The Ca_v β Subunit Protects the I-II Loop of the Voltage-gated Calcium Channel Ca_v 2.2 from Proteasomal Degradation but Not Oligoubiquitination*

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Ca_v β subunits interact with the voltage-gated calcium channel Ca_v 2.2 on a site in the intracellular loop between domains I and II (the I-II loop). This interaction influences the biophysical properties of the channel and leads to an increase in its trafficking to the plasma membrane. We have shown previously that a mutant Ca_v 2.2 channel that is unable to bind Ca_v β subunits (Ca_v 2.2 W391A) was rapidly degraded (Waithe, D., Ferron, L., Page, K. M., Chaggar, K., and Dolphin, A. C. (2011) J. Biol. Chem. 286, 9598–9611). Here we show that, in the absence of Ca_v β subunits, a construct consisting of the I-II loop of Ca_v 2.2 was directly ubiquitinated and degraded by the proteasome system. Ubiquitination could be prevented by mutation of all 12 lysine residues in the I-II loop to arginines. Including a palmitoylation motif at the N terminus of Ca_v 2.2 I-II loop was insufficient to target it to the plasma membrane in the absence of Ca_v β subunits (1). Inclusion of additional motifs, consisting of 18 amino acids starting 24 residues after the CAV1 sequence were not retained in the ER (13), and evidence found that attaching a palmitoylation motif to the N terminus of the I-II loop is insufficient to target it to the plasma membrane (1). The binding site for Ca_v β subunits on Ca_v α 1 was shown to be a highly conserved region of the intracellular loop between domains I and II (I-II loop) of high voltage-activated channels that became known as the α-interaction domain (AID) (7). This conserved motif, consisting of 18 amino acids starting 24 residues after the ER6 transmembrane segment of Ca_v 2.1, was found to bind to all four Ca_v β subunits (8).

The mechanism by which the Ca_v β subunit promotes trafficking of the Ca_v α 1 to the plasma membrane is under debate. Apart from Ca_v β2, which can associate with the plasma membrane either through palmitoylation (9) or by a polybasic region in the case of Ca_v β2e (10), the Ca_v β subunits are cytoplasmic proteins. It was first suggested that the Ca_v α 1 I-II loop contained an ER retention motif that was masked in the presence of a Ca_v β (11), but more recently it has been postulated that the I-II loops contain an acidic ER export signal (12). Fusion proteins containing the Ca_v α 1-I-II loops fused to a transmembrane CD4 sequence were not retained in the ER (13), and evidence suggests that binding of the Ca_v β subunits to the Ca_v 1.2 and Ca_v 2.2 I-II loop prevented ubiquitination and degradation of these channels (1, 13). Our previous work has shown that a mutant Ca_v 2.2 channel that is unable to bind Ca_v β subunits (Ca_v 2.2 W391A) was subjected to increased proteasomal degradation relative to the wild-type channel (1).

Efficient degradation by the proteasome requires the substrate to be ubiquitinated. Ubiquitin is a protein of 76 amino acids that is attached by a covalent bond to a lysine residue in the substrate; this then serves as a sorting signal (14). A single ubiquitin molecule can bind, resulting in monoubiquitination. More commonly, as ubiquitin contains seven lysine residues and can itself be ubiquitinated, polyubiquitination occurs. Different types of ubiquitination lead to different fates of the proteins; for example, Lys-48-linked ubiquitination is a signal to target the substrate for degradation via the proteasome (14).

In this study, we show that, in the absence of Ca_v β subunit, the isolated I-II loop of Ca_v 2.2 is directly ubiquitinated on a lysine residue within the loop and is rapidly degraded. Binding of a Ca_v β subunit prevents degradation of the ubiquitinated I-II loop but does not prevent oligoubiquitination. Surprisingly, we found that attaching a palmitoylation motif to the N terminus of the I-II loop is insufficient to target it to the plasma membrane in the absence of the Ca_v β even when protein degradation is inhibited. This suggests that membrane targeting

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3 The abbreviations used are: Ca_v, voltage-gated calcium; AID, α-interaction domain; Ub, ubiquitin; palm, palmitoylated; Bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; pF, picofarad; nS, nanosiemens; ER, endoplasmic reticulum; WCL, whole cell lysate.
requires the I-II loop to be correctly folded in the presence of the CaV/H9252 subunit.

