

Erbin Enhances Voltage-Dependent Facilitation of Ca_v1.3 Ca²⁺ Channels through Relief of an Autoinhibitory Domain in the Ca_v1.3 α_1 Subunit

Irina Calin-Jageman,^{1,2} Kuai Yu,^{1,2} Randy A. Hall,¹ Lin Mei,³ and Amy Lee^{1,2}

¹Department of Pharmacology and ²Center for Neurodegenerative Disease, Emory University, Atlanta, Georgia 30322, and ³Program of Developmental Neurobiology, Institute of Molecular Medicine and Genetics and Department of Neurology, Medical College of Georgia, Augusta, Georgia 30912

Ca_v1.3 (L-type) voltage-gated Ca²⁺ channels have emerged as key players controlling Ca²⁺ signals at excitatory synapses. Compared with the more widely expressed Ca_v1.2 L-type channel, relatively little is known about the mechanisms that regulate Ca_v1.3 channels. Here, we describe a new role for the PSD-95 (postsynaptic density-95)/Discs large/ZO-1 (zona occludens-1) (PDZ) domain-containing protein, erbin, in directly potentiating Ca_v1.3. Erbin specifically forms a complex with Ca_v1.3, but not Ca_v1.2, in transfected cells. The significance of erbin/Ca_v1.3 interactions is supported by colocalization in somatodendritic domains of cortical neurons in culture and coimmunoprecipitation from rat brain lysates. In electrophysiological recordings, erbin augments facilitation of Ca_v1.3 currents by a conditioning prepulse, a process known as voltage-dependent facilitation (VDF). This effect requires a direct interaction of the erbin PDZ domain with a PDZ recognition site in the C-terminal domain (CT) of the long variant of the Ca_v1.3 α_1 subunit (α_1 1.3). Compared with Ca_v1.3, the Ca_v1.3b splice variant, which lacks a large fraction of the α_1 1.3 CT, shows robust VDF that is not further affected by erbin. When coexpressed as an independent entity with Ca_v1.3b or Ca_v1.3 plus erbin, the α_1 1.3 CT strongly suppresses VDF, signifying an autoinhibitory function of this part of the channel. These modulatory effects of erbin, but not α_1 1.3 CT, depend on the identity of the auxiliary Ca²⁺ channel β subunit. Our findings reveal a novel mechanism by which PDZ interactions and alternative splicing of α_1 1.3 may influence activity-dependent regulation of Ca_v1.3 channels at the synapse.

Key words: L-type; voltage-gated Ca²⁺ channel; PDZ domain; facilitation; plasticity; excitability

Introduction

In the nervous system, L-type voltage-gated Ca²⁺ channels play essential roles in controlling membrane excitability (Marrión and Tavalin, 1998), gene expression (Bading et al., 1993; Weick et al., 2003), and synaptic plasticity (Johnston et al., 1992). Of multiple L-type Ca²⁺ channels that have been identified (Ca_v1.1–Ca_v1.4), Ca_v1.2 and Ca_v1.3 are widely expressed in the brain and in many of the same neuronal cell groups (Hell et al., 1993b; Ludwig et al., 1997). However, a number of experimental mouse models indicate neural functions for Ca_v1.3 that differ from those of Ca_v1.2. Ca_v1.2 is important for processes underlying spatial memory (Moosmang et al., 2005), whereas Ca_v1.3 activation selectively causes depression-like behavior (Sinnegger-Brauns et al., 2004), promotes synaptic changes associated with Parkinson's disease (Day et al., 2006), and is essential for auditory transduction

(Platzer et al., 2000; Dou et al., 2004). In addition, Ca²⁺ influx through Ca_v1.2 and Ca_v1.3 causes transcriptional activation in different brain regions (Hetzenauer et al., 2006). These findings suggest that Ca_v1.3 participates in distinct cellular and subcellular tasks compared with Ca_v1.2 and therefore may be subject to unique modes of regulation.

In support of this hypothesis, Ca_v1.3 but not Ca_v1.2 channels interact with Shank, a postsynaptic protein associated with excitatory synapses (Zhang et al., 2005). This interaction targets Ca_v1.3 to postsynaptic sites in neurons (Zhang et al., 2005, 2006) and permits Ca_v1.3 inhibition by G-protein-coupled receptors (Olson et al., 2005). Shank contains a single PSD-95 (postsynaptic density-95)/Discs large/ZO-1 (zona occludens-1) (PDZ) domain that binds to a C-terminal sequence in the α_1 subunit of Ca_v1.3 (α_1 1.3) (Zhang et al., 2005). Although PDZ domains are generally known to localize and scaffold membrane proteins with intracellular signaling partners, some PDZ interactions directly regulate the gating properties of ion channels (Ou et al., 2003). Given the wealth and diversity of PDZ proteins in the brain (Kim and Sheng, 2004), PDZ partners other than Shank could differentially modulate Ca_v1.3 and the role of these channels in various neurobiological contexts.

Here, we identify a novel regulatory mechanism for Ca_v1.3 channels involving erbin, a multifunctional PDZ protein concentrated at the neuromuscular junction and at excitatory synapses

Received June 23, 2006; revised Dec. 27, 2006; accepted Dec. 29, 2006.

This work was supported by National Institutes of Health Grants R01 NS044922 (A.L.), T32 DA015040 (I.C.-J.), and R01 NS044521 (L.M.), the W. M. Keck Foundation (R.A.H.), and the Whitehall Foundation (A.L.). We thank Drs. D. Lipscombe and E. Perez-Reyes for Ca_v1.3 and β_4 cDNAs, respectively; Dr. Jörg Striessnig for permission to use Ca_v1.3 ^{-/-} mice; Drs. Geoff Murphy and Anjali Rajadhyaksha for providing Ca_v1.3 ^{-/-} mouse brains; Amanda Castleberry for preparing the PDZ domain proteomic array used in the initial screen; and Dr. Frank Gordon for advice on statistical analyses.

Correspondence should be addressed to Amy Lee, Department of Pharmacology, Emory University School of Medicine, 5123 Rollins Research Building, 1510 Clifton Road, Atlanta, GA 30322. E-mail: alee@pharm.emory.edu.

DOI:10.1523/JNEUROSCI.5191-06.2007

Copyright © 2007 Society for Neuroscience 0270-6474/07/271374-12\$15.00/0

in the CNS (Huang et al., 2001; Kolch, 2003). Erbin binding to the C-terminal domain (CT) of $\alpha_1.3$ augments voltage-dependent facilitation (VDF) of Ca_v1.3 channels containing the auxiliary β_{1b} but not the β_4 subunit by relieving an inhibitory influence of the CT on this process. Ca_v1.3b channels in which the CT is removed by alternative splicing (Safa et al., 2001; Xu and Lipscombe, 2001) show robust VDF that is not further influenced by erbin. Erbin coimmunoprecipitates with and colocalizes with Ca_v1.3 in neurons, in which it may regulate postsynaptic Ca_v1.3 signaling. Our results define a new role for PDZ proteins in directly modulating Ca_v1.3 channels through intermolecular suppression of an auto-inhibitory domain in the $\alpha_1.3$ CT.

Materials and Methods

Constructs and molecular biology. The $\alpha_1.3b$ and $\alpha_1.3$ variants of the rat brain Ca_v1.3 α_1 subunit (GenBank accession numbers AF370009 and AF370010; provided by Dr. D. Lipscombe, Brown University, Providence, RI) and auxiliary channel subunits β_{1b} (GenBank accession number NM017346) or β_4 (GenBank accession number L02315; provided by Dr. E. Perez-Reyes, University of Virginia, Charlottesville, VA) and $\alpha_2\delta$ (GenBank accession number M21948) were used in this study. FLAG- $\alpha_1.3$ was generated by PCR amplification of a FLAG-tagged fragment (nucleotides 1–660 of $\alpha_1.3$) and cloned into *NheI* and *AleI* sites of rat $\alpha_1.3$ -pcDNA6/V5-His (Xu and Lipscombe, 2001). FLAG- $\alpha_1.2$ was described previously (Zhou et al., 2004). For pull-down assays, glutathione S-transferase (GST)-tagged constructs containing the cytoplasmic C-terminal domain of rat $\alpha_1.3$ [GST- $\alpha_1.3$ CT, nucleotides 5886–6465 (see Fig. 1) and 6360–6465 (see Fig. 8B)] or rat $\alpha_1.3b$ [GST- $\alpha_1.3b$ CT, nucleotides 4822–4930 (see Fig. 8B)] were subcloned into *BamHI/NotI* sites of pGEX4T.1 (GE Healthcare, Piscataway, NJ). Green fluorescent protein-tagged protein containing the C-terminal 500 aa of $\alpha_1.3$ not present in $\alpha_1.3b$ (GFP-CT₅₀₀) corresponds to nucleotides 4903–6465 of rat $\alpha_1.3$ subcloned into *HindIII/SacII* of pEGFPN1 (Clontech, Mountain View, CA). Hexahistidine- and S-tagged constructs (his-S-erbinPDZ, amino acids 1275–1371; his-S-MAG1PDZ1, amino acids 316–456) were described previously (Fam et al., 2005). Myc-erbin, myc-erbin Δ PDZ, GFP-erbin₉₆₅, and GFP-erbin₉₆₅ Δ PDZ were also described previously (Huang et al., 2001).

Antibodies. For the generation of rabbit polyclonal $\alpha_1.3$ antibodies, a GST fusion protein containing amino acids 1–41 (MQHQRQQQED-HANEANYARGLRPLISGEGPSTQPNSSKQTV) of rat $\alpha_1.3$ (GenBank accession number AF370009), GST- $\alpha_1.3$ NT_{1–41}, was used as an immunogen, and the resulting antiserum was generated by a commercial source (Covance, Denver, PA). For goat polyclonal $\alpha_1.3$ antibodies, two peptides corresponding to an N-terminal sequence (amino acids 24–37, PISGEGPSTQPNSS) and a sequence in the cytoplasmic loop linking domains II and III (amino acids 810–827, DNKVTIDYQEEAEDKD) were used as dual immunogens for antisera generated by a commercial source (ProSci, Poway, CA). The N-terminal sequence was chosen because it shows relatively low sequence homology with the corresponding regions of other L-type channel α_1 subunits (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). The loop II–III sequence was selected because it has been used previously to generate $\alpha_1.3$ -selective antibodies (Hell et al., 1993b). $\alpha_1.3$ antibodies were purified on affinity columns (AminoLink Coupling Gel; Pierce Biotechnology, Rockford, IL) coupled with the immunogens according to manufacturer's protocol. Rabbit $\alpha_1.3$ antibodies were purified first by passing antiserum through the GST column to remove anti-GST antibodies, and the flow-through was loaded onto the GST- $\alpha_1.3$ NT_{1–41} column overnight. Goat $\alpha_1.3$ antibodies were purified by passing antiserum over a column coupled with both immunogenic peptides. Antibodies were eluted with 5 M MgCl₂, dialyzed against PBS, and stored at 4°C in PBS containing 0.02% sodium azide.

The specificity of $\alpha_1.3$ antibodies was characterized by the following criteria. First, the antibodies recognized $\alpha_1.3$, but not other L-type channel α_1 subunits, in transfected cell lysates by immunoprecipitation (see Fig. 2B), Western blot (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material), and immunocytochemistry

(supplemental Fig. 1C,D, available at www.jneurosci.org as supplemental material). Second, these antibodies immunoprecipitated and detected a protein consistent in size with $\alpha_1.3$ in brain lysates of wild-type mice but not mice with targeted disruption of the $\alpha_1.3$ gene (Platzer et al., 2000) (see Fig. 2D). Third, immunofluorescent staining of primary neurons with both rabbit and goat antibodies was abolished by preadsorption of the antibodies with the immunogen (supplemental Fig. 2A,B, available at www.jneurosci.org as supplemental material). Together, these results validated the specificity and sensitivity of the antibodies for detection of transfected and native $\alpha_1.3$ in our experiments.

Rabbit polyclonal $\alpha_1.2$ antibodies were generated against a peptide corresponding to a sequence in rat $\alpha_1.2$ (KYTTKINMDDLQPS-ENEDKS) used previously for the generation of $\alpha_1.2$ -specific antibodies (Hell et al., 1993b). The criteria for determining specificity of these antibodies for $\alpha_1.2$ were similar to those for the $\alpha_1.3$ antibodies and will be described in a later report. Anti-erbin polyclonal antibodies were produced and characterized previously (Huang et al., 2001).

Other antibodies used were mouse monoclonal antibodies against FLAG M2 and myc (Sigma-Aldrich, St. Louis, MO), GFP (Santa Cruz Biotechnology, Santa Cruz, CA), and HRP-conjugated antibodies against S-protein (Novagen, La Jolla, CA).

