Molecular Determinants of the Ca_vβ-induced Plasma Membrane Targeting of the Ca_v1.2 Channel*§

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Ca_vβ subunits modulate cell surface expression and voltage-dependent gating of high voltage-activated (HVA) Ca_v1 and Ca_v2 α1 subunits. High affinity Ca_vβ binding onto the so-called α interaction domain of the I-II linker of the Ca_vα1 subunit is required for Ca_vβ modulation of HVA channel gating. It has been suggested, however, that Ca_vβ-mediated plasma membrane targeting could be uncoupled from Ca_vβ-mediated modulation of channel gating. In addition to Ca_vβ, Ca_vα2δ and calmodulin have been proposed to play important roles in HVA channel targeting. Indeed we show that co-expression of Ca_vα2δCa_vβ caused a 5-fold stimulation of the whole cell currents measured with Ca_v1.2 and Ca_vβ. To gauge the synergetic role of auxiliary subunits in the steady-state plasma membrane expression of Ca_v1.2, extracellularly tagged Ca_v1.2 proteins were quantified using fluorescence-activated cell sorting analysis. Co-expression of Ca_v1.2 with either Ca_vα2δ, calmodulin wild type, or apocalmodulin (alone or in combination) failed to promote the detection of fluorescently labeled Ca_v1.2 subunits. In contrast, co-expression with Ca_vβ stimulated plasma membrane expression of Ca_v1.2 by a 10-fold factor. Mutations within the α interaction domain of Ca_v1.2 or within the nucleotide kinase domain of Ca_vβ3 disrupted the Ca_vβ3-induced plasma membrane targeting of Ca_v1.2. Altogether, these data support a model where high affinity binding of Ca_vβ to the I-II linker of Ca_vα1 largely accounts for Ca_vβ-induced plasma membrane targeting of Ca_v1.2.

Voltage-dependent Ca^{2+} channels (Ca_v) are membrane proteins that play a key role in promoting Ca^{2+} influx in response to membrane depolarization in excitable cells. To this date, molecular cloning has identified the primary structures for 10 distinct calcium channel Ca_vα1 subunits (1–7) that are classified into three main subfamilies according to their high voltage-activated (HVA)2 gating (Ca_v1 and Ca_v2) or low voltage-activated gating (Ca_v3). In addition to the transmembrane pore-forming Ca_vα1 subunit, Ca_v1 and Ca_v2 channels arise from the multimerization of three other proteins (7): a cytoplasmic Ca_vβ subunit, a mostly extracellular Ca_vα2δ subunit, and calmodulin constitutively bound to the C terminus of Ca_vα1 (8–12).

A considerable body of work documents the interaction and modulation of the Ca_vα1 subunit of Ca_v1 and Ca_v2 channels (13–18) by the auxiliary Ca_vβ. The high affinity Ca_vα1-Ca_vβ interaction site on the pore-forming Ca_vα1 subunit is a conserved 18-residue sequence in the I-II linker called the α interaction domain (AID) (19, 20) that has been structurally resolved by high resolution x-ray crystallography (21–23). Structural work showed that the AID forms a α-helix that binds to the α binding pocket (ABP) in the Ca_vβ nucleotide kinase (NK) domain. It has been proposed that the MMQKAL cluster of residues within the latter determines the high affinity nanomolar interaction between the two proteins (24–29). Numerous mutational analyses of the AID residues have correlated the Ca_vβ-induced biophysical modulation with the high affinity binding of Ca_vβ to the AID peptide in a variety of Ca_vα1 isofoms for Ca_v1 and Ca_v2 channels (25, 29–32).

The association of Ca_vα1 and Ca_vβ subunits is also critical for proper channel maturation and cell surface expression of Ca_v2.2 (17), Ca_v1.2 (33, 34), and Ca_v2.3 (35). In Ca_v2.2, the I-II linker is presumed to play a role in this process (17, 18), and mutations within the AID motif eliminated its cell surface expression and biophysical modulation by Ca_vβ1b and Ca_vβ3 (32). In addition, the Ca_vβ2-induced increase in Ca_v1.2 whole cell currents was abolished with the AID-defective YW1/AAA mutant (29), suggesting that high affinity binding of Ca_vβ onto AID is required to traffic Ca_vα1 to the plasma membrane. Nonetheless, the unique character of the high affinity AID-ABP interface in the membrane targeting of Ca_vα1 has been questioned (27, 36–40). In particular, it has been suggested that Ca_vβ-mediated plasma membrane targeting could be uncoupled from Ca_vβ-mediated modulation of channel gating (26, 41) with important contributions from other intracellular regions (33, 39, 42–44).