Results

Trafficking of the Palmitoylated I-II Loop of CaV2.2 in tsA-201 Cells—To investigate the mechanism by which the CaV/β subunit protects CaV2.2 from degradation and is involved in trafficking it to the plasma membrane, a construct was made containing the intracellular I-II loop of CaV2.2 (amino acids 356–483), which is known to interact with the CaV/β via the AID (7). A palmitoylation motif, MTLESIMACCL, the first 11 amino acids of the guanine nucleotide-binding protein Gq (15), was added to its N terminus (palm CaV2.2 I-II) to target it to the plasma membrane, and a tag, either GFP or HA, was added to the C terminus (Fig. 1A).

The expression and localization of the palmitoylated I-II loop of CaV2.2 tagged with GFP (palm CaV2.2 I-II-GFP) was investigated using confocal microscopy. In the presence of CaV/β1b tagged with mCherry, palm CaV2.2 I-II-GFP was efficiently trafficked to the plasma membrane as shown by its colocalization with the plasma membrane marker CM-Dil (Fig. 1B, top panel). In the absence of CaV/β subunits, palm CaV2.2 I-II-GFP showed very low expression levels in tsA-201 cells and was not found at the plasma membrane (Fig. 1B, middle panel).
despite the presence of the palmitoylation sequence. Either the palm CaV2.2 I-II-GFP was being expressed at much lower levels in the absence of CaV1b/H9252, or more likely it was being degraded.

When expressed alone, CaV1b-mCherry was found uniformly distributed throughout the cytoplasm (Fig. 1B, bottom left panel), but when co-expressed with palm CaV2.2 I-II-GFP,
it too was found mainly at the plasma membrane (Fig. 1B, top left panel). Surprisingly, the palmitoylation motif alone was insufficient to target mCherry (Fig. 1B, middle row) or GFP (Fig. 1B, bottom row) to the plasma membrane; trafficking to the plasma membrane required both palm CaV₂₂ I-II and the CaV₂₂ β subunit (Fig. 1B).

Whether protein degradation was responsible for reduced palm CaV₂₂ I-II-GFP expression in the absence of CaV₂₂ β was
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investigated by measuring the levels of fluorescence in the absence and presence of the proteasomal inhibitor MG132 (Fig. 2, A and B). In the presence of MG132, the levels of palm Ca\textsubscript{v}2.2 I-II-GFP were significantly increased (Fig. 2, A and B), suggesting that it is usually rapidly degraded by the proteasome. However, it was still not associated with the plasma membrane.

In the presence of Ca\textsubscript{v}1b tagged with mCherry, palm Ca\textsubscript{v}2.2 I-II-GFP was expressed at increased levels and was trafficked to the plasma membrane (Fig. 2C, middle panel). There was no increase in fluorescence in the presence of MG132 (Fig. 2D), suggesting that Ca\textsubscript{v}1b protects palm Ca\textsubscript{v}2.2 I-II-GFP from being degraded. The same result was found using untagged Ca\textsubscript{v}1b (data not shown). Control experiments showed that expression of either Ca\textsubscript{v}1b-mCherry or palm GFP alone was not increased by MG132 (Fig. 2, E and F).

Evidence for Multiple Stages of Ubiquitination of the Ca\textsubscript{v}2.2 I-II Loop—To investigate the ubiquitination of the Ca\textsubscript{v}2.2 I-II loop in more detail, palmitoylated I-II loop constructs were tagged with hemagglutinin (HA) at the C terminus (palm Ca\textsubscript{v}2.2 I-II-HA) and expressed in tsA-201 cells in the presence or absence of Ca\textsubscript{v}1b as well as in the presence or absence of the proteasomal inhibitor MG132. The anti-HA antibody identified palm Ca\textsubscript{v}2.2 I-II-HA from whole cell lysates (Fig. 3A). Quantification showed that there was significantly less palm Ca\textsubscript{v}2.2 I-II-HA in the absence of Ca\textsubscript{v}1b than in the presence of Ca\textsubscript{v}1b (Fig. 3B) in both the absence of MG132 (Fig. 3B, compare columns 1 and 2, which correspond to Fig. 3A, lanes 2 and 3) and the presence of MG132 (Fig. 3B, columns 3 and 4). Ca\textsubscript{v}1b, therefore, is likely to protect palm Ca\textsubscript{v}2.2 I-II-HA from being degraded, although an effect on expression cannot be ruled out.

Palmitoylated Ca\textsubscript{v}2.2 I-II-HA proteins were immunoprecipitated from lysates with anti-HA antibody, and Western blotting analysis with both anti-HA and anti-ubiquitin antibodies was carried out. In this experiment, the protein A-Sepharose beads used for the pulldown were limiting; an excess of lysates was added in an attempt to immunoprecipitate equal amounts of Ca\textsubscript{v}2.2 I-II-HA construct containing 12 lysines (Fig. 4A, *). To determine whether ubiquitination occurs directly on the I-II loop, all 12 of the lysine residues were mutated to arginines (palm Ca\textsubscript{v}2.2 I-II K-R), and confocal and co-immunoprecipitation experiments were performed on the mutated constructs expressed in tsA-201 cells in the presence or absence of Ca\textsubscript{v}1b subunits.

