Proteolytic cleavage of the hydrophobic domain in the
CaVα2δ1 subunit improves assembly and activity of cardiac
CaV1.2 channels

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Voltage-gated L-type CaV1.2 channels in cardiomyocytes exist as heteromeric complexes with the pore-forming CaVα1, CaVβ, and CaVα2δ1 subunits. The full complement of subunits is required to reconstitute the native-like properties of L-type Ca2+ currents, but the molecular determinants responsible for the formation of the heteromeric complex are still being studied. Enzymatic treatment with phosphatidylinositol-specific phospholipase C, a phospholipase C specific for the cleavage of glycosylphosphatidylinositol (GPI)-anchored proteins, disrupted plasma membrane localization of the cardiac CaVα2δ1 prompting us to investigate deletions of its hydrophobic transmembrane domain. Patch-clamp experiments indicated that the C-terminally cleaved CaVα2δ1 proteins up-regulate CaV1.2 channels. In contrast, deleting the residues before the single hydrophobic segment (CaVα2δ1 Δ1059–1063) impaired current up-regulation. CaVα2δ1 mutants G1060I and G1061I nearly eliminated the cell-surface fluorescence of CaVα2δ1, indicated by two-color flow cytometry assays and confocal imaging, and prevented CaVα2δ1-mediated increase in peak current density and modulation of the voltage-dependent gating of CaV1.2. These impacts were specific to substitutions with isoleucine residues because functional modulation was partially preserved in CaVα2δ1 G1060A and G1061A proteins. Moreover, C-terminal fragments exhibited significantly altered mobility in denatured immunoblots of CaVα2δ1 G1060I and CaVα2δ1 G1061I, suggesting that these mutant proteins were impaired in proteolytic processing. Finally, CaVα2δ1 Δ1059–1063, but not CaVα2δ1 G1060A, failed to co-immunoprecipitate with CaV1.2. Altogether, our data support a model in which small neutral hydrophobic residues facilitate the post-translational cleavage of the CaVα2δ1 subunit at the predicted membrane interface and further suggest that preventing GPI anchoring of CaVα2δ1 averts its cell-surface expression, its interaction with CaVα1, and modulation of CaV1.2 currents.

In cardiac cells, Ca2+ signals control the force necessary for the myocardium to meet the physiological needs of the body (1). During diastole, the intracellular free ionized Ca2+ is maintained in the nanomolar range by the concerted action of mechanisms that prevent Ca2+ entry, promote its extrusion (mostly via the Na+/Ca2+ exchanger), and ensure its storage in the sarcoplasmic reticulum (2). Upon depolarization, Ca2+ enters the cell through the cardiac high-voltage-activated L-type CaV1.2 channel and initiates the myocardium contraction via Ca2+-induced Ca2+ release from the sarcoplasmic reticulum. Regulation of the L-type Ca2+ current has profound physiological significance. Alterations in density or the activation/inactivation gating of L-type CaV1.2 channels have been implicated in a variety of cardiovascular diseases such as hypertension (3), atrial fibrillation (4–7), heart failure (8, 9), and congenital arrhythmias (10–12).

L-type CaV1.2 channels are heteromultimeric protein complexes composed of the main pore-forming CaVα1 subunit non-covalently bound to the cytoplasmic CaVβ auxiliary subunit (CaVβ1–β4), the EF-hand protein calmodulin (constitutively bound to the C-terminus of CaVα1), and CaVα2δ1 subunit (13–17). The full complement of auxiliary subunits is required to produce CaV1.2 channels with the typical biophysical and biochemical properties of the native cardiac channels (18). CaVβ promotes the cell-surface trafficking of CaV1.2 channels through a high-affinity interaction (19) in part by preventing its degradation by the ubiquitin/proteasome system (20). Ca2+-dependent inactivation of CaV1.2 channels ensues following the interaction of Ca2+ with intracellular calmodulin (21). Co-expression of CaVα2δ1 with CaVβ-bound CaVα1 promotes the activation of CaV1.2 at more physiological voltages (22–27) by stabilizing the channel voltage sensors (28).

Over the last 15 years, structural studies have revealed the high-affinity interaction between CaVβ and CaVα1 as well as the Ca2+-calmodulin/CaVα1 association by X-ray crystallography (29). By contrast, there was until recently little structural information on CaVα2δ1. The reason can be found in the extreme complexity of the CaVα2δ1 protein topology that results from multiple co- and post-translational modifications.
GPI anchoring of the L-type Ca\textsubscript{v}2.61 subunit

Ca\textsubscript{v}2.61 arises from a single gene and is post-translationally cleaved into the large extracellular Ca\textsubscript{v}\alpha2 and the single-pass transmembrane Ca\textsubscript{v}\delta proteins bound by disulfide bridges (30–33). Ca\textsubscript{v}2.61 includes 20 cysteine residues, and it has been argued that intra-molecular disulfide bonds are required to stabilize its higher order structure. In addition, Ca\textsubscript{v}2.61 is glycosylated at 16 asparagine sites, which are required for the protein folding and stability (27, 34). These features represent significant hurdles for carrying the conventional protein expression and purification in bacterial systems. The three-dimensional (3D) structure of the skeletal muscle Ca\textsubscript{v}1.1 channel, recently solved by single particle cryo-electron microscopy (cryo-EM), provides so far the best high-resolution look at the native Ca\textsubscript{v}2.61 protein purified from a rabbit skeletal muscle T-tubule preparation in complex with the pore-forming Ca\textsubscript{v}\alpha1 from Ca\textsubscript{v}1.1 (Fig. 1A) (17, 35). The extracellular portion of the Ca\textsubscript{v}2.61 protein is constructed around five structural domains as follows: Cache1, von Willebrand factor A, Cache2, Cache3, and Cache4. There was, however, insufficient electron density to support amino acid assignment in a few specific regions, most notably in the “913–972” region and at the C-terminal domain between residues 1074 and 1106 (rabbit numbering) (17). These gaps in the 3D structure might be a consequence of the unintended action of proteases during the purification process, intrinsically disordered segments, or from an actual post-translational deletion. Although more than 11 different proteases can theoretically target 250 different sites in Ca\textsubscript{v}2.61 (36), the two deletions identified in the structure correspond to regions previously identified. From the first report of its purification, Ca\textsubscript{v}2.61 is known to be cleaved in the “913–972” region into Ca\textsubscript{v}2.2 and Ca\textsubscript{v}\delta proteins (30, 32, 37). The cleavage of the C-terminal region is also compatible with the proposed attachment of Ca\textsubscript{v}2.61 at the cell membrane through a glycosylphosphatidylinositol (GPI)\textsuperscript{4} anchor (32, 38, 39) at/or around residue Cys-1074 (rabbit isof orm). To date, more than 150 different human proteins are known to be GPI-anchored (40). This process, also referred to as glypia tion, occurs in the endoplasmic reticulum where the C terminus of type 1 transmembrane proteins is cleaved to be replaced by a GPI anchor (41–49). GPI modification promotes localization with membrane microdomains (40, 50) and/or confers biological activity by espousing the optimal conformation for protein-protein interaction (51).

Herein, we show that the native Ca\textsubscript{v}2.61 protein from rat cardiomyocytes is a substrate for prokaryotic phosphatidylinositol-phospholipase C (PI-PLC). Deletion of the last 24 C-terminal residues, including the hydrophobic domain of the rat Ca\textsubscript{v}2.61, had little impact on Ca\textsubscript{v}1.2 currents demonstrating that the C-terminally cleaved Ca\textsubscript{v}2.61 proteins achieve the conformation congruent with the modulation of Ca\textsubscript{v}1.2 channels. In contrast, deletion of four residues surrounding Cys-1059 (rat isof orm), which was the last amino acid identified in the 3D structure, impaired up-regulation of Ca\textsubscript{v}1.2 currents.

The migration profile of the C-terminal fragments was also significantly altered by single mutations in the “1059–1061” region, suggesting that the proteolytic cleavage was influenced by the chemical nature of the side chain in the site. More importantly, mutations of the predicted GPI-anchor sites markedly reduced the plasma membrane localization of Ca\textsubscript{v}2.61 proteins and prevented its co-immunoprecipitation with Ca\textsubscript{v}1.2. Altogether, our data are compatible with a model where GPI-anchored Ca\textsubscript{v}2.61 proteins are preferentially assembled and trafficked to the cell surface with the Ca\textsubscript{v}1.2 channel complex.