In addition to Ca_vβ, the ancillary subunit Ca_vα2δ and the ubiquitous calmodulin (CaM) protein have also been proposed to modulate HVA channel maturation and targeting (9). For instance, co-expression of Ca_vα2δ promoted the trafficking of the Ca_v1α1 subunit of Ca_v2.2 in COS-7 cells (45), suggesting that Ca_vα2δ could promote targeting of all HVA Ca_vα1 sub-
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units. CaM is a soluble, 17-kDa Ca\(^{2+}\)-binding protein that serves as a critical Ca\(^{2+}\) sensor for Ca\(^{2+}\)-dependent inactivation and facilitation upon Ca\(^{2+}\) binding in many Ca\(_{\text{V}}\),1 and Ca\(_{\text{V}}\),2 channels (46), of which Ca\(_{\text{V}}\),1.2 and Ca\(_{\text{V}}\),2.1 have been best characterized (8, 47). Constitutive calmodulin binding was reported on multiple sites in the Ca\(_{\text{V}}\),α1 subunit of Ca\(_{\text{V}}\),1.2 (48) of which the C-terminal pre-IQ and IQ domains are best characterized (49). Mutations (TLE/AAA and I/E) in the pre-IQ and the IQ CaM-binding domains of the C terminus decreased the whole cell current density of Ca\(_{\text{V}}\),1.2, suggesting that Ca\(^{2+}\)/CaM could modulate channel trafficking through its interaction with the C terminus (50) as it has been shown for small activated potassium channels (51).

To gauge the synergetic role of intracellular domains and auxiliary subunits in the steady-state plasma membrane expression of Ca\(_{\text{V}}\),1.2, we used a flow cytometry assay with an extracellularly HA-tagged Ca\(_{\text{V}}\),1.2 protein. Co-expression with Ca\(_{\text{V}}\),β3 produced a robust enhancement in the plasma membrane targeting of the Ca\(_{\text{V}}\),α1 subunit of Ca\(_{\text{V}}\),1.2. The WI residues in the AID helix of the I-II linker of Ca\(_{\text{V}}\),1.2 were critical for Ca\(_{\text{V}}\),β-stimulated plasma membrane targeting of Ca\(_{\text{V}}\),1.2. No other combination with or without the auxiliary calmodulin and/or the Ca\(_{\text{V}}\),α2bδ subunit produced any significant increase in the plasma membrane targeting of Ca\(_{\text{V}}\),1.2. Hence, Ca\(_{\text{V}}\),β appears to be the most potent determinant in the plasma membrane targeting of Ca\(_{\text{V}}\),1.2. Altogether, our data support a model where high affinity binding of the ABP of Ca\(_{\text{V}}\),β to the AID helix of Ca\(_{\text{V}}\),α1 largely accounts for Ca\(_{\text{V}}\),β-induced plasma membrane targeting of Ca\(_{\text{V}}\),1.2.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Techniques**—The rabbit Ca\(_{\text{V}}\),1.2 (GenBank\textsuperscript{TM} accession number X15539), the rat Ca\(_{\text{V}}\),β3 (GenBank\textsuperscript{TM} accession number M88751) (52), the rat brain Ca\(_{\text{V}}\),α2bδ (GenBank\textsuperscript{TM} accession number NM_000722) (53), and the human CaM (GenBank\textsuperscript{TM} accession number M27319) were used. All of the subunits were subcloned in commercial vectors under the control of the cytomegalovirus promoter (see supplemental text for details).

For the Ca\(_{\text{V}}\),β3 deletion mutants, flanking NotI sites were inserted around the region(s) to be deleted. Following restriction digest of the NotI fragment and religation of the cohesive ends, the resulting NotI site was mutated back to the wild type amino acids. The Ca\(_{\text{V}}\),β3 fragments (numbered from their deduced amino acid sequence) were subcloned into the NotI sites of the pCMV-Tag5a vector (see supplemental text for details) that is a C-terminal c-Myc tagging vector. A Kozak sequence and an ATG initiation codon were inserted at the 5’-end of the nucleotide sequence.

The calmodulin wild type cDNA was subcloned in the pMT21 vector (54). The dominant negative mutant of CaM (CaM\(_{1,2,3,4}\)) that impaired high affinity Ca\(^{2+}\) binding is D20A/D56A/D93A/D129A, which has been described elsewhere (55).

**Insertion of the HA Tag in the Ca\(_{\text{V}}\),α1 Subunit**—The hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted in the first extracytoplasmic predicted loop in Domain I at position 574 (nucleotide) for Ca\(_{\text{V}}\),1.2. The biophysical properties of the HA-tagged Ca\(_{\text{V}}\),α1 subunit of Ca\(_{\text{V}}\),1.2 expressed in HEK cells with the auxiliary Ca\(_{\text{V}}\),β3 subunit were found not to be significantly different from the wild type Ca\(_{\text{V}}\),1.2 channel expressed under the same conditions (see Fig. 1). In addition, cDNA injection of Ca\(_{\text{V}}\),1.2-HA constructions in concert with Ca\(_{\text{V}}\),α2bδ and Ca\(_{\text{V}}\),β3 subunits in Xenopus oocytes yielded a biophysical profile not significantly different from that reported previously for the Ca\(_{\text{V}}\),1.2 (56) channels expressed under the same conditions. Hence, the HA-tagged version of the Ca\(_{\text{V}}\),α1 subunit of Ca\(_{\text{V}}\),1.2 will be referred to as Ca\(_{\text{V}}\),1.2 wt throughout the text.

