

# Ca<sup>2+</sup>-Binding Protein-1 Facilitates and Forms a Postsynaptic Complex with Ca<sub>v</sub>1.2 (L-Type) Ca<sup>2+</sup> Channels

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Ca<sup>2+</sup>-binding protein-1 (CaBP1) is a Ca<sup>2+</sup>-binding protein that is closely related to calmodulin (CaM) and localized in somatodendritic regions of principal neurons throughout the brain, but how CaBP1 participates in postsynaptic Ca<sup>2+</sup> signaling is not known. Here, we describe a novel role for CaBP1 in the regulation of Ca<sup>2+</sup> influx through Ca<sub>v</sub>1.2 (L-type) Ca<sup>2+</sup> channels. CaBP1 interacts directly with the  $\alpha_1$  subunit of Ca<sub>v</sub>1.2 at sites that also bind CaM. CaBP1 binding to one of these sites, the IQ domain, is Ca<sup>2+</sup> dependent and competitive with CaM binding. The physiological significance of this interaction is supported by the association of Ca<sub>v</sub>1.2 and CaBP1 in postsynaptic density fractions purified from rat brain. Moreover, in double-label immunofluorescence experiments, CaBP1 and Ca<sub>v</sub>1.2 colocalize in numerous cell bodies and dendrites of neurons, particularly in pyramidal cells in the CA3 region of the hippocampus and in the dorsal cortex. In electrophysiological recordings of cells transfected with Ca<sub>v</sub>1.2, CaBP1 greatly prolonged Ca<sup>2+</sup> currents, prevented Ca<sup>2+</sup>-dependent inactivation, and caused Ca<sup>2+</sup>-dependent facilitation of currents evoked by step depolarizations and repetitive stimuli. These effects contrast with those of CaM, which promoted strong Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 with these same voltage protocols. Our findings reveal how Ca<sup>2+</sup>-binding proteins, such as CaM and CaBP1, differentially adjust Ca<sup>2+</sup> influx through Ca<sub>v</sub>1.2 channels, which may specify diverse modes of Ca<sup>2+</sup> signaling in neurons.

**Key words:** calcium; calmodulin; channel; postsynaptic; facilitation; inactivation

## Introduction

Voltage-gated Ca<sup>2+</sup> channels are major routes of Ca<sup>2+</sup> entry in neurons and are crucial for many forms of synaptic plasticity because they couple membrane depolarization to Ca<sup>2+</sup>-dependent signal transduction. In the postsynaptic membrane, Ca<sub>v</sub>1.2 (L-type) Ca<sup>2+</sup> channels selectively trigger activity-dependent gene expression (Murphy et al., 1991; Bading et al., 1993; Ginty et al., 1993; Dolmetsch et al., 2001; Weick et al., 2003), mRNA targeting into dendrites (Tongiorgi et al., 1997), and depression of R-type Ca<sup>2+</sup> channels in dendritic spines (Yasuda et al., 2003). The mechanism by which Ca<sup>2+</sup> influx through Ca<sub>v</sub>1.2 channels specifically activates these pathways remains unclear but may depend on the interaction of these channels with postsynaptic proteins that regulate their function and/or proximity to key effectors (Deisseroth et al., 1998; Davare et al., 2001).

Mounting experimental evidence supports a role for calmodulin (CaM) in regulating the signaling capability of postsynaptic Ca<sub>v</sub>1.2 channels via two mechanisms. First, CaM limits excessive Ca<sup>2+</sup> entry through Ca<sub>v</sub>1.2 in a process known as Ca<sup>2+</sup>-

dependent inactivation, which is mediated by CaM binding directly to the main  $\alpha_1$  subunit of Ca<sub>v</sub>1.2 (Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999). Second, physical coupling between CaM and Ca<sub>v</sub>1.2 is important for translocation of CaM to the nucleus and activation of transcription factors such as cAMP response element-binding protein and MEF-2 (myocyte enhancer factor 2) (Deisseroth et al., 1998; Dolmetsch et al., 2001). Such abilities of CaM to modulate and transduce Ca<sup>2+</sup> signals have led to its proposed role as a multifunctional Ca<sup>2+</sup> sensor (Chin and Means, 2000).

However, CaM is but one member of a family of Ca<sup>2+</sup>-binding proteins, some of which are expressed primarily in neurons (Burgoyne and Weiss, 2001; Haeseleer et al., 2002). Because of their structural similarity, CaM and these neuron-specific Ca<sup>2+</sup>-binding proteins (NCBPs) may interact with the same target molecules but mediate distinct forms of target regulation (Yang et al., 2002; Haynes et al., 2004). One such NCBP, Ca<sup>2+</sup>-binding protein-1 (CaBP1), interacts with Ca<sub>v</sub>2.1 (P/Q-type) Ca<sup>2+</sup> channels, which are also regulated directly by CaM. However, whereas CaM mediates Ca<sup>2+</sup>-dependent inactivation and facilitation of these channels (Lee et al., 1999, 2000; DeMaria et al., 2001), CaBP1 strongly enhances inactivation independent of Ca<sup>2+</sup> (Lee et al., 2002).

Although CaBP1 colocalizes with Ca<sub>v</sub>2.1 (P/Q-type) channels in a subset of presynaptic nerve terminals (Lee et al., 2002), antibodies recognizing CaBP1 and caldendrin, a splice variant of CaBP1, label primarily somatodendritic regions of neurons throughout the brain (Seidenbecher et al., 1998; Laube et al.,

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2002), a pattern that more closely parallels that of Ca<sub>v</sub>1.2 (L-type) channels (Westenbroek et al., 1990; Hell et al., 1993). Therefore, we hypothesize that CaBP1 may play an important role in regulating postsynaptic Ca<sub>v</sub>1.2 channels. We show that CaBP1 physically associates with Ca<sub>v</sub>1.2 at the postsynaptic density (PSD) and competes with CaM for binding to the channel. However, compared with the inhibitory effects of CaM, CaBP1 greatly prolongs and facilitates Ca<sup>2+</sup> currents conducted by Ca<sub>v</sub>1.2. These distinct regulatory effects of CaBP1 may contribute to the diverse properties of Ca<sub>v</sub>1.2 channels in neurons and determine how Ca<sup>2+</sup> signals are encoded and decoded in the postsynaptic neuron.

## Materials and Methods

**Constructs and molecular biology.** For electrophysiological and biochemical experiments, Ca<sub>v</sub>1.2 subunits [ $\alpha_1$ 1.2 (rbclI),  $\beta_{2A}$ , and  $\alpha_2\delta$  (Ellis et al., 1988; Snutch et al., 1991; Perez-Reyes et al., 1992)] were expressed from the pcDNA3.1+ vector (Invitrogen, Carlsbad, CA). For the FLAG- $\alpha_1$ 1.2 construct, a Kozak sequence and FLAG epitope (DYKDDDDK) were added to the N terminus of  $\alpha_1$ 1.2 by PCR of nucleotides 4–1272 of rbclI with a forward primer containing the additional sequences and the resulting PCR product cloned into *Hind*III sites in the parent plasmid (rbclI/pcDNA3.1+). FLAG- $\alpha_1$ 1.2<sub>IQ-AA</sub> and FLAG- $\alpha_1$ 1.2<sub>IQ-EE</sub> were generated by quick-change mutagenesis with primers incorporating nucleotide changes to substitute alanine or glutamate residues for I1624 and Q1625. The cDNA corresponding to the entire coding region of the short isoform of human CaBP1 (GenBank accession number AF169148) was subcloned into pcDNA3.1+ or pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA) (Haeseleer et al., 2000). For binding assays, constructs encoding fusion proteins of the C-terminal domain of  $\alpha_1$ 1.2 were generated by PCR and subcloned into pGEX 4T-1 (Amersham Biosciences, Piscataway, NJ) for glutathione S-transferase (GST)- or pTrcHisA (Invitrogen) for His-tagged proteins. The cDNA encoding neuronal Ca<sup>2+</sup> sensor-1 (NCS-1) was generated by PCR with specific primers from rat brain cDNA and subcloned into pcDNA3.1+.

**Cell culture and transfection.** Human embryonic kidney HEK293T cells were maintained in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere under 7% CO<sub>2</sub>. Cells were grown to ~70–80% confluence and transfected with Gene Porter reagent (Gene Therapy Systems, San Diego, CA) according to protocols of the manufacturer.

**Binding assays.** CaBP1 used for binding assays was obtained by transfecting HEK293T cells plated on 150 mm dishes with a total of 5  $\mu$ g of CaBP1 subcloned into pcDNA3.1+. Two days later, cells were homogenized in 1 ml of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1 mM benzamide, and 0.5% Triton X-100, pH 7.4), and membrane proteins were solubilized by rotating at 4°C for 30 min. Insoluble material was removed by ultracentrifugation at 100,000  $\times$  g for 30 min, and the supernatant was used immediately or aliquotted and stored at –80°C. This procedure separated CaBP1, which is primarily membrane associated in HEK293T cells (data not shown), and CaM, which is cytoplasmic and endogenously expressed by these cells. The absence of CaM, which would have complicated binding analyses with transfected CaBP1, was confirmed by Western blot of these solubilized membrane preparations with anti-CaM antibodies.

Fusion proteins containing fragments of the  $\alpha_1$ 1.2 C-terminal domain were expressed in BL21 *Escherichia coli* by isopropyl- $\beta$ -D-thiogalactopyranoside induction and immobilized on glutathione- or Ni<sup>2+</sup>-agarose beads for GST or His fusion proteins, respectively. Purified CaM (5  $\mu$ g; Sigma-Aldrich, St. Louis, MO) or lysates of cells transfected with CaBP1 were added to 50  $\mu$ l of a 50% slurry of immobilized fusion protein and brought to a total volume of 1 ml with binding buffer ([Tris-buffered saline (TBS): 20 mM Tris, pH 7.3, and 150 mM NaCl], 0.1% Triton X-100, and protease inhibitors) containing either 2 mM CaCl<sub>2</sub> or 10 mM EGTA. Binding reactions were incubated at 4°C, rotating, for 1 hr. The beads were washed three times with 1 ml of ice-cold binding buffer, and bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose. Bound CaBP1, CaM, or NCS-1 was detected by Western blot with rabbit polyclonal antibodies against CaBP1 [UW72, 1:2000

(Haeseleer et al., 2000)], monoclonal anti-CaM antibodies (1:1000; Upstate Biotechnologies, Waltham, MA), or rabbit polyclonal anti-NCS-1 antibodies (1:1000; Zymed, San Francisco, CA). Blots were processed with HRP-conjugated secondary antibodies (anti-rabbit IgG 1:4000, anti-mouse IgG 1:2000) and ECL reagents (Amersham Biosciences). For some experiments, His-tagged fusion proteins were detected by Western blotting with rabbit polyclonal anti-His antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).

