Molecular Determinants of the Ca\textsubscript{v}\(\beta\)-induced Plasma Membrane Targeting of the Ca\textsubscript{v}1.2 Channel\textsuperscript{a}\textsuperscript{b}特色小镇

Benoîte Bourdin\textsuperscript{1}, Fabrice Marger\textsuperscript{1}, Sébastien Wall-Lacelle\textsuperscript{2}, Toni Schneider\textsuperscript{3}, Hélène Klein\textsuperscript{1}, Rémy Sauvé\textsuperscript{1}, and Lucie Parent\textsuperscript{1,1}\textsuperscript{1}\textsuperscript{1}

From the \textsuperscript{1}Institute of Neurophysiology and Center for Molecular Medicine, Cologne University, D-50931 Cologne, Germany and the \textsuperscript{2}Département de Physiologie et Membrane Protein Research Group, Université de Montréal, Montréal, Québec H3C 3J7, Canada

Ca\textsubscript{v}\(\beta\) subunits modulate cell surface expression and voltage-dependent gating of high voltage-activated (HVA) Ca\textsubscript{v}1 and Ca\textsubscript{v}2 \(\alpha\) subunits. High affinity Ca\textsubscript{v}\(\beta\) binding onto the so-called \(\alpha\) interaction domain of the I-II linker of the Ca\textsubscript{v}\(\alpha\)1 subunit is required for Ca\textsubscript{v}\(\beta\) modulation of HVA channel gating. It has been suggested, however, that Ca\textsubscript{v}\(\beta\)-mediated plasma membrane targeting could be uncoupled from Ca\textsubscript{v}\(\beta\)-mediated modulation of channel gating. In addition to Ca\textsubscript{v}\(\beta\), Ca\textsubscript{v}\(\alpha\)2D and calmodulin have been proposed to play important roles in HVA channel targeting. Indeed we show that co-expression of Ca\textsubscript{v}\(\alpha\)2D caused a 5-fold stimulation of the whole cell currents measured with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}3. To gauge the synergetic role of auxiliary subunits in the steady-state plasma membrane expression of Ca\textsubscript{v}1.2, extracellularly tagged Ca\textsubscript{v}1.2 proteins were quantified using fluorescence-activated cell sorting analysis. Co-expression of Ca\textsubscript{v}1.2 with either Ca\textsubscript{v}\(\alpha\)2D, calmodulin wild type, or apocaltmodulin (alone or in combination) failed to promote the detection of fluorescently labeled Ca\textsubscript{v}1.2 subunits. In contrast, co-expression with Ca\textsubscript{v}\(\beta\)3 stimulated plasma membrane expression of Ca\textsubscript{v}1.2 by a 10-fold factor. Mutations within the \(\alpha\) interaction domain of Ca\textsubscript{v}1.2 or within the nucleotide kinase domain of Ca\textsubscript{v}\(\beta\)3 disrupted the Ca\textsubscript{v}\(\beta\)-induced plasma membrane targeting of Ca\textsubscript{v}1.2. Altogether, these data support a model where high affinity binding of Ca\textsubscript{v}\(\beta\) to the I-II linker of Ca\textsubscript{v}\(\alpha\)1 largely accounts for Ca\textsubscript{v}\(\beta\)-induced plasma membrane targeting of Ca\textsubscript{v}1.2.

Voltage-dependent Ca\textsuperscript{2+} channels (Ca\textsubscript{v}) are membrane proteins that play a key role in promoting Ca\textsuperscript{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\textsubscript{v}\(\alpha\) subunits (1–7) that are classified into three main subfamilies according to their high voltage-activated (HVA)\textsuperscript{2} gating (Ca\textsubscript{v}1 and Ca\textsubscript{v}2) or low voltage-activated gating (Ca\textsubscript{v}3). In addition to the transmembrane pore-forming Ca\textsubscript{v}\(\alpha\)1 subunit, Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels arise from the multimerization of three other proteins (7): a cytoplasmic Ca\textsubscript{v}\(\beta\) subunit, a mostly extracellular Ca\textsubscript{v}\(\alpha\)28 subunit, and calmodulin constitutively bound to the C terminus of Ca\textsubscript{v}\(\alpha\)1 (8–12).