**Cell Culture and Transfections**—tsA-201 (HEK293T or HEK7), a subclone of the human embryonic kidney cell line HEK-293 that expresses the simian virus 40 T-antigen, and COS1 cells were grown in Dulbecco’s high glucose minimum essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin at 37 °C under 5% CO\(_{2}\) atmosphere. COS1, HEK7, stable Ca\(_{\text{V}}\),β3, and Ca\(_{\text{V}}\),α2bδ cells lines were transiently transfected with HA-tagged Ca\(_{\text{V}}\),1.2 cDNA using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Protein expression of the auxiliary subunits in the stable and transient cell lines were confirmed routinely by Western blotting (see Fig. 2B). Transfection rate of the control pEGFP plasmid was estimated to be 66 ± 2% (n = 4) as assessed by flow cytometry from the fluorescence of the green fluorescent protein. Preliminary tests showed that Ca\(_{\text{V}}\),1.2 protein expression peaked 24–36 h after transfection.

**Western Blots**—Protein expression of all constructs was confirmed by Western blotting in total cell lysates. HA-tagged Ca\(_{\text{V}}\),1.2 constructs were detected with anti-HA. The procedures are detailed in the supplemental text. Briefly, the membranes were incubated with anti-HA (1:500) (Covance Biotechnology, Quebec, Canada) and revealed with an anti-mouse horseradish peroxidase secondary antibody (1:10000; Jackson Immunoresearch).

**Fluorescence-activated Cell Sorting (FACS) Experiments**—Cell surface expression of the Ca\(_{\text{V}}\),1.2 subunits was determined by flow cytometry using a FACScalibur\textsuperscript{®} flow cytometer (Becton Dickinson) at the flow cytometry facility of the Department of Microbiology of the Université de Montréal. The cells expressing the extracellular HA tag were detected using an anti-HA-conjugated FITC fluorophore with a FITC filter (530 nm). The relative intensity of staining provided a metric to quantify cell surface expression of the HA-tagged Ca\(_{\text{V}}\),1.2 proteins (see supplemental Figs. S1 and S2 and supplemental text for details). The HA-tagged Ca\(_{\text{V}}\),1.2 construct was systematically tested as a control with the mutant channels.

**Immunofluorescence**—For fluorescence microscopy, Ca\(_{\text{V}}\),β3 stable cells were grown on sterile poly-d-lysine-coated coverslips. The cells were fixed 24 h after transfection in 4% paraformaldehyde, permeabilized with 0.075% saponin for 10 min at room temperature, washed in phosphate-buffered saline, and blocked in IgG-free 2% bovine serum albumin in phosphate-buffered saline for 20 min. The cells were incubated with FITC-conjugated anti-HA antibody (1:100) for 1 h at room temperature prior to the cells being mounted (Prolong antifade kit; Invitrogen) on glass microscope slides. HA-tagged Ca\(_{\text{V}}\),1.2 channels (wild type and mutant) were visualized (×60) using an Olympus microscope IX-81 microscope along with Image-Pro Plus 5.0 software.
in Fig. 1B, average whole cell current density increased from $-7 \pm 2$ nA/pF $(n = 6)$ for the wild type Ca1,2 channel in the stable Ca1,2β3 stable cell line as compared with a current density of $-41 \pm 9$ nA/pF $(n = 7)$ for the wild type Ca1,2 channel measured in the same cell line after transient transfection with Caα2βδ subunit. Similar results were obtained for the HA-tagged Ca1,2β3 channels (Fig. 1B).

Ca1,2 Promotes Membrane Targeting of Ca1,2—To determine whether Caα2βδ stimulates plasma membrane targeting of Ca1,2 channels, protein density of the extracellularly HA-tagged Ca1,2 channel was quantified with an anti-HA-conjugated FITC fluorophore. Fig. 2A shows the histogram of the fluorescent signal measured after transient expression of the Caα1 and the auxiliary subunit (either Ca1,2β3 or Caα2βδ) in nonpermeabilized cells. Protein expression was confirmed by Western blotting (Fig. 2B). As seen, less than 0.5% of the cell population produced autofluorescence, whereas only 1% of the cells were fluorescent after the addition of the FITC antibody to control nontransfected cells (see raw data in supplemental Figs. S1 and S2). Transient co-expression of the HA-tagged Ca1,2β3 subunit in the stable Ca1,2β3 cell line increased the number of proteins detected at the membrane from a value of 4.5 ± 0.5% $(n = 25)$ in the nontransfected cell line to 23 ± 1% $(n = 29)$ with Ca1,2β3 $(p < 0.001)$ (Table 1).

The results obtained with Ca1,2β3 contrast with the effect observed when co-expressing HA-tagged Ca1,2 with Caα2βδ $(p > 0.1)$. No further increase in the fluorescent signal was observed in the combined presence of the two auxiliary subunits. Similar results were obtained when Ca1,2 was transiently expressed, either in a background of stably transfected Ca1,2β3 or in a background of stably transfected Caα2βδ cells (see Table 1 for numerical values). The maximum fluorescence obtained with Ca1,2β3 confirms that Caα2βδ has little effect by itself on the Ca1,2β3 protein density at the plasma membrane. Among Ca1,2β subunits, transient co-expression of Ca1,2-β4 with Ca1,2β4 caused a similar boost in plasma membrane expression, whereas Ca1,2β2a was found to be slightly less potent for a Ca1,2β3 $\approx$ Ca1,2β4 > Ca1,2β2a ranking (Table 1). Altogether, these results validated the fluorescence sorting analysis of HA-tagged Caα1 proteins to evaluate steady-state protein level in intact cells independently of channel gating.