Cell culture, transfection, and lysate preparation. A human embryonic kidney cell line transformed with SV40 T antigen (HEK293T) was maintained in DMEM with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37°C in a humidified atmosphere under 5% CO₂. Cells were grown to 70–80% confluence and transfected using Gene Porter reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocols. For immunoprecipitation, HEK293T cells were transfected with cDNAs encoding Ca_v1.2 (4 μ g of FLAG- $\alpha_1.2$, 2 μ g of β_{1b} , and 2 μ g of $\alpha_2\delta$) or Ca_v1.3 (6 μ g of FLAG- $\alpha_1.3$, 2 μ g of β_{1b} , and 2 μ g of $\alpha_2\delta$) and myc-erbin (4 μ g). For electrophysiological experiments, HEK293T cells plated on 35 mm dishes were transfected with \sim 5 μ g of total DNA (1.5 μ g of FLAG- $\alpha_1.3$ or FLAG- $\alpha_1.3b$, 0.5 μ g of β_{1b} or β_4 , and 0.5 μ g of $\alpha_2\delta$ with or without 1 μ g of erbin) and GFP expression plasmid (0.01 μ g) for fluorescent detection of transfected cells.

Pull-down binding assays. GST- or his-S-tagged fusion proteins were prepared and purified on glutathione-agarose beads or Ni²⁺-nitrilotriacetic acid-agarose beads, respectively, as described previously (Zhou et al., 2004). GST- $\alpha_1.3$ CT or - $\alpha_1.3b$ CT proteins immobilized on beads were incubated with purified his-S-erbinPDZ or his-S-MAG1PDZ1 proteins or GFP-erbin₉₆₅-transfected cell lysate and brought to a total volume of 400 μ l with binding buffer [50 mM Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl)/0.1% Triton X-100/protease inhibitors (1 μ g/ml each of PMSF, pepstatin, aprotinin, and leupeptin)]. Binding reactions were incubated, rotating at 4°C for 1–2 h. Beads were washed three times with ice-cold binding buffer (1 ml), and bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose. Western blotting was performed with appropriate antibodies followed by HRP-conjugated secondary antibodies and enhanced chemiluminescent detection reagents (GE Healthcare). Ponceau staining was used to verify that equal levels of immobilized GST- $\alpha_1.3$ CT proteins were used in each experimental group. Interpretations of results from pull-down assays were based on at least three independent experiments.

Coimmunoprecipitation assays. Two days after transfection, lysates from transfected HEK293 cells were harvested and solubilized in 1 ml/100 mm cell culture plate of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, and protease inhibitors) at 4°C for 30 min and subjected to centrifugation at 100,000 \times g (30 min) to remove insoluble material. Lysates were incubated with $\alpha_1.3$ or $\alpha_1.2$ antibodies (5 μ g) and 50 μ l of protein A-Sepharose (50% slurry) for 4 h, rotating at 4°C. After three washes with RIPA buffer (1 ml), proteins were eluted with SDS-containing sample buffer and subjected to SDS-PAGE. Coimmunoprecipitated proteins were detected by Western blotting with specific antibodies as described for pull-down assays. Conclusions were drawn from results of three independent experiments.

For coimmunoprecipitation assays from rat brain, crude membrane fractions were generated as follows: three frozen rat brains were homog-

enized in buffer [(in mM) 320 sucrose, 4 HEPES, 5 EDTA, 5 EGTA, pH 7.4 with NaOH, and protease inhibitors]. After homogenization, large debris was removed by spinning samples at 1000 × *g* for 6 min at 4°C. The supernatant was then subjected to ultracentrifugation at 100,000 × *g* (35 min), and the resulting pellet was solubilized for 3 h in 9 ml of buffer containing 1.2% digitonin, 300 mM KCl, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, and protease inhibitors at 4°C. A final centrifugation step (100,000 × *g* for 30 min) was performed to remove insoluble material. The resulting supernatant was precleared for 1 h with protein G agarose (200 μl; 50% slurry) and goat IgG (~100 μg). The samples (~9 ml) were then incubated with goat anti-α_{1.3} antibodies (~120 μg) or an equivalent amount of goat IgG (final antibody concentration, ~13 μg/ml) and protein G agarose (200 μl; 50% slurry) for 4 h, rotating at 4°C. After three washes with 0.1% digitonin/PBS buffer (1 ml), proteins were eluted with SDS-containing sample buffer and subjected to SDS-PAGE. Coimmunoprecipitated proteins were detected by Western blotting with erbin or rabbit α_{1.3}-specific antibodies. For immunoprecipitation experiments from brain lysates of Ca_v1.3^{+/+} or Ca_v1.3^{-/-} mice, an identical protocol was used, except that one-half of a mouse brain was used per immunoprecipitation with 30 μg of antibodies. Coimmunoprecipitation experiments from brain tissue were repeated at least twice with consistent results.

Immunocytochemistry. For double-label immunofluorescence, primary cultures of neurons were prepared as follows. Neocortical tissue was dissected from rat embryos (embryonic day 19). The tissue was then digested with papain for 1 h at 37°C and triturated in inactivation solution [minimum essential medium (MEM; Invitrogen) and 10% FBS]. The resulting cell suspension was plated at a density of ~300,000 neurons per 60 mm plate containing glass coverslips precoated with poly-D-lysine. Neurons were maintained in medium containing MEM, 1 mM pyruvate, 0.6% dextrose, 5% FBS, 1 × B-27 (Invitrogen), 0.5 mM penicillin/streptomycin/glutamine, and 0.001% MITO+ serum extender (BD Biosciences, San Jose, CA). For immunostaining, coverslips (8–14 d in culture) were fixed in 4% paraformaldehyde/4% sucrose/PBS and incubated in blocking buffer [10% normal goat serum (NGS) or 10% donkey serum (DS), 0.1% Triton X-100, in TBS]. All antibodies were diluted in TBS containing 2.5% NGS or DS and 0.1% Triton X-100, and samples were rinsed three times for 5–10 min between each step. After blocking, samples were incubated with rabbit anti-α_{1.3} antibodies (1:50) 4 h to overnight at 4°C and, after rinsing in TBS, with donkey anti-rabbit rhodamine-conjugated Fab fragments (1:300; Jackson ImmunoResearch, West Grove, PA) for 60 min. Sections were rinsed and blocked with goat anti-rabbit Fab fragments (1:50; Jackson ImmunoResearch) for ~2.5 h to block any rabbit IgG sites before the second labeling step. Sections were then incubated overnight at 4°C with anti-erbin antibodies (1:100–1:400), rinsed, and incubated with donkey FITC-anti-rabbit IgG (1:300; Jackson ImmunoResearch). The double-labeled sections were mounted and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) for viewing on a Zeiss (Oberkochen, Germany) LSM510 Meta confocal microscope. Image processing was with Zeiss LSM Image Browser and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Because the double-labeling procedure involved two rabbit antibodies, a number of control experiments were performed to confirm the pattern of erbin and α_{1.3} colocalization. In particular, to ensure that the incubation with goat anti-rabbit Fab fragments was sufficient to prevent recognition of the first primary antibody (α_{1.3}) by the second secondary antibody (FITC-anti-rabbit IgG), anti-erbin antibodies were omitted (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Loss of FITC fluorescence in this control experiment (supplemental Fig. 2D, available at www.jneurosci.org as supplemental material) argued against the possibility that overlap in α_{1.3} and erbin immunofluorescence resulted from insufficient blocking and therefore reactivity of both secondary antibodies with α_{1.3}-labeled sites. Interpretations from immunocytochemical experiments were based on consistent results obtained from more than five independent experiments.

Electrophysiological recordings. At least 48 h 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, 1 MgCl₂, and 10 BaCl₂ or 10 CaCl₂. Intracellular solutions consisted of the following (in mM): 140 *N*-methyl-D-glucamine, 10 HEPES, 2 MgCl₂, 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 MΩ in the bath solution, and series resistance was ~2–4 MΩ, compensated up to 80%. Normalized tail current–voltage and prepulse voltage-dependence data were fit with a single Boltzmann function: $A/[1 + \exp[(V - V_{1/2})/k] + b]$, where *V* is the test or prepulse voltage, *V*_{1/2} is the half-maximal voltage, *k* is a slope factor, *A* is the amplitude, and *b* is the baseline. Curve fits and data analyses were done with Igor Pro software (Wavemetrics, Lake Oswego, OR). All averaged data are presented as the mean ± SEM. Statistical significance of differences between two groups was determined by Student's *t* test as indicated (SigmaPlot; Systat Software, San Jose, CA). For statistical analyses of prepulse voltage and *I*–*V* curves, data were compared by two-way repeated-measures ANOVA (SigmaStat; Systat Software).

Results

Erbin binds to the α_{1.3} C-terminal domain

The extreme C-terminal residues of α_{1.3} (ITTL) (Fig. 1A) are consistent with a signature sequence favored by class I PDZ domains, (S/T)-X-ϕ-COOH, where ϕ is a hydrophobic amino acid, and X is any amino acid (Songyang et al., 1997). To identify PDZ proteins interacting with this sequence in α_{1.3}, we used a GST fusion protein containing the last 200 aa of α_{1.3} CT (Fig. 1A) to screen a proteomic array of 96 putative class I PDZ domains, as described previously (Fam et al., 2005). Several PDZ domains on the array interacted with α_{1.3} (data not shown), including one belonging to erbin, a member of the LAP (leucine-rich region and PDZ) family (Borg et al., 2000; Huang et al., 2001) (Fig. 1B). The interaction between erbin and α_{1.3} was confirmed in a pull-down assay with immobilized GST-α_{1.3} CT and a truncated version of erbin containing the PDZ domain (GFP-erbin₉₆₅) (Fig. 1C). GST-α_{1.3} CT, but not the GST control, precipitated GFP-erbin₉₆₅ from transfected cell lysates (Fig. 1C). To discount the possibility that GFP-erbin₉₆₅ could have associated indirectly with α_{1.3} CT via a second protein in the transfected cell lysate, we performed pull-down assays with purified protein corresponding to the PDZ domain of erbin (erbinPDZ) (Fig. 1D). Although GST-α_{1.3} CT showed dose-dependent binding to erbinPDZ, the class I PDZ of a different protein [membrane-associated guanylate kinase inverted 1 (MAGI1)] did not bind to GST-α_{1.3} CT (Fig. 1D). These results confirm that α_{1.3} CT interacts directly and specifically with the PDZ domain of erbin.

Although most PDZ domains interact with a canonical sequence at the extreme CT of their ligands, some also recognize internal motifs not at the CT (Penkert et al., 2004). Because the GST-α_{1.3} CT fusion protein encompassed a relatively large region (~200 aa) (Fig. 1A), binding to erbin could have been mediated by residues other than the C-terminal PDZ recognition site. To confirm the importance of the PDZ signature sequence (ITTL) in α_{1.3} CT for binding to erbin, we tested how substitution of alanine for the final leucine in this sequence affected the interaction (α_{1.3} CT_{L-A}) (Fig. 1E). This substitution eliminates the hydrophobic nature of the final residue, which is critical for maintaining PDZ–ligand interactions (Songyang et al., 1997). If erbin binds to α_{1.3} CT via a conventional class I PDZ interaction, the L–A substitution should significantly weaken the interaction with erbin. Consistent with this prediction, binding of erbinPDZ to GST-α_{1.3} CT_{L-A} was greatly reduced relative to GST-α_{1.3} in pull-down experiments (Fig. 1E). Therefore, erbin binding to α_{1.3} CT depends on the C-terminal PDZ recognition site.