A considerable body of work documents the interaction and modulation of the Ca\textsubscript{v}\(\alpha\)1 subunit of Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels (13–18) by the auxiliary Ca\textsubscript{v}\(\beta\). The high affinity Ca\textsubscript{v}\(\alpha\)1-Ca\textsubscript{v}\(\beta\) interaction site on the pore-forming Ca\textsubscript{v}\(\alpha\)1 subunit is a conserved 18-residue sequence in the I-II linker called the \(\alpha\) 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 \(\alpha\)-helix that binds to the \(\alpha\) binding pocket (ABP) in the Ca\textsubscript{v}\(\beta\) 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\textsubscript{v}\(\beta\)-induced biophysical modulation with the high affinity binding of Ca\textsubscript{v}\(\beta\) to the AID peptide in a variety of Ca\textsubscript{v}\(\alpha\)1 isoforms for Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels (25, 29–32).

The association of Ca\textsubscript{v}\(\alpha\)1 and Ca\textsubscript{v}\(\beta\) subunits is also critical for proper channel maturation and cell surface expression of Ca\textsubscript{v}2.2 (17), Ca\textsubscript{v}1.2 (33, 34), and Ca\textsubscript{v}2.3 (35). In Ca\textsubscript{v}2.2, 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\textsubscript{v}\(\beta\)1b and Ca\textsubscript{v}\(\beta\)3 (32). In addition, the Ca\textsubscript{v}2.2-induced increase in Ca\textsubscript{v}1.2 whole cell currents was abolished with the AID-defective YWI/AAA mutant (29), suggesting that high affinity binding of Ca\textsubscript{v}\(\beta\) onto AID is required to traffic Ca\textsubscript{v}\(\alpha\)1 to the plasma membrane. Nonetheless, the unique character of the high affinity AID-ABP interface in the membrane targeting of Ca\textsubscript{v}\(\alpha\)1 has been questioned (27, 36–40). In particular, it has been suggested that Ca\textsubscript{v}\(\beta\)-mediated plasma membrane targeting could be uncoupled from Ca\textsubscript{v}\(\beta\)-mediated modulation of channel gating (26, 41) with important contributions from other intracellular regions (33, 39, 42–44).

In addition to Ca\textsubscript{v}\(\beta\), the ancillary subunit Ca\textsubscript{v}\(\alpha\)2D and the ubiquitous calmodulin (CaM) protein have also been proposed to modulate HVA channel maturation and targeting (9). For instance, co-expression of Ca\textsubscript{v}\(\alpha\)2D promoted the trafficking of the Ca\textsubscript{v}\(\alpha\)1 subunit of Ca\textsubscript{v}2.2 in COS-7 cells (45), suggesting that Ca\textsubscript{v}\(\alpha\)2D could promote targeting of all HVA Ca\textsubscript{v}\(\alpha\)1 subunits.
united. 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 CaV1 and CaV2 channels (46), of which CaV1.2 and CaV2.1 have been best characterized (8, 47). Constitutive apicalmodulin binding was reported on multiple sites in the CaVα1 subunit of CaV1.2 (48) of which the C-terminal pre-IQ and IQ domains are best characterized (49). Mutations (TLF/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 CaV1.2, 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 CaV1.2, we used a flow cytometry assay with an extracellularly HA-tagged CaV1.2 protein. Co-expression with CaVα3 produced a robust enhancement in the plasma membrane targeting of the CaVα1 subunit of CaV1.2. The WI residues in the AID helix of the I-II linker of CaV1.2 were critical for CaVα3-stimulated plasma membrane targeting of CaV1.2. No other combination with or without the auxiliary calmodulin and/or the CaVα2β subunit produced any significant increase in the plasma membrane targeting of CaV1.2. Hence, CaVαβ appears to be the most potent determinant in the plasma membrane targeting of CaV1.2. Altogether, our data support a model where high affinity binding of the ABP of CaVαβ to the AID helix of CaVα1 largely accounts for CaVαβ-induced plasma membrane targeting of CaV1.2.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Techniques**—The rabbit CaV1.2 (GenBank accession number X15539), the rat CaVα3 (GenBank accession number M88751) (52), the rat brain CaVα2β (GenBank accession number NM_000722) (53), and the human CaM (GenBank accession number X15539), the rat brain CaV2b (GenBank accession number M88751) (52), the rat brain CaV2b (GenBank accession number NM_000722) (53), and the human CaM (GenBank accession number M88751) (52) 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 CaVα3 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 CaVα3 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 (CaM1-2,3-4) 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 CaVα1 Subunit**—The hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted in the first extracytoplasmic predicted loop in Domain I at position 574 (nucleotide) for CaV1.2. The biophysical properties of the HA-tagged CaVα1 subunit of CaV1.2 expressed in HEKT cells with the auxiliary CaVα3 subunit were found not to be significantly different from the wild type CaV1.2 channel expressed under the same conditions (see Fig. 1). In addition, cDNA injection of CaV1.2-HA constructions in concert with CaVα2β and CaVα3 subunits in Xenopus oocytes yielded a biophysical profile not significantly different from that reported previously for the CaV1.2 (56) channels expressed under the same conditions. Hence, the HA-tagged version of the CaVα1 subunit of CaV1.2 will be referred to as CaV1.2 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% CO2 atmosphere. COS1, HEK7, stable CaVαβ, and CaVα2β cells lines were transiently transfected with HA-tagged CaV1.2 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 blotsing (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 CaV1.2 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 CaV1.2 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, Québec, Canada) and revealed with an antimouse horseradish peroxidase secondary antibody (1:10000; Jackson ImmunoResearch).