Ubiquitination occurs on lysine residues within the Ca\textsubscript{v}2.2 I-II Loop—Ubiquitination involves the formation of a covalent bond between the C terminus of ubiquitin and the e-amino of a lysine residue on the substrate (17). The palmitoylated Ca\textsubscript{v}2.2 I-II-HA construct contains 12 lysines (Fig. 4A, *). To determine whether ubiquitination occurs directly on the I-II loop, all 12 of the lysine residues were mutated to arginines (palm Ca\textsubscript{v}2.2 I-II K-R), and confocal and co-immunoprecipitation experiments were performed on the mutated constructs expressed in tsA-201 cells in the presence or absence of Ca\textsubscript{v}1b subunits.

Confluent imaging experiments showed that, when expressed alone in tsA-201 cells, the palmitoylated Ca\textsubscript{v}2.2 I-II K-R mutant tagged with GFP (palm Ca\textsubscript{v}2.2 I-II K-R-GFP) was either expressed at higher levels or was less degraded than the wild-type I-II loop (Fig. 4B compared with wild type in Fig. 2A). However, it was not trafficked to the plasma membrane in the
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A. Amino acid sequence of palm CaV2.2 I-II-HA showing the palmitoylation motif (underlined) and HA tag (boxed). The CaVβ-binding domain is shown in bold, and lysine residues, which are all mutated to arginines in the K-R mutant, are marked with an asterisk. B. Representative confocal images of tsA-201 cells co-expressing palm mCherry (without CaVβ; grey scale; left) and palm CaV2.2 I-II K-R-GFP (grey scale; middle) in the absence (top panels) and presence (bottom panels) of MG132 (4 μm). The panel on the right shows the merged image; DAPI was used to visualize the nucleus (blue). The scale bar is 20 μm, and the grayscale calibration bar is shown below. These images were taken from the same experiment using the same settings as those shown in Fig. 2A. C, box and whisker plots showing fluorescence intensity of palm mCherry (left) or palm CaV2.2 I-II K-R-GFP (right) in the absence of MG132 (DMSO control; cyan bar; n = 238 cells) or presence of MG132 (yellow bar; n = 236) of the cells represented in B. The box shows the range of 25–75%, whiskers range from 10 to 90%, the median is shown as a solid line, and the mean is shown as a dotted line. GFP fluorescence was measured in all cells that were expressing mCherry. MG132 had no significant effect on the mean fluorescence of mCherry or palm CaV2.2 I-II K-R-GFP (mean fluorescence ± S.E.: for mCherry, 6089 ± 272 (DMSO) and 5421 ± 296 arbitrary units (a.u.) (MG132); for GFP, 1618 ± 122 (DMSO) and 1507 ± 110 arbitrary units (MG132)). D, as for B but for cells co-expressing palm CaV2.2 I-II K-R-GFP with CaVβ1b-mCherry. E, box and whisker plots (as in C) showing fluorescence intensity of the cells represented in D; palm CaVβ1b-mCherry (left) or palm CaV2.2 I-II K-R-GFP (right) in the absence of MG132 (cyan; n = 214 cells) or presence of MG132 (yellow; n = 226). MG132 had no significant effect on the mean fluorescence of CaVβ1b-mCherry (4219 ± 241 (DMSO) and 3846 ± 272 arbitrary units (MG132)) or palm CaV2.2 I-II K-R-GFP (3064 ± 193 (DMSO) and 3523 ± 226 arbitrary units (MG132)). F and G, normalized confocal data for cells represented in Figs. 2A and C, and 4B and D, expressed as box and whisker plots (as in C). Data have been taken from three independent experiments involving three separate transfections and normalized for mean fluorescence intensity of mCherry and GFP of palm CaV2.2 I-II-GFP WT in the presence of CaVβ1b-mCherry (shown in G). In the absence of CaVβ1b (F), mean free mCherry fluorescence (left) was not significantly different between WT (1.74 ± 0.07) and the K-R mutant (1.68 ± 0.06), whereas GFP fluorescence (right) was significantly lower for WT (0.09 ± 0.011; light blue; n = 441 cells) than for K-R mutant (0.581 ± 0.040; orange; n = 552; *** p < 0.0001, Student’s t test). In the presence of CaVβ1b (G), mean CaVβ1b-mCherry fluorescence was slightly lower for the K-R mutant (0.912 ± 0.027; n = 534) than for WT (1.00 ± 0.034; n = 518; *, p = 0.04, Student’s t test), whereas GFP fluorescence was slightly higher (1.189 ± 0.045 (K-R) and 1.00 ± 0.040 (WT); **, p = 0.002, Student’s t test), ns, non-significant.