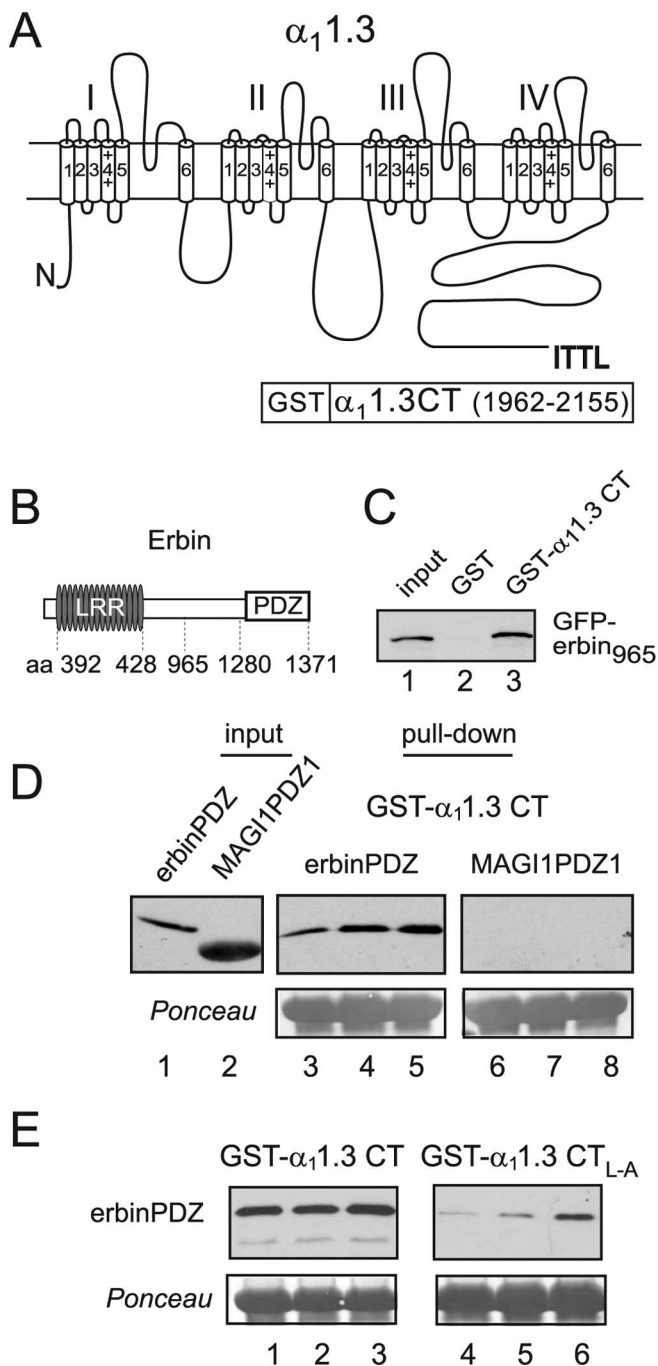


Figure 1. The PDZ domain of erbin binds to the C-terminal domain of $\alpha_1.3$. **A**, Schematic of rat $\alpha_1.3$ showing class I PDZ-binding consensus sequence (ITTL) in the cytoplasmic C-terminal domain and the region used for GST- $\alpha_1.3$ CT in pull-down assays (amino acids 1962–2155). **B**, Schematic of mouse erbin indicating position of LRR (amino acids 392–428) and the PDZ domain (amino acids 1280–1371). **C**, Binding of erbin to $\alpha_1.3$ CT. GST- $\alpha_1.3$ CT (lane 3) or GST alone (lane 2) was immobilized on glutathione-agarose beads and incubated with lysates from cells transfected with GFP-erbin₉₆₅ (amino acids 965–1371). Bound GFP-erbin₉₆₅ was detected by Western blot with anti-GFP antibodies. Lane 1 shows ~15% of GFP-erbin₉₆₅ input used in the pull-down assay. **D**, Binding of erbinPDZ but not MAGI1PDZ1 to $\alpha_1.3$ CT. GST- $\alpha_1.3$ CT was incubated with purified his-S-erbinPDZ (lanes 3–5) or his-S-MAGI1PDZ1 (lanes 6–8). Input his-S-tagged protein was the following (in μ g): 1 (lanes 3, 6), 2 (lanes 4, 7), and 4 (lanes 5, 8). Input lanes 1 and 2 show his-S-tagged proteins used in the assay (1 μ g). **E**, Impaired binding of erbinPDZ to $\alpha_1.3$ CT_{L-A}. His-S-erbinPDZ protein (lanes 1, 4, 1.5 μ g; lanes 2, 5, 3 μ g; lanes 3, 6, 6 μ g) was incubated with GST- $\alpha_1.3$ CT (lanes 1–3) or GST- $\alpha_1.3$ CT_{L-A} (lanes 4–6). In **D** and **E**, bound proteins were detected by Western blot (top) with anti-S-protein antibodies, and Ponceau staining (bottom) shows equal levels of GST- $\alpha_1.3$ CT or GST- $\alpha_1.3$ CT_{L-A} in each group.

To confirm that erbin interacts not only with the isolated CT fragment of $\alpha_1.3$ but also with the intact channel in mammalian cells, coimmunoprecipitation experiments were performed with lysates of HEK293T cells cotransfected with $Ca_v1.3$ subunits (FLAG- $\alpha_1.3$, β_{1b} , and $\alpha_2\delta$) and myc-tagged erbin. Because the α_1 subunit of $Ca_v1.2$ ($\alpha_1.2$) also contains a class I PDZ-binding element (VSNL) (Fig. 2A), we determined whether myc-erbin might selectively interact with either channel. Using $\alpha_1.3$ antibodies, we found that myc-erbin coimmunoprecipitated with $\alpha_1.3$ (Fig. 2B, lane 1). Myc-erbin was not detected when $\alpha_1.3$ antibodies were applied to lysates from cells cotransfected with $\alpha_1.2$ and erbin, arguing against nonspecific immunoprecipitation of erbin by $\alpha_1.3$ antibodies (Fig. 2B, lane 2). This result also confirmed that the antibodies specifically recognized $\alpha_1.3$ and not $\alpha_1.2$. However, the amount of myc-erbin that coimmunoprecipitated with $Ca_v1.3$ was relatively small (lane 1) compared with the total amount of myc-erbin that was detected in the cell lysates (lane 6). This could be explained by the presence of erbin endogenously expressed in HEK293 cells (Huang et al., 2001), which might have competed with transfected myc-erbin for interacting with $Ca_v1.3$. In addition, previous analyses indicate that the erbin PDZ domain has a strong preference for ligands with the CT sequence (E/D)-(S/T/V)-X-V and binds relatively weakly to sequences more similar to the ITTL $\alpha_1.3$ motif (Jaulin-Bastard et al., 2001). Such low-affinity interactions might be difficult to maintain during immunoprecipitation procedures that required strong detergent solubilization conditions.

With $\alpha_1.2$ antibodies, myc-erbin could not be coimmunoprecipitated with $\alpha_1.2$, despite the fact that $\alpha_1.2$ expression and immunoprecipitation was consistently greater than that observed for $\alpha_1.3$ (Fig. 2B, lanes 1, 3, 5, 6). The $\alpha_1.2$ antibodies did not immunoprecipitate $\alpha_1.3$, confirming the specificity of these antibodies for $\alpha_1.2$ (Fig. 2B, lane 2). Inability of myc-erbin to coimmunoprecipitate with $\alpha_1.2$ was not related to insufficient levels of myc-erbin, because Western blots of the transfected cell lysates indicated that equal levels of myc-erbin were coexpressed with $Ca_v1.3$ and $Ca_v1.2$ (Fig. 2B, lanes 5, 6). These results confirm that erbin selectively forms a complex with $Ca_v1.3$ channels and support previous observations that $Ca_v1.2$ and $Ca_v1.3$ are distinguished by the types of PDZ proteins with which they interact (Weick et al., 2003; Zhang et al., 2005).

$Ca_v1.3$ and erbin coimmunoprecipitate and colocalize in neurons

To address the neurobiological significance of erbin/ $Ca_v1.3$ interactions, we determined whether erbin and $Ca_v1.3$ formed a complex in rat brain in coimmunoprecipitation experiments. $Ca_v1.3$ channels were immunoprecipitated from solubilized rat brain membrane fractions with goat polyclonal $\alpha_1.3$ antibodies. $Ca_v1.3$ and associated erbin were detected by Western blot with rabbit polyclonal $\alpha_1.3$ and erbin antibodies, respectively. In these experiments, erbin was coimmunoprecipitated with $\alpha_1.3$ using $\alpha_1.3$ antibodies (Fig. 2C). The coimmunoprecipitation was specific in that a similar result was not obtained with an equivalent concentration of control IgG (Fig. 2C). The ~200 kDa protein immunoprecipitated and immunoblotted with our $\alpha_1.3$ antibodies corresponded to $\alpha_1.3$, because it was observed in brain lysates from wild-type mice ($Ca_v1.3^{+/+}$) but not mice lacking $\alpha_1.3$ ($Ca_v1.3^{-/-}$) (Fig. 2D), which were characterized previously (Platzer et al., 2000). These results confirm the existence of erbin and $Ca_v1.3$ interactions in the brain.

If erbin is a significant partner of neuronal $Ca_v1.3$ channels, these proteins should show similar cellular and subcellular local-

ization in the brain. To test this, we used confocal microscopy and double-label immunofluorescence of cortical neurons in culture. We observed erbin and Ca_v1.3 immunofluorescence in neuronal cell bodies (Fig. 3*A–C,G–I*). In addition, punctate erbin and Ca_v1.3 immunofluorescence was observed in proximal dendrites (Fig. 3*D–F*). In developing neurons, erbin and Ca_v1.3 immunofluorescence coincided strongly at the distal ends of dendritic processes (Fig. 3*G–I*). Colocalization of erbin and Ca_v1.3 in the soma and proximal dendrites was extensive in virtually all neurons examined (Fig. 3*C,F,I*). However, colocalization of erbin and Ca_v1.3 was not complete, and there were many puncta that showed only Ca_v1.3 or erbin labeling (Fig. 3*D–F*). In addition to control experiments (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), these results discounted the possibility that overlapping fluorescence was artifactual as a result of cross-reactivity of the secondary reagents in the double-labeling protocol. The nucleus of most neurons was labeled with erbin but not Ca_v1.3 antibodies (Fig. 3*A–C,G–I*), which is not unexpected, because erbin is involved in multiple cellular signaling functions (Kolch, 2003). Nevertheless, the strong colocalization of erbin and Ca_v1.3 in neurons and coimmunoprecipitation from brain lysates support the potential for erbin to physiologically interact with neuronal Ca_v1.3 channels.

Erbin augments voltage-dependent facilitation of Ca_v1.3

The cytoplasmic C-terminal domain of the Ca_v1.2 α₁ subunit interacts with a variety of regulatory proteins that directly modulate channel activation or inactivation (Catterall, 2000). To test the hypothesis that erbin binding to α₁1.3 CT might similarly influence the function of Ca_v1.3, we screened for modulatory effects of erbin in electrophysiological recordings of HEK293T cells cotransfected with Ca_v1.3 subunits (α₁1.3, β_{1b}, and α₂δ). This system allows transfection and electrophysiological analysis of recombinant channels in the relative absence of competing influences. Similar experiments in primary neurons are complicated by the existence of multiple classes of voltage-gated Ca²⁺ channels and subsequent difficulty of isolating modulatory effects specific for Ca_v1.3 channels. Despite the low levels of endogenous erbin in HEK293 cells (Huang et al., 2001), functional effects of erbin on signaling have been studied in this cell line through transfection of exogenous erbin cDNAs (Dai et al., 2006), which we found to increase erbin expression significantly over endogenous levels (data not shown). Therefore, modulatory effects of erbin could be resolved by comparing Ca_v1.3 properties in cells cotransfected with Ca_v1.3 and erbin and in cells transfected with Ca_v1.3 alone.

Consistent with previous reports (Koschak et al., 2001; Safa et al., 2001; Scholze et al., 2001; Xu and Lipscombe, 2001), Ca_v1.3 showed a relatively hyperpolarized range of activation (Fig. 4).