**Coimmunoprecipitation assays.** HEK293T cells were transfected with equimolar amounts of cDNAs encoding Ca<sub>v</sub>1.2 subunits (FLAG- $\alpha_1$ 1.2,  $\beta_{2A}$ , and  $\alpha_2\delta$ ) with or without CaBP1. At least 48 hr later, cell lysates were prepared as described above and incubated with 40  $\mu$ l of anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 hr, rotating at 4°C. After five washes with 1 ml of lysis buffer, proteins were eluted with sample buffer and subjected to SDS-PAGE, and coimmunoprecipitated proteins were detected by Western blotting with UW72 or monoclonal anti-FLAG antibodies (M2, 1:2000; Sigma-Aldrich), followed by secondary antibodies and ECL detection as described above.

For coimmunoprecipitation from rat brain, fractions containing the PSD were isolated by a modified method described previously (Carlin et al., 1980). These procedures, as well as those described for Immunohistochemistry, were done in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Freshly removed rat brains were homogenized in ice-cold buffer [homogenization buffer (HB): 4 mM HEPES, 1 mM EDTA, and 0.32 M sucrose, pH 7.4] with protease inhibitors (1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A, 17  $\mu$ g/ml PMSF, 8  $\mu$ g/ml calpain inhibitor I, 8  $\mu$ g/ml calpain inhibitor II, and 1 mM benzamide). After centrifugation at 1000  $\times$  g for 10 min, the membrane pellet was resuspended in HB and layered onto a sucrose step gradient (0.8 and 1.2 M), which was centrifuged at 150,000  $\times$  g for 1 hr. Material at the interface of the gradient was removed, centrifuged at 150,000  $\times$  g for 30 min, resuspended in HB containing 0.5% Triton X-100, incubated for 15 min, and centrifuged at 35,000  $\times$  g for 20 min. The final pellet containing the PSD fraction was resuspended in HB and stored at –80°C. Before an experiment, PSD fractions were pelleted and solubilized in HB containing 1% Triton X-100 for 20 min at 4°C with continuous stirring, and insoluble material was removed by centrifugation at 150,000  $\times$  g for 30 min. The presence of PSD-95 in the resulting supernatant, which was detected by Western blotting with anti-PSD-95 antibodies (1:2000; Affinity Bioreagents, Golden, CO) was used to confirm that postsynaptic density proteins were solubilized by this procedure. For coimmunoprecipitations, the supernatant (1 ml) was incubated for 1.5 hr, rotating at 4°C with 10  $\mu$ g of rabbit polyclonal antibodies against  $\alpha_{1C}$  (Alomone Labs, Jerusalem, Israel) or purified rabbit IgG (Jackson ImmunoResearch, West Grove, PA) as a control. Immune complexes were isolated by incubating with 3–5 mg of preswollen Protein A-Sepharose for 2 hr at 4°C. The beads were washed three times with 0.1% Triton X-100 in TBS and subjected to SDS-PAGE, and immunoprecipitated proteins were detected by Western blotting with  $\alpha_{1C}$  (1:200) or UW72 antibodies as described above.

**Double-label immunohistochemistry.** Adult male Sprague Dawley rats were anesthetized deeply (4% chloral hydrate, i.p.) and intracardially perfused with 3% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were removed and cryoprotected overnight at 4°C in 30% sucrose, 0.1 M PB. Tissue sections (30–40  $\mu$ m) were cut on a freezing sliding microtome, rinsed in TBS, and blocked in TBS containing 5% normal goat serum (NGS) and 0.025% Triton X-100 [blocking buffer (BB)]. All antibodies were diluted in TBS containing 2.5% NGS and 0.025% Triton X-100, and sections were rinsed three times for 20 min each after each step. Blocked brain sections were incubated with anti- $\alpha_{1C}$  antibodies (1:100; generously provided by Dr. Johannes Hell, University of Iowa, Iowa City, IA) overnight at 4°C and, after rinsing in TBS, with 1:400 rhodamine-conjugated donkey anti-rabbit Fab fragments (Jackson ImmunoResearch) for 60 min. Before double labeling, sections were again blocked at 37°C for 60 min with BB, 120 min with goat anti-rabbit Fab fragments (1:50; Jackson ImmunoResearch), 30 min in 2% avidin-TBS, and 30 min in 2% biotin-TBS. Sections were then incubated overnight at 4°C with UW72 (1:1000), the next day with biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch), and subsequently

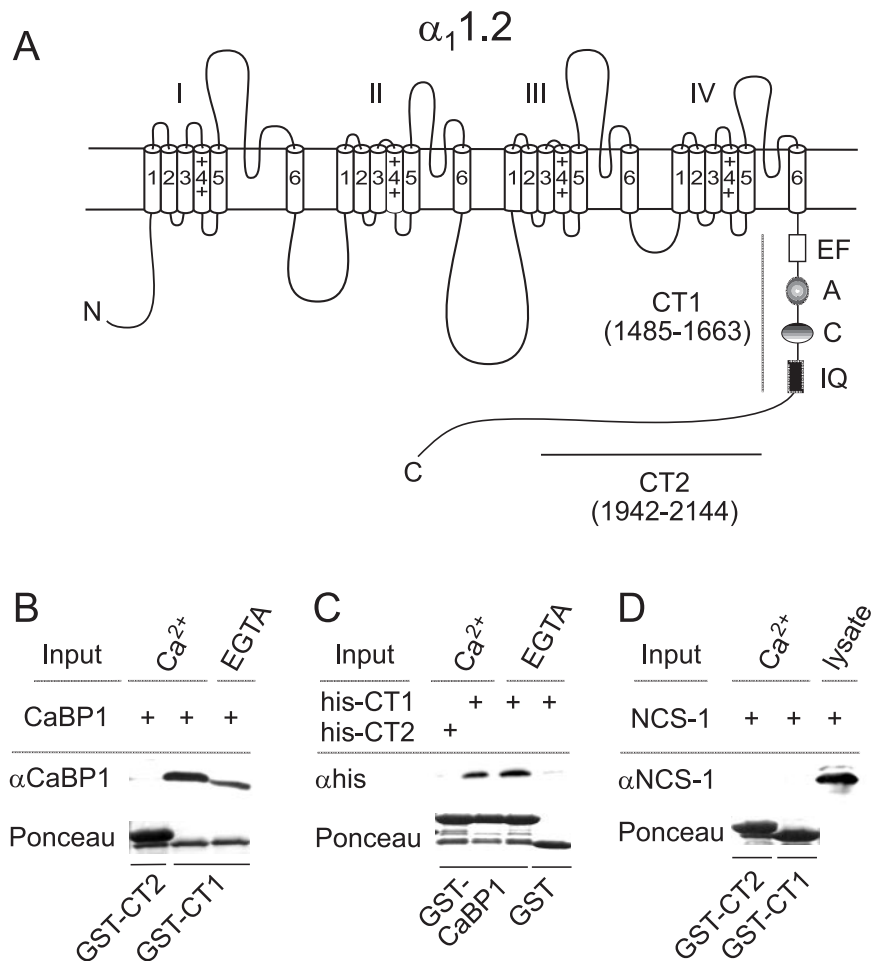
with FITC-avidin D in TBS (1:1600; Vector Laboratories, Burlingame, CA). The double-labeled sections were mounted and coverslipped with Vectashield (Vector Laboratories) for viewing under a Zeiss (Oberkochen, Germany) LSM510 Meta confocal microscope.

**Electrophysiological recordings.** HEK293T cells plated on 35 mm dishes were transfected with a total of 5  $\mu$ g of DNA including 0.3  $\mu$ g of a pEGFP-N1 or CaBP1/pEGFPN1 for fluorescent detection of transfected cells. At least 48 hr after transfection, whole-cell patch-clamp recordings of transfected cells were acquired with a HEKA Elektronik (Lambrecht/Pfalz, Germany) EPC-9 patch-clamp amplifier. Data acquisition and leak subtraction using a P/−4 protocol were done with Pulse software (HEKA Elektronik). Extracellular recording solutions contained the following (in mM): 150 Tris, 2 MgCl<sub>2</sub>, and 10 CaCl<sub>2</sub> or BaCl<sub>2</sub>. Intracellular solutions consisted of the following (in mM): 140 N-methyl-D-glucamine, 10 HEPES, 2 MgCl<sub>2</sub>, 2 Mg-ATP, and 5 EGTA. The pH of intracellular and extracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Electrode resistances were typically 1–2 M $\Omega$  in the bath solution, and series resistance was ~2–4 M $\Omega$ , compensated up to 70%. In recordings of Ba<sup>2+</sup> currents, voltage protocols were adjusted by −10 mV to compensate for the corresponding shift in voltage dependence of activation with substitution of extracellular Ba<sup>2+</sup> for Ca<sup>2+</sup>. The time course of *I*<sub>Ca</sub> decay was fit by  $A_{\text{slow}}[\exp(-t/\tau_{\text{slow}})] + A_{\text{fast}}[\exp(-t/\tau_{\text{fast}})]$ , where *t* is time, *A*<sub>slow</sub> and *A*<sub>fast</sub> are the amplitudes of the slow and fast exponentials, respectively, at *t* = 0, and  $\tau_{\text{slow}}$  and  $\tau_{\text{fast}}$  are the time constants of the decay of the two processes. Data were analyzed using Igor software (WaveMetrics, Lake Oswego, OR), and graphs and statistical analysis were done with Sigma Plot (SPSS, Chicago, IL).