Results

Residues at the membrane interface in Ca\textsubscript{v}2.61 are essential for the functional modulation of Ca\textsubscript{v}1.2 currents

The high-resolution 3D cryo-EM structure of the purified rabbit Ca\textsubscript{v}2.61 subunit did not resolve the last 31 C-terminal residues, deduced from the nucleotide sequence suggesting that these residues are cleaved in the mature protein and replaced by a GPI anchor in the skeletal L-type Ca\textsuperscript{2+} channel (Fig. 1A) (17). To examine the presence of the GPI-anchored Ca\textsubscript{v}2.61 subunit in cardiomyocytes, rat ventricular myocytes were treated with prokaryotic phosphatidylinositol-phospholipase C (PI-PLC), which cleaves GPI anchors and releases lipid-anchored proteins (52). As seen, the cell-surface fraction of the PLC-treated cardiac Ca\textsubscript{v}2.61 proteins was significantly decreased (41 ± 5%, n = 3) but not completely eradicated (Fig. 2), suggesting that transmembrane and GPI-anchored forms of Ca\textsubscript{v}2.61 may co-exist in native tissues as shown for other proteins (53). To evaluate the functional impact of the C-terminal residues, deletion mutants of the rat mCherry-Ca\textsubscript{v}2.61-HA construct (Fig. 1B) were produced, and whole-cell currents were measured after recombinant expression with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}β3 (Fig. 3A). The mCherry-Ca\textsubscript{v}2.61-HA construct, described earlier (25, 27), sports a constitutive fluorescent mCherry signal at the C terminus allowing the robust detection of C-terminal fragments on Western blots as well as confirming the translation of the protein. A 9-residue hemagglutinin (HA) tag from the human influenza virus, inserted in the extracellular domain, enables the identification of Ca\textsubscript{v}2.2 at the cell surface using a FITC-conjugated anti-HA antibody.

As reported before (25, 27), co-expression of the rat Ca\textsubscript{v}2.61 WT construct with Ca\textsubscript{v}1.2 WT enhanced whole-cell peak current densities from −2.5 ± 0.3 pA/pF (n = 34) (mock vector) to −15 ± 1 pA/pF (n = 109) in the presence of 2 mM Ca\textsuperscript{2+} as the charge carrier (Fig. 3B). The increase in peak current density was associated with a ≈20-mV leftward shift in the activation potential of Ca\textsubscript{v}1.2 suggesting that Ca\textsubscript{v}2.61 WT might increase channel function in part by improving the channel open state (Fig. 3C). Deletion of 24 residues within the C terminus (mCherry-Ca\textsubscript{v}2.61-HA Δ1061–1085, Fig. 1B) produced proteins that expressed at the cell surface (Fig. 3D) and up-regulated Ca\textsubscript{v}1.2 currents in a typical fashion with a ≈5-fold increase in peak current density and a ≈16-mV shift in the activation potential (Table 1 for details). Smaller deletions of Δ1062–1068, Δ1062–1085, and Δ1069–1085 were also consistently found to modulate Ca\textsubscript{v}1.2 currents. In contrast, the deletion of four residues in the “1059–1063” region produced volt-
GPI anchoring of the L-type Ca\textsubscript{\textalpha}2\delta1 subunit

Figure 1. A, three-dimensional cryo-electron microscopy (3D cryo-EM) structure of the rabbit Ca\textsubscript{\textalpha}2\delta1 protein. Schematic representation of the rabbit Ca\textsubscript{\textalpha}2\delta1 protein in complex with the pore-forming subunit of Ca\textsubscript{\textalpha}1.1 (green) (Protein Data Bank code 5GJV). The extracellular core of Ca\textsubscript{\textalpha}2\delta1 is shown in blue-tinted white and the von Willebrand factor type A domain (residues 251–443) is shown in cyan. N-Glycosylation sites are shown as pink sticks, and putative disulfide bonds are identified by blue spheres. The presumed location of the plasma membrane is indicated with a white dotted line. The image was produced using PyMOL. B, primary sequence alignment of the rabbit and the rat Ca\textsubscript{\textalpha}2\delta1 protein. The rat Ca\textsubscript{\textalpha}2\delta1 (GenBank\textsuperscript{TM} NM_012919) was subcloned in the pmCherry-N1 vector to express the fluorophore at the C-terminal end of the protein. The hemagglutinin (HA) tag (YPYDVPDYA) was inserted in the extracellular domain of Ca\textsubscript{\textalpha}2\delta1 between Asp-676 and Arg-677 in the Cache2 domain (25). The color code in the primary sequence of the transmembrane \delta protein identifies the putative regions required for recognition by the glycosylphosphatidylinositol transamidase to carry the post-translational modification. These regions were determined by combining results obtained with multiple algorithms (Big-PI, PredGPI, and GPI-SOM) (49, 55). The transmembrane domain was determined from a GlobPlot analysis (73) (please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third-party hosted site.). Single-letter codes were used to represent the amino acids.

age-activated currents with biophysical properties indistinguishable from currents measured in mock-transfected cells, although Ca\textsubscript{\textalpha}1.2/Ca\textsubscript{\textalpha}3 subunits activated at voltages significantly more negative in the presence of \Delta1059–1063 construct than in the complete absence of Ca\textsubscript{\textalpha}2\delta1. All constructs, even the non-functional Ca\textsubscript{\textalpha}2\delta1 \Delta1059–1063, produced proteins with the expected molecular masses (Western blotting data, not shown) demonstrating that proteins were appropriately translated. In addition, all constructs were expressed at the cell surface as attested by the surface fluorescence signals measured using a flow cytometry assay (25, 27, 54). Nonetheless, the cell-surface fluorescence signals measured for mCherry-Ca\textsubscript{\textalpha}2\delta1-HA WT (Table 2). Although C1059S mutant was also tested because it is predicted to form a disulfide bridge with the neighboring Cys-404 residue in the 3D cryo-EM structure of Ca\textsubscript{\textalpha}1.1 (17). As seen, C1059A produced typical voltage-activated inward Ca\textsuperscript{2+} currents, whereas substitutions with serine or glycine impaired up-regulation of Ca\textsubscript{\textalpha}1.2 currents despite the observation that all these mutants were detected at the cell surface with similar intensities (Fig. 4 and Tables 1 and 2).

Alanine-substituted Ca\textsubscript{\textalpha}2\delta1 mutants are compatible with up-regulation of Ca\textsubscript{\textalpha}1.2 currents

GPI anchoring usually includes a precise sequence of residues formed by a linker region localized 11 residues before the cleavage site “\omega,” and a spacer region of 5–7 amino acids before the transmembrane hydrophobic domain (Fig. 1B) (49, 53). The “\omega” cleavage site is usually formed by small amino acids (glycine, alanine, cysteine, valine, or serine) such as the ones found in the 1059–1063 region, which includes Cys-1059, Gly-1060, Gly-1061, Val-1062, and Ser-1063. Algorithm searches conducted with PredGPI (55) and Big-PI (49) suggest that Gly-1061 or Val-1062 could form a functional \omega cleavage site in Ca\textsubscript{\textalpha}2\delta1. Given that the post-translational modification by the GPI-transamidase requires small amino acids, residues 1059–1062 were substituted either with the larger amino acid isoleucine (124 Å\textsuperscript{3}) or with the smaller alanine residue (67 Å\textsuperscript{3}) (56). In addition, the C1059S mutant was also tested because it is predicted to form a disulfide bridge with the neighboring Cys-404 residue in the 3D cryo-EM structure of Ca\textsubscript{\textalpha}1.1 (17). As seen, C1059A produced typical voltage-activated inward Ca\textsuperscript{2+} currents, whereas substitutions with serine or glycine impaired up-regulation of Ca\textsubscript{\textalpha}1.2 currents despite the observation that all these mutants were detected at the cell surface with similar intensities (Fig. 4 and Tables 1 and 2).

Ca\textsubscript{\textalpha}2\delta1 G1060A, G1061A, and V1062A within the putative \omega cleavage site for GPI-anchoring produced mutant proteins that functionally modulated Ca\textsubscript{\textalpha}1.2 currents (Fig. 5 and Table 1). These mutants were largely detected at the cell surface with the relative fluorescence signals being 60–80% of the signals measured for mCherry-Ca\textsubscript{\textalpha}2\delta1-HA WT (Table 2). Although channel function is compatible with single substitution with alanine residues at either position 1060 or 1061, the double alanine-substituted mCherry-Ca\textsubscript{\textalpha}2\delta1-HA construct G1060A/G1061A failed to increase Ca\textsubscript{\textalpha}1.2/Ca\textsubscript{\textalpha}3 currents (Fig. 5 and Table 1). The cell-surface fluorescence was, how-
The decrease of whole-cell currents measured in the presence of the PI-PLC at the plasma membrane. A. ventricular myocytes were isolated from adult rats. Cell lysates were incubated for 2 h either with 5 units/ml phosphatidylinositol-phospholipase C or the vehicle solution. Protein fractions (total cell lysates, cytosolic, total membrane, and plasma membrane fraction) were electrophoresed on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with an anti-CaV1.2 (Alomone, 1:1000) and anti-pan-cadherin (Invitrogen, 1:5000). Each lane was loaded with 20 µg of protein. The plasma membrane fraction was identified as the cadherin-enriched fraction. Cadherin was used as a loading control. B. expression of the native CaV1.2 was normalized to the density of cadherin ([CaV1.2]/[cadherin]) in all fractions in the absence and presence of PLC. The relative intensity of [CaV1.2]/[cadherin] at the plasma membrane fraction was then estimated as the ratio of [CaV1.2]/[cadherin] over the sum of the [CaV1.2]/[cadherin] protein density signals measured in all fractions and normalized by the relative intensity of the [CaV1.2]/[cadherin] in the plasma membrane fraction in the absence of PLC. As seen, there was a 41 ± 5% (n = 3) decrease in the relative intensity of the CaV1.2 proteins found in the PLC-treated plasma membrane fraction.