**Fluorescence-activated Cell Sorting (FACS) Experiments**—Cell surface expression of the CaV1.2 subunits was determined by flow cytometry using a FACSCalibur® 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 CaV1.2 proteins (see supplemental Figs. S1 and S2 and supplemental text for details). The HA-tagged CaV1.2 construct was systematically tested as a control with the mutant channels.

**Immunofluorescence**—For fluorescence microscopy, CaVα3 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 CaV1.2 channels (wild type and mutant) were visualized (×60) using an Olympus microscope IX-81 microscope along with Image-Pro Plus 5.0 software.
Role of the AID Helix in Channel Targeting

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

**Ca\(_{\alpha2}\) Increases Whole Cell Currents of Ca\(_{\gamma1.2}\)—**Co-expression of Ca\(_{\gamma1.2}\) and Ca\(_{\gamma2.1}\) with the auxiliary Ca\(_{\alpha2}\) subunit was shown to stimulate whole cell currents (45) in COS-7 cells, suggesting that Ca\(_{\alpha2}\) could promote plasma membrane targeting of HVA Ca\(_{\gamma}\) subunits. In Ca\(_{\gamma1.2}\), the gating charge appears to be unaffected by co-expression with Ca\(_{\alpha2}\), suggesting that Ca\(_{\alpha2}\) stimulates channel facilitation by setting Ca\(_{\gamma1.2}\) channels in a conformational state very close to the open state without increasing protein density (57). To evaluate the functional role of Ca\(_{\alpha2}\), Ca\(_{\gamma1.2}\) wt and HA-tagged Ca\(_{\gamma1.2}\) a1 subunits were transiently transfected in the Ca\(_{\gamma3}\) stable HEK7 cell line in the absence and in the presence of Ca\(_{\alpha2}\). As shown in Fig. 1A, whole cell currents, recorded in the presence of a physiological solution containing 2 mM Ca\(^{2+}\) (see the supplemental text), were significantly larger when measured in the presence of the Ca\(_{\alpha2}\), confirming that Ca\(_{\alpha2}\) stimulates whole cell currents of Ca\(_{\gamma1.2}\) (9). As shown in Fig. 1B, average whole cell current density increased from \(-7 \pm 2\) pA/pF (\( n = 6 \)) for the wild type Ca\(_{\gamma1.2}\) channel in the stable Ca\(_{\gamma3}\) stable cell line as compared with a current density of \(-41 \pm 9\) pA/pF (\( n = 7 \)) for the wild type Ca\(_{\gamma1.2}\) channel measured in the same cell line after transient transfection with Ca\(_{\alpha2}\) subunit. Similar results were obtained for the HA-tagged Ca\(_{\gamma1.2}\) channels (Fig. 1B).