## Results

### CaBP1 binds to the C-terminal domain of $\alpha_1.2$

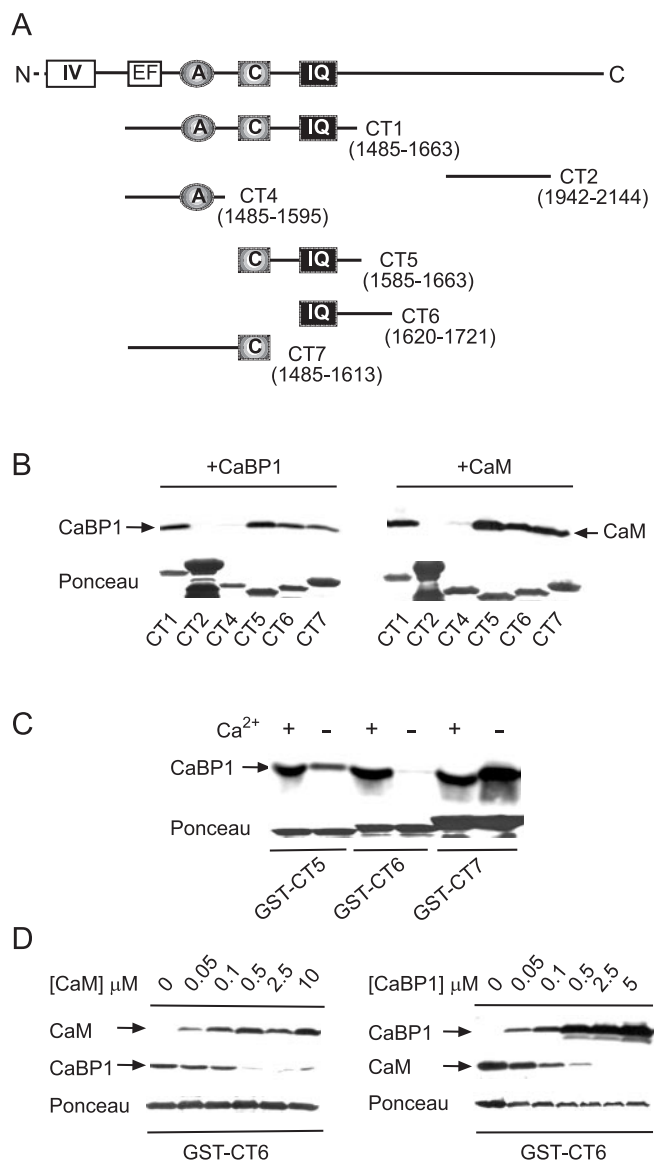
Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 depends on CaM binding to multiple sequence elements in the C-terminal domain of  $\alpha_1.2$  (Soldatov et al., 1998; Zühlke and Reuter, 1998; Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999, 2000). These sites (A, C, and IQ) (Fig. 1A) mediate binding to either Ca<sup>2+</sup>-free (apo)CaM or Ca<sup>2+</sup>-bound CaM and are in the proximal portion of the cytoplasmic C-terminal domain just downstream of an EF-hand motif, also implicated in Ca<sup>2+</sup>-dependent inactivation (Zühlke and Reuter, 1998; Pate et al., 2000; Peterson et al., 2000; Romanin et al., 2000; Pitt et al., 2001; Erickson et al., 2003; Tang et al., 2003). On the basis of its similarity with CaM, we hypothesized that CaBP1 might also interact with these C-terminal CaM-binding sites. To test this, we generated a GST fusion protein including these regions (CT1) (Fig. 1A) and analyzed binding to CaBP1 in pull-down assays. CaBP1 was brought down with CT1 but not a more distal region of the C-terminal domain used as a negative control (CT2) (Fig. 1A,B). CaBP1 binding to CT1 was stronger in the presence of Ca<sup>2+</sup> but was not abolished when EGTA was included in the assay, indicating that CaBP1 binding to  $\alpha_1.2$  is partially Ca<sup>2+</sup> independent. This result was confirmed in reverse experiments in which GST-tagged CaBP1, but not



**Figure 1.** CaBP1 interacts with the C-terminal domain of the  $\alpha_1$  subunit of Ca<sub>v</sub>1.2. *A*, Schematic of  $\alpha_1.2$  with C-terminal domain showing CT1 and CT2 regions used for GST fusion constructs in binding experiments. Amino acid boundaries are indicated in parentheses. CT1 included sequences involved in Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2: the EF-hand (EF) and CaM-interacting sites A, C, and the IQ domain (IQ). N, N terminus of  $\alpha_1.2$ . *B*, Binding of CaBP1 to CT1 but not CT2. GST-CT1 or CT2 fusion proteins were immobilized on glutathione-agarose beads and incubated with lysates from cells transfected with CaBP1 in the presence of 2 mM Ca<sup>2+</sup> or 10 mM EGTA. Bound CaBP1 was detected by Western blot. Ponceau staining of the blot indicated levels of GST fusion protein used in each binding assay. *C*, Binding of His-tagged CT1 to GST-tagged CaBP1. GST or GST-CaBP1 was immobilized on glutathione-agarose beads and incubated with purified His-CT1 or His-CT2. Bound fusion proteins were detected by Western blot with anti-His antibodies. *D*, NCS-1 does not bind to CT1. Binding assay was performed as in *B*, except that lysates from cells transfected with NCS-1 were used and immunoblotting was done with rabbit polyclonal antibodies against NCS-1. NCS-1 was detected in transfected cell lysates (last lane) but not in samples pulled down by GST-CT1 or CT2.

GST, pulled down His-tagged CT1, but not CT2, both with and without Ca<sup>2+</sup> (Fig. 1C). This interaction with CaBP1 was specific in that a related NCBP, NCS-1, did not bind CT1 under the same conditions (Fig. 1D).

To further refine the binding determinants for CaBP1, we compared CaBP1 interactions with GST fusion proteins containing various subsets of the A, C, and IQ sites (Fig. 2A). As shown in Figure 2B, CaBP1 was pulled down by all fusion proteins that contained the C and/or IQ sites (CT1, CT5, CT6, and CT7) but not CT4, which had only the A site. An identical pattern of binding was observed for CaM (Fig. 2B). However, previous studies show binding of CaM to peptides but not fusion proteins containing the A site, perhaps because of the lower affinity of CaM binding to this site, which may not be detectable in our pull-down assay (Pate et al., 2000; Romanin et al., 2000; Pitt et al., 2001; Tang et al., 2003). Therefore, although we cannot rule out the possibility that CaBP1 also interacts with low affinity at the A site, our results confirm that both C and IQ sites contain sequences important for binding CaBP1 and CaM.



**Figure 2.** CaBP1 binds to putative CaM-binding sites in the C-terminal domain of  $\alpha_1.2$ . *A*, Schematic of C-terminal domain of  $\alpha_1.2$  showing regions tested for binding CaM or CaBP1 in binding assays. *B*, Binding of CaM and CaBP1 to multiple sites in  $\alpha_1.2$ . GST-tagged  $\alpha_1.2$  fragments shown in *A* were incubated with CaBP1 from transfected cells (left) or purified CaM (right) in the presence of 2 mM Ca<sup>2+</sup>. Bound proteins were detected by Western blotting with specific antibodies. Integrity and levels of GST-tagged proteins were confirmed by Ponceau staining. *C*, Ca<sup>2+</sup>-dependent and -independent binding of CaBP1. Binding of CaBP1 to GST-CT5, GST-CT6, or GST-CT7 was performed as in *B* and compared when assay was done with 2 mM Ca<sup>2+</sup> (+) or 10 mM EGTA (−). *D*, Competitive binding of CaM and CaBP1 to CT6. Binding of CaBP1 or CaM to GST-CT6 was performed as in *B* with 2 mM Ca<sup>2+</sup> and compared in the presence of increasing concentrations of purified CaM (left) or CaBP1 (right).

Current models of CaM interactions with Ca<sub>v</sub>1.2 involve constitutive association of CaM with the A and C sites with Ca<sup>2+</sup> binding to CaM promoting interactions with the IQ, which then transduces Ca<sup>2+</sup>-dependent inactivation (Pate et al., 2000; Pitt et al., 2001) (but see Romanin et al., 2000; Erickson et al., 2003; Tang et al., 2003). To determine whether the same was true for CaBP1, Ca<sup>2+</sup>-dependent binding of CaBP1 to these sites was analyzed with Ca<sup>2+</sup> (2 mM) or EGTA (10 mM) in the assay. In these experiments, CaBP1 binding to the C site was Ca<sup>2+</sup> independent, whereas binding to CT6, which contained the IQ, occurred only in the presence of Ca<sup>2+</sup> (Fig. 2C). Moreover, binding

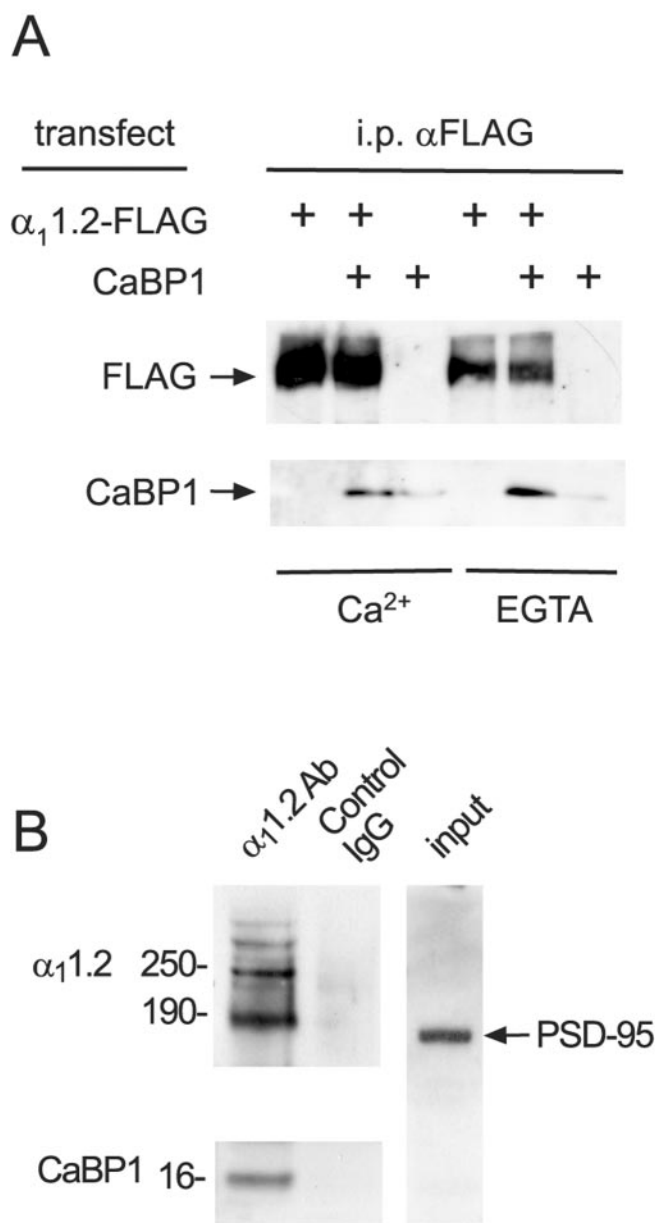
of CaM and CaBP1 to the IQ was mutually exclusive in that CaM effectively displaced CaBP1 binding to CT6 at a concentration between 100 and 500 nM, and CaBP1 competed with CaM for binding to CT6 at a similar range of concentrations (Fig. 2D). These results confirm that similar molecular determinants mediate binding of CaBP1 and CaM. As has been reported for CaM, CaBP1 may be tethered to the C site at resting Ca<sup>2+</sup> concentrations, with elevations in Ca<sup>2+</sup> promoting conformational changes in CaBP1 that permit binding to IQ.