Absence of CaVβ subunit, again suggesting that the palmitoylation motif alone is not sufficient for trafficking the I-II loop to the plasma membrane. In the presence of MG132, the K-R mutant I-II loop still accumulated in aggregates (Fig. 4B) like the wild-type I-II loop, indicating that the mutant is also misfolded in the absence of CaVβ. Unlike the wild-type, however, palm CaV2.2 I-II K-R-GFP fluorescence was not increased by MG132 (Fig. 4C), suggesting that the K-R mutant undergoes less proteasomal degradation than wild type. In the presence of CaVβ1b, both the palmitoylated CaV2.2 I-II K-R-GFP and the mCherry-tagged CaVβ1b were found at the plasma membrane (Fig. 4D), indicating that CaVβ was still able to interact with the I-II loop when lysine residues were mutated to arginines. Neither palm CaV2.2 I-II K-R-GFP nor CaVβ1b-mCherry fluorescence was increased in the presence of MG132 (Fig. 4E). The fluorescence intensity of palm CaV2.2 I-II K-R-GFP when expressed without CaVβ1b was significantly higher than that of wild-type CaV2.2 I-II-GFP (Fig. 4F), whereas in the presence of

FIGURE 4. Mutation of lysine residues in palm CaV2.2 I-II-GFP reduces degradation. A, amino acid sequence of palm CaV2.2 I-II-HA showing the palmitoylation motif (underlined) and HA tag (boxed). The CaVβ-binding domain is shown in bold, and lysine residues, which are all mutated to arginines in the K-R mutant, are marked with an asterisk. B, Representative confocal images of tsA-201 cells co-expressing palm mCherry (without CaVβ; grey scale; left) and palm CaV2.2 I-II K-R-GFP (grey scale; middle) in the absence (top panels) and presence (bottom panels) of MG132 (4 μm). The panel on the right shows the merged image; DAPI was used to visualize the nucleus (blue). The scale bar is 20 μm, and the grayscale calibration bar is shown below. These images were taken from the same experiment using the same settings as those shown in Fig. 2A. C, box and whisker plots showing fluorescence intensity of palm mCherry (left) or palm CaV2.2 I-II K-R-GFP (right) in the absence of MG132 (DMSO control; cyan bar; n = 238 cells) or presence of MG132 (yellow bar; n = 236) of the cells represented in B. The box shows the range of 25–75%, whiskers range from 10 to 90%, the median is shown as a solid line, and the mean is shown as a dotted line. GFP fluorescence was measured in all cells that were expressing mCherry. MG132 had no significant effect on the mean fluorescence of mCherry or palm CaV2.2 I-II K-R-GFP (mean fluorescence ± S.E.: for mCherry, 6089 ± 272 (DMSO) and 5421 ± 296 arbitrary units (a.u.) (MG132); for GFP, 1618 ± 122 (DMSO) and 1507 ± 110 arbitrary units (MG132)). D, as for B but for cells co-expressing palm CaV2.2 I-II K-R-GFP with CaVβ1b-mCherry. E, box and whisker plots (as in C) showing fluorescence intensity of the cells represented in D; palm CaVβ1b-mCherry (left) or palm CaV2.2 I-II K-R-GFP (right) in the absence of MG132 (cyan; n = 214 cells) or presence of MG132 (yellow; n = 226). MG132 had no significant effect on the mean fluorescence of CaVβ1b-mCherry (4219 ± 241 (DMSO) and 3846 ± 272 arbitrary units (MG132)) or palm CaV2.2 I-II K-R-GFP (3064 ± 193 (DMSO) and 3523 ± 226 arbitrary units (MG132)). F and G, normalized confocal data for cells represented in Figs. 2A and C, and 4B and D, expressed as box and whisker plots (as in C). Data have been taken from three independent experiments involving three separate transfections and normalized for mean fluorescence intensity of mCherry and GFP of palm CaV2.2 I-II-GFP WT in the presence of CaVβ1b-mCherry (shown in G). In the absence of CaVβ1b (F), mean free mCherry fluorescence (left) was not significantly different between WT (1.74 ± 0.07) and the K-R mutant (1.68 ± 0.06), whereas GFP fluorescence (right) was significantly lower for WT (0.09 ± 0.011; light blue; n = 441 cells) than for K-R mutant (0.581 ± 0.040; orange; n = 552; *** p < 0.0001, Student’s t test). In the presence of CaVβ1b (G), mean CaVβ1b-mCherry fluorescence was slightly lower for the K-R mutant (0.912 ± 0.027; n = 534) than for WT (1.00 ± 0.034; n = 518; *, p = 0.04, Student’s t test), whereas GFP fluorescence was slightly higher (1.189 ± 0.045 (K-R) and 1.00 ± 0.040 (WT); **, p = 0.002, Student’s t test), ns, non-significant.
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Ca,β₁b-mCherry, the difference in GFP fluorescence intensities was much reduced (Fig. 4G).