**Interaction Domain: the Role of the WI Pair**—Crystallographic analyses have shown that the AID-Ca1,2 interaction is

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**Statistical Analysis**—Statistical analyses were performed using the built-in one-way analysis of variance fitting routine for two independent populations of Origin 7.0. The data were considered statistically significant at $p < 0.01$.

**RESULTS**

Ca1,2α2δ Increases Whole Cell Currents of Ca1,2—Co-expression of Ca1,2 and Ca1,2 with the auxiliary Ca1,2α2δ subunit was shown to stimulate whole cell currents (45) in COS-7 cells, suggesting that Ca1,2α2δ could promote plasma membrane targeting of HVA Caα1 subunits. In Ca1,2, the gating charge appears to be unaffected by co-expression with Ca1,2α2δ, suggesting that Ca1,2α2δ stimulates channel facilitation by setting Ca1,2 channels in a conformational state very close to the open state without increasing protein density (57). To evaluate the functional role of Ca1,2α2δ, Ca1,2 wt and HA-tagged Ca1,2α1 subunits were transiently transfected in the Ca1,2β3 stable HEKT cell line in the absence and in the presence of Ca1,2βδ. As shown in Fig. 1A, whole cell currents, recorded in the presence of a physiological solution containing 2 mM Ca2+ (see the supplemental text), were significantly larger when measured in the presence of the Ca1,2α2δ, confirming that Ca1,2α2δ stimulates whole cell currents of Ca1,2 (9). As shown
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anchored through a set of six residues, Asp, Leu, Gly, Tyr, Trp, and Ile, distributed among three α-helical turns of the I-II linker of Ca1.2 (21–23), with the WI pair of residues being most critical for the AID-CaVβ protein interaction (29). To evaluate whether the AID-CaVβ interaction controls the plasma membrane targeting of Ca1.2, the HA-tagged Ca1.2 subunit was transiently co-expressed in HEKT cells or in CaVβ stable cells. CaVβ3 stimulated the plasma membrane targeting of N-terminal mutants: L464A, G466A, G466F, Y467G, Y467A, Y467S, and Y467F (Fig. 3A, supplemental Fig. S4, and supplemental Table SII). When compared with the control situation (±CaVβ3), there was a 4–8-fold stimulation in the plasma membrane targeting mutations in the order G466A > G466F > Y467F > Y467G > L464A > Y467A > Y467S. Hence, no single point mutation in the N-terminal region of the AID completely abolished the CaVβ3-induced stimulation in the plasma membrane targeting of Ca1.2. In contrast, the CaVβ3 stimulation effect was completely eradicated in the double mutant G466A/ Y467F, even though each individual mutation behaved like the wild type channel, suggesting that each residue contributes to the high affinity interaction with CaVβ3.

Point mutations in the C-terminal WI pair yielded a different picture. I471L was the only mutant that was detected at the membrane to the same extent as the wild type channel in the presence of CaVβ3. However, CaVβ3 stimulated significantly the plasma membrane targeting of I471A and I471F mutants. W470Y, W470F, W470A, W470G, I417G, and I417R were not significantly different in the presence or in the absence of CaVβ3 (Fig. 3C and supplemental Table SII). Western blots carried out in total cell lysates with the anti-HA confirmed that all of the Ca1.2 mutants tested produced proteins with the expected molecular weight (Fig. 3, B and D). Immunofluorescence microscopy confirmed that W470A disrupted the plasma membrane targeting of Ca1.2 in the presence of CaVβ3 (supplemental Fig. S5). Membrane expression of Ca1.2 in cultured hippocampal neurons was also disrupted after mutation of the key tryptophan residue to alanine (58).

Furthermore, double mutations in the same region completely eradicated the CaVβ3 stimulation of

![Image](https://example.com/image.png)