Although CaBP1 effectively competed with CaM for interaction with the GST fragment containing the IQ (Fig. 2D), it was important to determine whether CaBP1 could associate with the intact channel in cells in which CaM is endogenously expressed at high concentrations. Therefore, we tested whether CaBP1 could coimmunoprecipitate with FLAG-tagged  $\alpha_1.2$  (FLAG- $\alpha_1.2$ ) in transfected HEK293T cells. As shown in Figure 3A, FLAG antibodies coimmunoprecipitated CaBP1 from cells cotransfected with FLAG- $\alpha_1.2$  but not from cells transfected with CaBP1 or FLAG- $\alpha_1.2$  alone. Moreover, the association of CaBP1 and FLAG- $\alpha_1.2$  did not require Ca<sup>2+</sup>, which is consistent with the Ca<sup>2+</sup>-independent binding of CaBP1 to the C site (Fig. 2B). These results confirm that CaBP1 is constitutively associated with Ca<sub>v</sub>1.2 channels in cells, which may involve displacing CaM from binding sites in the cytoplasmic C-terminal domain of  $\alpha_1.2$ .

### CaBP1 forms a postsynaptic complex with Ca<sub>v</sub>1.2

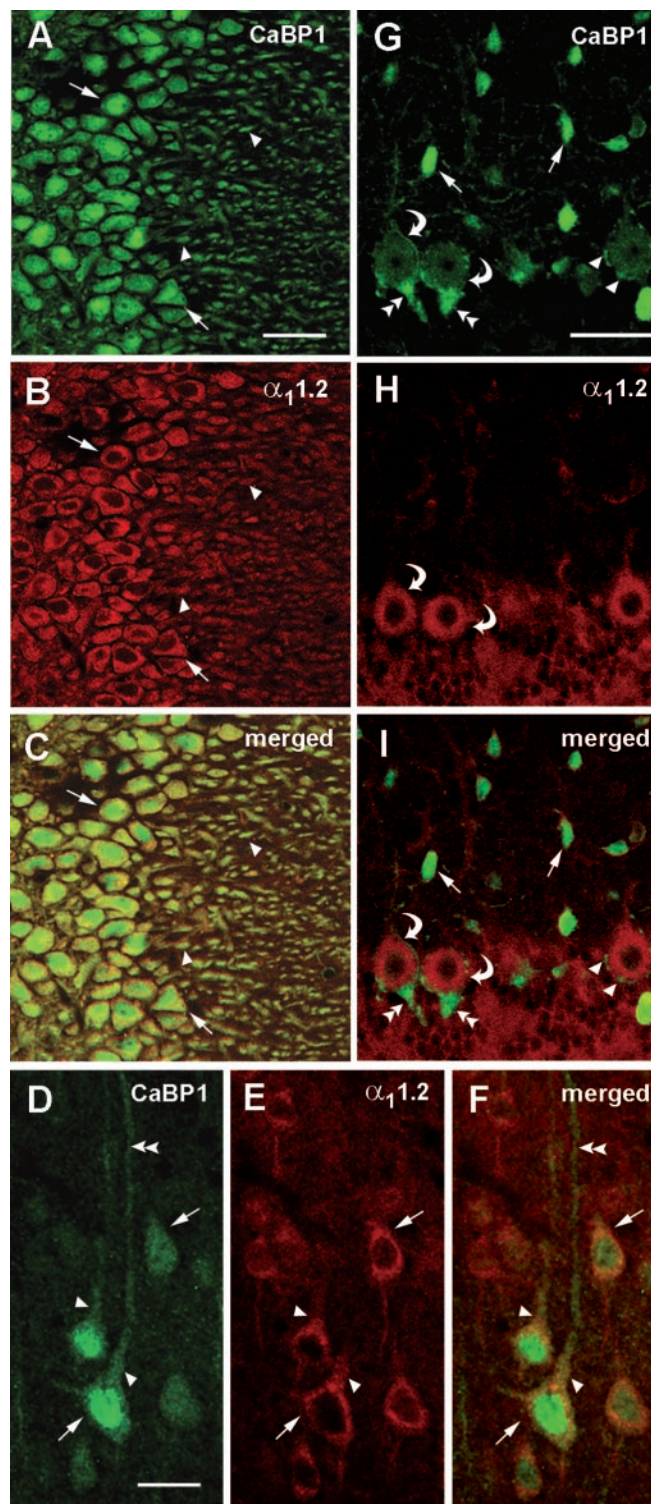
Previous immunohistochemical studies of CaBP1 localization in the brain indicated a cellular and subcellular distribution (Seidenbecher et al., 1998; Laube et al., 2002) that overlapped considerably with that for Ca<sub>v</sub>1.2 (Westenbroek et al., 1990; Hell et al., 1993). However, initial attempts to coimmunoprecipitate Ca<sub>v</sub>1.2 and CaBP1 from whole brain homogenates were unsuccessful, perhaps because of a preferential interaction of both CaBP1 and Ca<sub>v</sub>1.2 at the PSD, in which both proteins are enriched (Hell et al., 1996; Seidenbecher et al., 1998). Because many protein complexes in the PSD are difficult to extract with standard detergent concentrations (Lau et al., 1996), it is possible that interactions between Ca<sub>v</sub>1.2 and CaBP1 at the PSD may have been undetectable in whole brain lysates attributable to their insolubility. Therefore, we focused on whether CaBP1 and Ca<sub>v</sub>1.2 channels associated in PSD fractions that were solubilized and checked for expression of postsynaptic markers such as PSD-95 by immunoblotting (Fig. 3B). From these fractions, anti- $\alpha_1.2$  antibodies, but not rabbit IgG used as a control, consistently coimmunoprecipitated the short isoform of CaBP1, which was the isoform used in our biochemical studies (Fig. 3B). The isolation of a postsynaptic complex containing CaBP1 and Ca<sub>v</sub>1.2 indicates that the molecular interactions between these proteins we characterized *in vitro* may be physiologically relevant *in vivo*.

To determine the extent to which CaBP1/Ca<sub>v</sub>1.2 interactions occur in the brain, we used double-label immunofluorescence for colocalizing both proteins in sections of rat brain. In general, CaBP1 and Ca<sub>v</sub>1.2 were strongly colocalized in numerous cell groups throughout the brain. In the hippocampus, labeling for CaBP1 was prominent in the soma and dendrites of CA3 pyramidal cells and almost completely overlapped with that for Ca<sub>v</sub>1.2 (Fig. 4A–C). In the dorsal cortex, CaBP1 and Ca<sub>v</sub>1.2 were colocalized in pyramidal cells, particularly in layer V. However in these neurons, Ca<sub>v</sub>1.2 labeling was restricted to the soma and proximal dendrites, whereas CaBP1 labeling extended far into the distal dendrites (Fig. 4D–F). Furthermore, whereas Ca<sub>v</sub>1.2 immunoreactivity was excluded from the nucleus, all neurons that

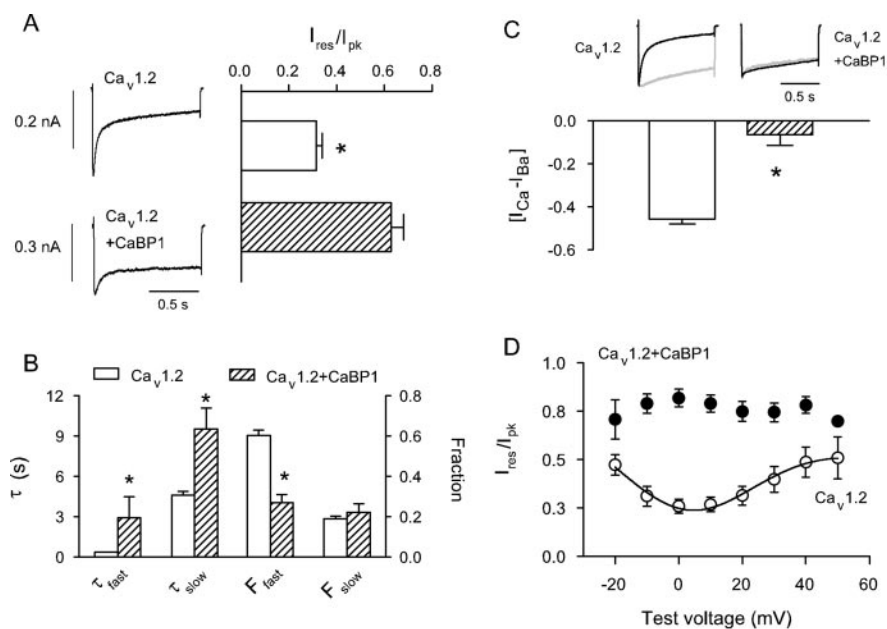


**Figure 3.** Coimmunoprecipitation of CaBP1 and  $\alpha_1$ 1.2 from transfected cells and rat brain. *A*, Cells transfected with Ca<sub>v</sub>1.2 ( $\alpha_1$ 1.2-FLAG,  $\beta_{2A}$ , and  $\alpha_2\delta$ ), Ca<sub>v</sub>1.2 plus CaBP1, or CaBP1 alone were subject to lysis and immunoprecipitation using anti-FLAG antibodies. Experiments were done in the presence of 2 mM Ca<sup>2+</sup> or 10 mM EGTA. Immunoprecipitated proteins were detected by Western blotting with antibodies recognizing the FLAG epitope (top) or CaBP1 (bottom). *B*, Immunoprecipitation of a complex containing both  $\alpha_1$ 1.2 and CaBP1 from PSD fractions of rat brain. PSD proteins were isolated on sucrose gradients, solubilized, and incubated with  $\alpha_1$ 1.2 antibodies. Immunoprecipitated proteins were detected by Western blotting with antibodies against  $\alpha_1$ 1.2 (top) or CaBP1 (bottom). Immunoblotting of the PSD fraction was also performed with antibodies against PSD-95 (right).