**Ca\(_{\alpha}\) Promotes Membrane Targeting of Ca\(_{\gamma1.2}\)—**To determine whether Ca\(_{\alpha2}\) stimulates plasma membrane targeting of Ca\(_{\gamma1.2}\) channels, protein density of the extracellularly HA-tagged Ca\(_{\gamma1.2}\) 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 Ca\(_{\alpha1}\) and the auxiliary subunit (either Ca\(_{\beta3}\) or Ca\(_{\alpha2}\)) 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 Ca\(_{\gamma1.2}\) subunit in the stable Ca\(_{\gamma3}\) cell line increased the number of proteins detected at the membrane from a value of 4.5 \pm 0.5% (\( n = 25 \)) in the nontransfected cell line to 23 \pm 1% (\( n = 29 \)) with Ca\(_{\gamma3}\)/Ca\(_{\gamma1.2}\) (\( p < 0.001 \)) (Table 1).

The results obtained with Ca\(_{\gamma3}\) contrast with the effect observed when co-expressing HA-tagged Ca\(_{\gamma1.2}\) with Ca\(_{\alpha2}\) (\( p > 0.1 \)). No further increase in the fluorescent signal was observed in the combined presence of the two auxiliary subunits. Similar results were obtained when Ca\(_{\gamma2.1}\) was transiently expressed, either in a background of stably transfected Ca\(_{\gamma3}\) or in a background of stably transfected Ca\(_{\alpha2}\) cells (see Table 1 for numerical values). The maximum fluorescence obtained with Ca\(_{\gamma3}\) confirms that Ca\(_{\alpha2}\) has little effect by itself on the Ca\(_{\gamma1.2}\) protein density at the plasma membrane. Among Ca\(_{\alpha}\) subunits, transient co-expression of Ca\(_{\gamma1.2}\)-HA with Ca\(_{\gamma3}\) caused a similar boost in plasma membrane expression, whereas Ca\(_{\gamma2a}\) was found to be slightly less potent for a Ca\(_{\gamma3}\) \( \approx \) Ca\(_{\gamma2}\) ranking (Table 1). Altogether, these results validated the fluorescence sorting analysis of HA-tagged Ca\(_{\gamma3}\) proteins to evaluate steady-state protein level in intact cells independently of channel gating.

**\( \alpha \) Interaction Domain: the Role of the WI Pair—**Crystallographic analyses have shown that the AID-Ca\(_{\gamma}\) interaction is...
Role of the AID Helix in Channel Targeting

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 Ca\textsubscript{v}1.2 (21–23), with the W1 pair of residues being most critical for the AID-Ca\textsubscript{v}β protein interaction (29). To evaluate whether the AID-Ca\textsubscript{v}β interaction controls the plasma membrane targeting of Ca\textsubscript{v}1.2, the HA-tagged Ca\textsubscript{v}1.2 subunit was transiently co-expressed in HEKT cells or in Ca\textsubscript{v}α2bβ stable cells. Ca\textsubscript{v}β3 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 (±Ca\textsubscript{v}β3), 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 Ca\textsubscript{v}β3-induced stimulation in the plasma membrane targeting of Ca\textsubscript{v}1.2. In contrast, the Ca\textsubscript{v}β3 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 Ca\textsubscript{v}β3.

Point mutations in the C-terminal W1 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 Ca\textsubscript{v}β3. However, Ca\textsubscript{v}β3 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 Ca\textsubscript{v}β3 (Fig. 3C and supplemental Table SII). Western blots carried out in total cell lysates with the anti-HA confirmed that all of the Ca\textsubscript{v}1.2 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 Ca\textsubscript{v}1.2 in the presence of Ca\textsubscript{v}β3 (supplemental Fig. S5). Membrane expression of Ca\textsubscript{v}1.2 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 Ca\textsubscript{v}β3 stimulation of

![FIGURE 2. Ca\textsubscript{v}β stimulated Ca\textsubscript{v}1.2 membrane expression in HEKT cells.](image)

**TABLE 1**

Fluorescence-activated cell sorting analysis of Ca\textsubscript{v}1.2 ± auxiliary subunits

FACS results obtained after the transient transfection of Ca\textsubscript{v}1.2-HA wt in either HEKT control cells, stable Ca\textsubscript{v}β3 cells, or stable Ca\textsubscript{v}α2bβ 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 cytometer (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.