**FIGURE 2. CaVβ stimulated Ca1.2 membrane expression in HEKT cells.** A, HA-tagged Ca1.2 wt was co-expressed transiently either in the stable CaVβ3 or the stable CaVα2β6 cell line. Cell surface expression of Ca1.2 wt was determined in intact cells by flow cytometry using the anti-HA FITC conjugate antibody (Ab). The histogram shows the number of fluorescent cells as a function of the experimental conditions. Cell fluorescence (HEKT no Ab) was 0.01% throughout, and the addition of the FITC did not significantly increase the level of fluorescence in HEKT cells not transfected with the HA-tagged Ca1.2 (HEKT with Ab). As seen, no co-expression of Ca1.2 with CaVβ3 significantly promoted membrane expression of Ca1.2 (p < 0.001). Co-expression of Ca1.2 with CaVα2β6 did not alter the number of Ca1.2 channels at the membrane (p > 0.1). Co-expression with both auxiliary subunits did not further improve the membrane expression of Ca1.2. The Ca1.2 + CaVβ3 + CaVα2β6 condition (either Ca1.2 + CaVβ3 expressed transiently in the stable CaVα2β6 cell line or Ca1.2 + CaVα2β6 expressed transiently in the CaVβ3 cell line) was not significantly different from the Ca1.2 + CaVβ3 condition (p > 0.1). Similar results were obtained with transient expression systems. The numerical values can be found in Table 1. B, Western blot analyses of HEKT cells transiently or stably transfected with CaVα2β6 or CaVβ3, using CaVα2β6-1 (1:200) and CaVβ3 (1:500) antibodies. Each lane was loaded with 50 μg of protein. Panel A, HEKT cells were transiently or stably transfected with CaVβ3. Lane 1, control nontransfected cells. Lane 2, transient transfection of CaVβ3. Lane 3, control nontransfected cells. Lane 4, stable CaVβ3 cell line. Lane 5 transient co-transfection of CaVβ3 and CaVα2β6. Panel B, HEKT cells were transiently or stably transfected with CaVα2β6. Lane 1, control nontransfected cells. Lane 2, transient transfection of CaVα2β6. Lane 3, control nontransfected cells. Lane 4, stable CaVα2β6 cell line.

**TABLE 1**

Fluorescence-activated cell sorting analysis of Ca1.2 ± auxiliary subunits

FACS results obtained after the transient transfection of Ca1.2-HA wt in either HEKT control cells, stable CaVβ3 cells, or stable CaVα2β6 cells. One day (24 h) after transfection, the cells were incubated with anti-HA FITC conjugate (10 μg/ml) at room temperature for 45 min. FACS separation of FITC-positive cells was performed on a FACScalibur® flow cyrometer (Becton Dickinson), and fluorescence was quantified using CellQuest software (Becton Dickinson). The results are reported as percentage values of cells in M2. The data were pooled from experiments carried out over a period of 8 months. The data are shown as the means ± S.E. of the individual experiments, and the number of experiments appears in parentheses. ND, not determined.

| Construct transient expression | HEKT | HEKT CaVβ3 stable | HEKT CaVα2β6 stable | HEKT CaVβ3 + CaVα2β6 stable |
|-------------------------------|------|------------------|---------------------|-----------------------------|
| Cells without antibody        | 0.3 ± 0.1 (32) | 0.18 ± 0.04 (20) | 0.54 ± 0.07 (3) | 2.8 ± 0.2 (3) |
| Cells with antibody           | 1.1 ± 0.3 (19) | 1.0 ± 0.4 (19) | 0.93 ± 0.05 (3) | 4.2 ± 0.4 (3) |
| Ca1.2-HA wt                  | 4.5 ± 0.5 (25) | 21 ± 1 (29) | 6 ± 1 (3) | 19 ± 2 (3) |
| Ca1.2-HA wt + CaVβ3          | 25 ± 2 (3) | ND | 21 ± 2 (3) | ND |
| Ca1.2-HA wt + CaVβ2a         | 14 ± 1 (3) | ND | ND | ND |
| Ca1.2-HA wt + CaVβ4          | 22 ± 3 (3) | ND | ND | ND |
| Ca1.2-HA wt + CaVα2β6 + CaM wt | 2 ± 1 (3) | Ca | ND | ND |
| Ca1.2-HA wt + CaVα2β6        | 8 ± 3 (5) | 26 ± 1 (3) | ND | ND |
| Ca1.2-HA wt + CaM wt          | 1.5 ± 0.3 (3) | 19.1 ± 0.3 (3) | 2.7 ± 0.4 (3) | ND |
| Ca1.2-HA wt + CaM1,2,3,4     | 1.4 ± 0.1 (3) | 19.1 ± 0.6 (3) | ND | ND |
| Ca1.2-HA wt + CaVβ3 + CaVα2β6| 23 ± 2 (6) | ND | ND | ND |
CaV1.2 plasma membrane targeting (supplemental Fig. S6 and Table S1). Partial (458–463) or complete (458–475) removal of the AID-binding site within the I-II loop yielded similar results, confirming that no other low affinity CaV1 subunit could promote the plasma membrane targeting of CaV1.2 in the absence of the AID region.

Isothermal titration calorimetry assays have substantiated that the affinity of CaV2a for the AID region of CaV1.2 decreased after single-point mutations of these residues. There was a 1000-fold increase in the $K_d$ with the W/A and I/A mutants, whereas alanine mutation of the Leu and Gly residues imparted a smaller 5–10-fold decrease in the CaV2a affinity (29). Substitution of the tryptophan residue by either tyrosine or phenylalanine only partly compensated for the mutation, confirming the requirement of a residue containing a double aromatic ring at this position. For the neighboring isoleucine position, mutation with the conserved leucine residue was found to preserve the CaV3-induced membrane targeting of CaV1.2. For comparison, I387L in CaV2.3 was the only mutant tested in the WI pair that supported CaV3 binding and CaV3 modulation of gating (31). In contrast, none of the CaV1.2 mutations identified in the short QT syndrome, an inherited form of cardiac arrhythmia (59), was shown to affect the CaV3 plasma membrane targeting of CaV1.2 (supplemental Fig. S7 and Table SII).