Figure 2. CaV1.2 protein from adult rat cardiomyocytes is cleaved by PI-PLC at the plasma membrane.

GPI anchoring of the L-type CaVα2δ1 subunit

Figure 2. CaVα2δ1 protein from adult rat cardiomyocytes is cleaved by PI-PLC at the plasma membrane. A. ventricular myocytes were isolated from adult rats. Cell lysates were incubated for 2 h either with 5 units/ml phosphatidylinositol-phospholipase C or the vehicle solution. Protein fractions (total cell lysates, cytosolic, total membrane, and plasma membrane fraction) were electrophoresed on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with an anti-CaV1.2 (Alomone, 1:1000) and anti-pan-cadherin (Invitrogen, 1:5000). Each lane was loaded with 20 µg of protein. The plasma membrane fraction was identified as the cadherin-enriched fraction. Cadherin was used as a loading control. B. expression of the native CaVα2δ1 was normalized to the density of cadherin ([CaV1.2]/[cadherin]) in all fractions in the absence and presence of PLC. The relative intensity of [CaVα2δ1]/[cadherin] at the plasma membrane fraction was then estimated as the ratio of [CaVα2δ1]/[cadherin] over the sum of the [CaVα2δ1]/[cadherin] protein density signals measured in all fractions and normalized by the relative intensity of the [CaVα2δ1]/[cadherin] in the plasma membrane fraction in the absence of PLC. As seen, there was a 41 ± 5% (n = 3) decrease in the relative intensity of the CaVα2δ1 proteins found in the PLC-treated plasma membrane fraction.

Alanine-substituted CaVα2δ1 mutants are cleaved by PLC

GPI-linked proteins are tethered to the outer leaflet of the cell plasma membrane resulting in a protein that can be released in the medium by the action of the PI-PLC enzyme (46, 57, 58). The surface distribution of mCherry-CaVα2δ1-HA, GFP-GPI, and a non-GPI-anchored GFP-tagged protein (LDLR-GFP) was analyzed by imaging live cells. LDLR-GFP is a fusion protein of the low-density lipoprotein receptor, which does not form a GPI anchor but rather uses the full transmembrane domain, in tandem with a green fluorescent protein (GFP) tag at its C terminus (59–61). The GFP-GPI construct was used as a positive control (60, 62). As seen, the LDLR-GFP, GFP-GPI, and mCherry-CaVα2δ1-HA proteins all co-localized with wheat germ agglutinin (WGA) at the cell surface under control conditions (Fig. 7A) as illustrated by the bright white border produced by merging the fluorescence intensities for the test construct and the cell-surface marker. Compared with typical transmembrane proteins, GPI-anchored proteins reside in higher proportion on the cell surface (53). Incubation with 3 units/ml PI-PLC significantly impaired the surface localization of mCherry-CaVα2δ1-HA WT proteins (Fig. 7, A and B, panels ii) with a global reduction of 47 ± 7% (∼2500 cells) in the HA-FITC fluorescence relative to the fluorescence signal for WGA. This reduction was similar to the impact of the PI-PLC treatment on the cell-surface fluorescence of the GPI-anchored GFP protein. In contrast, the cell-surface fluorescence of LDLR-GFP was relatively constant (90–120% of the fluorescence signal measured in control dishes) (Fig. 7, A and B, panels iii). Cell-surface expression of the alanine-substituted CaVα2δ1 G1060A and G1061A proteins was also significantly reduced after incubation with PI-PLC (Fig. 7, A and B, panels iv and v) suggesting that these mutations do not prevent the formation of the GPI anchor.

The release of plasma membrane-bound CaVα2δ1 by PI-PLC was further confirmed quantitatively using large-volume high-
Figure 3. Deletion of the 1059–1063 region in Ca\textsubscript{\(\alpha\)2\(\delta\)1} prevents functional up-regulation of Ca\textsubscript{1.2} currents. A stable recombinant HEK cell expressing Ca\textsubscript{\(\alpha\)2\(\beta\)3} was transiently transfected simultaneously with pCMV-Ca\textsubscript{\(\alpha\)2\(\beta\)3}, WT and pmCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT or mutants in all experiments described below. A, representative whole-cell Ca\textsuperscript{2+} current traces obtained after recombinant expression of mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT, mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA Δ1059–1063, Δ1061–1085, Δ1062–1068, and Δ1069–1085. Currents were recorded in the presence of 2 mM Ca\textsuperscript{2+} from a holding potential of −100 mV. Time scale is 100 ms throughout. The current density scale is either 5 or 10 pA/pF, as indicated. B, averaged current-voltage relationships for the deleted constructs. The absolute peak current densities measured with mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT varied from −9 to −44 pA/pF over the 8-month recording period with a mean of −15 ± 1 pA/pF (n = 109). Averaged peak current densities obtained with the mock mCherry vector are shown. Co-expression with Ca\textsubscript{\(\alpha\)2\(\beta\)3} left-shifted the voltage dependence of activation of Ca\textsubscript{1.2} WT/Ca\textsubscript{\(\alpha\)\(\beta\)3} from \(E_{\text{act}}\) = +9 ± 2 mV (n = 34) to \(E_{\text{act}}\) = −8.6 ± 0.3 mV (n = 109) for Ca\textsubscript{1.2} WT/Ca\textsubscript{\(\alpha\)\(\beta\)3} with mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT. Except for Δ1059–1063, the peak current densities of the deleted constructs were similar to values measured in the presence of mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT. Statistical analyses were performed with a one-way ANOVA test: *, \(p < 0.01\) and **, \(p < 0.001\) against the mock vector. See Table 1 for details.

### Table 1

| Ca\textsubscript{1.2} WT + Ca\textsubscript{\(\alpha\)\(\beta\)3} with | Electrophysiological properties in 2 mM Ca\textsuperscript{2+} |
|---|---|
| | Peak current density | \(E_{\text{act}}\) | ΔGact |
| | pA/pF | mV | kcal mol\(^{-1}\) |
| Mock mCherry vector | −2.5 ± 0.3 (34) | 9 ± 2 | 0.2 ± 0.1 |
| mCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT | −15 ± 1 (109)** | −8.6 ± 0.3** | −0.69 ± 0.03** |
| Δ1059–1063 | −2.7 ± 0.4 (18) | −4 ± 2 | −0.3 ± 0.1** |
| Δ1061–1085 | −11 ± 3 (9)** | −7.0 ± 0.1** | −0.6 ± 0.1** |
| Δ1062–1068 | −10 ± 3 (6)** | −3 ± 1 | −0.5 ± 0.1** |
| Δ1069–1085 | −12 ± 3 (6)** | −19 ± 1 | −0.7 ± 0.1** |
| C1059A | −9 ± 2 (4)** | −7.6 ± 0.5 | −0.6 ± 0.1** |
| C1059G | −9 ± 2 (5)** | −8.7 ± 0.4** | −0.68 ± 0.03** |
| C1059H | −4 ± 1 (5) | −3 ± 2 | −0.2 ± 0.3 |
| C1059I | −2.6 ± 0.3 (6) | 0 ± 2 | 0.2 ± 0.2 |
| C1059S | −5 ± 1 (8)** | −1 ± 6 | −0.3 ± 0.2 |
| G1060A | −12 ± 2 (8)** | −11 ± 2** | −0.8 ± 0.2** |
| G1060C | −12 ± 2 (8)** | 6 ± 4 | 0.2 ± 0.1 |
| G1060G | −8 ± 2 ±10)** | −5 ± 1** | −0.36 ± 0.08** |
| G1061A | −4.0 ± 0.8 (11)** | −2 ± 2 | −0.4 ± 0.2 **|
| G1060A/G1061A | −4 ± 1 (8)** | −2 ± 4 | −0.1 ± 0.2** |
| V1062A | −26.1 ± 0.7 (10)** | −9.4 ± 0.8** | −0.8 ± 0.1** |
| V1062I | −9 ± 2 (10)** | −9 ± 1** | −0.71 ± 0.06** |

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GPI anchoring of the L-type CaV_{α2δ1} subunit

throughput flow cytometry assays (Fig. 8) (54). The cell-surface expression of the mCherry-CaV_{α2δ1} construct was tested in the presence of CaV_{1.2} and CaV_{β3}. Incubation with 3 units/ml PI-PLC significantly disrupted but did not abolish the

Table 2
Cell surface expression of CaV_{α2δ1} constructs

| CaV_{1.2} + CaV_{β3} | Intact cells | Permeabilized cells |
|----------------------|-------------|---------------------|
| +mCherry-CaV_{α2δ1}-HA WT | 94 ± 7 (12) | 94 ± 8 (12) |
| Δ1059-1063 | 29 ± 3 (6)** | 101 ± 4 (6) |
| Δ1061-1085 | 14 ± 1 (3)** | 58 ± 3 (1)** |
| Δ1062-1068 | 11 ± 0.9 (3)** | 41 ± 2 (3)** |
| Δ1062-1068 | 12 ± 2 (3)** | 45 ± 3 (3)** |
| Δ1069-1085 | 10 ± 0.9 (3)** | 38 ± 2 (3)** |
| C1059A | 33 ± 0.4 (3)** | 105 ± 2 (3) |
| C1059G | 16 ± 0.6 (3)** | 71 ± 3 (3)** |
| C1059S | 16 ± 0.6 (3)** | 94 ± 1 (3) |
| C1059S | 36 ± 0.3 (3)** | 108 ± 2 (3) |
| G1060A | 85 ± 5 (9) | 91 ± 3 (9) |
| G1060I | 1 ± 0.9 (9)** | 28 ± 1 (9)** |
| G1061A | 60 ± 10 (9)** | 80 ± 20 (9) |
| G1061A | 11 ± 1 (9)** | 46 ± 2 (9)** |
| G1061A/G1061A | 34 ± 7 (6)** | 71 ± 7 (6)** |
| V1062A | 92 ± 9 (6) | 93 ± 5 (6) |
| V1062I | 53 ± 2 (6)** | 71 ± 4 (6)** |

