            | 22          |                  |

| SNTα1Cβ2n | | |
| \(G_{\text{max}}\) | 48.9 ± 8.72 | 48.29 ± 8.35 |                  |
| \(V_{\text{rev}}\) | 71.48 ± 3.04 | 79.29 ± 8.15 |                  |
| \(V_{p}\) | 6.1 ± 1.19   | 14.34 ± 1.01 | 8.24*            |
| \(K_p\) | 6.52 ± 0.32  | 6.72 ± 0.16  |                  |
| \(n\) | 7             | 9           |                  |

| Δ20-LNTα1Cβ2n | | |
| \(G_{\text{max}}\) | 27.21 ± 5.33 | 36.57 ± 2.59 |                  |
| \(V_{\text{rev}}\) | 76.18 ± 4.78 | 71.60 ± 1.32 |                  |
| \(V_{p}\) | 5.40 ± 1.12  | 11.66 ± 0.82 | 6.26*            |
| \(K_p\) | 6.71 ± 0.76  | 6.64 ± 0.26  |                  |
| \(n\) | 4             | 7           |                  |

| Δ20-46-LNTα1Cβ2n | | |
| \(G_{\text{max}}\) | 24.63 ± 3.93 | 21.69 ± 2.61 |                  |
| \(V_{\text{rev}}\) | 72.36 ± 5.68 | 72.97 ± 4.26 |                  |
| \(V_{p}\) | 5.38 ± 2.20  | 15.48 ± 0.70 | 10.11*           |
| \(K_p\) | 7.60 ± 0.54  | 6.79 ± 0.10  |                  |
| \(n\) | 4             | 5           |                  |

* \(p < 0.001\)  
** \(p < 0.01\)

module and has the low \(P_{p}\) characteristic of LNTα1C (35), the biophysical properties of these four deletion constructs are generally similar to those of the SNTα1C isofrom.

Our previous work suggested that VDI was accelerated in the Δ139-LNTα1C, mutant (29). In agreement with this, the kinetics of VDI in Δ139-LNTα1C and, to a smaller extent, in SNTα1C and Δ46-LNTα1C are faster than those in wild-type LNTα1C (Fig. 5, Table 1, and supplemental Fig. S5A). Changes in VDI in Δ20-LNTα1C and Δ20–46-LNTα1C were less pronounced and not statistically significant (supplemental Fig. S5A). Together, these results implicate the N terminus of α1C in the regulation of VDI.

Unexpectedly, Δ46-LNTα1C, which still contains the CaBP1-binding site (Fig. 3A), showed little effect of CaBP1 on VDI (Fig. 5A and supplemental Fig. S6A). CaBP1 caused no significant change in \(r_{2000}\) of Δ46-LNTα1C, compared with an ∼45–50% reduction in \(r_{2000}\) in LNTα1C or SNTα1C (at +10 or +20 mV) (Table 1). Similarly, in Δ139-LNTα1C, CaBP1 barely accelerated VDI, although a small residual effect of CaBP1 was apparent, especially in \(r_{400}\) (Fig. 5A, Table 1, and supplemental Fig. S6C).

Considering that the approximately +10-mV shift in activation caused by CaBP1 may alter VDI kinetics, we compared VDI of the various channel constructs at +10 mV in the absence of CaBP1 and at +20 mV in the presence of CaBP1 (Fig. 5, A and B). This comparison also revealed a strong attenuation of the effect of CaBP1 on Δ46-LNTα1C and Δ139-LNTα1C channels. The small residual effect of CaBP1 on \(r_{2000}\) seen in both mutants (correlated with residual changes in \(A_{\text{fast}}\) and \(A_{\text{slow}}\) (supplemental Fig. S6) was significantly smaller than that seen in the wild-type channels (\(p < 0.001\)) compared with LNTα1C, one-way analysis of variance) (Fig. 5C). We conclude that the initial segments of both LNTα1C and SNTα1C isoforms play a major role in transducing the CaBP1-induced acceleration of VDI.

We confirmed these observations in HEK293T cells expressing either the rat SNTα1C isoform (rbcII) or the rabbit LNTα1C isoform and several of the N-terminal deletion mutants (supplemental Fig. S5B). Inactivation was measured as \(r_{1000}\) in HEK293T cells as described previously (6). In the wild-type channels, CaBP1 significantly accelerated VDI, decreasing \(r_{1000}\) by 31% in rbcII and by 27% in rabbit LNTα1C. Deletion of the first 64 aa in rbcII or of 46 aa in LNTα1C completely abolished the CaBP1-induced change in \(r_{1000}\) of \(I_{\text{nat}}\).