FIGURE 3. AID = 458QQLLEDLKGYLDWITQAE475

A. AID = 458QQLLEDLKGYLDWITQAE475

1. non-transfected cells
2. CaV1.2-HA
3. CaV1.2-HA + CaVβ3
4. CaV1.2-HA W470A
5. CaV1.2-HA W470A + CaVβ3
6. CaV1.2-HA G466A/Y467A
7. CaV1.2-HA G466A/Y467A + CaVβ3

B. Western blot analyses of HEKT cells transiently transfected with CaV1.2 wt or mutants in stable CaV3 cells using HA (1:500) and CaV3 (1:500) antibodies. Lane 1: control nontransfected cells. Lane 2: CaV1.2-HA. Lane 3: CaV1.2-HA + CaVβ3. Lane 4: CaV1.2-HA W470A. Lane 5: CaV1.2-HA W470A + CaVβ3. Lane 6: CaV1.2-HA G466A/Y467A. Lane 7: CaV1.2-HA G466A/Y467A + CaVβ3.

C. Western blot analyses confirmed that the W470A mutant was expressed in total cell lysates and recognized by the anti-HA (1:500). Each lane was loaded with 50 μg of protein. Lane 1: control nontransfected cells. Lane 2: CaV1.2-HA W470A. Lane 3: CaV1.2-HA I471A. Lane 4: CaV1.2-HA I471L. Lane 5: CaV1.2-HA I471R.

D. Western blot analyses confirmed that the CaV2a protein was quantified as described for supplemental Fig. S2. The residues targeted in these experiments are underlined within the primary sequence of the AID region of CaV1.2 shown in A. The number of fluorescent cells decreased in the order CaV1.2-HA wt > I471L > I471F > I471A > I471S > I471R > 458, 464, 466, 467, 470, 471, 475. The numerical values can be found in supplemental Table S1.
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Altogether our results support a strong correlation between Ca$_{\beta3}$ binding affinity to the AID region as determined from fusion proteins and from isothermal titration calorimetry assays (29) and its role in promoting the targeting of Ca$_{\alpha1,2}$ proteins at the plasma membrane. More importantly our results suggest that the molecular determinants that account for Ca$_{\beta3}$ binding to the AID region are also responsible for the Ca$_{\alpha1,2}$-induced stimulation of the plasma membrane targeting of Ca$_{\alpha1,2}$ proteins.

The NK Domain of Ca$_{\alpha1}\cdot$Ca$_{\beta}$ Is Essential for Plasma Membrane Targeting of Ca$_{\alpha1,2}$ — The observation that different Ca$_{\alpha}$s, which all share a conserved core containing the SH3 and NK domains, cause different biophysical effects on Ca$_{\alpha1}\cdot$Ca$_{\beta}$ subunits suggests that other regions besides the conserved AID-ABP interaction, could influence channel conformational changes (13). The SH3 domain of Ca$_{\alpha1}$,Ca$_{\beta2}$ was found to bind to the I-II linker of Ca$_{\alpha2,1}$ channels, suggesting that low affinity interactions outside of the AID-ABP interface could contribute to the full functional effects of the Ca$_{\alpha1}$,Ca$_{\beta}$ subunits (40). A few years later, however, the conserved AID-NK domain interaction was found to be necessary for Ca$_{\alpha}$,Ca$_{\beta}$-stimulated Ca$_{\alpha2,1}$ channel surface expression (60). To evaluate whether the AID-NK interaction controls the plasma membrane targeting of Ca$_{\alpha1,2}$, the HA-tagged Ca$_{\alpha1,2}$ subunit was transiently co-expressed in HEKT cells in the presence of Ca$_{\alpha1}$,Ca$_{\beta}$ full-length or deleted constructs as well as with Ca$_{\alpha1}$,Ca$_{\beta}$ fragments.

We found that the NK domain of Ca$_{\alpha1}$,Ca$_{\beta3}$ (180–364) (Fig. 4A) was required for the plasma membrane targeting of Ca$_{\alpha1,2}$ (Fig. 4B and supplemental Table SIII). The deletion of the SH3 domain between residues 57 and 123 preserved 80% of the Ca$_{\alpha1,2}$ protein detected at the membrane (Fig. 4B). The deleted Ca$_{\alpha1}$,Ca$_{\beta3}$ 180–364 construct preserved the typical Ca$_{\alpha1}$,Ca$_{\beta}$ modulation of channel gating and inactivation current kinetics. Peak whole cell current density was not significantly affected (Fig. 5).

The Ca$_{\alpha1}\cdot$Ca$_{\beta1}$ interaction appears to require the MMKQAL motif in the $\alpha3$ helix of the NK domain and was identified in the crystal structure (21–23) as critical for the high affinity AID-ABP interaction. Indeed deletion of the 195–200 residue region of Ca$_{\alpha1}$,Ca$_{\beta3}$ completely abolished plasma membrane targeting of Ca$_{\alpha1,2}$ (Fig. 4B), and the single point mutation M196A in Ca$_{\alpha1}$,Ca$_{\beta3}$, equivalent to M245 in Ca$_{\alpha1}$,Ca$_{\beta2}$ (29), significantly decreased plasma membrane targeting with only 13 ± 1% ($n = 3$) fluorescent cells (supplemental Table SIII). Nonetheless, the NK domain (181–362 fragment) alone was not sufficient for targeting Ca$_{\alpha1,2}$ to the membrane (Fig. 4D). Only the larger fragment (58–362) that includes part of the SH3 domain was verified by Western blot (Fig. 4, C and E).