Surface localization of mCherry-CaV_{α2δ1}-HA WT, C1059S, and V1062I proteins (Fig. 8, A and B). In particular, the treatment with PI-PLC specifically reduced the FITC cell-surface fluorescence of the WT construct by 35 ± 3% (n = 6) without altering the total mCherry fluorescence (Fig. 8C). Similar results were obtained for mCherry-CaV_{α2δ1}-HA C1059S and V1062I mutants. PI-PLC released, respectively, 45 ± 4% (n = 3) and 33 ± 2% (n = 3) of the C1059S and V1062I surface-bound proteins supporting the view that C1059S and V1062I do not prevent the formation of a GPI anchor. Altogether these data were in line with the fraction of native CaV_{α2δ1} proteins expressed at the cell surface that were cleaved by PLC in rat cardiomyocytes.

Mobility of the C-terminal fragments is altered in isoleucine-substituted glycine mutants

Our data suggest that preventing the proteolytic cleavage at the level of Gly-1060 and Gly-1061 impairs cell-surface localization of CaV_{α2δ1} and functional modulation of CaV_{1.2} currents. To confirm that these glycine residues play a role in the proteolytic cleavage of CaV_{α2δ1}, the mobility of the C-terminal fragments was examined in denaturing immunoblots of the mCherry-CaV_{α2δ1}-HA fusion protein (Fig. 9). As seen in Fig. 9A, the N-terminal fragment of the highly glycosylated mCherry-CaV_{α2δ1}-HA WT construct was detected by the CaV_{α2} antibody at 170 kDa. Given that N-linked glycosylation alters the protein mobility by ≈50 kDa (27), this result supports the cleavage of the protein at the level of the 1059–1063 region leaving out ≈30–35 kDa equivalent to the small hydrophobic domain (3.7-kDa fragment) attached to the 28.8-kDa mCherry protein. Cell fractionation assays (27) confirmed that the 170-

Figure 4. Alanine- and serine-substituted CaV_{α2δ1} Cys-1059 mutants modulate channel function. A, representative whole-cell Ca^{2+} current traces obtained after recombinant expression of mCherry-CaV_{α2δ1}-HA WT, mCherry-CaV_{α2δ1}-HA C1059S, C1059A, and C1059I. Recordings were made in the presence of 2 mM Ca^{2+} from a holding potential of –100 mV. Time scale is 100 ms throughout. The current density scale is either 5 or 10 pA/pF. B, averaged current-voltage relationships. Averaged peak current densities obtained with the mock mCherry vector are shown. The peak current densities of mCherry-CaV_{α2δ1}-HA WT, C1059S, and C1059A were not significantly different from one another, whereas mCherry-CaV_{α2δ1}-HA C1059I behaved like the mock vector. Statistical analyses were performed with a one-way ANOVA test: *, p < 0.01, and ***, p < 0.001 against the mock vector. See Table 1 for details. C, distribution of the free energies of activation. The free energy of activation (ΔGact) for C1059S and C1059A overlapped with the values measured in the presence of mCherry-CaV_{α2δ1}-HA WT, whereas the C1059I mutant behaved like the mock vector. D, representative two-dimensional plots of mCherry versus FITC fluorescence are shown for the mutants shown in A. Cell-surface expression decreased significantly for mCherry-CaV_{α2δ1}-HA C1059I. Analysis was carried out as described in Fig. 3. The single straight line drawn at the maximum of the fluorescence signal for the WT construct was reported in all bar graphs to better visualize the changes in the signal. Numerical values are shown in Table 2.
Figure 5. Alanine-substituted mutants in the 1060–1062 region behaved essentially like CaV_{1.2} WT. HEK CaV_{1.2} stable cells were transiently transfected with pCMV-CaV_{1.2} WT and pmCherry-CaV_{1.2} HA WT, pmCherry-CaV_{1.2} G1060A, pmCherry-CaV_{1.2} G1061A, pmCherry-CaV_{1.2} G1060A-G1061A (abbreviated G1060A/61A). A, representative whole-cell Ca^{2+} current traces were obtained in the presence of 2 mM Ca^{2+} from a holding potential of −100 mV. Time scale is 100 ms throughout, and the peak current density scale is 5 or 10 pA/pF. B, averaged current-voltage relationships. The peak current densities of mCherry-CaV_{1.2} HA WT, G1060A, G1061A, and V1062A were not significantly different from one another, whereas mCherry-CaV_{1.2} HA G1060A/G1061A behaved like the mock vector. Statistical analyses were performed with a one-way ANOVA test: *, p < 0.01, and **, p < 0.001 against the mock vector. See Table 1 for details. C, distribution of the free energies of activation. The free energy of activation (ΔGact) for all mutants, except the double G1060A/G1061A, overlapped with the values measured in the presence of mCherry-CaV_{1.2} HA WT. D, representative two-dimensional plots of mCherry versus FITC fluorescence are shown for the alanine-substituted mutants shown in A. Cell-surface fluorescence was robust except for the double G1060A/G1061A (abbreviated G1060A/61A). Analysis was carried out as described in Fig. 3. The single straight line drawn at the maximum of the fluorescence signal for the WT construct was reported in all bar graphs to better visualize the changes in the signal. Numerical values are shown in Table 2.

Figure 6. Isoleucine-substituted CaV_{1.2} G1060I and G1061I mutants prevent up-regulation of CaV_{1.2} currents. HEK CaV_{1.2} stable cells were transiently transfected with pCMV-CaV_{1.2} WT and pmCherry-CaV_{1.2} I1060I-HA WT, pmCherry-CaV_{1.2} I1060I G1060I, pmCherry-CaV_{1.2} I1060I G1061I, or pmCherry-CaV_{1.2} I1060I-V1062I. A, representative whole-cell Ca^{2+} current traces were recorded in the presence of 2 mM Ca^{2+} from a holding potential of −100 mV. Time scale is 100 ms throughout. The current density scale is either 5 or 10 pA/pF as indicated. B, averaged current-voltage relationships. Peak current densities versus voltage relationships were measured for the WT construct and the mutants (as shown). Statistical analyses were performed with a one-way ANOVA test: *, p < 0.01, and **, p < 0.001 against the mock vector. See Table 1 for details. C, distribution of the free energies of activation. The free energy of activation (ΔGact) for G1060I and G1061I overlapped with the values measured for the mock vector. D, confocal live cell imaging of CaV_{1.2} WT, G1060I, G1061I, and V1062I mutants are shown at a ×63 magnification. As seen, there was little HA-FITC surface fluorescence for G1060I and G1061I, although the FITC signal was detected. In addition, the FITC signal and the plasma membrane marker were strongly co-localized in cells expressing mCherry-CaV_{1.2} WT and mCherry-CaV_{1.2} G1060A/G1061A V1062I indicating that these proteins are found at the plasma membrane. Scale bar corresponds to 10 μm.
GPI anchoring of the L-type Ca\textsubscript{v}\textsubscript{1.2} subunit

**Figure 7.** Phospholipase C-mediated cleavage of Ca\textsubscript{v}\textsubscript{1.2} G1060A and G1061A prevents cell-surface expression of Ca\textsubscript{v}\textsubscript{1.2}. Stable Ca\textsubscript{v}3 cells were transfected cells with pmCherry-Ca\textsubscript{v}\textsubscript{1.2}-HA WT + pCMV-Ca\textsubscript{1.2} (panel ii); pCMV-GFP-GPI + pmCherry mock vector (panel iii); LDLR-GFP + mCherry mock plasmid (panel iv); mCherry-Ca\textsubscript{v}\textsubscript{1.2}-HA G1060A (panel v). Transfected cells were incubated overnight either with the vehicle (20 mM Tris-HCl buffer) or with PI-PLC at 3 units/ml and stained for imaging as described under “Experimental procedures.” The GFP-GPI protein confirmed the activity of PI-PLC, whereas the pmCherry mock vector monitored the cell transfection efficiency (fluorescence density at the cell surface relative to the fluorescence signal for WGA in control dishes. For comparison, PI-PLC caused a 40 ± 7% (22–66% of the control signal) reduction in the cell-surface fluorescence for GFP-GPI, and no significant change for the cell-surface fluorescence of LDLR-GFP (90–127% of the control signal). Furthermore, PI-PLC impaired the cell-surface expression of pmCherry-Ca\textsubscript{v}\textsubscript{1.2} G1060A with a 50 ± 8% reduction in the relative intensity for the HA-FITC surface labeling (n = 507 cells without PI-PLC and n = 436 cells with PI-PLC) and a smaller 32 ± 10% reduction for G1061A (n = 733 cells without PI-PLC and n = 382 cells with PI-PLC). Altogether, these data suggest that mCherry-Ca\textsubscript{v}\textsubscript{1.2}-HA G1060A and G1061A proteins are GPI-anchored. Scale bar, 10 μm.

kDa protein is the main species detected in the plasma membrane fractions (Fig. 9B).