were labeled with the CaBP1 antibody showed strong immunoreactivity in the nuclear as well as cytoplasmic compartments, which may signify a role for CaBP1 also in regulating gene expression. In the cerebellum, weak-to-moderate labeling for both proteins was detected in Purkinje cell bodies and proximal dendrites (Fig. 4*G–I*). In addition, intense labeling for CaBP1 was found in interneurons resembling basket and stellate cells in the molecular layer and in the axon terminals and large Pinceaux synapses formed from basket cells onto Purkinje cell bodies (Fig. 4*G, I*)



**Figure 4.** Colocalization of CaBP1 and  $\alpha_1$ 1.2 in rat brain. *A–I*, Confocal images of rat brain sections sequentially double labeled with antibodies against CaBP1 and  $\alpha_1$ 1.2. Immunofluorescence was viewed under optics for fluorescein (for CaBP1; *A, D, G*) or rhodamine (for  $\alpha_1$ 1.2; *B, E, H*). Regions of colocalization appear yellow in the merged images (*C, F, I*). Extensive colocalization of CaBP1 and  $\alpha_1$ 1.2 was detected in cell bodies (arrows) and dendrites (arrowheads) of neurons in the CA3 region of the hippocampus (*A–C*). In layer V of the cerebral cortex (*D–F*), CaBP1 and  $\alpha_1$ 1.2 colocalized in pyramidal cell soma (arrows) and proximal dendrites (arrowheads) but not more distal dendrites (double arrowheads). In the cerebellum (*G–I*), CaBP1 and  $\alpha_1$ 1.2 were colocalized in Purkinje cell bodies (curved arrows), but only CaBP1 was found in interneurons in the molecular layer (arrows) and in small perisomatic synapses (arrowheads) and large Pinceaux synapses (double arrowheads) onto Purkinje cell bodies. Scale bars: *A–C, G–I*, 100  $\mu$ m; *D–F*, 50  $\mu$ m.



**Figure 5.** CaBP1 prolongs Ca<sup>2+</sup> currents through Ca<sub>v</sub>1.2 channels and does not support Ca<sup>2+</sup>-dependent inactivation. *A*, Effect of CaBP1 on Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents evoked by step depolarizations. Traces represent Ca<sup>2+</sup> currents evoked by a 1 sec step from  $-80$  to  $+10$  mV in whole-cell patch-clamp recordings of HEK293T cells transfected with Ca<sub>v</sub>1.2 subunits with or without CaBP1.  $I_{res}/I_{pk}$  was determined by dividing the residual current amplitude at the end of the pulse by the peak current amplitude. Extracellular solution contained 10 mM Ca<sup>2+</sup>, and intracellular solution contained 5 mM EGTA. Data represent mean  $\pm$  SEM for Ca<sub>v</sub>1.2 ( $n = 17$ ) and Ca<sub>v</sub>1.2 plus CaBP1 ( $n = 11$ ) ( $*p < 0.001$ ). *B*, Impact of CaBP1 on fast and slow inactivation. Ca<sup>2+</sup> currents obtained in *A* were fit with a double-exponential function. The fast and slow time constants ( $\tau_{fast}$ ,  $\tau_{slow}$ ) and fraction of channels showing fast and slow components of inactivation ( $F_{fast}$ ,  $F_{slow}$ ) were averaged and shown for cells transfected with Ca<sub>v</sub>1.2 alone or Ca<sub>v</sub>1.2 plus CaBP1 ( $*p < 0.001$ ). *C*, Comparison of Ca<sup>2+</sup>-dependent inactivation in cells transfected with Ca<sub>v</sub>1.2 alone or cotransfected with CaBP1. Traces show currents evoked by 1 sec depolarizing step from  $-80$  to  $+10$  mV for  $I_{Ca}$  (black trace) or 0 mV for  $I_{Ba}$  (gray trace) to compensate for  $-10$  mV voltage shift for  $I_{Ba}$ . Extracellular solution contained either 10 mM Ca<sup>2+</sup> or Ba<sup>2+</sup>.  $[I_{Ca} - I_{Ba}]$  represents the difference between  $I_{res}/I_{pk}$  for  $I_{Ca}$  and the mean  $I_{res}/I_{pk}$  for  $I_{Ba}$  for Ca<sub>v</sub>1.2 channels alone ( $n = 17$ ; open bar) or Ca<sub>v</sub>1.2 plus CaBP1 ( $n = 11$ ; hatched bar) ( $*p < 0.001$ ). *D*, U-shaped voltage dependence of  $I_{res}/I_{pk}$  for  $I_{Ca}$  in cells transfected with Ca<sub>v</sub>1.2 alone but not in cells cotransfected with CaBP1.  $I_{res}/I_{pk}$  was determined for  $I_{Ca}$  evoked by 1 sec test pulses from  $-80$  mV to various voltages, averaged, and plotted against test voltage for Ca<sub>v</sub>1.2 alone (open circles;  $n = 9$ ) or Ca<sub>v</sub>1.2 plus CaBP1 (filled circles;  $n = 8$ ).

(Sotelo and Llinas, 1972). In contrast, no Ca<sub>v</sub>1.2 labeling was detected in these interneurons or presynaptic terminals (Fig. 4*H,I*). These cellular and subcellular distinctions between CaBP1 and Ca<sub>v</sub>1.2 immunostaining exclude the possibility that the extensive colocalization in the hippocampus and the cortex was attributable to nonspecific reactivity of the secondary antibodies used in the double-labeling procedure. Although CaBP1 may have activities independent of interactions with Ca<sub>v</sub>1.2, the coimmunoprecipitation of CaBP1 with Ca<sub>v</sub>2.1, and their colocalization in many of the same cells, suggest that the two may interact in a subset of principal neurons in the brain.

#### CaBP1 prolongs Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents and does not support Ca<sup>2+</sup>-dependent inactivation

Because CaBP1 and CaM share similar binding determinants in  $\alpha_1.2$ , CaBP1 could simply replicate the modulatory effects of CaM. Alternatively, CaBP1 may differentially regulate the channel as we showed for Ca<sub>v</sub>2.1 (P/Q-type) channels (Lee et al., 2002). To distinguish between these possibilities, we analyzed inactivation of Ca<sup>2+</sup> currents through Ca<sub>v</sub>1.2 channels transfected in HEK293T cells in whole-cell patch-clamp recordings. Inactivation was quantitatively compared as the residual current amplitude at the end of the test pulse normalized to the peak current ( $I_{res}/I_{pk}$ ). In these experiments, Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents ( $I_{Ca}$ ) inactivated rapidly and with a biphasic time course during a

1 sec step depolarization, as a result of endogenous Ca<sup>2+</sup>/CaM interacting with the channel (Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999) (Fig. 5*A*). In contrast, cotransfection with CaBP1 caused  $I_{Ca}$  to inactivate more slowly, resulting in a significant increase in  $I_{res}/I_{pk}$  compared with in cells transfected with Ca<sub>v</sub>1.2 alone [ $0.63 \pm 0.05$  ( $n = 11$ ) for Ca<sub>v</sub>1.2 plus CaBP1 vs  $0.32 \pm 0.02$  ( $n = 17$ ) for Ca<sub>v</sub>1.2 alone;  $p < 0.001$ ] (Fig. 5*A*). Biexponential fits of  $I_{Ca}$  inactivation revealed a significant effect of CaBP1 in slowing the fast time constant ( $\tau_{fast} = 2.9 \pm 1.5$  sec for Ca<sub>v</sub>1.2 plus CaBP1 vs  $0.4 \pm 0.03$  sec for Ca<sub>v</sub>1.2 alone;  $p < 0.05$ ) and decreasing the fraction of channels inactivating with  $\tau_{fast}$  ( $F_{fast} = 0.27 \pm 0.04$  for Ca<sub>v</sub>1.2 plus CaBP1 vs  $0.60 \pm 0.03$  for Ca<sub>v</sub>1.2 alone;  $p < 0.001$ ) (Fig. 5*B*). In addition, CaBP1 nearly doubled the slow time constant ( $\tau_{slow} = 9.5 \pm 1.6$  sec for Ca<sub>v</sub>1.2 plus CaBP1 vs  $4.6 \pm 0.6$  sec for Ca<sub>v</sub>1.2 alone;  $p < 0.001$ ) but did not affect the fraction of channels inactivating with  $\tau_{slow}$  ( $F_{slow} = 0.22 \pm 0.04$  for Ca<sub>v</sub>1.2 plus CaBP1 vs  $0.19 \pm 0.01$  for Ca<sub>v</sub>1.2 alone;  $p = 0.4$ ) (Fig. 5*B*).