<table>
<thead>
<tr>
<th>Construct transient expression</th>
<th>Cell lines</th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEKT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells without antibody</td>
<td></td>
<td>0.3</td>
<td>±0.1 (32)</td>
<td>0.18 ± 0.04 (20)</td>
<td>0.54 ± 0.07 (3)</td>
</tr>
<tr>
<td>Cells with antibody</td>
<td></td>
<td>1.1</td>
<td>±0.3 (19)</td>
<td>1.0 ± 0.4 (19)</td>
<td>0.93 ± 0.05 (3)</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}β3</td>
<td></td>
<td>4.5</td>
<td>±0.5 (25)</td>
<td>2.3 ± 1.29 (29)</td>
<td>6 ± 1.3 (3)</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}β2a</td>
<td></td>
<td>25</td>
<td>±2 (3)</td>
<td>ND</td>
<td>21 ± 2 (3)</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}β4</td>
<td></td>
<td>14</td>
<td>±1 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}α2bβ</td>
<td></td>
<td>22</td>
<td>±3 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}α2bβ + CaM wt</td>
<td></td>
<td>2</td>
<td>±1 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}α2b6</td>
<td></td>
<td>8</td>
<td>±3 (5)</td>
<td>ND</td>
<td>26 ± 1 (3)</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + CaM wt</td>
<td></td>
<td>1.5</td>
<td>±0.3 (3)</td>
<td>19.1 ± 0.3 (3)</td>
<td>2.7 ± 0.4 (3)</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + CaM wt</td>
<td></td>
<td>1.4</td>
<td>±0.1 (3)</td>
<td>19.1 ± 0.6 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2-HA wt + Ca\textsubscript{v}β3 + Ca\textsubscript{v}α2b8</td>
<td></td>
<td>22</td>
<td>±2 (6)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
CaV1.2 plasma membrane targeting (supplemental Fig. S6 and Table SI). Partial (L458–463) or complete (L458–475) removal of the AID-binding site within the I-II loop yielded similar results, confirming that no other low affinity CaV2.2a site within the CaV1.2 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 CaV2.2a for the AID region of CaV1.2 decreased after single-point mutations of these residues. There was a 1000-fold increase in the Kd 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 CaV2.2a 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 CaV2.2a-induced membrane targeting of CaV1.2. For comparison, I387L in CaV2.3 was the only mutant tested in the WI pair that supported CaV2.3 binding and CaV2.3 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 CaV2.3 plasma membrane targeting of CaV1.2 (supplemental Fig. S7 and Table SII).