Two C-terminal fragments of 46 and 33 kDa were detected by the anti-mCherry in the total cell lysates (Fig. 9, A and B). The more intense 33-kDa band is compatible with the production of the hydrophobic domain (3.7 kDa) attached to mCherry (28.8 kDa) = 32.5 kDa. The fainter 46-kDa band is compatible with the protein being cleaved in the “898–956” region (rat numbering) (17), thus producing a band at 45.8 kDa (17-kDa Ca\textsubscript{v}\textsubscript{1.2} G1060A, 28.8-kDa mCherry). Ca\textsubscript{v}\textsubscript{1.2} G1061A hence appears to be simultaneously cleaved at the level of the GPI anchor and at the level of the Ca\textsubscript{v}\textsubscript{1.2} protein. The C-terminal fragments of the alanine-substituted G1060A and G1061A mutants behaved essentially like the WT construct with the more intense band showing up at 33 kDa and a weaker band at 46 kDa. In contrast, the 33-kDa band was absent or much weaker in the isoleucine-substituted G10601 and G10611 mutants, although the larger C-terminal 46-kDa fragment was clearly present. In addition, both proteins were detected by the anti-Ca\textsubscript{v}\textsubscript{1.2} in total cell lysates at the expected molecular mass of 205 kDa but not in the plasma membrane fraction from the G10611-transfected cells (n = 3 different transfections and cell fractionation assays) (Fig. 9B). These data suggest that the presence of an isoleucine residue at positions 1060 or 1061 (G1060I and G1061I) results into a larger fraction of Ca\textsubscript{v}\textsubscript{1.2} proteins not being cleaved at the level of the GPI-anchor.

**Heteromeric protein assembly is impaired in the Ca\textsubscript{v}\textsubscript{1.2} \textDelta{1059–1063} construct**

Given that the decrease in channel function observed with the GPI-impaired mutants could not solely result from a diminution in the cell-surface expression, we last examined whether GPI-anchored Ca\textsubscript{v}\textsubscript{1.2} proteins were preferentially interacting with Ca\textsubscript{v}1.2/Ca\textsubscript{v}3 channels. The uncoupling between cell-surface expression and channel modulation suggests indeed that Ca\textsubscript{v}\textsubscript{1.2} might be expressed at the surface without interacting with Ca\textsubscript{v}1.2 as suggested by recent single-molecule imaging studies (64).
Co-immunoprecipitation assays were carried out by pulling down Ca\(_{\alpha2\delta1}\) WT, G1060A, and Δ1059–1063 from the anti-c-Myc-coated beads in the presence of Ca\(_{\alpha1.2}/Ca_{\beta3}\)-c-Myc (Fig 10). As seen, Ca\(_{\alpha2\delta1}\) WT (Fig. 10A) and G1060A (Fig. 10B) were well expressed (input lanes) and yielded robust signals after column elution. In contrast, Ca\(_{\alpha2\delta1}\) Δ1059–1063 (Fig. 10C) did not seem to interact strongly with Ca\(_{\alpha1.2}/Ca_{\beta3}\), although the input signal was quite strong. In fact, the pulldown assays were performed after loading onto the beads twice the amount of starting material for Ca\(_{\alpha2\delta1}\) Δ1059–1063 (10 μg in Fig. 10C compared with 5 μg of protein for A and B). Even under these conditions, the signal for Ca\(_{\alpha2\delta1}\) WT was stronger than for Ca\(_{\alpha2\delta1}\) Δ1059–1063. The weaker interaction is further emphasized when comparing the different exposure times (Fig. 10C, panels i–iii). The relative intensity of the signal in the bound fraction was about ~60 times stronger for the WT than for the Δ1059–1063 construct despite similar intensities in the input lanes. Control Western blotting of proteins found in the unbound fraction, otherwise known as the flow-through fraction, confirmed that Δ1059–1063 was present in the protein mixture as were Ca\(_{\alpha2\delta1}\) WT and G1060A (Fig. 10C, panel iv). These data suggest that the cleavage and subsequent GPI modification may confer in part the biological activity of Ca\(_{\alpha2\delta1}\) by promoting the optimal conformation for the interaction between Ca\(_{\alpha1.2}\) and Ca\(_{\alpha2\delta1}\).

**Discussion**

**Residues Cys-1059–Gly-1062 in Ca\(_{\alpha2\delta1}\) are essential for the functional modulation of L-type Ca\(_{\alpha1.2}\) channels**

The Ca\(_{\alpha2\delta1}\) protein is known to increase whole-cell currents of high voltage-activated Ca\(^{2+}\) channels. In particular, Ca\(_{\alpha2\delta1}\) promotes the channel activation gating of the L-type Ca\(_{\alpha1.2}\) channel (22–27) by stabilizing the channel voltage sensors in repeats I–III (28, 35). In contrast, Ca\(_{\alpha2\delta1}\) augments whole-cell currents of Ca\(_{\alpha1.2}\) and Ca\(_{\alpha2.3}\) channels with little changes in their activation potential (24, 37, 63, 67) supporting
membranes (shown within the present in all but in the plasma membrane fraction. No significant signal was found in the cytoplasmic fraction for CaVα1.2). GAPDH was plasma membrane fraction. Note that the relative intensity of the 46- and 33-kDa bands mirrored the pattern observed in total cell lysates (transfected with pCMV-Tag5-CaV1.2 and pCMV-CaV1.2). Immunoblotting was carried out on total WT (20 µg of proteins). From left to right, lane 1, mCherry CaVα1.2-HA WT; lane 2, mCherry CaVα1.2-HA G1060A; lane 3, mCherry CaVα1.2-HA G1060I; lane 4, mCherry CaVα1.2-HA WT G1061A; lane 5, mCherry CaVα1.2-HA G1061I. The molecular masses of the different bands were estimated by linear regression and interpolation from the molecular mass markers using the Image Lab™ Software 5.2 (Bio-Rad). Lane 1, 170, 46, and 33 kDa; lane 2, 170, 46, and 33 kDa; lane 3, 205 and 45 kDa; lane 4, 170, 46, and 33 kDa; lane 5, 205, 170, 45, and 33 kDa. The 205-kDa band corresponds to the N-glycosylated form of the mCherry-CaVα1.2-HA protein, and the 170-kDa band is compatible with the presence of the N-glycosylated form of CaVα1.2-GPI-anchored protein. The 33-kDa band is compatible with the calculated molecular mass of the C terminus cleaved around the ω site and attached to the mCherry fluorophore. B, cell fractions were obtained through preparative ultracentrifugation of G1061A- and G1061I-transfected cells, and Western blotting was carried out (from left to right). Lane 1, whole-cell lysates; lane 2, cytoplasmic fractions; lane 3, total membranes, and lane 4, plasma membranes (shown within the gray rectangle for better visualization). Each lane was loaded with 2 µg of proteins. Proteins were probed as described in A. Cadherin (pan-cadherin, 1:5000) was used as a marker for the plasma membrane. The mCherry-CaVα1.2-HA WT and mCherry-CaVα1.2-HA G1061A proteins are detected as 170-kDa species in the plasma membrane fraction. The mCherry-containing bands identified at 46 and 30 kDa were absent from the plasma membrane fraction. Note that the relative intensity of the 46- and 33-kDa bands mirrored the pattern observed in total cell lysates (A). GAPDH was present in all but in the plasma membrane fraction. A significant signal was found in the cytoplasmic fraction for CaVα1.2 (lane 2).