These effects of CaBP1 on slowing inactivation of  $I_{Ca}$  are similar to results obtained with CaM mutants unable to bind Ca<sup>2+</sup>. These CaM mutants interact with the tethering site on the channel but are unable to support Ca<sup>2+</sup>-dependent inactivation (Peterson et al., 1999; Zühlke et al., 1999). To determine whether a similar mechanism could account for the effects of CaBP1, we compared inactivation with

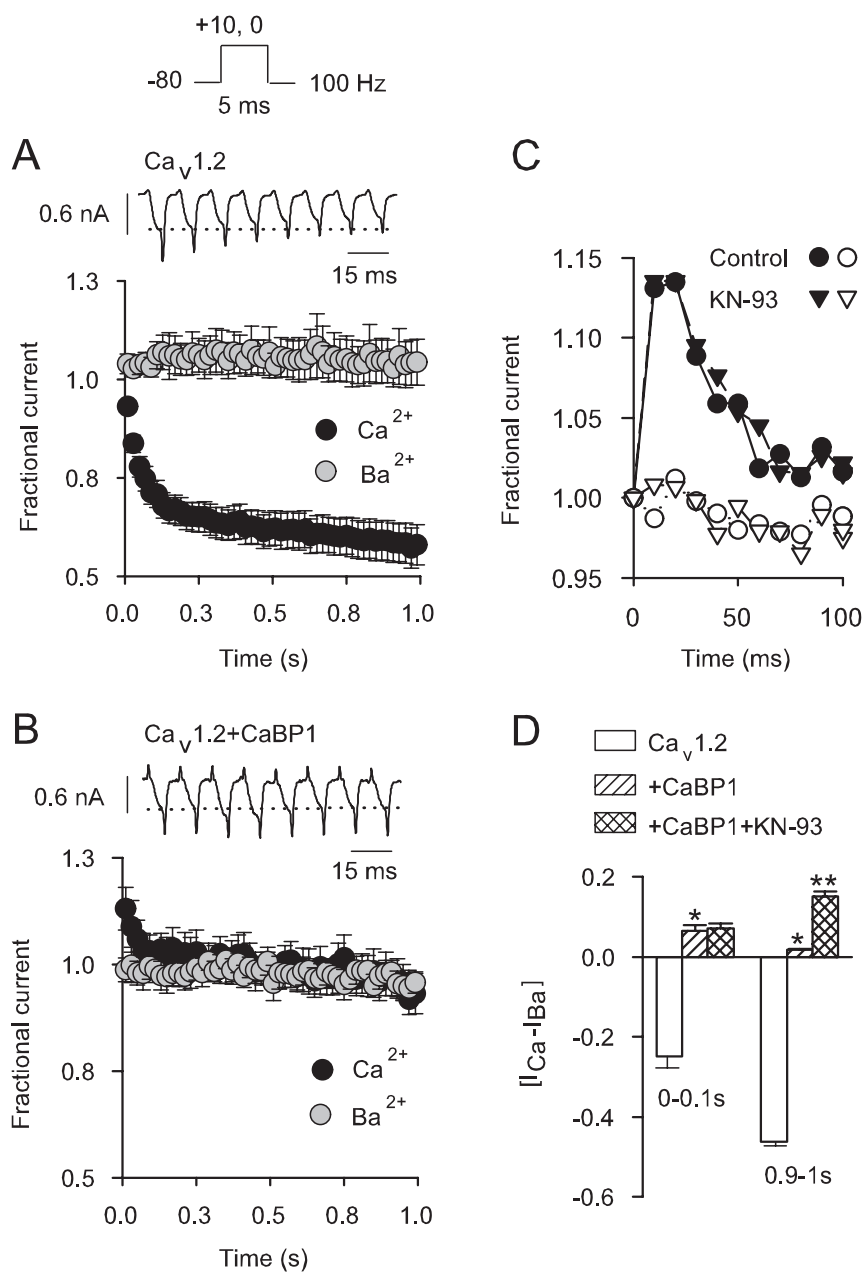
Ca<sup>2+</sup> and Ba<sup>2+</sup> as the charge carriers, because Ba<sup>2+</sup> does not substitute favorably for Ca<sup>2+</sup> in binding to EF-hand motifs and therefore blocks Ca<sup>2+</sup>-dependent effects of CaM and other Ca<sup>2+</sup> sensors (Wang, 1985). In cells transfected with Ca<sub>v</sub>1.2 alone, Ca<sup>2+</sup>/CaM-dependent inactivation was evident as a sizeable difference in  $I_{res}/I_{pk}$  for  $I_{Ca}$  compared with  $I_{Ba}$  [ $(I_{Ca} - I_{Ba}) = -0.46 \pm 0.02$ ;  $n = 17$ ] (Fig. 5*C*). In comparison, in cells cotransfected with CaBP1, this difference in  $I_{Ca}$  and  $I_{Ba}$  inactivation was nearly abolished [ $(I_{Ca} - I_{Ba}) = -0.06 \pm 0.05$ ;  $n = 11$ ;  $p < 0.01$ ] (Fig. 5*C*). In addition, for Ca<sub>v</sub>1.2 alone, a plot of  $I_{res}/I_{pk}$  versus test voltage was U-shaped, with maximal inactivation at  $+5$  mV, which was near the peak of the  $I$ - $V$  curve, as would be expected of a process depending on incoming Ca<sup>2+</sup> (Brehm et al., 1980) (Fig. 5*D*). In contrast, the extent of  $I_{Ca}$  inactivation in cells cotransfected with CaBP1 did not change appreciably with different test voltages (Fig. 5*D*). These results are similar to those obtained previously with the dominant-negative CaM mutants (Peterson et al., 1999; Zühlke et al., 1999) and demonstrate a novel effect of CaBP1 on prolonging Ca<sup>2+</sup> currents and opposing Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2.

#### CaBP1 causes Ca<sup>2+</sup>-dependent facilitation and prevents inactivation during repetitive stimuli

To characterize the significance of CaBP1 regulation of Ca<sub>v</sub>1.2 channels during more physiological stimuli, we evoked Ca<sub>v</sub>1.2 currents with trains of short (5 msec) depolarizations using both

Ca<sup>2+</sup> and Ba<sup>2+</sup> as permeant ions. With this voltage protocol, inactivation of  $I_{Ca}$  proceeds rapidly and causes an ~50% reduction in current amplitude by the end of the train (Fig. 6A). This strong inactivation is Ca<sup>2+</sup> dependent because the amplitude of  $I_{Ba}$  remains relatively constant throughout the protocol. Consistent with the prolonged currents during the 1 sec step depolarizations (Fig. 5A, C), neither  $I_{Ca}$  nor  $I_{Ba}$  inactivated appreciably during the train in cells cotransfected with CaBP1 (Fig. 6B). Moreover, CaBP1 caused an initial facilitation in  $I_{Ca}$  current amplitude, which was Ca<sup>2+</sup> dependent in that no similar change in  $I_{Ba}$  was observed (Fig. 6B, C). The facilitated  $I_{Ca}$  was maximal by the third pulse in the train and declined rapidly to initial levels by the sixth pulse. Ca<sup>2+</sup>-dependent regulation of Ca<sub>v</sub>1.2 with and without CaBP1 was quantitatively expressed as the difference in  $I_{Ca}$  and  $I_{Ba}$  [ $(I_{Ca} - I_{Ba})$ ] during the first (0–0.1 sec) and last (0.9–1 sec) periods of the stimulus train. CaBP1 caused a significant increase (~6%) in  $I_{Ca}$  compared with  $I_{Ba}$  in the first 100 msec, in contrast to ~25% decrease in  $I_{Ca}$  relative to  $I_{Ba}$  over this same time period with Ca<sub>v</sub>1.2 alone (Fig. 6D). The effect of CaBP1 was especially dramatic in the last 100 msec, in which Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 caused  $I_{Ca}$  to decay to nearly 50% of the amplitude of  $I_{Ba}$ , whereas cells cotransfected with CaBP1 showed virtually no difference in  $I_{Ca}$  and  $I_{Ba}$  amplitudes (Fig. 6D).

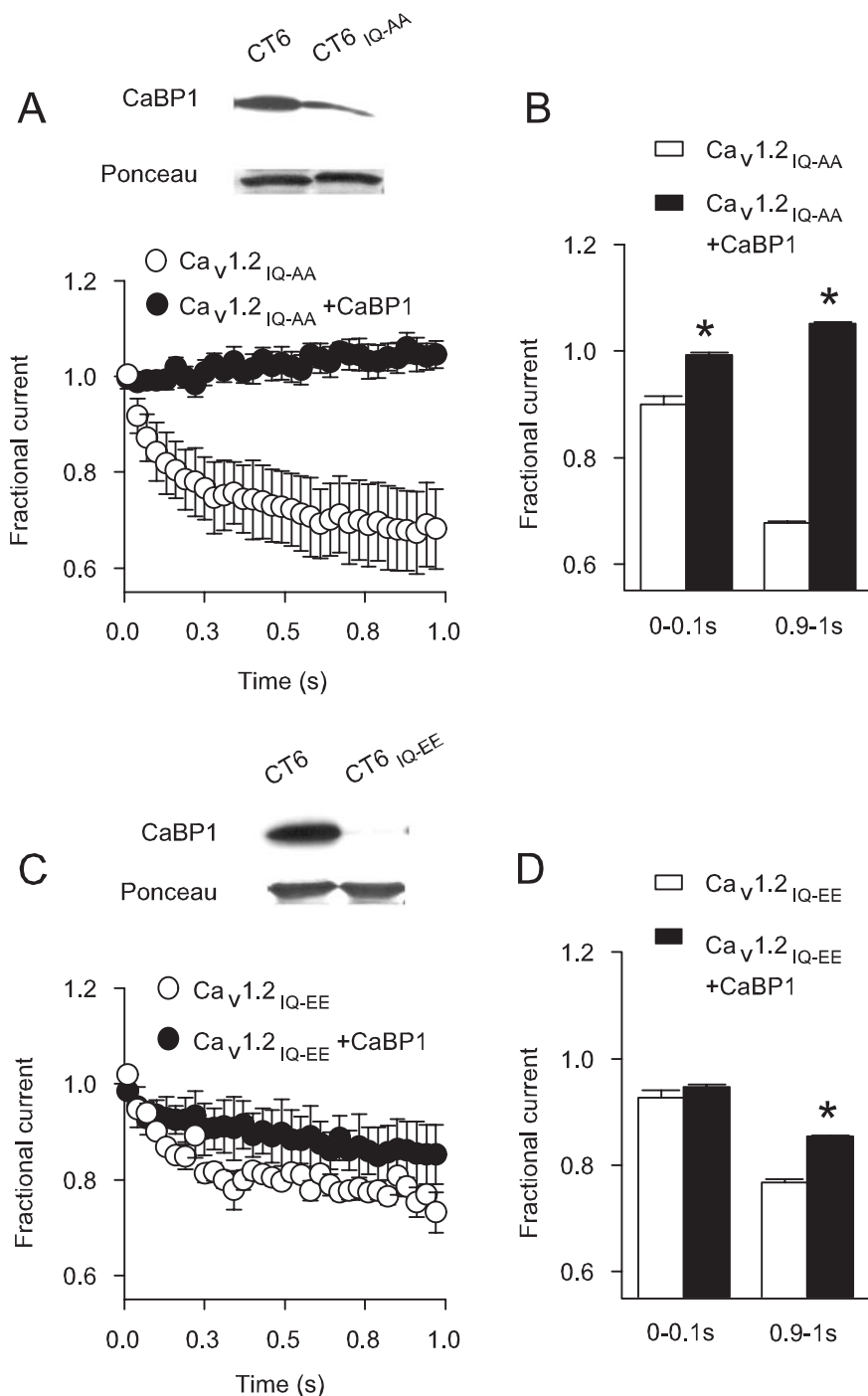
Ca<sup>2+</sup>-dependent facilitation caused by CaBP1 could result from a direct interaction with Ca<sub>v</sub>1.2 or through an indirect pathway, such as through the activation of CaM kinase II, which causes Ca<sup>2+</sup>-dependent facilitation of cardiac Ca<sub>v</sub>1.2 channels (Xiao et al., 1994; Yuan and Bers, 1994; Dzshura et al., 2000). To rule out the latter mechanism, we tested the effect of the CaM kinase II inhibitor KN-93 on  $I_{Ca}$  and  $I_{Ba}$  in cells cotransfected with Ca<sub>v</sub>1.2 and CaBP1. KN-93 did not significantly reduce the initial facilitation of  $I_{Ca}$  [ $(I_{Ca} - I_{Ba}) = 0.06 \pm 0.01$  ( $n = 5$ ) for control vs  $0.07 \pm 0.01$  ( $n = 5$ ) for KN-93;  $p = 0.58$ ] and actually augmented the difference in  $I_{Ca}$  and  $I_{Ba}$  in the later part of the train in cells cotransfected with CaBP1 [ $(I_{Ca} - I_{Ba}) = 0.02 \pm 0.002$  for control vs  $0.15 \pm 0.01$  for KN-93;  $p < 0.01$ ] (Fig. 6C, D). This later effect of KN-93 suggests that CaM kinase II may oppose the effects of CaBP1 on  $I_{Ca}$  facilitation. Alternatively, enhanced Ca<sup>2+</sup>-dependent facilitation with KN-93 may be related to nonspecific actions independent of CaM kinase II, which have been reported previously (Smyth et al., 2002). Regardless of a potential modulatory role for CaM kinase II, these results clearly show that CaM kinase II activation is not required for CaBP1-dependent facilitation of Ca<sub>v</sub>1.2, which may rely instead on direct Ca<sup>2+</sup>-dependent interactions of CaBP1 with Ca<sub>v</sub>1.2.