Western blotting of the palmitoylated Ca,β₂.2 I-II K-R-HA-tagged construct immunoprecipitated with anti-HA antibody showed that the direct binding of ubiquitin to the I-II loop was abolished by mutation of all lysine residues (Fig. 5, A and B). The ladder of bands corresponding to the size expected for the addition of one or more ubiquitins to the palmitoylated I-II loop (Fig. 5A, lane 3) was absent for both the anti-HA (Fig. 5A, lanes 4–7) and anti-ubiquitin antibodies (Fig. 5B).

The Ca,β subunit is known to interact with the AID within the first half of the I-II loop (7). To identify which part of the I-II loop is involved in ubiquitination, two further K-R mutant HA-tagged constructs were made: one in which the first four lysine residues were mutated to arginines, referred to as 5’ K-R, and the second in which the last eight lysines were mutated to arginines, 3’ K-R. Western blots of the palm Ca,β₂.2 I-II K-R-HA-tagged constructs immunoprecipitated with the anti-HA and blotted with anti-HA and anti-ubiquitin antibodies show that both constructs were able to bind ubiquitin (Fig. 5, C and D). This indicates either that ubiquitin binds to more than one lysine residue or that it is able to switch to another lysine if the preferred lysine is no longer available.

To test whether the ladder of bands in Figs. 3 and 5 represents monoubiquitination of multiple lysine residues or oligoubiquitination of a single residue, we used a GFP-tagged mutant ubiquitin construct in which all seven lysine residues were mutated to arginines, GFP-UbKO (18). This mutant ubiquitin can no longer form polyubiquitinated chains but can still compete with endogenous ubiquitin to monoubiquitinate the substrate. When included in the transfactions, a single GFP-UbKO was found to bind to the palmitoylated Ca,β₂.2 I-II loop (Fig. 6A, lanes 3 and 4; data quantified in Fig. 6B), whereas the ladder of multiple GFP-ubiquitin (Ub) moieties bound was absent (Fig. 6, A and B, compare lanes 4 and lanes 6). Taken together, these data suggest that only a single lysine residue on the I-II loop is oligoubiquitinated sequentially, but if the preferred lysine has been mutated to arginine, this oligoubiquitination can occur on another available lysine.

Ca,β₁b Does Not Protect Ca,β₂.2 I-II Loop from Oligoubiquitination—Ca,β₁b protected palm Ca,β₂.2 I-II-GFP from being degraded (Fig. 2, A–D). Western blotting, however, showed that palm Ca,β₂.2 I-II-HA was ubiquitinated even when co-transfected with Ca,β₁b (Fig. 3, C and E). Although ubiquitination is usually the initial step on the proteasomal degradation pathway, mono- or oligoubiquitination can also lead to different outcomes for the protein (14). We therefore asked whether the I-II loop that was interacting with the Ca,β₁b was protected from ubiquitination or was also ubiquitinated. To do this, we co-expressed palm Ca,β₂.2 I-II-HA with either GFP-tagged Ca,β₁b (Ca,β₁b-GFP) or with GFP (without Ca,β) as a control in tsA-201 cells, immunoprecipitated the Ca,β with anti-GFP antibody, and immunoblotted with anti-HA and anti-Ub antibodies.

Ca,β₁b-GFP was able to co-immunoprecipitate non-ubiquitinated palm Ca,β₂.2 I-II-HA (Fig. 6C, lanes 4 and 5, shown with an arrow). In addition, the anti-HA antibody also detected a ladder of bands likely to represent ubiquitinated I-II loop products (Fig. 6C, top, lanes 4 and 5) that were also detected using the anti-Ub antibody (Fig. 6C, bottom, marked with asterisks). Both non-ubiquitinated and ubiquitinated palm Ca,β₂.2 I-II-HA products immunoprecipitated directly with the anti-HA antibody are shown in lanes 6–9 for comparison. These results, quantified in Fig. 5D, could indicate that the I-II loop does not need to be ubiquitinated to interact with Ca,β subunits but that Ca,β is able to interact with both ubiquitinated and non-ubiquitinated I-II loop. Alternatively, ubiquitin may interact with the Ca,β₂.2 I-II after it has bound to Ca,β.