**Figure 9.** Mobility of the C-terminal fragments is altered in mCherry-CaVα2Δ61-HA G1060I and G1061I proteins. A, HEKT cells transiently transfected with pmCherry-CaVα2Δ61-HA WT (referred to as WT), G1060A (referred to as 60A), G1060I (referred to as 60I), G1061A (referred to as 61A), or G1061I (referred to as 61I), were lysed, and total proteins were fractionated on SDS-PAGE (8%). Western blot analysis was carried out with the antibodies against CaVα2 (1:1000 Alomone) (top panel) and mCherry (1:25,000 Biovision) (middle panel). Membranes were then repropid with antibodies against the housekeeping protein GAPDH (1:10,000 Sigma) (lower panel). Each lane was loaded with 30 µg of proteins. From left to right, lane 1, mCherry CaVα1.2-HA WT; lane 2, mCherry CaVα1.2-HA G1060A; lane 3, mCherry CaVα1.2-HA G1060I; lane 4, mCherry CaVα1.2-HA WT G1061A; lane 5, mCherry CaVα1.2-HA G1061I. The molecular masses of the different bands were estimated by linear regression and interpolation from the molecular mass markers using the Image Lab™ Software 5.2 (Bio-Rad). Lane 1, 170, 46, and 33 kDa; lane 2, 205, 170, 46, and 33 kDa; lane 3, 205 and 45 kDa; lane 4, 170, 46, and 33 kDa; lane 5, 205, 170, 45, and 33 kDa. The 205-kDa band corresponds to the N-glycosylated form of the mCherry-CaVα1.2-HA protein, and the 170-kDa band is compatible with the presence of the N-glycosylated form of CaVα1.2-GPI-anchored protein. The 33-kDa band is compatible with the calculated molecular mass of the C terminus cleaved around the ω site and attached to the mCherry fluorophore. B, cell fractions were obtained through preparative ultracentrifugation of G1061A- and G1061I-transfected cells, and Western blotting was carried out (from left to right). Lane 1, whole-cell lysates; lane 2, cytoplasmic fractions; lane 3, total membranes, and lane 4, plasma membranes (shown within the gray rectangle for better visualization). Each lane was loaded with 2 µg of proteins. Proteins were probed as described in A. Cadherin (pan-cadherin, 1:5000) was used as a marker for the plasma membrane. The mCherry-CaVα2Δ61-HA WT and mCherry-CaVα2Δ61-HA G1061A proteins are detected as 170-kDa species in the plasma membrane fraction. The mCherry-containing bands identified at 46 and 30 kDa were absent from the plasma membrane fraction. Note that the relative intensity of the 46- and 33-kDa bands mirrored the pattern observed in total cell lysates (A). GAPDH was present in all but in the plasma membrane fraction. A significant signal was found in the cytoplasmic fraction for CaVα1.2 (lane 2).

**Figure 10.** Deletion of the cleavage site impairs the co-immunoprecipitation of CaV1.2/CaVβ3 with CaVα2Δ61 proteins. HEKT cells were transiently transfected with pCMV-Tag5-CaVβ3 and pCMV-CaVα2Δ61-c-Myc and either pmCherry-CaVα2Δ61-HA WT (A), G1060A (B), or Δ1059–1063 (C). Cell lysates were immunoprecipitated overnight with anti-c-Myc magnetic beads to capture CaVβ3, eluted in 2× Laemmli buffer, and fractionated by SDS-PAGE using 8% gels. Western blotting was carried out with anti-CaVβ3 (A), anti-CaVα1 (B), and anti-CaVα2 (C) antisera after the final wash (lane 1) and after the elution step (lane 2). Immunoblotting was carried out on total WT (20 µg), G1060A (20 µg), and Δ1059–1063 (40 µg) proteins before the immunoprecipitation assay (input lane). The signal for the housekeeping protein GAPDH is shown below each blot. Co-immunoprecipitation assays were carried out with mCherry-CaVα1.2-HA WT (5 µg), G1060A (5 µg), and Δ1059–1063 (10 µg) proteins. Images were captured after short (1 s, panels i) or longer exposure times (30 s and 200 s, panels ii and iii). CaVβ3 and CaVβ1.2 proteins migrated, respectively, at 60 and 250 kDa. All CaVα2Δ61 proteins migrated at ~175 kDa, which is consistent with cleavage at or around Cys-1059. CaVα2Δ61-HA WT and G1060A consistently yielded a stronger co-immunoprecipitation signal than CaVα2Δ61-HA WT and G1060A in the plasma membrane fraction, with fluorescence readings (input lane) over the GAPDH signal. All immunoblots were carried out in parallel under the same transfection and extraction conditions. The mCherry-CaVα2Δ61-HA Δ1059–1063 proteins that did not bind to the antibody-bead complex (referred to as the unbound fraction) were collected, diluted in a Laemmli buffer, and fractionated by SDS-PAGE using an 8% gel and revealed with the anti-CaVα2Δ61 (panel iv) as a marker for the plasma membrane. In contrast, the mCherry-CaVα2Δ61-HA Δ1059–1063 proteins that bound to the antibody-bead complex (referred to as the bound fraction) were collected, diluted in a Laemmli buffer, and fractionated by SDS-PAGE using an 8% gel and revealed with the anti-CaVα2Δ61 (panel iii) as a marker for the plasma membrane. The quantification was carried out by reporting the ratio of the intensity measured under non-saturating conditions (30 s exposure) at the input lane over the GAPDH signal. The mCherry-CaVα2Δ61-HA Δ1059–1063 proteins that did not bind to the antibody-bead complex were collected, diluted in a Laemmli buffer, and fractionated by SDS-PAGE using an 8% gel and revealed with the anti-CaVα2Δ61 (panel iv). As seen, mCherry-CaVα2Δ61-HA Δ1059–1063 migrated at 175 kDa confirming that the proteins were appropriately translated and were present in the preparation in detectable quantities throughout. All experiments, carried out four times with both mutants and eight times for the WT construct over a period of 2 months, yielded similar results.

the view that association of CaVα2Δ61 within the heteromeric complex might depend upon the molecular makeup of the CaVα1 subunit (64).

Herein we show that deleting five residues between Cys-1059 and Ser-1063 (Δ1059–1063) impaired the negative shift in the activation potential as well as the up-regulation of CaV1.2 cur-
GPI anchoring of the L-type Ca\textsubscript{\alpha2\delta1} subunit

The strongest biochemical evidence linking the mutations of the glycine residues to a modification in the proteolytic processing of Ca\textsubscript{\alpha2\delta1} protein came from investigating the mobility of the C-terminal fragments using the mCherry antibody. A short 33-kDa C-terminal fragment was reproducibly associated with Ca\textsubscript{\alpha2\delta1} WT and the alanine-substituted mutants (G1060A, G1061A, and V1062A), all forms of the protein that supported whole-cell current up-regulation. This fragment is compatible with the migration of the short hydrophobic domain coupled with the mCherry protein. In contrast, this fragment was absent in the isoleucine-substituted Ca\textsubscript{\alpha2\delta1} G1060l and G1061l proteins, suggesting that proteolytic cleavage of the C terminus was affected by the mutation. Of note, all proteins produced a C-terminal fragment of 46 kDa, compatible with an additional cleavage site at a location predicted to overlap with the N-terminal end of the Ca\textsubscript{\delta1} protein. Given that the isoleucine-substituted Ca\textsubscript{\alpha2\delta1} C1059I, G1060l, and G1061l but not V1062l failed to up-regulate Ca\textsubscript{\alpha2\delta1} currents, these data suggest that the sequence Cys-1059—Gly-1060—Gly-1061 forms the proteolytic cleavage site of Ca\textsubscript{\alpha2\delta1}. Deleting this region significantly reduced the co-immunoprecipitation of Ca\textsubscript{\alpha2\delta1} with Ca\textsubscript{\alpha2\delta1} proteins from cardiomyocytes was significantly reduced by an enzymatic treatment with PI-PLC. As reported herein, only some of the surface-bound Ca\textsubscript{\alpha2\delta1} WT proteins were released by PLC. GPI-anchored proteins are known to exist in vivo as heterogeneous mixtures with considerable variation in their glycophore modifications and lipid moieties (51, 53) and in the composition of the amide-linked fatty acid forming the GPI anchor (57), which in turn can influence the affinity of PLC for its substrate. It is thus impossible to conclude whether the remaining fraction of non-releasable proteins was localized in surface microdomains inaccessible to PI-PLC or whether the remaining pool of Ca\textsubscript{\alpha2\delta1} proteins located at the cell surface was simply resistant to PI-PLC and thus not GPI-anchored. It is important to note, however, that surface-bound Ca\textsubscript{\alpha2\delta1} WT, C1059S, and V1062I proteins, which modulated Ca\textsubscript{\alpha1,2} currents, were cleaved by PI-PLC to a similar extent. This observation suggests that a similar fraction of the Ca\textsubscript{\alpha2\delta1} proteins at the cell membrane is PLC-cleavable despite individual alterations in their cell-surface trafficking.

Molecular determinants underlying the cleavage of Ca\textsubscript{\alpha2\delta1}

The proposed proteolytic cleavage of the hydrophobic domain in Ca\textsubscript{\alpha2\delta1} agrees with the 3D structure of the Ca\textsubscript{\alpha1,1} channel complex (17) and supports the GPI anchoring of the protein. GPI anchoring is generally inferred from the predicted primary amino acid sequence, but enzymatic cleavage of the membrane-bound protein by PI-PLC further supports GPI anchoring (53). As shown in this work, the cell-surface expression of recombinant Ca\textsubscript{\alpha2\delta1} in HEKT cells and native Ca\textsubscript{\alpha2\delta1} proteins from cardiomyocytes was significantly reduced by an enzymatic treatment with PI-PLC. As reported herein, only some of the surface-bound Ca\textsubscript{\alpha2\delta1} WT proteins were released by PLC. GPI-anchored proteins are known to exist in vivo as heterogeneous mixtures with considerable variation in their glycophore modifications and lipid moieties (51, 53) and in the composition of the amide-linked fatty acid forming the GPI anchor (57), which in turn can influence the affinity of PLC for its substrate. It is thus impossible to conclude whether the remaining fraction of non-releasable proteins was localized in surface microdomains inaccessible to PI-PLC or whether the remaining pool of Ca\textsubscript{\alpha2\delta1} proteins located at the cell surface was simply resistant to PI-PLC and thus not GPI-anchored. It is important to note, however, that surface-bound Ca\textsubscript{\alpha2\delta1} WT, C1059S, and V1062I proteins, which modulated Ca\textsubscript{\alpha1,2} currents, were cleaved by PI-PLC to a similar extent. This observation suggests that a similar fraction of the Ca\textsubscript{\alpha2\delta1} proteins at the cell membrane is PLC-cleavable despite individual alterations in their cell-surface trafficking.