**Figure 6.** CaBP1 blocks Ca<sup>2+</sup>-dependent inactivation and causes facilitation during repetitive stimuli. *A*, Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 during trains of depolarizations.  $I_{Ca}$  and  $I_{Ba}$  were evoked by 5 msec test pulses from -80 to +10 mV for  $I_{Ca}$  or 0 mV for  $I_{Ba}$  as shown in voltage protocol above. Shown are representative traces of  $I_{Ca}$  during the first eight pulses. Dotted line indicates initial current amplitude. Fractional current, plotted below, represents test current amplitude normalized to that for the first pulse in the train and plotted against time for  $I_{Ca}$  (black circles;  $n = 11$ ) and  $I_{Ba}$  (gray circles;  $n = 5$ ). Points represent the mean  $\pm$  SEM, and every other point is plotted. *B*, Loss of Ca<sup>2+</sup>-dependent inactivation but gain of  $I_{Ca}$  facilitation with CaBP1. Current traces (top) and fractional current (bottom) were obtained as in *A*, except that recordings were from cells cotransfected with CaBP1 ( $n = 5$  for  $I_{Ca}$ ;  $n = 4$  for  $I_{Ba}$ ). *C*, Ca<sup>2+</sup>-dependent facilitation in cells cotransfected with CaBP1 is not prevented by KN-93. Facilitation of  $I_{Ca}$  (filled symbols) but not  $I_{Ba}$  (open symbols) is shown for cells cotransfected with Ca<sub>v</sub>1.2 plus CaBP1 recorded with control intracellular solution (circles) or that containing the CaM kinase II inhibitor KN-93 (2  $\mu$ M; triangles). Results were obtained as in *A*; and data from single representative cells were plotted for the first 100 msec of the train. *D*, Effects of CaBP1 on Ca<sup>2+</sup>-dependent modulation of Ca<sub>v</sub>1.2.  $[I_{Ca} - I_{Ba}]$  represents the difference between the average fractional current for  $I_{Ca}$  and  $I_{Ba}$  for the first 10 (0–0.1 sec) or last 10 (0.9–1 sec) pulses. Shown are results obtained from cells transfected with Ca<sub>v</sub>1.2 alone ( $n = 11$ ), Ca<sub>v</sub>1.2 plus CaBP1 ( $n = 5$ ), and cells cotransfected with Ca<sub>v</sub>1.2 plus CaBP1 that were treated with KN-93 ( $n = 5$ ) ( $*p < 0.05$  compared with Ca<sub>v</sub>1.2 alone;  $**p < 0.01$  compared with Ca<sub>v</sub>1.2 plus CaBP1).

#### The IQ mediates facilitation of Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents by CaBP1

CaM binding to the IQ is critical for Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 (Qin et al., 1999; Romanin et al., 2000; Zühlke et al., 2000; Erickson et al., 2003). To determine whether facilitation of



**Figure 7.** Essential role for the IQ in CaBP1 binding and modulation of Ca<sub>v</sub>1.2 during repetitive stimuli. *A*, Effect of alanine substitutions in the IQ on binding of CaBP1 and modulation of Ca<sub>v</sub>1.2. Top panels show CaBP1 binding to GST-tagged fragments containing the IQ with or without IQ-AA substitutions (CT6, CT6<sub>IQ-AA</sub>). Binding was done with 2 mM Ca<sup>2+</sup> as described in Figure 2B. Bottom panel shows pattern of *I*<sub>Ca</sub> evoked by repetitive stimuli in cells transfected with Ca<sub>v</sub>1.2<sub>IQ-AA</sub> alone (open circles) or cotransfected with CaBP1 (filled circles). Data were obtained with voltage protocols and plotted as in Figure 6. Points represent mean ± SEM (*n* = 4–6), with every second point plotted. *B*, Fractional current obtained in *A* was averaged for the first 10 (0–0.1 sec) and last 10 (0.9–1 sec) pulses in the train and shown for Ca<sub>v</sub>1.2<sub>IQ-AA</sub> transfected alone (open bars) or cotransfected with CaBP1 (filled bars) (\**p* < 0.005 compared with Ca<sub>v</sub>1.2<sub>IQ-AA</sub>). *C*, IQ to EE substitutions prevent binding and inhibit functional effects of CaBP1. Top panel shows loss of CaBP1 binding to GST-tagged CT6 with IQ-EE substitutions. Bottom panel shows *I*<sub>Ca</sub>, obtained as in *A*, in cells transfected with Ca<sub>v</sub>1.2 channels containing the IQ-EE mutation alone (*n* = 5; open circles) or cotransfected with CaBP1 (*n* = 6; filled circles). *D*, Fractional current for data plotted in *C* was averaged as in *B* for Ca<sub>v</sub>1.2<sub>IQ-EE</sub> alone (open bars) or Ca<sub>v</sub>1.2<sub>IQ-EE</sub> plus CaBP1 (filled bars) (\**p* < 0.05 compared with Ca<sub>v</sub>1.2<sub>IQ-EE</sub>).

*I*<sub>Ca</sub> in cells cotransfected with CaBP1 similarly depended on the IQ, we analyzed channels in which the first two residues of this site (I1624 and Q1625) were replaced with either alanine (Ca<sub>v</sub>1.2<sub>IQ-AA</sub>) or glutamate (Ca<sub>v</sub>1.2<sub>IQ-EE</sub>). Previous studies showed that the IQ-AA mutation significantly impairs Ca<sup>2+</sup>-dependent inactivation and, interestingly, unmasks a second effect of Ca<sup>2+</sup>/CaM in causing facilitation in trains of depolarizations given at significantly lower frequencies (3.3 Hz) than that used in Figure 6 (100 Hz) (Zühlke et al., 2000). This Ca<sup>2+</sup>/CaM-dependent facilitation would complicate analyses of the effect of the IQ-AA mutation on CaBP1-dependent facilitation but was absent in recordings with the 100 Hz stimulus protocol, which favored inactivation of *I*<sub>Ca</sub> rather than facilitation (Fig. 7*A*). Unlike its impact on Ca<sup>2+</sup>/CaM-dependent modulation of Ca<sub>v</sub>1.2, the IQ-AA mutation was only partially effective in preventing the functional effects of CaBP1. During the first 100 msec of the train, overt facilitation of *I*<sub>Ca</sub> was not observed in cells cotransfected with Ca<sub>v</sub>1.2<sub>IQ-AA</sub> and CaBP1, in that the amplitude of *I*<sub>Ca</sub> did not increase above initial levels (compare Figs. 6*B*, 7*A*, first five pulses). However, CaBP1 still caused a significant increase in *I*<sub>Ca</sub> during both the first 10 pulses [0.99 ± 0.01 (*n* = 6) for Ca<sub>v</sub>1.2<sub>IQ-AA</sub> plus CaBP1 vs 0.90 ± 0.01 (*n* = 5) for Ca<sub>v</sub>1.2<sub>IQ-AA</sub> alone; *p* < 0.05] and last 10 pulses of the stimulus train (1.05 ± 0.004 for Ca<sub>v</sub>1.2<sub>IQ-AA</sub> plus CaBP1 vs 0.68 ± 0.003 for Ca<sub>v</sub>1.2<sub>IQ-AA</sub> alone; *p* < 0.001) (Fig. 7*A*, *B*). Binding analyses showed that the IQ-AA mutation weakened, but did not abolish, CaBP1 binding to the IQ (Fig. 7*A*), which may explain the residual modulation of Ca<sub>v</sub>1.2<sub>IQ-AA</sub> by CaBP1.