Mutation of all Lysine Residues in the I-II Loop Reduces Degradation of Full-length Ca,β₂.2 Channels—To determine whether ubiquitination of the I-II loop plays a role in degradation of the full-length channel, the 12 lysine residues in the I-II loop of Ca,β₂.2 (Fig. 4A) were mutated to arginines. The wild-type and mutated channels were expressed in tsA-201 cells and examined by confocal imaging. In this experiment, the Ca,β₂.2 α₁ subunits contained an extracellular HA tag (6), and GFP was fused to the N terminus to give GFP-Ca,β₂.2-HA (wild type (WT)) or GFP-Ca,β₂.2 K-R-HA (containing 12 lysine-to-arginine mutations). Full-length channels were expressed with auxiliary Ca,α₂.₃δ-1 subunits in the presence or absence of MG132 and the presence or absence of Ca,β₁b-mCherry (Fig. 7, A and C). As with the palm Ca,β₂.2 I-II loop, MG132 caused a significant increase in the total GFP-Ca,β₂.2-HA fluorescence measured in the absence of Ca,β₁b (Fig. 7, A and B), whereas MG132 had no effect on the GFP fluorescence of GFP-Ca,β₂.2 K-R-HA (Fig. 7, C and D). In the absence of Ca,β₁b, total GFP fluorescence intensity was significantly lower for GFP-Ca,β₂.2-HA WT than for GFP-Ca,β₂.2 K-R-HA (Fig. 7E). In contrast, in the presence of Ca,β₁b, GFP fluorescence intensities were not significantly different between the two conditions (Fig. 7F). This suggests that the K-R mutations within the I-II loop protect the full-length channel from degradation in the absence of Ca,β.

We next wanted to determine whether mutation of the lysines in the I-II loop had any effect on trafficking of the full-length channel. Full-length GFP-Ca,β₂.2-HA (either WT or K-R mutant) was expressed in tsA-201 cells together with Ca,β₁b and Ca,α₂.₃δ-1. In non-permeabilized conditions, an anti-HA antibody was used to label the channel in the proximity of the plasma membrane (Fig. 8A). HA labeling was measured on a line (width of 10 pixels) drawn around the cell, and this was compared with the intracellular GFP fluorescence of the same construct measured within the cell and excluding that at the plasma membrane. Although intracellular GFP fluorescence was slightly higher for GFP-Ca,β₂.2 K-R-HA than for the WT channel in this experiment, the HA fluorescence intensities at the plasma membrane were unchanged (Fig. 8B), indicating that the 12 mutations in the I-II loop have little effect on Ca,β₂.2 trafficking.

Electrophysiological examination of Ca,β₂.2-HA channels expressed in tsA-201 cells together with Ca,β₁b-GFP and Ca,α₂.₃δ-1 showed that the K-R mutant produced a decrease in current density compared with the WT channel (Fig. 8, C and D). The reduction in current density attributed to the K-R mutation was paralleled by a reduction in whole cell conductance (G_max) through the mutated channel (Fig. 8D, left).
K-R mutant also had a significantly depolarized $V_{\text{SO}_{\text{act}}}$ (Fig. 8D, right) compared with WT Ca$_V$2.2. As a hyperpolarization in $V_{\text{SO}_{\text{act}}}$ is observed in the presence of the Ca$_V$β1b (19), the effect of the K-R mutation on Ca$_V$2.2 activation kinetics plausibly reflects a perturbed interaction between the Ca$_V$ α1 and Ca$_V$ β subunits.

**Discussion**

It is well established that the Ca$_V$β subunit binds to the Ca$_V$α1 channel via the AID in the intracellular I-II loop (7) and that this interaction is required for trafficking the Ca$_V$α1 subunit to the plasma membrane (4). Previously we have shown that a mutant Ca$_V$2.2 channel that is unable to bind Ca$_V$β subunits (Ca$_V$2.2 W391A (19)) was rapidly degraded (1). In the present study, we have shown that expression of a construct containing the isolated I-II loop of Ca$_V$2.2 with a palmitoylation motif was also rapidly degraded when expressed alone but was efficiently trafficked to the plasma membrane in the presence of the Ca$_V$β1b subunit.