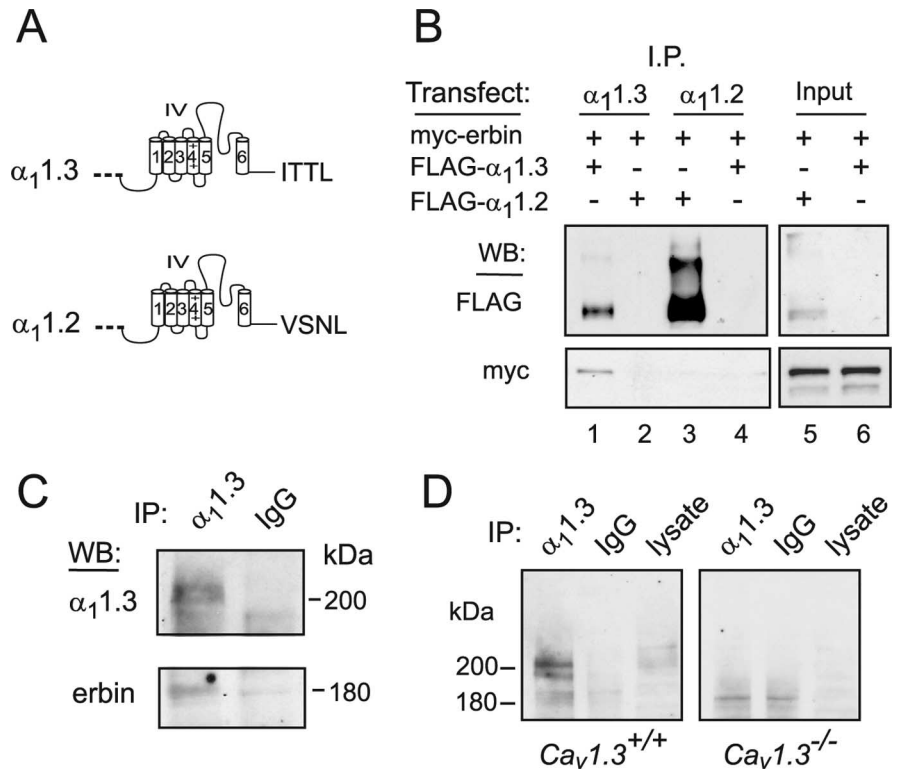


Figure 2. Coimmunoprecipitation of erbin with Ca_v1.3 from transfected cells and brain. **A**, Schematic of α₁1.3 and α₁1.2 showing C-terminal PDZ-binding motifs. **B**, Coimmunoprecipitation of erbin and Ca_v1.3 from transfected cells. HEK293T cells were cotransfected with myc-erbin and Ca_v1.3 (FLAG-α₁1.3, β_{1b}, and α₂δ; lanes 1, 4, 6) or Ca_v1.2 (FLAG-α₁1.2, β_{1b}, and α₂δ; lanes 2, 3, 5) and subjected to lysis and immunoprecipitation using rabbit antibodies against α₁1.3 (lanes 1, 2) or α₁1.2 (lanes 3, 4). Immunoprecipitated proteins (I.P.; lanes 1–4) were detected by Western blotting (WB) with antibodies recognizing FLAG (top) or myc (bottom) epitopes. Lanes 5 and 6 represent 5% of the cell lysate input used for coimmunoprecipitation. **C**, Coimmunoprecipitation of erbin and Ca_v1.3 from the brain. Rat brain lysates were incubated with goat α₁1.3 antibodies or control goat IgG for immunoprecipitation, and α₁1.3 and erbin were detected by Western blotting with rabbit α₁1.3 and erbin antibodies, respectively. **D**, Lack of α₁1.3 immunoprecipitation in Ca_v1.3^{-/-} mouse brain. α₁1.3 immunoprecipitation protocol used in **C** was applied to brain lysates from Ca_v1.3^{+/+} or Ca_v1.3^{-/-} mice. An ~200 kDa protein corresponding to α₁1.3 was detected in the brain lysate, and samples were immunoprecipitated with α₁1.3 antibodies from Ca_v1.3^{+/+} but not Ca_v1.3^{-/-} mice. Lysate lanes represent 3% of the input used for coimmunoprecipitation.

However, parameters for tail current activation curves were not significantly different in cells transfected with Ca_v1.3 alone ($V_{1/2} = -21.7 \pm 1.6$ mV; $k = -5.8 \pm 0.5$; $n = 9$) and those cotransfected with erbin ($V_{1/2} = -22.7 \pm 1.9$ mV; $k = -6.8 \pm 0.7$; $n = 6$; $p = 0.70$ for $V_{1/2}$; $p = 0.25$ for k) (Fig. 4*A,B*). In addition, erbin did not influence the average amplitude of the Ca_v1.3 peak current evoked by various test voltages (Fig. 4*C,D*). There was no significant difference in the peak current amplitudes in current–voltage (I – V) relationships in cells transfected with Ca_v1.3 alone and those cotransfected with erbin ($F_{(1,12)} = 0.0005$; $p = 0.983$) (Fig. 4*C,D*). These results show that erbin does not regulate the expression level or activation properties of Ca_v1.3 channels in mammalian cells.

Ca_v1.3 channels undergo prominent VDF that, in recombinant systems, is independent of heterotrimeric G-proteins or phosphorylation (Safa et al., 2001). To determine whether erbin influenced this aspect of Ca_v1.3 function, we used a paired-pulse protocol in which the amplitude of two test currents (P1 and P2) evoked by the same voltage step was compared before and after a conditioning (facilitating) prepulse (Fig. 5*A*). Consistent with previous studies (Safa et al., 2001), a short (20 ms) conditioning prepulse from -90 to $+60$ mV caused a significant increase ($30.3 \pm 4.1\%$; $n = 12$; $p < 0.01$) in Ca_v1.3 currents evoked by a test pulse from -90 to -20 mV (Fig. 5*A*). With the same voltage

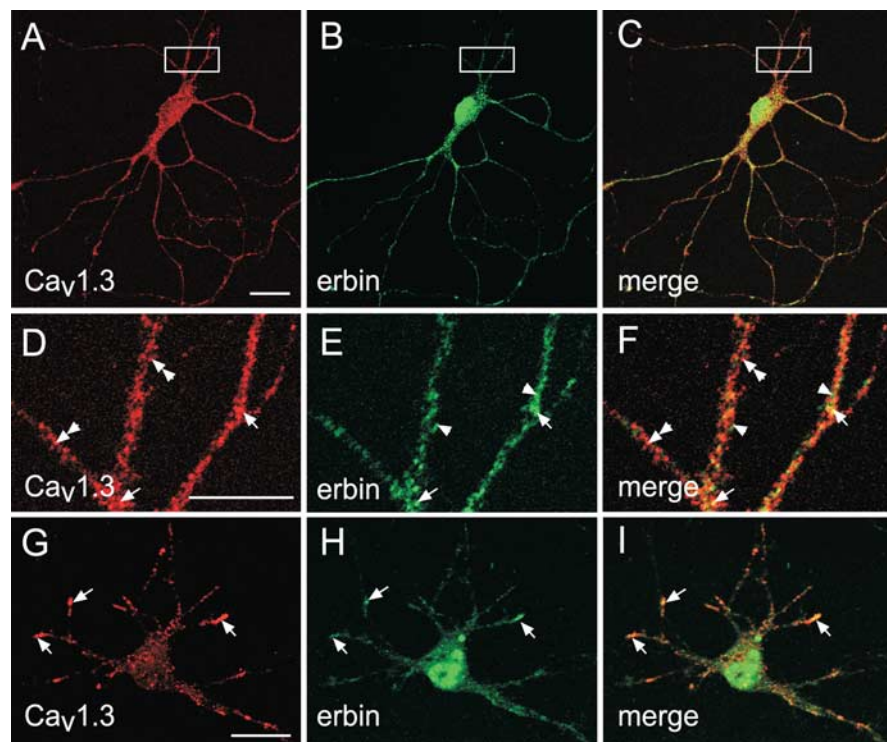


Figure 3. Ca_v1.3 and erbin colocalize in somatodendritic domains of cortical neurons in culture. **A–I**, Confocal images of primary cortical neuron cultures (**A–F**, 14 d in culture; **G–I**, 8 d in culture) sequentially double labeled with antibodies against $\alpha_1.3$ (to detect Ca_v1.3) and erbin are shown. Immunofluorescence was viewed under optics for rhodamine for Ca_v1.3 (**A, D, G**) or fluorescein for erbin (**B, E, H**). Regions of colocalization appear yellow in the merged images (**C, F, I**). Extensive colocalization of erbin and Ca_v1.3 was detected in cell bodies (**A–C, G–I**) and dendrites (**D–F**; higher-magnification view of boxed region in panels **A–C**). In **D–I**, elements immunoreactive for Ca_v1.3 alone (double arrowheads), erbin alone (arrowheads), or Ca_v1.3 plus erbin (arrows) are indicated. Scale bars: **A** (for **A–C**), **G** (for **G–I**), 20 μ m; (in **D**) **D–F**, 10 μ m.

protocol, the amount of facilitation in cells cotransfected with erbin was dramatically increased ($47.8 \pm 6.6\%$; $n = 10$) and was significantly greater than in cells transfected with Ca_v1.3 alone ($p < 0.01$) (Fig. 5A). The enhanced facilitation attributable to erbin could have resulted from a negative shift or steeper dependence of facilitation on prepulse voltage. To test this, we compared the relationship between prepulse voltage and percentage of maximal facilitation in cells transfected with Ca_v1.3 alone or those cotransfected with erbin (Fig. 5B). However, Boltzmann fits of prepulse voltage curves revealed no significant effect of erbin on either $V_{1/2}$ (-12.2 ± 3.4 for Ca_v1.3 alone vs -8.3 ± 3.4 for Ca_v1.3 plus erbin; $p = 0.62$) or slope (-19.0 ± 2.7 for Ca_v1.3 alone vs -17.6 ± 1.7 for Ca_v1.3 plus erbin; $p = 0.71$). A second mechanism by which erbin could augment VDF is by increasing the number of channels undergoing facilitation as a result of a given prepulse. This was tested by plotting net facilitation, which was the amplitude of the P2 relative to the P1 current [F_{ratio} (P2/P1)] (Fig. 5C) against prepulse voltage. With prepulse voltages ranging from -20 to $+60$ mV, net facilitation was significantly greater ($F_{(1,20)} = 5.25$; $p < 0.05$) in cells cotransfected with erbin than in cells with Ca_v1.3 alone (Fig. 5C). These results confirm that erbin promotes VDF by increasing the pool of channels undergoing prepulse-induced facilitation, rather than enhancing voltage dependence of entry into the facilitated state.

In these experiments, Ba²⁺ rather than Ca²⁺ was used as the charge carrier to resolve VDF without the competing effects of Ca²⁺-dependent inactivation, which would significantly diminish the amplitude of the P2 test current in double-pulse protocols. However, because Ca²⁺ is the physiologically relevant charge

carrier, we also tested whether erbin influenced facilitation of Ca_v1.3 Ca²⁺ currents (Fig. 6). With a modified double-pulse protocol, Ca²⁺-dependent inactivation of Ca_v1.3 currents was evident as a decrease in the P2 relative to the P1 current ($F_{\text{ratio}} < 1$) with prepulse voltages ranging from -20 to $+30$ mV (Fig. 6B). As would be expected of a Ca²⁺-dependent process, inactivation paralleled the amount of Ca²⁺ influx during the prepulse such that F_{ratio} was smallest with a prepulse to 0 mV (Fig. 6B), which evoked the maximal inward Ca²⁺ current (data not shown). Although erbin did not affect the extent to which Ca_v1.3 Ca²⁺ currents inactivated ($p > 0.18$, for prepulses from -40 mV to $+30$ mV), prepulse steps from $+50$ to $+80$ mV caused facilitation ($F_{\text{ratio}} > 1$) of Ca_v1.3 Ca²⁺ currents that was significantly greater ($p < 0.05$) in cells cotransfected with erbin than in cells with Ca_v1.3 alone (Fig. 6A,B). That erbin increased facilitation across this voltage range for Ca_v1.3 when either Ca²⁺ (Fig. 6) or Ba²⁺ (Fig. 5) was used as the permeant ion supports modulation of voltage- rather than Ca²⁺-dependent facilitation. As for Ca_v1.3 Ba²⁺ currents, the effect of erbin on facilitation of Ca²⁺ currents was not accompanied by effects on current amplitude, because I - V relationships for Ca²⁺ currents in cells with Ca_v1.3 alone and Ca_v1.3 plus erbin were not significantly different ($p = 0.16$; data not shown). Because the effects of erbin on facilitation of Ca_v1.3 Ca²⁺ currents were smaller and occurred across a narrower set of prepulse voltages than for Ba²⁺ currents, the molecular mechanism underlying erbin-dependent increases in VDF was analyzed further with Ba²⁺ as the charge carrier.