In contrast to these results, the IQ-EE mutation completely prevented binding of CaBP1 to the IQ (Fig. 7*C*). Moreover, this mutation significantly suppressed the facilitatory actions of CaBP1 on *I*<sub>Ca</sub> evoked in both the initial and later periods of the 100 Hz stimulus train (Fig. 7*C*, *D*). During the first 100 msec, there was no significant difference in the fractional amplitude of *I*<sub>Ca</sub> in cells transfected with Ca<sub>v</sub>1.2<sub>IQ-EE</sub> alone (0.93 ± 0.01; *n* = 5) and in cells cotransfected with CaBP1 (0.95 ± 0.01; *n* = 6; *p* = 0.22). The IQ-EE mutation nearly abolished the effect of CaBP1 on prolonging *I*<sub>Ca</sub> during the train, although there was still a small but statistically significant difference between the average fractional current during the last 100 msec in cells transfected with Ca<sub>v</sub>1.2<sub>IQ-EE</sub> alone (0.77 ± 0.01) and in cells cotransfected with CaBP1 (0.85 ± 0.002; *p* < 0.01). These findings are consis-



tent with a model in which low-affinity interactions of CaBP1 with the IQ, which are lost in Ca<sub>v</sub>1.2<sub>IQ-AA</sub> (Fig. 7A), trigger the initial phase of facilitation, whereas high-affinity binding of CaBP1, which is spared by the IQ-AA mutation (Fig. 7A) but eliminated by IQ-EE (Fig. 7C), could mediate the effects of CaBP1 on prolonging Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents during a train of depolarizations. Thus, a complex mechanism accounts for how CaBP1 stabilizes the open state of Ca<sub>v</sub>1.2 channels, involving dynamic interactions of CaBP1 with multiple determinants in the C-terminal domain of α<sub>1</sub>1.2.

## Discussion

Our results provide new insights into the role of CaBP1 in the regulation of neuronal Ca<sup>2+</sup> signaling. CaBP1 binds to the same sites as CaM in the C-terminal domain of the α<sub>1</sub> subunit of Ca<sub>v</sub>1.2 but has opposite effects on channel function. Whereas CaM enhances inactivation of Ca<sub>v</sub>1.2 in a Ca<sup>2+</sup>-dependent manner, CaBP1 causes overt Ca<sup>2+</sup>-dependent facilitation and prolongs Ca<sup>2+</sup> currents by inhibiting inactivation. The association and colocalization of CaBP1 and Ca<sub>v</sub>1.2 postsynaptically in neurons throughout the brain implicate Ca<sup>2+</sup> channel regulation by CaBP1 as an important determinant of voltage-gated Ca<sup>2+</sup> influx in neurons.

### Molecular basis for opposing regulation of Ca<sub>v</sub>1.2 by CaBP1 and CaM

Several lines of evidence imply that CaBP1 is constitutively associated with α<sub>1</sub>1.2 via the C site at resting Ca<sup>2+</sup> levels, with Ca<sup>2+</sup> influx through the channel promoting a Ca<sup>2+</sup>-dependent shift of CaBP1 to the IQ and subsequent facilitation of Ca<sub>v</sub>1.2. First, CaBP1 coimmunoprecipitates with the intact channel under Ca<sup>2+</sup>-free conditions, and CaBP1 binding only to the C site is Ca<sup>2+</sup> independent (Figs. 2, 3). Second, Ca<sup>2+</sup>-dependent facilitation caused by CaBP1 occurs within the first 10–100 msec in repetitive pulse protocols and is prevented by mutations in the IQ that also prevent binding of CaBP1 (Figs. 6B, 7C,D). These results implicate the IQ as an important effector site mediating CaBP1-dependent facilitation, the rapid onset of which is consistent with a secondary interaction of preassociated CaBP1 with the IQ. It is important to note that CaM is endogenously expressed in our transfected cells, which raises the additional possibility that CaM and CaBP1 binding to separate sites simultaneously in α<sub>1</sub>1.2 could jointly modulate Ca<sub>v</sub>1.2. This seems unlikely given that CaBP1 displaces CaM binding to the IQ (Fig. 2D), and preassociated CaBP1 rather than free CaM would presumably have the advantage in modulating the channel via interactions with the IQ. Although additional experiments will be required to fully exclude the involvement of CaM, our current findings support a model in which CaBP1 enhances Ca<sub>v</sub>1.2 currents through sequential interactions at the tethering region (C site) and IQ.

Because CaBP1 does bind to the same sites in α<sub>1</sub>1.2 as CaM, a major question is how CaBP1 causes such distinct regulation of Ca<sub>v</sub>1.2. One possibility is that CaBP1 simply acts as a dominant negative in blocking the effects of endogenous CaM. Although dominant-negative CaM mutants prevent Ca<sup>2+</sup>-dependent inactivation of Ca<sub>v</sub>1.2 (Peterson et al., 1999; Zühlke et al., 1999), they do not cause overt facilitation of Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents like that in cells transfected with CaBP1 (Fig. 6B,C). Therefore, we propose that CaBP1 acts not simply by displacing endogenous CaM but rather induces different conformational changes during binding α<sub>1</sub>1.2 compared with CaM. Although CaBP1 and CaM are ~50% similar at the amino acid level, CaBP1 differs in that it is N-terminally myristoylated, a modification that mediates its as-

sociation with the plasma membrane (Haynes et al., 2004). It is possible, then, that myristoylated CaBP1 may facilitate interactions of the α<sub>1</sub>1.2 C-terminal domain with the plasma membrane that suppress inactivation. CaBP1 also has mutations in EF-hand 2 that prevent Ca<sup>2+</sup> binding and an extra α-helical turn in the linker between N- and C-terminal lobes (Haeseleer et al., 2000). These differences collectively could permit interactions of CaBP1 with molecular contacts within the C-terminal domain of α<sub>1</sub>1.2 that directly stabilize channel opening rather than inactivation.

### Neuronal Ca<sup>2+</sup> binding proteins as functional subunits of voltage-gated Ca<sup>2+</sup> channels

Increasing evidence supports a role for NCBPs, such as CaBP1, in diversifying the properties of voltage-gated Ca<sup>2+</sup> channels in neurons (Wang et al., 2001; Weiss and Burgoyne, 2001; Lee et al., 2002; Tsujimoto et al., 2002; Weiss and Burgoyne, 2002). Given that CaM binding and regulation appears to be a common mechanism among different Ca<sup>2+</sup> channel classes (Liang et al., 2003), NCBPs, like CaBP1, may contribute to the heterogeneous properties of these channels in neurons through direct interactions with the α<sub>1</sub> subunit. In some cases, the modulatory effect of an NCBP may be similar to that of CaM (Tsujimoto et al., 2002). In others, differences in how the NCBP interacts with the CaM-binding site may lead to alternate forms of channel regulation. For example, we showed previously that CaBP1 also interacts with the CaM-binding domain of Ca<sub>v</sub>2.1 (P/Q-type) channels, but unlike CaM, has strong inhibitory effects on Ca<sub>v</sub>2.1 currents (Lee et al., 2002). This provides an interesting contrast to our present results showing a facilitatory effect of CaBP1 on Ca<sub>v</sub>1.2 channels, suggesting fundamental differences in the structure and function of CaM/CaBP1-binding sites in Ca<sub>v</sub>1.2 compared with Ca<sub>v</sub>2.1 channels.

### Physiological significance of CaBP1 in regulating the L-type conductance in neurons

Ca<sub>v</sub>1.2 channels in many neurons show little inactivation during sustained depolarizations, leading to their original classification as L-type for their “long-lasting” conductance (Nowycky et al., 1985). Because most characterizations of these channels used Ba<sup>2+</sup> as a charge carrier, it is not clear to what extent they undergo rapid Ca<sup>2+</sup>-dependent inactivation similar to Ca<sub>v</sub>1.2 channels in the heart (Yue et al., 1990; Neely et al., 1994). Although prominent Ca<sup>2+</sup>-dependent inactivation typifies L-type channels in thalamocortical neurons (Meuth et al., 2002), Ca<sub>v</sub>1.2 currents carried by Ca<sup>2+</sup> ions in a variety of neuronal cell types are prolonged and slowly inactivating (Fisher and Bourque, 1995; Avery and Johnston, 1996; von Gersdorff and Matthews, 1996; Beck et al., 1997). Furthermore, L-type channels with properties similar to those we observed in cells cotransfected with Ca<sub>v</sub>1.2 and CaBP1 have been characterized in neurons in which we found CaBP1 and Ca<sub>v</sub>1.2 to be strongly colocalized. Slowly inactivating L-type Ca<sup>2+</sup> currents have been described in neocortical pyramidal neurons (Brown et al., 1993; Sayer et al., 1993), and L-type channels in some hippocampal neurons exhibit sustained activation after tetanic stimulation (Schjött and Plummer, 2000). In contrast, pyramidal cells specifically from the CA1 region of the hippocampus, in which CaBP1 is not expressed (Laube et al., 2002), possess L-type Ca<sup>2+</sup> channels with strong Ca<sup>2+</sup>-dependent inactivation (Kay, 1991). Although facilitation of neuronal L-type channels by cAMP- and voltage-dependent mechanisms is well documented (Parri and Lansman, 1996; Song and Surmeier, 1996; Davare et al., 2001; Meuth et al., 2002), our findings are the first to show that Ca<sub>v</sub>1.2 channels undergo Ca<sup>2+</sup>-dependent facilitation mediated by CaBP1, which may contrib-

ute to the observed phenotypic diversity of L-type channels in neurons (Forti and Pietrobon, 1993; Kavalali and Plummer, 1994).

L-Type Ca<sup>2+</sup> channels have long been implicated in mechanisms of activity-dependent synaptic plasticity. Ca<sup>2+</sup> influx through postsynaptic L-type channels can promote long-term potentiation (LTP) or long-term depression (LTD) at various synapses (Grover and Teyler, 1990; Huang and Malenka, 1993; Christie et al., 1996). How Ca<sup>2+</sup> influx through L-type channels specifically activates pathways leading to LTP or LTD is not clear but may depend on the nature of the Ca<sup>2+</sup> signal itself. For example, LTD at mossy fiber–CA3 pyramidal cell synapses requires significant increases in postsynaptic Ca<sup>2+</sup> mediated by L-type channels localized to the proximal dendrites of CA3 neurons (Lei et al., 2003), in which we found Ca<sub>v</sub>1.2 channels to strongly colocalize with CaBP1 (Fig. 4A–C). At these and other synapses, CaBP1 interactions with Ca<sub>v</sub>1.2 may play an important role in amplifying postsynaptic Ca<sup>2+</sup> signals, thus regulating the strength and direction of activity-dependent synaptic change.

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