The I-II loops of Ca$_V$1.3 (20) and other L-type Ca$_V$α1 subunits (21) have been shown to be targeted to the plasma membrane even in the absence of Ca$_V$β subunits. Trafficking required the presence of a polybasic plasma membrane binding motif consisting of four arginine residues in the distal portion of the Ca$_V$1.2 I-II loop (21). In contrast, our experiments show that the palmitoylated I-II loop of Ca$_V$2.2 is not targeted to the plasma membrane unless Ca$_V$β is present despite the fact that it too has similar clusters of basic amino acids in its distal portion (Fig. 4A).

To target the I-II loop to the plasma membrane, we therefore included a palmitoylation motif derived from the G protein $\text{G}_{\alpha_q}$, $\text{G}_{\alpha_q}$ subunits contain two cysteines close to the N termin-
FIGURE 6. Oligoubiquitination, occurring on a single lysine within the I-II loop, does not prevent an interaction with CaVβ1b-GFP. A, Western blot showing tsA-201 cells expressing WT palm CaV2.2 I-II-HA in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of CaVβ1b and in the presence of GFP-UbKO (lanes 3 and 4) or GFP-Ub (lanes 5 and 6) immunoprecipitated (IP) and immunoblotted (IB) with anti-HA antibody. Lanes 1 and 2 show palm CaV2.2 I-II-HA bound to endogenous ubiquitins (shown with asterisks). GFP-Ub competes with endogenous ubiquitin to give larger products (arrows for + 1 x or + 2 x GFP-Ub), but only one dominant band is seen when GFP-UbKO is used. B, quantification of band intensities of ubiquitinated palm CaV2.2 I-II-HA expressed as a percentage of total HA-detected products per lane. Samples are all in the presence of 4 μM MG132. Band intensities were measured using ImageJ and expressed as scatter plots and show the percentage of HA-products bound to two or three GFP-tagged ubiquitins (top), a single GFP-Ub (middle), or one to three endogenous ubiquitins (bottom). Lanes are the same as those shown in A. In the presence of GFP-UbKO, there is a significant reduction in the amount of endogenous ubiquitins bound to palm CaV2.2 I-II-HA; in the absence of CaVβ1b, mean band intensities (solid lines) ± S.E. were 29.6 ± 2.68 (WT alone; black symbols), 4.92 ± 1.51 (WT + GFP-UbKO; green), and 7.24 ± 1.16% (WT + GFP-Ub; cyan), and in the presence of CaVβ1b, mean band intensities were 35.26 ± 2.35 (WT; red), 5.84 ± 2.26 (WT + GFP-UbKO; blue; **, p < 0.0001), and 6.39 ± 0.95% (WT + GFP-Ub; pink). Statistical analysis was carried out using one-way analysis of variance with Bonferroni post hoc test. For WT alone, there is no measurable amount of GFP-ubiquitin bound (middle and top plots). Transfecting GFP-UbKO gives a statistically significant increase in the binding of a single GFP-Ub to palm CaV2.2 I-II-HA in both the absence and presence of CaVβ1b (middle plot; 19.86 ± 2.34%; green; **, p = 0.0001 and 18.40 ± 4.06%; blue; *, p = 0.011), whereas there is no increase in the amount of palm CaV2.2 I-II-HA bound to two or three GFP-Ub moieties (top, 1.31 ± 0.66 and 1.70 ± 0.88%). In contrast, in the presence of GFP-Ub, a single GFP-Ub bound (middle plot) increases to 17.91 ± 1.7 and 14.78 ± 0.92% in the absence and presence of CaVβ1b, respectively, and the addition of two to three GFP-Ub bound (top) also increases to 12.87 ± 3.47 and 15.89 ± 2.35% (**, p = 0.0048). C, Western blots showing tsA-201 cells expressing WT palm CaV2.2 I-II-HA in the presence of free GFP (lanes 2, 3, 6, and 7) or CaVβ1b tagged with GFP (lanes 4, 5, 8, and 9) immunoprecipitated with anti-GFP (lanes 1–5) or anti-HA (lanes 6–9) and immunoblotted with anti-HA (top) or anti-Ub (bottom) antibodies. Untransfected (unt) cells are shown in lane 1. MG132 (4 μM) is included where shown. The size of palm CaV2.2 I-II-HA that is not ubiquitinated is shown with an arrow. Asterisks show ubiquitinated products detected with both anti-HA and anti-Ub antibodies. ns, non-significant.
Ubiquitination and Degradation of the CaV Channel I-II Loop