Erbin binding to $\alpha_1.3$ supports VDF of Ca_v1.3

Based on our biochemical evidence supporting a direct interaction between erbin and $\alpha_1.3$ (Figs. 1, 2), we hypothesized that this interaction is required for the modulation of Ca_v1.3 VDF by erbin. If so, then preventing erbin from binding to $\alpha_1.3$ should abolish the increased VDF in cells with Ca_v1.3 plus erbin. This prediction was first tested with truncated erbin constructs that either lacked or were generally restricted to the PDZ domain (erbin $_{\Delta\text{PDZ}}$ and erbin $_{965}$, respectively) (Fig. 7). Compared with cells cotransfected with full-length erbin, VDF was significantly reduced when Ca_v1.3 was cotransfected with erbin $_{\Delta\text{PDZ}}$ ($F_{(1,16)} = 6.46$; $p < 0.05$) (Fig. 7A,B). This result confirms the importance of the erbin PDZ domain for VDF. However, erbin contains other functional domains, including a leucine-rich repeat region (LRR) (Fig. 1B) implicated in subcellular trafficking of membrane proteins (Legouis et al., 2003). Thus, it was possible that the PDZ domain acted in concert with the LRR of erbin to promote VDF of Ca_v1.3 through a mechanism independent of binding to $\alpha_1.3$. This was not the case, because erbin $_{965}$, which did not contain the LRR, caused increased VDF compared with cells with Ca_v1.3 alone ($F_{(1,15)} = 5.44$; $p < 0.05$) (Fig. 7A,C). Moreover, the effect of erbin $_{965}$ on VDF was not significantly different from full-length erbin ($F_{(1,13)} = 0.05$; $p = 0.83$) (Fig. 7C). These results

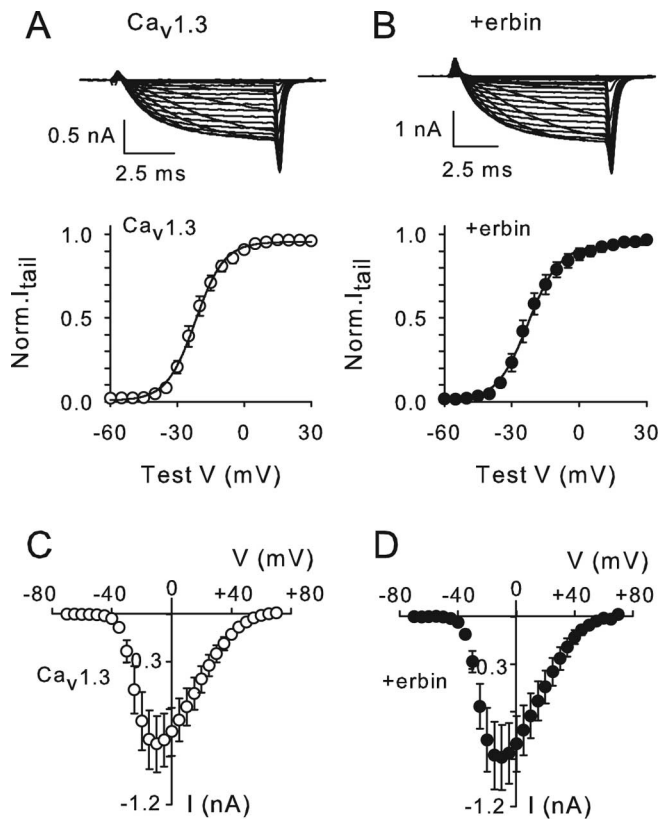


Figure 4. Erbin does not affect voltage-dependent activation or mean amplitude of $Ca_v1.3$ currents. **A, B**, Normalized tail current (Norm. I_{tail}) activation curves for HEK293T cells transfected with $Ca_v1.3$ alone ($n = 9$) (**A**) or cotransfected with $Ca_v1.3$ plus erbin ($n = 6$) (**B**). Test pulses (10 ms) were applied from a holding voltage of -90 mV to various voltages, and peak tail currents were measured after repolarization to -70 mV for 2 ms. Tail currents were normalized to the largest in the series and plotted against test voltage. Representative current traces are shown at top. **C, D**, Current–voltage relationships for $Ca_v1.3$ alone (**C**) and $Ca_v1.3$ plus erbin (**D**). Data were from same voltage protocol as in **A** and **B**, except that peak current amplitudes during the test pulse were plotted against test voltage. Error bars represent SEM.

show that the PDZ domain of erbin is necessary and sufficient for modulation of $Ca_v1.3$ VDF.

To characterize the importance of the PDZ-binding site in the $\alpha_1.3$ CT for erbin modulation, we took advantage of a naturally occurring $\alpha_1.3$ variant ($\alpha_1.3b$) in which inclusion of an alternatively spliced exon leads to truncation of the C-terminal domain and subsequent elimination of the PDZ-binding sequence (Safa et al., 2001; Xu and Lipscombe, 2001) (Fig. 8A). If erbin binding specifically to $\alpha_1.3$ CT was required for increasing VDF, channels containing $\alpha_1.3b$ ($Ca_v1.3b$) should be insensitive to the effects of cotransfected erbin. The potential of erbin to interact with $\alpha_1.3b$ was first tested in pull-down assays with his-tagged erbinPDZ and GST-tagged $\alpha_1.3$ CT or $\alpha_1.3b$ CT (Fig. 8A). Relatively weak binding of erbinPDZ was observed with $\alpha_1.3b$ CT compared with $\alpha_1.3$ CT (Fig. 8B). Consistent with these results, VDF of $Ca_v1.3b$ channels was not significantly affected by cotransfected erbin ($F_{(1,20)} = 0.06$; $p = 0.81$) (Fig. 8C). This was not attributable to the inability of $Ca_v1.3b$ channels to undergo VDF, because VDF was quite robust in cells transfected with $Ca_v1.3b$ alone ($F_{ratio} = 1.42 \pm 0.09$ with a $+60$ mV prepulse; $n = 14$). Together, our results support a mechanism in which the PDZ domain of erbin binding to the C-terminal domain $\alpha_1.3$ mediates enhanced VDF that is specific for $Ca_v1.3$ L-type channels.

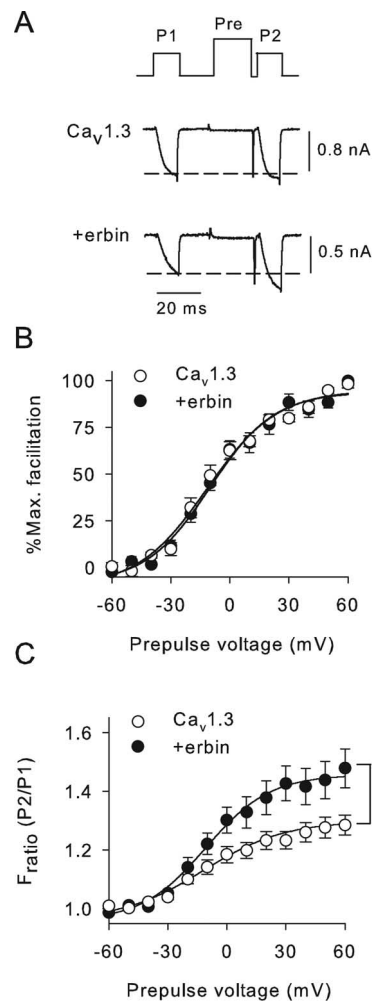


Figure 5. Erbin augments VDF of $Ca_v1.3$ Ba^{2+} currents. **A**, Representative Ba^{2+} current traces and double-pulse voltage protocol for measuring VDF in cells transfected with $Ca_v1.3$ alone or cotransfected with erbin. Currents were evoked by 10 ms test pulses from -90 to -20 mV before (P1) and after (P2) a conditioning 20 ms prepulse (Pre) to $+60$ mV. **B**, No effect of erbin on prepulse voltage dependence of facilitation. Percentage of facilitation was calculated as $[(I_{P2}/I_{P1}) - 1] \times 100$ for cells for different prepulse voltages, normalized to that for a $+60$ mV prepulse, and plotted as percentage of maximum (Max.) facilitation against prepulse voltage for cells transfected with $Ca_v1.3$ alone ($n = 11$) or cotransfected with erbin ($n = 10$). **C**, Increased net facilitation attributable to erbin. F_{ratio} represents the ratio of the P2 and P1 test currents and is plotted against prepulse voltage for $Ca_v1.3$ alone ($n = 12$) or $Ca_v1.3$ plus erbin ($n = 10$). * $p < 0.05$. Error bars represent SEM.

Erbin relieves the inhibitory effect of $\alpha_1.3$ CT on VDF

In recordings of the short $Ca_v1.3b$ variant, we found that VDF was on average greater than for $Ca_v1.3$ and was not different from that caused by erbin in cells cotransfected with the long $Ca_v1.3$ variant ($F_{(1,22)} = 0.04$; $p = 0.85$) (Figs. 5C, 8C). This result suggested an intriguing mechanism in which the $\alpha_1.3$ CT, which is missing in $Ca_v1.3b$, may act as an inhibitory module for VDF. The absence of the inhibitory CT domain in $\alpha_1.3b$ might therefore permit enhanced facilitation of $Ca_v1.3b$, which cannot be influenced further by erbin (Fig. 8C). If so, then $\alpha_1.3$ CT when expressed as a separate entity should suppress VDF of $Ca_v1.3b$. We tested this prediction with GFP-CT₅₀₀ (Fig. 9). Compared with cells with $Ca_v1.3b$ alone, VDF was significantly impaired by cotransfection with CT₅₀₀ ($F_{(1,16)} = 14.32$; $p < 0.01$) (Fig. 9A). In contrast, CT₅₀₀ did not significantly affect VDF for $Ca_v1.3$ ($F_{(1,6)} = 0.49$; $p = 0.51$) (Fig. 9B). These results confirm an inhibitory role for the CT of $\alpha_1.3$ in VDF.

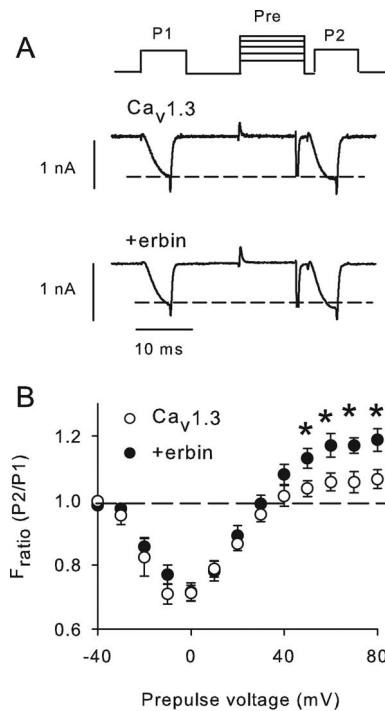


Figure 6. Erbin increases VDF of Ca_v1.3 Ca²⁺ currents. **A**, Representative Ca²⁺ current traces and double-pulse voltage protocol for measuring VDF in cells transfected with Ca_v1.3 alone ($n = 6$) or cotransfected with erbin ($n = 7$). Currents were evoked by 5 ms test pulses from -90 to -10 mV before (P1) and after (P2) a conditioning 10 ms prepulse (Pre) to varying voltages. **B**, Increased net facilitation for prepulse voltages greater than $+40$ mV in cells cotransfected with erbin. F_{ratio} was determined and plotted against prepulse voltage as in Figure 5C. The dotted line indicates $F_{\text{ratio}} = 1$. Points falling below the line result from Ca²⁺-dependent inactivation with prepulse voltages evoking significant Ca²⁺ entry. * $p < 0.05$ by two-factor ANOVA and Tukey's post-test. Error bars represent SEM.

Erbin interactions might facilitate voltage-dependent removal of the α_1 1.3 CT, thus disinhibiting VDF as in Ca_v1.3b channels. If so, then CT₅₀₀ should similarly “reinhibit” VDF of Ca_v1.3 channels modulated by erbin. As predicted, coexpression of CT₅₀₀ strongly suppressed the increase in VDF caused by erbin ($F_{(1,19)} = 23.18$; $p < 0.001$) (Fig. 9C). However, in this experiment, VDF inhibition could have resulted simply from CT₅₀₀ competitively binding to erbin and displacing it from the channel. This would then allow the endogenous CT of the channel to inhibit VDF, as in cells transfected with Ca_v1.3 alone. If this were the case, inhibition by CT₅₀₀ in cells cotransfected with Ca_v1.3 plus erbin should be relatively invariant with prepulse voltage and similar to that seen in cells transfected with Ca_v1.3 alone. Alternatively, if CT₅₀₀ inhibition involved interaction with an inhibitory site in Ca_v1.3 that was increasingly accessible in facilitated channels, CT₅₀₀ inhibition in cells cotransfected with Ca_v1.3 plus erbin should increase with prepulse voltage, as in cells transfected with Ca_v1.3b alone. To resolve the underlying mechanism, we compared the prepulse voltage dependence of VDF inhibition by CT₅₀₀ for Ca_v1.3, Ca_v1.3b, and Ca_v1.3 plus erbin (Fig. 9D). In cells transfected with Ca_v1.3 plus erbin, VDF inhibition by CT₅₀₀ increased steeply with prepulse voltage and was not significantly different from that in cells transfected with Ca_v1.3b alone ($F_{(1,15)} = 0.29$; $p = 0.60$) (Fig. 9D). In contrast, percentage of inhibition of VDF by CT₅₀₀ in cells with Ca_v1.3 alone did not vary greatly with prepulse voltage and was significantly different from that in cells with either Ca_v1.3b ($F_{(1,8)} = 31.0$; $p < 0.001$) or with Ca_v1.3 plus erbin ($F_{(1,13)} = 25.0$; $p < 0.001$) (Fig. 9D). These