To further define the determinants within the initial 46 aa required for CaBP1 modulation of VDI, we analyzed the Δ20-LNTα1C and Δ20–46-LNTα1C deletion mutants. However, both Δ20-LNTα1C and Δ20–46-LNTα1C manifested enhancement of VDI by CaBP1 similar to the wild-type channels (Fig. 5 and supplemental Fig. S7), suggesting that either the first or last half of this proximal N-terminal region is sufficient for modulation of VDI by CaBP1.

DISCUSSION

Here, we have demonstrated that the neuronal Ca²⁺-binding protein CaBP1 profoundly regulates multiple aspects of gating of the CaV1.2 channel. In addition to the previously described regulation of CDI, we have shown that CaBP1 also regulates activation gating and VDI. The N terminus of α1C is important for the regulation of VDI by CaBP1. CaBP1 binds to the distal third of the N-terminal tail, adjacent to the first transmembrane domain of the channel, yet deletion mutagenesis showed that the functionally important part of N terminus is its initial segment, which does not bind CaBP1 in a direct biochemical assay. Importantly, the initial segments of both LNTα1C and SNTα1C isoforms, encoded by either one of the alternative first exons of the α1C gene CACNA1C (exon 1a or 1, respectively), function as...
CaBP1 Regulates Voltage-dependent Inactivation of CaV1.2

FIGURE 5. N terminus of α1C is involved in the regulation of VDI by CaBP1. A, comparison of the time course of decay of \( I_{\text{Ca}} \) in various channel constructs tested. Currents were recorded at +10 (black) and +20 (dark gray) mV in the indicated constructs expressed in oocytes without CaBP1. In addition, currents recorded at +20 mV in the presence of CaBP1 are presented (light gray). Currents were normalized and averaged (\( n = 14 \)). S.E. values are shown for each 20th point (\( n = 4–14 \) oocytes). B, the CaBP1-induced shift in activation does not account for its effect on VDI. Normalized \( I_{\text{Ca}} \) recorded at +10 mV without CaBP1 (black) is compared with \( I_{\text{Ca}} \) recorded at +20 mV in the presence of CaBP1 (gray) in representative cells. C, summary of the experiments shown in A. Values of \( r_{2000} \), measured at +10 mV (black bars) or +20 mV (dark gray bars), and +20 mV when coexpressing CaBP1 (light gray bars) are summarized. The statistical significance of the differences between the control at +10 or +20 mV and CaBP1 at +20 mV (t test) is shown above each pair of bars. The net effect of CaBP1, i.e., the difference in \( r_{2000} \) in CaBP1 (20 mV) versus the control (LNTα1C at 10 mV) is shown by white bars. Negative values indicate acceleration of VDI by CaBP1. Compared with LNTα1C, the effect of CaBP1 on Δ46-LNTα1C and Δ139-LNTα1C was significantly different, as determined by one-way analysis of variance followed by the Bonferroni t test. These statistical differences are indicated under the corresponding white bars. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ns, not significant.

regulators of the effect of CaBP1 on VDI. This demonstrates, for the first time, a novel modulatory function for the initial segment of the N terminus of the short isoform of α1C.

Role of the N Terminus of α1C and Mechanism of Action—Our results indicate that the initial segment of α1C plays a crucial role in CaBP1 regulation of VDI. The channel construct lacking the initial segment (Δ46-LNTα1C), which starts essentially with the protein sequence encoded by the obligatory exon 2 (44, 45), retains only a residual small regulation of VDI by CaBP1. Importantly, the effect of CaBP1 is equally restored by the addition of either the short 16-aa initial segment encoded by exon 1 or the long 46-aa segment encoded by the alternative exon 1a. Thus, regulation by CaBP1 is observed in both isoforms of α1C. (A third N-terminal isoform of α1C starting with a 9-aa segment encoded by the alternative exon 1c is expressed in rat smooth muscle, but no such isoform was identified in humans (46).)