FIGURE 3. Point mutations within the C-terminal residues on the AID helix disrupted the Caβ stimulation of CaV1.2 plasma membrane targeting. A, HA-tagged CaV1.2 wt and mutants were expressed transiently either in the HEKT cells or in the stable Caβ3 cell line. Cell surface expression of the CaV1.2 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. The number of fluorescent cells decreased in the order CaV1.2-HA wt ~ L464A, G466A, G466F, Y467G > Y467A, Y467F, G466A/Y467A > Y467S, G466A/Y467G > G466A/Y467F. The numerical values can be found in supplemental Table SI. B, Western blot analyses of HEKT cells transiently transfected with CaV1.2 wt or mutants in stable Caβ3 cells using HA (1:500) and Caβ3 (1:500) antibodies. Lane 1, control nontransfected cells. Lane 2, CaV1.2-HA. Lane 3, CaV1.2-HA + Caβ3. Lane 4, CaV1.2-HA W470A. Lane 5, CaV1.2-HA W470A + Caβ3. Lane 6, CaV1.2-HA G466A/Y467A. Lane 7, CaV1.2-HA G466A/Y467A + Caβ3. 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. C, HA-tagged CaV1.2 wt and mutants were expressed transiently either in the HEKT cells or in the stable Caβ3 cell line. Cell surface expression of the CaV1.2 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 => I471G, W470A, W470F, W470G, W470Y. The numerical values can be found in supplemental Table SI. D, Western blot analyses confirmed that the CaV1.2-W470A, I471A, I471L, and I471R mutants expressed with the expected molecular weight in total cell lysates and were 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.
The NK domain of Ca<sub>v</sub>β3 is critical for the plasma membrane targeting of Ca<sub>v</sub>1.2. A, schematic diagram of the domain organization of the Ca<sub>v</sub>β3 subunit based on the crystal structure and adapted from (21). B, HA-tagged Ca<sub>v</sub>1.2 wt and Ca<sub>v</sub>β3 mutants were expressed transiently in the HEKT cell lines. Cell surface expression of the Ca<sub>v</sub>1.2 protein was quantified as described for supplemental Fig. S2. The residues targeted in these experiments are identified in the primary sequence of Ca<sub>v</sub>β3. The Δ57–123 deletion removed the SH3 domain; the Δ170–175 removed the PYDVVP sequence; and the Δ195–200 removed the MMQKAL sequence, also termed the ABP domain. The numerical values are provided in supplemental Table SIII. C, Western blot analyses of HEKT cell lysates transiently transfected with Ca<sub>v</sub>β3 constructs using anti-Ca<sub>v</sub>β3 (1:500). Each lane was loaded with 10 μg of protein except for Ca<sub>v</sub>β3 Δ54–362 loaded with 50 μg of protein. Lane 1, control nontransfected cells. Lane 2, Ca<sub>v</sub>β3 wt. Lane 3, Ca<sub>v</sub>β3 M196A. Lane 4, Ca<sub>v</sub>β3 Δ170–175. Lane 5, Ca<sub>v</sub>β3 Δ195–200. Lane 6, Ca<sub>v</sub>β3 Δ54–120. Lane 7, Ca<sub>v</sub>β3 Δ57–175. Lane 8, Ca<sub>v</sub>β3 Δ180–364. Lane 9, Ca<sub>v</sub>β3 Δ54–362. Western blot analyses confirmed that the Ca<sub>v</sub>β3 deleted proteins were detected in total cell lysates with the expected molecular weight. D, HA-tagged Ca<sub>v</sub>1.2 wt and Ca<sub>v</sub>β3 fragments were expressed transiently in the HEKT cells. Cell surface expression of the Ca<sub>v</sub>1.2 protein was quantified as described for supplemental Fig. S2. The residues targeted in these experiments are identified in the primary sequence of the Ca<sub>v</sub>β3. The 181–362 fragment is equivalent to the NK domain. The numerical values are provided in supplemental Table SIII. E, Western blot analyses of HEKT cell lysates transiently transfected with the Ca<sub>v</sub>β3 fragments using anti-c-Myc (1:500). Each lane was loaded with 50 μg of protein. Lane 1, control nontransfected cells. Lane 2, Ca<sub>v</sub>β3 wt. Lane 3, Ca<sub>v</sub>β3 58–120. Lane 4, empty lane. Lane 5, Ca<sub>v</sub>β3 181–362. The Ca<sub>v</sub>β3 58–120 fragment formed a 7.4-kDa protein that cannot be seen in this figure. Western blot analyses confirmed that the Ca<sub>v</sub>β3 fragments were detected in total cell lysates with the expected molecular weight.

Altogether our results support a strong correlation between Ca<sub>v</sub>β3 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<sub>v</sub>1.2 proteins at the plasma membrane. More importantly our results suggest that the molecular determinants that account for Ca<sub>v</sub>β3 binding to the AID region are also responsible for the Ca<sub>v</sub>β3-induced stimulation of the plasma membrane targeting of Ca<sub>v</sub>1.2 proteins.