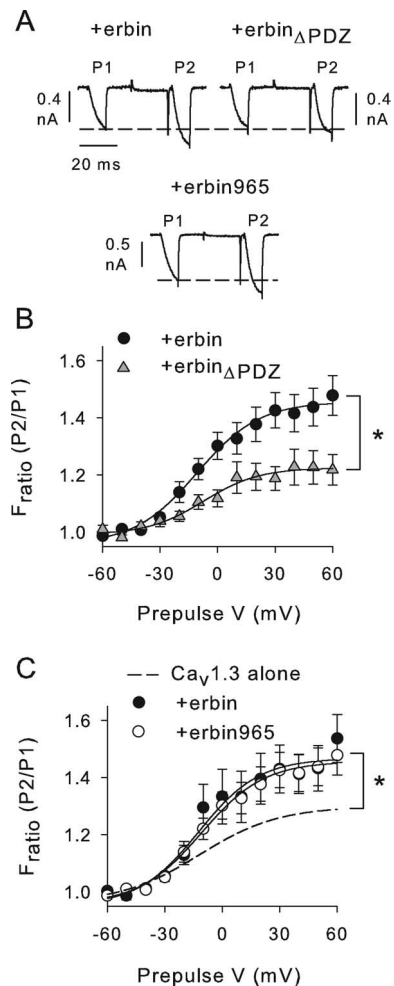


Figure 7. Increased VDF depends on PDZ domain of erbin. **A**, Representative current traces obtained with the same voltage protocol as in Figure 5A for cells cotransfected with Ca_v1.3 plus erbin, erbin Δ PDZ, or erbin965 (amino acids 965–1371). **B**, Deletion of erbinPDZ prevents effects on VDF. F_{ratio} is plotted against prepulse V for cells cotransfected with Ca_v1.3 plus erbin ($n = 10$) or erbin Δ PDZ ($n = 8$). * $p < 0.05$. **C**, Deletion of LRR of erbin does not prevent effects of erbin on VDF. F_{ratio} was plotted against prepulse voltage for cells cotransfected with Ca_v1.3 plus erbin ($n = 10$) or erbin965 ($n = 5$). The dashed line represents data replotted from Figure 5C for cells transfected with Ca_v1.3 alone. * $p < 0.05$ for Ca_v1.3 plus erbin965 compared with Ca_v1.3 alone. Error bars represent SEM.

results show that CT₅₀₀ does not reverse the effects of erbin by displacing it from the channel. Rather, our data suggest that CT₅₀₀ suppresses VDF in cells with Ca_v1.3b or Ca_v1.3 plus erbin through the same voltage-dependent mechanism.

Because VDF of L-type channels can be affected by the auxiliary Ca²⁺ channel β subunit (Cens et al., 1998), we determined whether substituting the β_{1b} subunit used in our experiments with the β_4 subunit influenced modulation of Ca_v1.3 VDF by erbin and α_1 1.3 CT. Like β_{1b} , β_4 is highly expressed in the brain (Castellano et al., 1993; Ludwig et al., 1997; Vendel et al., 2006) and has been shown to associate with a large fraction of brain L-type channels (Pichler et al., 1997), although the identity of the β subunits interacting specifically with Ca_v1.3 in the brain has yet to be characterized. Although Ca_v1.3 channels containing β_4 (Ca_v1.3- β_4) exhibited VDF similar to that with β_{1b} , there was no significant difference in VDF in cells with Ca_v1.3- β_4 alone and those cotransfected with erbin ($F_{(1,13)} = 0.05$; $p = 0.83$) (Fig. 10A). However, VDF of the Ca_v1.3b variant with β_4 (Ca_v1.3b- β_4) was significantly increased compared with Ca_v1.3- β_4 ($F_{(1,11)} =$

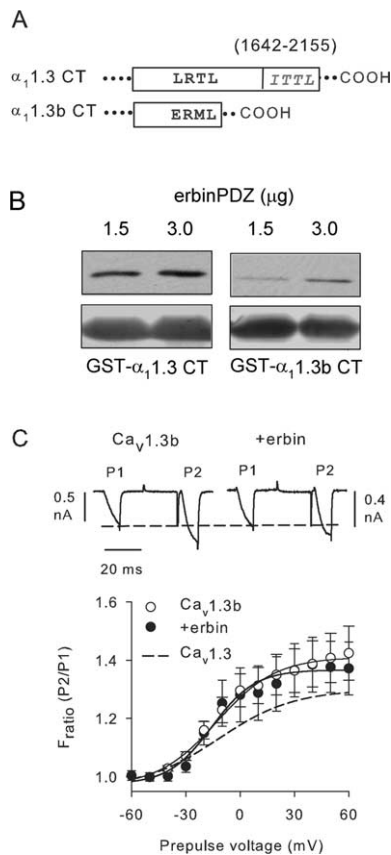


Figure 8. Erbin does not cause increased VDF of Ca_v1.3b channels. **A**, Schematic showing differences in C-terminal domain of $\alpha_1.3$ and $\alpha_1.3b$. Parentheses indicate unique sequence including the PDZ-binding site (ITTL) in $\alpha_1.3$ but not $\alpha_1.3b$. Splicing of this region in $\alpha_1.3b$ results in a truncated C-terminal domain with a distinct C-terminal sequence (ERML). **B**, Erbin-PDZ interacts weakly with $\alpha_1.3b$ CT. GST- $\alpha_1.3$ CT or GST- $\alpha_1.3b$ CT was used in pull-down assay with his-5-erbinPDZ (1.5 or 3.0 μ g). Top, Western blot detection of erbinPDZ. Bottom, Ponceau staining showing equal levels of immobilized GST- $\alpha_1.3$ CT (left) and GST- $\alpha_1.3b$ CT (right) used in assay. **C**, No effect of erbin on VDF of Ca_v1.3b. F_{ratio} was plotted against prepulse voltage for cells transfected with Ca_v1.3b alone ($n = 14$) or cotransfected with erbin ($n = 8$). The dashed line represents data replotted from Figure 5C for cells transfected with Ca_v1.3 alone. Representative current traces obtained with a +60 mV prepulse are shown above. Error bars represent SEM.

13.8; $p < 0.01$) (Fig. 10A,B). Moreover, coexpression of CT₅₀₀ with Ca_v1.3b- β_4 significantly blunted VDF ($F_{(1,11)} = 44.5$; $p < 0.001$) (Fig. 10B), similar to that observed for Ca_v1.3b channels containing β_{1b} (Fig. 9A). We conclude that VDF modulation by erbin depends on which β subunit is associated with Ca_v1.3, and the inhibition of VDF imposed by the $\alpha_1.3$ CT is a fundamental regulatory feature of $\alpha_1.3$, which is removed by alternative splicing of the CT in Ca_v1.3b.

Discussion

Our results reveal a novel mechanism controlling VDF of Ca_v1.3 Ca²⁺ channels. Erbin binding to the CT of the Ca_v1.3 α_1 subunit alleviates an inhibitory effect of the CT, causing increased facilitation of Ca_v1.3 currents after a conditioning prepulse. This effect is seen for Ca_v1.3 channels containing the auxiliary β_{1b} but not β_4 subunit (Figs. 5C, 10A). The Ca_v1.3b variant lacks the PDZ-binding site with which erbin interacts and, more importantly, the autoinhibitory CT domain. As a consequence, Ca_v1.3b channels are “disinhibited,” exhibiting more robust VDF than Ca_v1.3, regardless of the identity of the auxiliary β subunit (Figs. 8–10). Thus, the extent to which neuronal Ca_v1.3 channels undergo

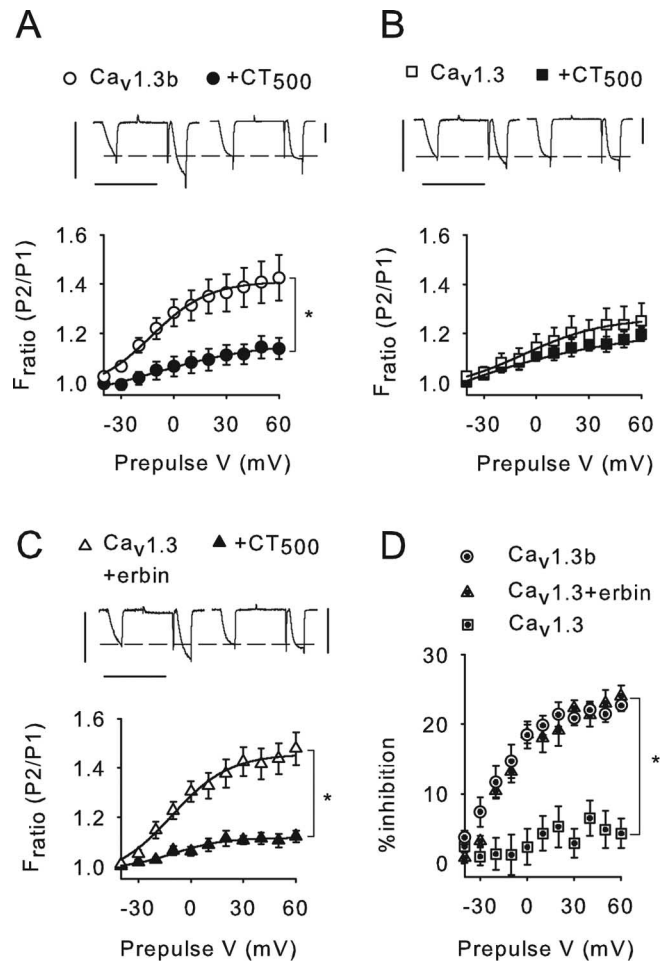


Figure 9. Autoinhibition of VDF by the CT of $\alpha_1.3$. **A–C**, Cotransfection of $\alpha_1.3$ CT fragment (CT₅₀₀; amino acids 1655–2155) suppresses VDF. Effect of CT₅₀₀ on F_{ratio} is plotted against prepulse V in cells with Ca_v1.3b ($n = 6$) (**A**), Ca_v1.3 ($n = 4$) (**B**), or Ca_v1.3 plus erbin ($n = 10$) (**C**). Representative current traces obtained with a +60 mV prepulse are shown above. Vertical scale bars, 0.8 nA; horizontal scale bars, 40 ms. * $p < 0.01$. **D**, Voltage-dependent inhibition of VDF by CT₅₀₀. Percentage inhibition was measured as $[1 - (F_{ratio+CT500}/F_{ratio})] \times 100$, where F_{ratio} is for Ca_v1.3b, Ca_v1.3, or Ca_v1.3 plus erbin, and $F_{ratio+CT500}$ represents the mean F_{ratio} for these groups cotransfected with CT₅₀₀. Stronger prepulse voltage dependence of CT₅₀₀ inhibition is observed for Ca_v1.3b and Ca_v1.3 plus erbin compared with Ca_v1.3. * $p < 0.001$. Error bars represent SEM.

facilitation in response to membrane depolarization may depend on $\alpha_1.3$ splice variation and cell-type specific interactions with different β subunits and PDZ proteins.