Calmodulin in the Plasma Membrane Targeting of Ca$_{\alpha1,2}$ — CaM interacts with multiple sites in the Ca$_{\alpha1}\cdot$Ca$_{\beta}$ subunit of Ca$_{\alpha1,2}$ (48, 61), of which the C-terminal pre-IQ and IQ...
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domains are best characterized. Constitutive CaM binding to the N terminus has also been reported (62). To determine whether low affinity binding of CaM to intracellular regions contributes to trafficking of CaV1.2 channels (50, 63), CaV1.2 was co-expressed with CaM wt or the dominant negative mutant of CaM (CaM1,2,3,4) in HEKT control cells (supplemental Fig. S8) and in CaVβ3 stable cells. Overexpression of CaM wt or its negative dominant mutant in CaVβ3 stable cells did not significantly alter whole cell currents measured in the presence of 2 mM Ca\(^{2+}\) with peak current densities of \(-8 \pm 2\) pA/pF (n = 9) (CaM wt) and of \(-9 \pm 3\) pA/pF (n = 9) (CaM1,2,3,4), whereas co-expression with the latter significantly decreased calcium-dependent inactivation kinetics (supplemental Fig. S9). Cytometry flux assays also failed to show a change in the plasma membrane expression of CaV1.2 with or without CaVβ3 (Fig. 6 and supplemental Table SIV). These data contrast with previous reports that CaM1,2,3,4 co-expression reduced peak CaV1.2 current amplitude in HEK cells compared with CaM co-expression (8). It suggests that CaVβ3 is the dominant subunit to promote plasma membrane targeting of CaV1.2 and that CaM does not act synergistically with CaVβ3 under these conditions.

Overexpression of CaM wt was reported to promote the plasma membrane targeting of CaV1.2 proteins in COS1 cells, provided there was a complete absence of CaVβ3 (63). To test the hypothesis that CaM could chaperone CaV1.2 to the membrane in the presence of CaVα2bδ in our expression system, FACS experiments were carried out in the stable CaVα2bδ cell line. As seen in Fig. 6B, CaM was unable to increase the number of CaV1.2 proteins at the membrane in the absence of CaVβ3 under these conditions. Overexpression of CaM wt with the double mutant W470A/I471A (supplemental Table SIV) also failed to promote plasma membrane targeting of CaV1.2, thus ruling out a mechanism whereby low affinity binding of CaVβ subunit to the AID region could mask the CaM effect.

The 1643–1666 fragment in the C terminus forms the high affinity (K\(_d\) > 3 nM) IQ-binding domain that co-crystallized...
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FIGURE 7. Mutations within the high affinity CaM binding motif did not alter the CaV1.2 stimulation of CaV1.2 plasma membrane targeting. A, flow cytometry data. HA-tagged CaV1.2 wt and mutants were expressed transiently either in HEKT cells or in the stable Cavβ3 cell line. Cell surface expression of the CaV1.2 protein was quantified as described for supplemental Fig. S2. The number of fluorescent cells decreased significantly for the mutants ΔC1623–1666, I1654A, I1654A/Q1655A, and T1591A/L1592A/F1593A (p < 0.01) as compared with the CaV1.2-HA wt protein under the same conditions. From left to right, the channels were CaV1.2-HA wt, ΔC1623–1666, ΔC1643–1666, I1654A, Q1655A, I1654A/Q1655A, Y1657A, F1658A, Y1657A/F1658A, and T1591A/L1592A/F1593A. The numerical values can be found in supplemental Table SIV. B, Western blot analyses confirmed that the CaV1.2 mutant proteins were detected in total cell lysates by the anti-HA (1:500) with the expected molecular weight. Lane 1, nontransfected cells. Lane 2, CaV1.2-HA. Lane 3, CaV1.2-HA ΔC1643. Lane 4, CaV1.2-HA I1654A. Lane 5, CaV1.2-HA ΔC1644–1666. Lane 7, CaV1.2-HA ΔC1623–1666. Each lane was loaded with 50 µg of protein. C, Western blot analyses confirmed that the CaV1.2 mutant proteins were detected in total cell lysates by the anti-HA (1:500) with the expected molecular weight. Lane 1, nontransfected cells. Lane 2, CaV1.2-HA wt. Lane 3, CaV1.2-HA T1591A/L1592A/F1593A. Each lane was loaded with 50 µg of protein.