The ability of the 16 aa-initial segment of the SNTα1C isoform to regulate CaBP1 modulation contrasts with the inability of this region to regulate the channel’s maximal open probability, a function that is unique to the partially homologous 20 aa of the LNTα1C isoform, the NTI module (35). This distinction indicates that the initial segment of the N terminus regulates maximal \( P_\infty \) and VDI by distinct molecular mechanisms. This concept is supported by the finding that another Ca\(^{2+}\)-binding protein, KChIP (\( K_\alpha \) channel-interacting protein), which interacts with the N terminus of α1C, regulates only \( P_\infty \), but not inactivation of the cardiac LNTα1C isoform (10).

Our finding that deletion of most of the N terminus does not influence the effect of CaBP1 on voltage dependence of activation highlights the specificity with which the N terminus regulates the VDI modulation by CaBP1. Specificity is also supported by the preservation of CaBP1-dependent regulation of VDI in mutants in which the first or second portion of the 46-aa segment was deleted, Δ20-LNTα1C and Δ20–46-LNTα1C. These two mutants differ by the presence of the NTI module (in Δ20–46-LNTα1C) and the corresponding ~7-fold difference in maximal \( P_\infty \) (35). Our finding that CaBP1 still modulates VDI in Δ20-LNTα1C and Δ20–46-LNTα1C raises the possibility that the length of the N terminus is the critical parameter. A similar proposal has been made for the N terminus of Ca\(\alpha\)\(\beta\) based on the ability of truncations of this region to prevent effects of Ca\(\alpha\)\(\beta\) on voltage-dependent inactivation (47, 48). However, we cannot rule out that there are two distinct regions that mediate the effect of CaBP1 on VDI, which are present in the first and second halves of the 46-aa initial segment and in the 16 aa of SNTα1C.
Interestingly, CaBP1 did not interact directly with the initial N-terminal segment of LNTα1C, although we cannot rule out that low affinity interactions may have evaded our pulldown assays. Because deletion of most of the N terminus including the CaBP1-binding site does not alter VDI regulation by CaBP1 any more than deletion of the initial segment alone, we propose that the initial segment plays a key role in transducing the effect of CaBP1. This effect may require CaBP1 binding to the distal N terminus and/or to the previously characterized site in the C-terminal domain (6). The initial segment of the N terminus may allosterically couple CaBP1 binding to VDI modulation. We proposed a similar mechanism to account for the ability of the CaV β-subunit to inhibit $P_v$ via the N-terminus of the NNTα1C module despite lack of a direct CaV β-N terminus interaction (30).

**Complex Regulation of VDI by the N Terminus of α1C** —The mechanism underlying inactivation of high voltage CaV channels is not completely understood but may involve “hinged lid” or pore block-type mechanisms (20, 49). In the N-terminal tail of α1C, there has been proposed to regulate the inactivation process (29, 31), in addition to other well established determinants (the C-terminal tail, cytosolic loop I, and the pore itself) CaV α subunit (Caβ and CaM) (18, 20, 21, 50). Our results further suggest a complex regulation of the inactivation process by multiple parts of the N-terminal tail of the CaV1.2 α1C-subunit. Thus, despite the crucial role of the initial segment in regulation of VDI by CaBP1, it only moderately affects the VDI process itself: the overall kinetics of VDI are only slightly accelerated by the presence of activation and the acceleration of VDI is reduced by CaBP1, which would enhance Ca$^{2+}$ entry (30).

**CaBP1 Regulates VDI in CaV1.2, and Possible Physiological Implications** —The depolarizing shift in the voltage dependence of activation and the acceleration of VDI are expected to reduce Ca$^{2+}$ entry in excitatory cells. On the other hand, CDI is reduced by CaBP1, which would enhance Ca$^{2+}$ entry (6). Dual opposite regulation of VDI and CDI resembles the effect of sorcin (8) and may be physiologically relevant. For CaV1.2 channels in ventricular cardiomyocytes, VDI may primarily regulate channel function under basal conditions, whereas CDI is dominant upon β-adrenergic stimulation (51, 52). The importance of VDI as a CaV1.2 regulatory mechanism is further underscored by findings that the Timothy syndrome mutation G406R, which leads to autism, cardiac arrhythmia, and developmental abnormalities (53), inhibits VDI but spares CDI (54). CaBP1 is colocalized with L-type Ca$^{2+}$ channels in somatodendritic areas of neurons in the brain (4) and is well posed to regulate their function. Therefore, CaBP1 regulation of VDI and CDI may contribute significantly toward activity-dependent regulation of neuronal Ca$^{2+}$ signals.

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CaBP1 Regulates Voltage-dependent Inactivation of Ca_{V}1.2

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