The NK Domain of Ca<sub>v</sub>β3 Is Essential for Plasma Membrane Targeting of Ca<sub>v</sub>1.2—The observation that different Ca<sub>v</sub>βs, which all share a conserved core containing the SH3 and NK domains, cause different biophysical effects on Ca<sub>v</sub>α1 subunits suggests that other regions besides the conserved AID-ABP interaction, could influence channel conformational changes (13). The SH3 domain of Ca<sub>v</sub>β2a was found to bind to the I–II linker of Ca<sub>v</sub>2.1 channels, suggesting that low affinity interactions outside of the AID-ABP interface could contribute to the full functional effects of the Ca<sub>v</sub>β subunit (40). A few years later, however, the conserved AID-NK domain interaction was found to be necessary for Ca<sub>v</sub>β-stimulated Ca<sub>v</sub>2.1 channel surface expression (60). To evaluate whether the AID-NK interaction controls the plasma membrane targeting of Ca<sub>v</sub>1.2, the HA-tagged Ca<sub>v</sub>1.2 subunit was transiently co-expressed in HEKT cells in the presence of Ca<sub>v</sub>β full-length or deleted constructs as well as with Ca<sub>v</sub>β3 fragments.

We found that the NK domain of Ca<sub>v</sub>β3 (180–364) (Fig. 4A) was required for the plasma membrane targeting of Ca<sub>v</sub>1.2 (Fig. 4B and supplemental Table SIII). Targeted deletion of the SH3 domain between residues 57 and 123 preserved 80% of the Ca<sub>v</sub>1.2 protein detected at the membrane (Fig. 4B). The deleted Ca<sub>v</sub>β3 Δ57–123 construct preserved the typical Ca<sub>v</sub>β3 modulation of channel gating and inactivation current kinetics. Peak whole cell current density was not significantly affected (Fig. 5).

The Ca<sub>v</sub>α1-Ca<sub>v</sub>β interaction appears to require the MMQKAL motif in the α3 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<sub>v</sub>β3 completely abolished plasma membrane targeting of Ca<sub>v</sub>1.2 (Fig. 4B), and the single point mutation M196A in Ca<sub>v</sub>β3, equivalent to M245 in Ca<sub>v</sub>β2a (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<sub>v</sub>1.2 to the membrane (Fig. 4D). Only the larger fragment (58–362) that includes part of the SH3 domain was found to stimulate significantly the plasma membrane targeting of Ca<sub>v</sub>1.2. The integrity of the constructions was verified by Western blot (Fig. 4, C and E).

Calmodulin in the Plasma Membrane Targeting of Ca<sub>v</sub>1.2—CaM interacts with multiple sites in the Ca<sub>v</sub>α1 subunit of Ca<sub>v</sub>1.2 (48, 61), of which the C-terminal pre-IQ and IQ
Role of the AID Helix in Channel Targeting

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 Ca_{v1.2} channels (50, 63), Ca_{v1.2} was co-expressed with CaM wt or the dominant negative mutant of CaM (CaM_{1,2,3,4}) in HEKT control cells (supplemental Fig. S8) and in Ca_{v3} stable cells. Overexpression of CaM wt or its negative dominant mutant in Ca_{v3} 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 ($n = 9$) (CaM_{1,2,3,4}), 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 Ca_{v1.2} with or without Ca_{v3} (Fig. 6 and supplemental Table SIV). These data contrast with previous reports that CaM_{1,2,3,4} co-expression reduced peak Ca_{v1.2} current amplitudes in HEK cells compared with CaM co-expression (8). It suggests that Ca_{v3} is the dominant subunit to promote plasma membrane targeting of Ca_{v1.2} and that CaM does not act synergistically with Ca_{v3} under these conditions.

Overexpression of CaM wt was reported to promote the plasma membrane targeting of Ca_{v1.2} proteins in COS1 cells, provided there was a complete absence of Ca_{v3} (63). To test the hypothesis that CaM could chaperone Ca_{v1.2} to the membrane in the presence of Ca_{v3}SUB-unit in our expression system, FACS experiments were carried out in the stable Ca_{v3} cell line. As seen in Fig. 6B, CaM was unable to increase the number of Ca_{v1.2} proteins at the membrane in the absence of Ca_{v3} under these conditions. Overexpression of CaM wt with the double mutant W470A/I471A (supplemental Table SIV) also failed to promote plasma membrane targeting of Ca_{v1.2}, thus ruling out a mechanism whereby low affinity binding of Ca_{v3} 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
Role of the AID Helix in Channel Targeting