Mechanisms of L-type channel facilitation

Facilitation of voltage-gated Ca²⁺ channels in response to a hormonal or depolarizing stimulus is an adaptive mechanism that can boost Ca²⁺ entry in excitable cells (Pietrobon and Hess, 1990; Dolphin, 1996). For Ca_v1.2 L-type channels, various mechanisms for facilitation have been characterized, including those involving phosphorylation of the channel by cAMP-dependent protein kinase (PKA) (Catterall, 2000) and association with calmodulin-dependent protein kinase II (CaMKII) (Dzhura et al., 2000; Hudmon et al., 2005; Grueter et al., 2006; Lee et al., 2006). Although PKA and CaMKII also cause facilitation of Ca_v1.3 channels (Qu et al., 2005; Gao et al., 2006), the enhanced facilitation caused by erbin and splice variation of the $\alpha_1.3$ CT in our study differs from these mechanisms in a number of ways. First, although PKA and CaMKII cause an increase in Ca_v1.3 peak

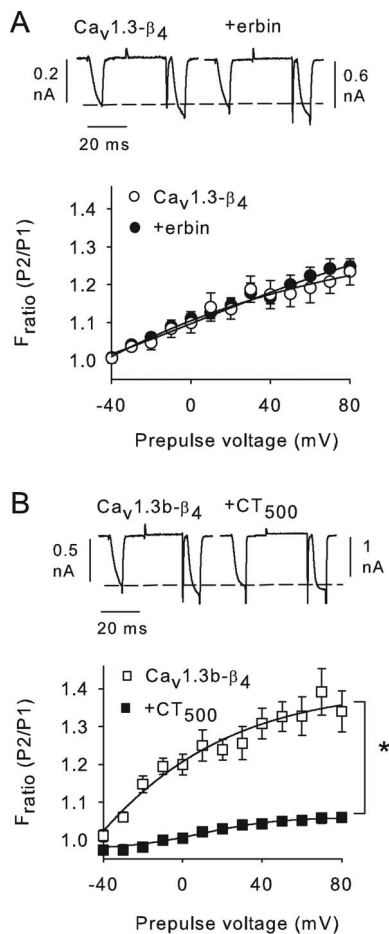


Figure 10. Effect of erbin but not α_1 .1.3 CT depends on auxiliary Ca²⁺ channel β subunit. **A**, Erbin does not increase VDF of Ca_v1.3 channels containing the β_4 subunit (Ca_v1.3- β_4). F_{ratio} was determined from double-pulse protocol as described in Figure 5, except that P1 and P2 currents were evoked by 7 ms test pulses from -90 to -20 mV in cells transfected with Ca_v1.3- β_4 ($n = 7$) or cotransfected with erbin ($n = 8$). **B**, CT₅₀₀ inhibits VDF of β_4 -containing Ca_v1.3b channels (Ca_v1.3b- β_4). F_{ratio} was determined and plotted as in **A** for cells transfected with Ca_v1.3b- β_4 ($n = 6$) or cotransfected with CT₅₀₀ ($n = 7$). * $p < 0.001$. For **A** and **B**, representative current traces obtained with +80 mV prepulse are shown above. Error bars represent SEM.

current amplitude (Qu et al., 2005) and negative shift in voltage-dependent activation (Gao et al., 2006), respectively, erbin had neither effect on Ca_v1.3 activation in our study (Fig. 4). Second, CaMKII activation by insulin-like growth factor causes facilitation of both Ca_v1.3 and Ca_v1.3b (Gao et al., 2006), whereas we find that Ca_v1.3 but not Ca_v1.3b channels are subject to erbin-dependent facilitation (Figs. 5, 8). Third, neither PKA nor CaMKII-dependent facilitation of Ca_v1.3 requires conditioning depolarization (Qu et al., 2005; Gao et al., 2006), unlike the clearly voltage-dependent effects of erbin on Ca_v1.3 facilitation (Fig. 5). Therefore, we conclude that erbin promotes facilitation of Ca_v1.3 channels via a different molecular pathway than that described previously for these channels by PKA or CaMKII. Although we cannot rule out the possibility that other second messengers are involved, our data implicate the α_1 .1.3 CT as a key regulatory element in this process.

Autoinhibitory function of the α_1 .1.3 CT and modulation by PDZ proteins

An autoinhibitory role for the CT of the Ca_v1.1 and Ca_v1.2 α_1 subunits is well established (Wei et al., 1994; Morrill and Cannon, 2000; Gao et al., 2001). Deletion of the distal C-terminal domain

of the α_1 subunit enhances activation of Ca_v1.1 (Morrill and Cannon, 2000) and Ca_v1.2 (Wei et al., 1994) when these channels are expressed in recombinant systems. This upregulation of channel activity is reversed by reintroduction of the distal CT fragment as a separate entity (Gao et al., 2001; Hulme et al., 2005, 2006), much like the inhibitory effects on VDF that we observed when the α_1 .1.3 CT (CT₅₀₀) was coexpressed with Ca_v1.3b (Figs. 9A,D, 10B) or Ca_v1.3 plus erbin (Fig. 9C,D). How the α_1 .1.3 CT inhibits VDF is not clear but may involve intramolecular interactions with other domains in α_1 .1.3. Previous studies have shown that the CT of α_1 .1.2 and α_1 .1.1 is subject to proteolytic processing *in vivo* (De Jongh et al., 1991; Hell et al., 1993a) and that the cleaved distal CT product remains associated with the α_1 subunit (Gerhardstein et al., 2000; Hulme et al., 2005, 2006). For Ca_v1.1 and Ca_v1.2, the distal CT interacts directly with the proximal CT of the α_1 subunit at sites that are conserved in the corresponding regions of α_1 .1.3 (Hulme et al., 2005, 2006). The putative proximal CT interaction site is included in the CT₅₀₀ fragment that inhibited VDF in our study (Figs. 9, 10B), whereas the distal CT interaction site is retained in the truncated CT of the α_1 .1.3b (Fig. 8A). Thus, it is possible that for Ca_v1.3, the interaction of distal and proximal CT sites forms an autoinhibitory module for VDF, which is disrupted when erbin binds to the distal CT of β_{1b} -containing channels or when the distal CT is removed by alternative splicing in Ca_v1.3b. Coexpression of the CT₅₀₀ fragment would inhibit VDF of Ca_v1.3b (Figs. 9A,D, 10B) or Ca_v1.3 plus erbin (Fig. 9C,D) through reassociation with the proximal CT, therefore restoring the autoinhibition. Although additional experiments are required to confirm this mechanism, our results are consistent with previous observations that interdomain interactions involving the α_1 .1.2 CT regulate Ca_v1.2 activation and inactivation (Ivanina et al., 2000; Kobrinisky et al., 2005), which might parallel the role of the CT in modulating Ca_v1.3 VDF.

Our findings that the Ca_v1.3 channels containing the β_4 subunit were insensitive to modulation by erbin further underscore the importance of auxiliary β subunits in differentially regulating not only gating but also modulation of voltage-gated Ca²⁺ channels (Richards et al., 2004). Although additional investigation is necessary, β_4 may impair physical association of erbin with Ca_v1.3 or channel transitions to facilitated states that are favored by erbin. Alternatively, given that β_4 variants interact with cellular signaling molecules other than voltage-gated Ca²⁺ channels (Hibino et al., 2003; Vendel et al., 2006), β_4 could recruit other modulators to the Ca_v1.3 channel complex that oppose the effects of erbin. Because both β_{1b} and β_4 are highly expressed in the brain (Ludwig et al., 1997; Pichler et al., 1997), whether neuronal Ca_v1.3 channels are modulated by erbin could vary according to which β subunit is associated with the channel complex.

PDZ proteins as modulators of voltage-gated Ca²⁺ channels

PDZ domain-containing proteins have emerged as multifaceted regulators of voltage-gated Ca²⁺ channel targeting and localization (Maximov et al., 1999; Zhang et al., 2005, 2006), modulation by G-protein-coupled receptors (Olson et al., 2005), and signal transduction (Weick et al., 2003). By selectively targeting and assembling Ca²⁺ channels with various signaling molecules, PDZ proteins can confer specific forms of Ca²⁺ channel regulation in different subcellular contexts. For example, the PDZ protein Shank clusters Ca_v1.3 but not Ca_v1.2 at postsynaptic sites with D₁ dopamine receptors, enabling dopaminergic suppression of Ca_v1.3 L-type currents (Olson et al., 2005; Zhang et al., 2005). Our study is the first to show a more direct role for a PDZ protein in regulating Ca²⁺ channel gating. We have not tested whether

related PDZ proteins, such as Shank, that interact with the α_1 1.3 CT have an effect similar to erbin in increasing VDF of Ca_v1.3- β_{1b} . Our findings that a truncated version of erbin containing primarily the PDZ domain (erbin₉₆₅) (Fig. 7A, C) was sufficient for increasing VDF suggest that a PDZ/ α_1 1.3 CT interaction is the essential determinant for the modulation. However, PDZ proteins, including erbin and Shank, differ significantly in their overall structure and can vary in terms of the number of PDZ domains and the existence of other protein interaction motifs (Kim and Sheng, 2004). Such structural variations may alter the modulatory potential of PDZ proteins, perhaps by allowing them to scaffold multiple distinct signaling molecules. This raises the possibility that a wide range of PDZ interactions may influence other aspects of Ca_v1.3 function, thus enhancing the signaling capabilities of these channels in neurons. In this context, it is important to note that peptide mimetics of the class I PDZ-binding site in α_1 1.3 (Fig. 1A) may competitively disrupt interactions with multiple PDZ proteins and thus may not reflect the functional consequences of disturbing a single PDZ interaction.

Physiological significance of Ca_v1.3 channel facilitation and modulation by erbin/ α_1 1.3 splicing

Compared with previous descriptions of native L-type channel VDF (Pietrobon and Hess, 1990; Forti and Pietrobon, 1993; Kavalali and Plummer, 1996), VDF of Ca_v1.3 channels in our experiments occurred with relatively moderate depolarizing pre-pulses (−20 to +60 mV) (Figs. 5–10). This feature of VDF is consistent with previous reports of Ca_v1.3 splice variants cloned from a pituitary cell line (Safa et al., 2001) and L-type channels in neostriatal medium spiny neurons (Song and Surmeier, 1996) and hippocampal pyramidal neurons (Kavalali and Plummer, 1996). In cortical neurons, L-type channels influence neuronal activity via Ca²⁺-dependent plateau potentials (Nasif et al., 2005) and regulation of interspike intervals during repetitive firing (Pineda et al., 1998). Increased VDF of Ca_v1.3- β_{1b} caused by erbin or expression of the Ca_v1.3b variant could amplify such activity-dependent Ca²⁺ signals, which normally control cortical excitability. Given that Ca_v1.3- β_{1b} but not Ca_v1.3- β_4 channels are subject to modulation by erbin (Figs. 5, 10), it is interesting to consider the lethargic mutation in the Ca²⁺ channel β_4 subunit, which causes seizures and ataxia (Burgess et al., 1997). This mutation prevents interactions of β_4 with the Ca²⁺ channel α_1 subunit and causes compensatory increases in the association of α_1 with other β subunits, including β_{1b} (Burgess et al., 1999). Our results suggest a mechanism in which such pathological increases in Ca_v1.3- β_{1b} channels might cause neurological defects through aberrant upregulation of Ca²⁺ signaling and neuronal excitability.