Experimental procedures

Recombinant DNA techniques

The rabbit Ca\textsubscript{\alpha1,2} (GenBank\textsuperscript{TM} X15539) and the rat Ca\textsubscript{\beta3} (GenBank\textsuperscript{TM} M88751) were subcloned in commercial vectors under the control of the CMV promoter as described elsewhere (25, 67). The coding sequence (1091 residues) of the rat brain
GPI anchoring of the L-type Ca\textsubscript{\(\alpha\)2\(\delta\)1} subunit

Ca\textsubscript{\(\alpha\)2\(\delta\)1} clone (GenBank\textsuperscript{TM} NM_012919) (68) was subcloned in the pmCherry-N1 vector, and the hemagglutinin (HA) epitope (YPYDVPDYA) was inserted in the extracellular domain of Ca\textsubscript{\(\alpha\)2} between Asp-676 and Arg-677, as described (Fig. 1B) (27, 54). Point mutations were produced with the Q5 site-directed mutagenesis kit (New England Biolabs Inc., Whitby, Ontario, Canada) in the pmCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA construct according to the manufacturer's instructions as described elsewhere (25, 27). Ca\textsubscript{\(\alpha\)2\(\delta\)1} deletion mutants were produced by PCR overlap extension using non-mutagenic for-domain of Ca\textsubscript{\(\alpha\)1} and pmCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1} and pmCherry-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT or mutants (G1060A and Δ1059–1063). Two days after transfection, cells were homogenized in 20 mM NaMOPS (pH 7.4), 300 mM NaCl, and 1% digitonin supplemented with protease inhibitors (Thermo Fisher Scientific). Homogenates were sonicated, incubated for 1 h at 4 °C, and centrifuged at 16,000 × g for 30 min. The supernatants containing 4–10 μg/μl total proteins were collected and diluted with an equal volume of 20 mM NaMOPS (pH 7.4), 300 mM NaCl (to 0.5% final concentration of digitonin), mixed by pipetting, and incubated overnight with 50 μl (Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT and G1060A) or 100 μl (Δ1059–1063) of anti-c-Myc magnetic beads (Thermo Fisher Scientific). Beads were collected using a PureProteome magnetic rack (Millipore) and then washed three times with a buffer containing 20 mM NaMOPS (pH 7.4), 300 mM NaCl, and 0.2% digitonin. The bound proteins were eluted with 20 μl of Laemmli buffer at 95 °C for 5 min, electrophoresed on an 8% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. Western blotting was carried out with either anti-Ca\textsubscript{\(\alpha\)1} (Alomone, 1:10,000), anti-Ca\textsubscript{\(\alpha\)1} (Alomone, 1:5000), or the anti-Ca\textsubscript{\(\alpha\)2\(\delta\)1} (Alomone, 1:1000) with an anti-rabbit as secondary antibody (Jackson ImmunoResearch, 1:10,000). Signals were detected with the ECL chemiluminescent substrate (Thermo Fisher Scientific), and blots were visualized with the ChemiDoc Touch system (Bio-Rad).

Isolation of the plasma membrane fraction from recombinant HEKT cells

Four different protein fractions (total cell lysates, cytosolic, total membrane, and plasma membrane fraction) were prepared as explained before (27, 69). Briefly, transfected HEKT cells cultured in 100-mm dishes were homogenized at 4 °C in a Tris-based solution containing a mixture of protease inhibitors (Sigma) and 1 mM EDTA at pH 7.4. The cell homogenate was aliquoted into three tubes. After a 2-h incubation period at 4 °C with 1% (v/v) Triton X-100, the first tube was centrifuged at 10,000 × g for 10 min to remove cell debris, nuclei, and mitochondria. The supernatant was kept as the total protein fraction (whole-cell lysates). The second tube was centrifuged at 200,000 × g for 20 min. The supernatant is referred to as the cytosolic fraction. The pellet was resuspended in homogenizing buffer containing 1% (v/v) Triton X-100. After 30 min of incubation on ice, a second centrifugation was done at 200,000 × g. The resulting supernatant is referred to as the total membrane protein fraction. The third tube was centrifuged at 10,000 × g for 10 min. The supernatant obtained was centrifuged at 200,000 × g and 4 °C for 20 min. The pellet was resuspended in the homogenizing buffer containing 0.6 M KCl. Subsequent centrifugations were performed at 200,000 × g and 4 °C for 20 min to wash out the KCl. The final pellet was resuspended in the homogenizing buffer and is considered to be enriched in plasma membrane proteins. Proteins were electrophoresed on an 8% SDS-polyacrylamide gel and blotted with the anti-Ca\textsubscript{\(\alpha\)2\(\delta\)1} (Alomone, 1:1000), anti-mCherry (Biovision, 1:10,000), anti-GAPDH (Sigma, 1:25,000), and anti-pan-cadherin (Life Technologies, Inc., 1:5000) as a marker of the plasma membrane fraction. The extracellular N-terminal fragments were identified using the anti-Ca\textsubscript{\(\alpha\)2\(\delta\)1}, whereas the anti-mCherry was used to detect C-terminal fragments.

Co-immunoprecipitation of Ca\textsubscript{\(\alpha\)1} and Ca\textsubscript{\(\alpha\)2\(\delta\)1} with Ca\textsubscript{\(\beta\)3-c-Myc}

HEKT cells were transiently transfected with pCMV-Ca\textsubscript{\(\alpha\)2\(\delta\)1}-HA WT or
Enzymatic cleavage and immunoblotting of adult rat cardiomyocytes

Experiments were approved by the Animal Protection Committee of the Montreal Heart Institute (protocol no. 2014-1775; 2014-10-01) and were performed in accordance with the guidelines of the Canadian Council for Animal Care and the Guide for the Care and Use of Laboratory Animals 8th Edition (2011). Male Sprague-Dawley adult rats (150–200 g) (Charles River Laboratories, St. Constant, Canada) were anesthetized with a mix of pentobarbital/heparin. Hearts were quickly removed and placed on ice-cold Tyrode’s solution containing 130 mm NaCl, 5.4 mm KCl, 1 mm MgCl, 0.33 mm Na₂HPO₄, 10 mm HEPES, 5.5 mm glucose, and 1 mm CaCl₂ (pH 7.4). Ventricular myocytes were then isolated as described elsewhere (70). Briefly, myocytes were homogenized at 4 °C in a Tris-based solution containing a mixture of protease inhibitors (Sigma), including 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotonin, bestatin, E-64, leupeptin, and 1 mm EDTA at pH 7.4 (69). Cell lysates were incubated 2 h either with 5 units/ml phosphatidylinositol-specific phospholipase C enzyme (Life Technologies, Inc.) (52) or with the vehicle solution. Protein fractions (total cell lysates, cytosolic, total membrane, and plasma membrane fraction) were isolated and immunoblotted as explained above (27, 69). The expression of the native Ca₂δ₁ was normalized to the density of cadherin in all fractions ([Ca₂δ₁]/[cadherin]). The relative intensity of [Ca₂δ₁]/[cadherin] at the plasma membrane fraction was then estimated as the ratio of [Ca₂δ₁]/[cadherin] over the sum of the [Ca₂δ₁]/[cadherin] protein density signals measured in all fractions and normalized by the relative intensity of the [Ca₂δ₁]/[cadherin] in the plasma membrane fraction in the absence of PLC.

Phosphatidylinositol-specific phospholipase C digestion of recombinant proteins

HEKT cells were transfected with Caᵥ1.2, Caᵥβ₃, and mCherry-Caᵥα₂δ₁-HA constructs. Cells were dissociated 24 h after transfection and for confocal imaging and seeded for 6 h in Dulbecco’s modified Eagle’s medium high glucose + 1% fetal bovine serum. Cells were incubated for 2–16 h with phosphatidylinositol-specific phospholipase C (Life Technologies, Inc.) at a concentration of 3 units/ml in a solution containing 20 mm Tris-HCl, 1 mm EDTA, 0.01% sodium azide, and 50% glycerol at pH 7.5 (52). Half the cells were incubated under the same conditions with the vehicle solution. PI-PLC-treated cells were used immediately for live-cell imaging or for flow cytometry assays.

Live-cell imaging

HEKT Caᵥβ₃ stable cells were transiently transfected with pCMV-Caᵥ1.2 WT and pmCherry-Caᵥα₂δ₁-HA WT or mutants and stained 24 h after transfection. The plasma membrane was visualized with wheat germ agglutinin-Alexa 647 (WGA-647) (1:200, Life Technologies, Inc.) (25, 27). WGA is a carbohydrate-binding protein that recognizes sialic acid and N-acetylglucosaminyl sugar residues. An anti-HA FITC-conjugated antibody (10 μg/ml) (clone HA-7, Sigma) was used to detect the HA-tagged Caᵥα₂δ₁ protein, and the nuclei were stained with DAPI (1:1000) (Life Technologies, Inc.) in 1× PBS for 45 min at 4 °C. Confocal fluorescent images were captured between 1 and 3 h after staining with a Zeiss LSM 710 confocal microscope system equipped with a ×63/1.4 oil objective. The 488-nm laser was used to detect either FITC or GFP fluorescence, and the mCherry fluorescence was measured at 594 nm. The fluorescence of the plasma membrane marker WGA-647 was measured at 633 nm. The images were analyzed using the FIJI software to delete background, subtract noise, and to produce co-localization pixel maps (shown in white) between the 488- and 633-nm channels using the co-localization finder plugin from ImageJ (71).