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, ΔC1644–1666. Lane 3, Cavβ3 stable. Lane 4, CaV1.2-HA ΔC1643. Lane 6, CaV1.2-HA ΔC1644–1666. Lane 7, CaV1.2-HA ΔC1623–1666. Each lane was loaded with 50 μg of protein. B, Western blot analyses confirmed that 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, ΔC1644–1666, ΔC1654A, Q1655A, Y1657A, F1658A, Y1657A/F1658A, and T1591A/L1592A/F1593A. The numerical values can be found in supplemental Table SIV. 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 ΔC1643. Lane 4, CaV1.2-HA ΔC1644–1666. Lane 5, CaV1.2-HA ΔC1623–1666. 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 (K_D ≈ 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/F1658A and Y1657A/F1658A, and I1654A/Y1657A/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/A mutation only moderately affected Ca^2+÷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 apicalmodulin-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 Ca^2+÷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 CaVα2δ6 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 CaVα2δ6 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 CaVα2δ6 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,
Role of the AID Helix in Channel Targeting

The mechanism whereby Ca$_\alpha$-B antagonizes ER retention of the Ca$_\alpha$-a1 subunit remains debated (73). In the two-site model, Ca$_\alpha$-B 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$_\alpha$-B-binding sites could contribute to plasma membrane targeting. Several observations could be suggestive of such a mechanism. Surface expression of Ca$_\alpha$-1.2-HA channels in Xenopus oocytes was not increased by injection of the Ca$_\alpha$-b2a protein, and even decreased gating currents and surface expression of the Ca$_\alpha$-1.2-ΔAID-expressing oocytes (36, 74). Covalently linking Ca$_\alpha$-b2b to the C terminus of Ca$_\alpha$-1.2 stimulated whole cell currents but failed to modulate channel gating in HEK cells (37, 38). Ca$_\alpha$-b2b-induced modulation of trafficking and gating was also uncoupled in N-terminally truncated Ca$_\alpha$-1.2 (39). Deletion of a low affinity interaction site between the SH3 module of Ca$_\alpha$-B and the I-II linker of the Ca$_\alpha$-2.1 subunit (outside the AID-GK interaction) did not affect Ca$_\alpha$-2.1 protein trafficking (40). Small fragments of Ca$_\alpha$-B arising from putative splice variants were also shown to bind to the C terminus of the Ca$_\alpha$-1.2 subunit where they promoted membrane targeting in the absence of the GK/SH3 module of Ca$_\alpha$-B 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$_\alpha$-B interacts sequentially with the Ca$_\alpha$-a1 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$_\alpha$-B onto the AID motif would account for both Ca$_\alpha$-B-induced modulation of gating and the Ca$_\alpha$-B-plasma membrane trafficking of Ca$_\alpha$-a1 (32, 77, 78). For Ca$_\alpha$-1.2 expressed in a mammalian cell system, the Trp$^{470}$ and Ile$^{472}$ residues previously shown to account for the high affinity binding of Ca$_\alpha$-B (29) onto the Ca$_\alpha$-1.2 subunit were herein found to account for the Ca$_\alpha$-B stimulation of Ca$_\alpha$-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$_\alpha$-1.2. Our data hence support a model whereby high affinity binding of the MMQKAL motif of Ca$_\alpha$-B to the AID helix of the Ca$_\alpha$-a1 subunit is required for chaperoning and modulating HVA Ca$_\alpha$ channels.

Acknowledgments—We thank Serge Sénéchal and Dr. Jacques Thibodeau (Department of Microbiology and Immunology) for help with the fluorescence-activated cell sorting experiments and analysis; Michel Lauzon and Dr. Pierre Bissonnette for help with confocal and fluorescent microscopy; Yolaine Dodier, Alexandra Raybaud, Florian LeCoz, and Guillaume Roussel for preliminary experiments; Julie Verner for cell culture; Michel Brunette for computer maintenance; and Claude Gauthier for artwork.

REFERENCES

3. Birnbaumer, L., Campbell, K. P., Catterall, W. A., Harpold, M. M., Hof-
Role of the AID Helix in Channel Targeting

JULY 23, 2010•VOLUME 285•NUMBER 30

Biol. 160, 127–140