with CaM (49). This high affinity binding site overlaps with the C-terminal “targeting domain” identified previously (42, 64). To test the hypothesis that constitutive calmodulin binding to the IQ motif is required for plasma membrane targeting, FACs experiments were carried out after mutations of the aromatic residues responsible for the high affinity (Kd ≈ 3 nM) CaM binding (49). Complete deletion of the 1643–1666 fragment did not alter surface labeling, whereas the W470A mutation in the ΔIQ channel eliminated plasma membrane targeting of CaV1.2 (supplemental Table SIV), suggesting that the IQ domain is not likely to act as a retention signal. Furthermore, point mutations Q1655A, Y1657A, and F1658A, as well as multiple mutations I1654A/Q1655A, I1654A/Q1655A/Y1657A/F1658A, and I1654A/I1655A/F1658A did not alter plasma membrane targeting of CaV1.2. Plasma membrane targeting was not affected by a triple mutation in the pre-IQ domain (CaV1.2 T1651A/F1652A/L1653A) and was modestly supported in the I1654A and I1654A/Q1655A mutants (Fig. 7 and supplemental Table SIV). It should be remembered that the I/E mutation only moderately affected Ca2+/CaM binding to the C-terminal peptide of CaV1.2 as compared with the I/E mutant (65). Deleting the larger 1623–1666 region, identified as an important targeting domain (42), completely eradicated the plasma membrane expression of CaV1.2 both in the presence and in the absence of CaVβ3 (Fig. 7 and supplemental Table SIV). Furthermore, as shown by others before (50), the CaV1.2 protein could not be detected at the membrane in the presence of the triple T1591A/L1592A/F1593A mutation. The three TLF residues are located in a pre-IQ apocalmodulin-binding site (peptide A) (48, 66), but overexpression with CaM wt or CaM1,2,3,4 did not rescue plasma membrane targeting (supplemental Table SIV). Altogether, these data highlight the role of the C terminus in the plasma membrane targeting of CaV1.2 and suggest that high affinity Ca2+/CaM binding is not critical for the plasma membrane targeting of CaV1.2.

DISCUSSION

To exhibit functional activity, ion channels must be targeted to the plasma membrane. Co-expression of CaV1.2 with either CaVα2δ or CaM (alone or in combination) failed to promote significantly the detection of fluorescently labeled CaV1.2-HA channels in intact cells by flow cytometry. Co-expression of CaV1.2 in the presence of CaVβ3 with either CaVα2δ or CaM failed to further increase the number of CaV1.2 proteins detected at the plasma membrane. Furthermore, plasma membrane targeting of AID-disrupted CaV1.2 mutants (thus in the absence of high affinity CaVβ binding) could not be recovered by overexpressing the calmodulin protein alone or in combination with the auxiliary CaVα2δ subunit, suggesting that CaVβ is the critical auxiliary subunit in the plasma membrane targeting of CaV1.2.

Plasma membrane targeting of CaV1.2 was decreased but not abolished in the double I1654A/Q1655A mutant in the presence of CaVβ3 and was not altered in the absence of CaVβ3,
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The mechanism whereby Ca/β antagonizes ER retention of the Ca/α1 subunit remains debated (73). In the two-site model, Ca/β stimulation of protein expression and modulation of gating are controlled by distinct sites through a two-to-one stoichiometry. This model opens up the possibility that secondary Ca/β-binding sites could contribute to plasma membrane targeting. Several observations could be suggestive of such a mechanism. Surface expression of Ca/1.2-ΔAID-expressing oocytes was not increased by injection of the Ca/β2a protein, and even decreased gating currents and surface expression of the Ca/1.2-ΔAID-expressing oocytes (36, 74). Covalently linking Ca/β2b to the C terminus of Ca/1.2 stimulated whole cell currents but failed to modulate channel gating in HEK cells (37, 38). Ca/β2b-induced modulation of trafficking and gating was also uncoupled in N-terminally truncated Ca/1.2 (39). Deletion of a low affinity interaction site between the SH3 module of Ca/β and the I-II linker of the Ca/2.1 subunit (outside the AID/GK interaction) did not affect Ca/2.1 protein trafficking (40). Small fragments of Ca/β2 arising from putative splice variants were also shown to bind to the C terminus of the Ca/1.2 subunit where they promoted membrane targeting in the absence of the GK/SH3 module of Ca/β subunits (75, 76). It remains, however, difficult to assess the physiological relevance of these findings, given that they result from in vitro interaction studies between isolated peptides.

In the one-site model, Ca/β interacts sequencially with the Ca/α1 subunit through a unique binding site in the I-II linker in a 1:1 stoichiometry to dislodge ER retention signals and modulate gating (25). In the intact channel, high affinity binding of Ca/β onto the AID motif would account for both Ca/β-induced modulation of gating and the Ca/β-plasma membrane trafficking of Ca/α1 (32, 77, 78). For Ca/1.2 expressed in a mammalian cell system, the Trp470 and Ile471 residues previously shown to account for the high affinity binding of Ca/β (29) onto the Ca/1.2 subunit were herein found to account for the Ca/β stimulation of Ca/1.2 plasma membrane targeting. As mentioned earlier, disruption of these residues alone or in combination had a dominant effect and abrogated cell surface labeling of Ca/1.2. Our data hence support a model whereby high affinity binding of the MMQKAK motif of Ca/β to the AID helix of the Ca/α1 subunit is required for chaperoning and modulating HVA Ca/ channels.

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