References

- Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* 260:181–186.
- Borg JP, Marchetto S, Le Bivic A, Ollendorff V, Jaulin-Bastard F, Saito H, Fournier E, Adelaide J, Margolis B, Birnbaum D (2000) ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nat Cell Biol* 2:407–414.
- Burgess DL, Jones JM, Meisler MH, Noebels JL (1997) Mutation of the Ca²⁺ channel β subunit gene *Cchb4* is associated with ataxia and seizures in the lethargic (*lh*) mouse. *Cell* 88:385–392.
- Burgess DL, Biddlecome GH, McDonough SI, Diaz ME, Zilinski CA, Bean BP, Campbell KP, Noebels JL (1999) β subunit reshuffling modifies N- and P/Q-type Ca²⁺ channel subunit compositions in lethargic mouse brain. *Mol Cell Neurosci* 13:293–311.
- Castellano A, Wei X, Birnbaumer L, Perez-Reyes E (1993) Cloning and expression of a neuronal calcium channel β subunit. *J Biol Chem* 268:12359–12366.
- Catterall WA (2000) Structure and regulation of voltage-gated Ca²⁺ channels. *Annu Rev Cell Dev Biol* 16:521–555.
- Cens T, Restituito S, Vallentin A, Charnet P (1998) Promotion and inhibition of L-type Ca²⁺ channel facilitation by distinct domains of the β subunit. *J Biol Chem* 273:18308–18315.
- Dai P, Xiong WC, Mei L (2006) Erbin inhibits RAF activation by disrupting the sur-8-Ras-Raf complex. *J Biol Chem* 281:927–933.
- Day M, Wang Z, Ding J, An X, Ingham CA, Shering AF, Wokosin D, Ilijic E, Sun Z, Sampson AR, Mugnaini E, Deutch AY, Sesack SR, Arbuthnott GW, Surmeier DJ (2006) Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat Neurosci* 9:251–259.
- De Jongh KS, Warner C, Colvin AA, Catterall WA (1991) Characterization of the two size forms of the α_1 subunit of skeletal muscle L-type calcium channels. *Proc Natl Acad Sci USA* 88:10778–10782.
- Dolphin AC (1996) Facilitation of Ca²⁺ current in excitable cells. *Trends Neurosci* 19:35–43.
- Dou H, Vazquez AE, Namkung Y, Chu H, Cardell EL, Nie L, Parson S, Shin HS, Yamoah EN (2004) Null mutation of α_1D Ca²⁺ channel gene results in deafness but no vestibular defect in mice. *J Assoc Res Otolaryngol* 5:215–226.
- Dzhura I, Wu Y, Colbran RJ, Balsler JR, Anderson ME (2000) Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. *Nat Cell Biol* 2:173–177.
- Fam SR, Paquet M, Castleberry AM, Oller H, Lee CJ, Traynelis SF, Smith Y, Yun CC, Hall RA (2005) P2Y1 receptor signaling is controlled by interaction with the PDZ scaffold NHERF-2. *Proc Natl Acad Sci USA* 102:8042–8047.
- Forti L, Pietrobon D (1993) Functional diversity of L-type calcium channels in rat cerebellar neurons. *Neuron* 10:437–450.
- Gao L, Blair LA, Salinas GD, Needleman LA, Marshall J (2006) Insulin-like growth factor-1 modulation of Ca_v1.3 calcium channels depends on Ca²⁺ release from IP3-sensitive stores and calcium/calmodulin kinase II phosphorylation of the α_1 subunit EF hand. *J Neurosci* 26:6259–6268.
- Gao T, Cuadra AE, Ma H, Bunemann M, Gerhardstein BL, Cheng T, Eick RT, Hosey MM (2001) C-terminal fragments of the α_1C (Ca_v1.2) subunit associate with and regulate L-type calcium channels containing C-terminal-truncated α_1C subunits. *J Biol Chem* 276:21089–21097.
- Gerhardstein BL, Gao T, Bunemann M, Puri TS, Adair A, Ma H, Hosey MM (2000) Proteolytic processing of the C terminus of the α_{1C} subunit of L-type calcium channels and the role of a proline-rich domain in membrane tethering of proteolytic fragments. *J Biol Chem* 275:8556–8563.
- Grueter CE, Abiria SA, Dzhura I, Wu Y, Ham AJ, Mohler PJ, Anderson ME, Colbran RJ (2006) L-type Ca²⁺ channel facilitation mediated by phosphorylation of the β subunit by CaMKII. *Mol Cell* 23:641–650.
- Hell JW, Yokoyama CT, Wong ST, Warner C, Snutch TP, Catterall WA (1993a) Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel α_1 subunit. *J Biol Chem* 268:19451–19457.
- Hell JW, Westenbroek RE, Warner C, Ahljianian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA (1993b) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel α_1 subunits. *J Cell Biol* 123:949–962.
- Hetzenauer A, Sinnegger-Brauns MJ, Striessnig J, Singewald N (2006) Brain activation pattern induced by stimulation of L-type Ca²⁺-channels: contribution of Ca_v1.3 and Ca_v1.2 isoforms. *Neuroscience* 139:1005–1015.
- Hibino H, Pironkova R, Onwumere O, Rousset M, Charnet P, Hudspeth AJ, Lesage F (2003) Direct interaction with a nuclear protein and regulation of gene silencing by a variant of the Ca²⁺-channel β_4 subunit. *Proc Natl Acad Sci USA* 100:307–312.
- Huang YZ, Wang Q, Xiong WC, Mei L (2001) Erbin is a protein concentrated at postsynaptic membranes that interacts with PSD-95. *J Biol Chem* 276:19318–19326.
- Hudmon A, Schulman H, Kim J, Maltez JM, Tsien RW, Pitt GS (2005) CaMKII tethers to L-type Ca²⁺ channels, establishing a local and dedicated integrator of Ca²⁺ signals for facilitation. *J Cell Biol* 171:537–547.
- Hulme JT, Konoki K, Lin TW, Gritsenko MA, Camp II DG, Bigelow DJ, Catterall WA (2005) Sites of proteolytic processing and noncovalent association of the distal C-terminal domain of Ca_v1.1 channels in skeletal muscle. *Proc Natl Acad Sci USA* 102:5274–5279.
- Hulme JT, Yarov-Yarovsky V, Lin TW, Scheuer T, Catterall WA (2006) Au-

- toinhibitory control of the Ca_v1.2 channel by its proteolytically processed distal C-terminal domain. *J Physiol (Lond)* 576:87–102.
- Ivanina T, Blumenstein Y, Shistik E, Barzilai R, Dascal N (2000) Modulation of L-type Ca²⁺ channels by Gβγ and calmodulin via interactions with N and C termini of alpha 1C. *J Biol Chem* 275:39846–39854.
- Jaulin-Bastard F, Saito H, Le Bivic A, Ollendorff V, Marchetto S, Birnbaum D, Borg JP (2001) The ERBB2/HER2 receptor differentially interacts with ERBIN and PICK1 PSD-95/DLG/ZO-1 domain proteins. *J Biol Chem* 276:15256–15263.
- Johnston D, Williams S, Jaffe D, Gray R (1992) NMDA-receptor-independent long-term potentiation. *Annu Rev Physiol* 54:489–505.
- Kavalali ET, Plummer MR (1996) Multiple voltage-dependent mechanisms potentiate calcium channel activity in hippocampal neurons. *J Neurosci* 16:1072–1082.
- Kim E, Sheng M (2004) PDZ domain proteins of synapses. *Nat Rev Neurosci* 5:771–781.
- Kobrinisky E, Tiwari S, Maltsev V, Harry JB, Lakatta E, Abernethy DR, Soldatov NM (2005) Differential role of the alpha 1C subunit tails in regulation of the Ca_v1.2 channel by membrane potential, beta subunits and Ca²⁺ ions. *J Biol Chem* 280:12474–12485.
- Kolch W (2003) Erbin: sorting out ErbB2 receptors or giving Ras a break? *Sci STKE* 2003:37.
- Koschak A, Reimer D, Huber I, Grabner M, Glossmann H, Engel J, Striessnig J (2001) α_{1D} (Ca_v1.3) subunits can form L-type Ca²⁺ channels activating at negative voltages. *J Biol Chem* 276:22100–22106.
- Lee TS, Karl R, Moosmang S, Lenhardt P, Klugbauer N, Hofmann F, Kleppisch T, Welling A (2006) Calmodulin kinase II is involved in voltage-dependent facilitation of the L-type Ca_v1.2 calcium channel: identification of the phosphorylation sites. *J Biol Chem* 281:25560–25567.
- Legouis R, Jaulin-Bastard F, Schott S, Navarro C, Borg JP, Labouesse M (2003) Basolateral targeting by leucine-rich repeat domains in epithelial cells. *EMBO Rep* 4:1096–1102.
- Ludwig A, Flockerzi V, Hofmann F (1997) Regional expression and cellular localization of the α₁ and β subunit of high voltage-activated calcium channels in rat brain. *J Neurosci* 17:1339–1349.
- Marrion NV, Tavalin SJ (1998) Selective activation of Ca²⁺-activated K⁺ channels by co-localized Ca²⁺ channels in hippocampal neurons. *Nature* 395:900–905.
- Maximov A, Sudhof TC, Bezprozvanny I (1999) Association of neuronal calcium channels with modular adaptor proteins. *J Biol Chem* 274:24453–24456.
- Moosmang S, Haider N, Klugbauer N, Adelsberger H, Langwieser N, Muller J, Stiess M, Marais E, Schulla V, Lacinova L, Goebbels S, Nave KA, Storm DR, Hofmann F, Kleppisch T (2005) Role of hippocampal Ca_v1.2 Ca²⁺ channels in NMDA receptor-independent synaptic plasticity and spatial memory. *J Neurosci* 25:9883–9892.
- Morrill JA, Cannon SC (2000) COOH-terminal truncated α_{1S} subunits conduct current better than full-length dihydropyridine receptors. *J Gen Physiol* 116:341–348.
- Nasif FJ, Hu XT, White FJ (2005) Repeated cocaine administration increases voltage-sensitive calcium currents in response to membrane depolarization in medial prefrontal cortex pyramidal neurons. *J Neurosci* 25:3674–3679.
- Olson PA, Tkatch T, Hernandez-Lopez S, Ulrich S, Ilijic E, Mugnaini E, Zhang H, Bezprozvanny I, Surmeier DJ (2005) G-protein-coupled receptor modulation of striatal Ca_v1.3 L-type Ca²⁺ channels is dependent on a Shank-binding domain. *J Neurosci* 25:1050–1062.
- Ou Y, Stregre P, Miller SM, Makielski J, Ackerman M, Gibbons SJ, Farrugia G (2003) Syntrophin gamma 2 regulates SCN5A gating by a PDZ domain-mediated interaction. *J Biol Chem* 278:1915–1923.
- Penkert RR, DiVittorio HM, Prehoda KE (2004) Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. *Nat Struct Mol Biol* 11:1122–1127.
- Pichler M, Cassidy TN, Reimer D, Haase H, Krause R, Ostler D, Striessnig J (1997) β subunit heterogeneity in neuronal L-type Ca²⁺ channels. *J Biol Chem* 272:13877–13882.
- Pietrobon D, Hess P (1990) Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature* 346:651–655.
- Pineda JC, Waters RS, Foecking RC (1998) Specificity in the interaction of HVA Ca²⁺ channel types with Ca²⁺-dependent AHPs and firing behavior in neocortical pyramidal neurons. *J Neurophysiol* 79:2522–2534.
- Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J (2000) Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca²⁺ channels. *Cell* 102:89–97.
- Qu Y, Baroudi G, Yue Y, El-Sherif N, Boutjdir M (2005) Localization and modulation of α_{1D} (Ca_v1.3) L-type Ca²⁺ channel by protein kinase A. *Am J Physiol Heart Circ Physiol* 288:H2123–H2130.
- Richards MW, Butcher AJ, Dolphin AC (2004) Ca²⁺ channel β-subunits: structural insights AID our understanding. *Trends Pharmacol Sci* 25:626–632.
- Safa P, Boulter J, Hales TG (2001) Functional properties of Ca_v1.3 (alpha1D) L-type Ca²⁺ channel splice variants expressed by rat brain and neuroendocrine GH3 cells. *J Biol Chem* 276:38727–38737.
- Scholze A, Plant TD, Dolphin AC, Nurnberg B (2001) Functional expression and characterization of a voltage-gated Ca_v1.3 (α_{1D}) calcium channel subunit from an insulin-secreting cell line. *Mol Endocrinol* 15:1211–1221.
- Sinnesger-Brauns MJ, Hetzenauer A, Huber IG, Renstrom E, Wietzorrek G, Berjukov S, Cavalli M, Walter D, Koschak A, Waldschutz R, Hering S, Bova S, Rorsman P, Pongs O, Singewald N, Striessnig J (2004) Isoform-specific regulation of mood behavior and pancreatic beta cell and cardiovascular function by L-type Ca²⁺ channels. *J Clin Invest* 113:1430–1439.
- Song WJ, Surmeier DJ (1996) Voltage-dependent facilitation of calcium channels in rat neostriatal neurons. *J Neurophysiol* 76:2290–2306.
- Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, Chishti AH, Crompton A, Chan AC, Anderson JM, Cantley LC (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* 275:73–77.
- Vendel AC, Terry MD, Striegel AR, Iverson NM, Leuranguer V, Rithner CD, Lyons BA, Pickard GE, Tobet SA, Horne WA (2006) Alternative splicing of the voltage-gated Ca²⁺ channel β₄ subunit creates a uniquely folded N-terminal protein binding domain with cell-specific expression in the cerebellar cortex. *J Neurosci* 26:2635–2644.
- Wei X, Neely A, Lacerda AE, Olcese R, Stefani E, Perez-Reyes E, Birnbaumer L (1994) Modification of Ca²⁺ channel activity by deletions at the carboxyl terminus of the cardiac α₁ subunit. *J Biol Chem* 269:1635–1640.
- Weick JP, Groth RD, Isaksen AL, Mermelstein PG (2003) Interactions with PDZ proteins are required for L-type calcium channels to activate cAMP response element-binding protein-dependent gene expression. *J Neurosci* 23:3446–3456.
- Xu W, Lipscombe D (2001) Neuronal Ca(V)1.3α₁ L-type channels activate at relatively hyperpolarized membrane potentials and are incompletely inhibited by dihydropyridines. *J Neurosci* 21:5944–5951.
- Zhang H, Maximov A, Fu Y, Xu F, Tang TS, Tkatch T, Surmeier DJ, Bezprozvanny I (2005) Association of Ca_v1.3 L-type calcium channels with Shank. *J Neurosci* 25:1037–1049.
- Zhang H, Fu Y, Altier C, Platzer J, Surmeier DJ, Bezprozvanny I (2006) Ca_v1.2 and Ca_v1.3 neuronal L-type calcium channels: differential targeting and signaling to pCREB. *Eur J Neurosci* 23:2297–2310.
- Zhou H, Kim SA, Kirk EA, Tippens AL, Sun H, Haeseleer F, Lee A (2004) Ca²⁺-binding protein-1 facilitates and forms a postsynaptic complex with Ca_v1.2 (L-type) Ca²⁺ channels. *J Neurosci* 24:4698–4708.