Flow cytometry assays

Flow cytometry experiments were conducted as described elsewhere (25, 27, 54). In all related figures, stable Caᵥβ₃ cells were transiently transfected simultaneously with pCMV-Caᵥ1.2 WT and pmCherry-Caᵥα₂δ₁-HA WT or mutants (54). To determine the cell-surface expression level of the mCherry-Caᵥα₂δ₁-HA proteins, cells were harvested 24 h after transfection, washed in 1× PBS buffer, and stained with the FITC-conjugated mouse monoclonal anti-HA epitope tag antibody at 5 μg/ml (Sigma) or with the control IgG1-FITC murine isotype control (5 μg/ml) at 4 °C for 30 min. To determine the total quantity of both intracellular and extracellular expression of the tagged proteins, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ fixation/permeabilization solution kit (BD Biosciences). Roughly 10,000 cells were counted using a FACSAria III® SORP flow cytometer (BD Biosciences) at the flow cytometry facility hosted by the Department of Microbiology, Infectiologie, and Immunologie at the Université de Montréal. The fluorescence intensity detected with the IgG1-FITC isotype control murine (5 μg/ml) or with the anti-HA FITC-conjugated antibody (5 μg/ml) in HEKT untransfected cells was not significantly different from the fluorescence measured in the complete absence of fluorophore (25). The following control conditions were carried out in triplicate with each series of experiments: (a) untransfected Caᵥβ₃ cells without the anti-HA FITC-conjugated antibody; (b) untransfected Caᵥβ₃ cells with the anti-HA FITC-conjugated antibody to assess the level of background staining; and (c) Caᵥβ₃ cells transfected with pCMV-Caᵥ1.2 and pmCherry-Caᵥα₂δ₁-HA WT. Expressing mCherry-Caᵥα₂δ₁-HA WT in HEKT cells produced a 3-log increase in the FITC (x axis) and mCherry fluorescence (y axis) on two-dimensional dot plots (leftmost plots in Figs. 3D, 4D, 5D, and 8, A and B), as shown previously (25, 27, 54).

Quantification of steady-state cell-surface expression by flow cytometry assays

Flow cytometry data were analyzed using the FlowJo software, version 10 (TreeStar, Ashland, OR) as described (25). Relative expression of Caᵥα₂δ₁ was calculated based on Δmedian fluorescence intensity (ΔMedFI) for each fluorophore (mCherry or FITC) as explained elsewhere (54). Briefly, the gates for the positive cells (P2) and the negative cells (P3) were set manually on the two-dimensional dot plots. On the fluorescence histograms shown in the middle and right panels of Figs.
GPI anchoring of the L-type Ca\(_{\alpha}2\delta1\) subunit

3D, 4D, 5D, and 8, A and B, the cell count (y axis) is shown as a function of the fluorescence intensity (x axis) within the region delineated by the P3 (negative cells) and the P2 (positive cells) gates. The \(\Delta\)MedFI for FITC was calculated by subtracting the FITC fluorescence density of the negative cells (P3) from the fluorescence density of the FITC-positive cells (P2). The same method was used to calculate the \(\Delta\)MedFI for mCherry (data not shown). \(\Delta\)MedFI for FITC measured in intact non-permeabilized cells was used as a relative index of the steady-state cell-surface expression of the HA-tagged Ca\(_{\alpha}2\delta1\) (middle panels in Figs. 3D, 4D, 5D, 8A, and 8B), whereas the \(\Delta\)MedFI for mCherry attested that the protein was translated until the C terminus. The \(\Delta\)MedFI values for FITC were also measured after cell permeabilization (right panels in Figs. 3D, 4D, 5D, 8A, and 8D). This procedure was especially important for the mutants that failed to generate significant cell-surface fluorescence as a means to confirm the accessibility of the HA epitope.

\(\Delta\)MedFI values were normalized to the maximum value measured the same day for mCherry-Ca\(_{\alpha}2\delta1\)-HA WT expressed under the same conditions to account for variations in the absolute fluorescence intensity of the anti-HA FITC-conjugated antibody. We are thus reporting changes in the relative cell-surface fluorescence as compared with the fluorescence intensity of the mCherry-Ca\(_{\alpha}2\delta1\)-HA WT. The normalized \(\Delta\)MedFI values for mCherry measured for each mutant in intact and permeabilized cells were not significantly different from one another (\(p > 0.1\)) (data not shown) suggesting that the cell permeabilization procedure did not distort significantly the relative fluorescence readout under most conditions.

### Patch-clamp experiments in HEKT cells

Whole-cell patch-clamp experiments were carried out on isolated cells after transfection in HEKT cells in the presence of the pEGFP vector coding for the GFP (0.2 \(\mu\)g) as a control for transfection efficiency. Electrodes were filled with a solution containing (in mm) the following: 140 CsCl; 0.6 NaGTP; 3 MgATP; 10 EGTA; 10 HEPES; titrated to pH 7.3 with NaOH with a resistance varying between 2.8 and 3.2 mehms. Cells were bathed in a modified Earle’s saline solution (in mm) as follows: 135 NaCl; 20 tetraethylammonium chloride; 2 CaCl\(_2\); 1 MgCl\(_2\); 10 HEPES, titrated to pH 7.3 with KOH. GFP-positive cells were selected for patching. On-line data acquisition was achieved with the Axopatch 200-B amplifier (Molecular Devices, Sunnyvale, CA) connected to the PClamp software Clampex 10.5 through the Digidata 1440A acquisition system (Molecular Devices) (25). A series of 450-ms voltage pulses were applied from a holding potential of \(-100\) mV at a frequency of 0.2 Hz, from \(-60\) to \(+70\) mV at 5-mV intervals. Series resistance was compensated to \(-85\%\) after on-line capacitive transient cancellation. Unless stated otherwise, whole-cell currents were sampled at 5 kHz and filtered at 1 kHz. PClamp software Clampfit 10.5 was used for data analysis. Mid-potentials of activation values (\(E_{0.5,\text{act}}\)) were estimated from the peak I–V curves obtained for each channel composition and were reported as the mean of individual measurements \(\pm\) S.E. (25, 72). The free energy of activation was calculated using the mid-activation potential shown in Equation 1,

\[
\Delta G_{\text{act}} = z \cdot F \cdot E_{0.5,\text{act}}
\]  

(Eq. 1)

where \(z\) is the effective charge displacement during activation, and \(F\) is the Faraday constant (65). The r100 ratio is defined as the ratio of peak whole-cell currents remaining after a depolarizing pulse of 100 ms (\(I_{\text{100 ms}}/I_{\text{peak}}\)) and was used as an indicator of the inactivation kinetics (reported in Table 2). To assess for internal consistency, the experiments carried out with novel mutants always included a control experiment performed with mCherry-Ca\(_{\alpha}2\delta1\)-HA WT (pCMV-Ca\(_{\alpha}1.2\) WT + pCMV-Ca\(_{\beta3}\) + pmCherry-Ca\(_{\alpha}2\delta1\)-HA WT) thus explaining the larger sample size for mCherry-Ca\(_{\alpha}2\delta1\)-HA WT. Previous experiments confirmed that mCherry-Ca\(_{\alpha}2\delta1\)-HA WT sustains the functional modulation of Ca\(_{\alpha}1.2\) currents (25). Experiments performed under the same conditions yielded peak current densities that could vary by as much as \(\pm 35\%\) between each series of experiments. This variation appeared to be essentially linked to minor changes in the cell density at the time of transfection. Data from all experiments performed under the same conditions over a period of 10 months were pooled, and physio
tical properties are reported in Table 2. Experiments were performed at room temperature (20°C).

### Statistics

Results were expressed as mean \(\pm\) S.E. Tests of significance were carried out using the unpaired ANOVA with the Tukey test embedded in the Origin 7.0 analysis software (OriginLab Corp., Northampton, MA). Data were considered statistically significant at *, \(p < 0.01\), and **, \(p < 0.001\).

### Author contributions

E. S. produced single and multiple mutants, performed and analyzed flow-cytometry experiments, conducted patch-clamp experiments, performed live-cell imaging, and carried out the immunoblotting of the cell fractions. B. B. conducted patch-clamp experiments and performed the rat cardiomyocyte experiments. M. P. T. performed and analyzed flow-cytometry experiments and provided immunoblotting of the mutants under denaturing conditions. J. B. prepared Fig. 1 and carried out the coinmunoprecipitation assays. B. G. A. provided the rat cardiomyocytes. G. M. provided constructs and supervised the phospholipase C experiments. L. P. designed and coordinated the study, interpreted the data, and wrote the manuscript. All authors reviewed the results and approved the final version of this manuscript.

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