FIGURE 7. Full-length CaV2.2 is protected from degradation by mutation of the lysine residues in its I-II loop. A, representative confocal images of tsA-201 cells co-expressing palm mCherry (without Caβ2, left panel) and full-length GFP-CaV2.2-HA (middle panel). The top row shows the WT CaV2.2 in the absence of Caβ2 and MG132, the middle row shows palm mCherry and GFP-CaV2.2-HA in the presence of MG132 (4 μM), and the third row shows Caβ1b-mCherry with mean normalized fluorescence values (middle panel) and full-length GFP-CaV2.2-HA (right panel) fluorescence was significantly increased by MG132 (***, p < 0.001, Student’s t test), whereas normalized GFP fluorescence (**, p < 0.01) was not signifi cantly different between WT (light blue; n = 1678 cells) and the K-R mutant (orange; n = 233) of the cells represented in A. The box shows the range of 25–75%, whiskers range from 10 to 90%, the median is shown as a solid line, and the mean is shown as a dotted line. GFP fluorescence was measured in all cells that were expressing mCherry. Palm mCherry fluorescence (left) was slightly increased in the presence of MG132 (*, p = 0.046, Student’s t test) with a mean fluorescence ± S.E. of 4155 ± 210 arbitrary units (a.u.) in DMSO versus 5403 ± 898 arbitrary units in MG132, whereas palm Caβ2-I-II-GFP (right) fluorescence was significantly increased by MG132 with mean ± S.E. of 2609 ± 153 (DMSO) and 4973 ± 598 arbitrary units (MG132) (***, p < 0.0001, Student’s t test). C, as for A but for cells expressing CaV2.2 that had all 12 lysine residues in its I-II loop mutated to arginines (K-R). Box and whisker plots (as in B) showing fluorescence intensity in the absence of MG132 (DMSO control; cyan bar; n = 269 cells) or in the presence of MG132 (yellow bar; n = 138) of the cells represented in C. Palm mCherry fluorescence (left) was slightly increased in the presence of MG132 (*, p = 0.02, Student’s t test) with mean fluorescence ± S.E. of 3787 ± 233 (DMSO) and 4822 ± 60 arbitrary units (MG132), whereas there was no difference in palm Caβ2-I-II-K-R-GFP fluorescence (right; mean ± S.E. of 6098 ± 449 (DMSO) and 5936 ± 520 arbitrary units (MG132)). E and F, box and whisker plots (as in B) of normalized fluorescence intensity of mCherry and GFP fluorescence of WT in the presence of Caβ1b-mCherry (shown in F). In the absence of Caβ1b (E), mCherry fluorescence (left) was not significantly different between WT (light blue; n = 1678 cells) and the K-R mutant (orange; n = 1046) with mean normalized fluorescence values ± S.E. of 1.138 ± 0.033 and 1.064 ± 0.037, whereas normalized GFP fluorescence (right) was significantly lower for WT than the K-R mutant (**, p < 0.001, Student’s t test) with mean values of 0.402 ± 0.032 and 1.132 ± 0.069. In the presence of Caβ1b (F), although Caβ1b-mCherry expression was slightly lower for K-R mutant (orange; n = 734; mean, 0.841 ± 0.033) than WT (light blue; n = 738; mean, 1.00 ± 0.039; **, p = 0.0018, Student’s t test), GFP fluorescence was not significantly different (mean values, 1.00 ± 0.065 for WT and 0.973 ± 0.065 for K-R mutant). ns, non-significant.

nus, and palmitoylation of both residues leads to greater stability at the plasma membrane (22). G protein α subunits follow a two-signal model for targeting to the plasma membrane (23). For non-myristoylated G proteins, such as Gαq, an α-helical polybasic motif at the N terminus provides the initial signal (24, 25), and this signal, in conjunction with an association with the G protein βγ subunit (Gβγ) (26), allows targeting to the plasma membrane and subsequent palmitoylation (25). Tethering of the CaVβ2e subunit to the plasma membrane also requires a polybasic motif at the N terminus (10). However, although the I-II loop of CaV2.2 contains multiple basic amino acids at the N terminus (Fig. 4A), this region alone is insufficient to target it to the plasma membrane, even when it is palmitoylated, in the absence of CaVβ subunits.
Ubiquitination and Degradation of the Ca\textsubscript{v} Channel I-II Loop

Binding of Ca\textsubscript{v}β to the AID is thought to induce a dramatic change in secondary structure of the I-II loop (27). In the absence of Ca\textsubscript{v}β, circular dichroism (CD) measurements showed that the AID exists as a random coil (28), whereas in the presence of Ca\textsubscript{v}β, the AID was shown to have an α-helical structure (28, 29). Secondary structure predictions suggested that the entire linker between the last transmembrane domain of domain I (IS6) and the AID could form a continuous α-helix (28), and CD measurements showed that this was the case (30). The helical content was found to be significantly higher for this region of Ca\textsubscript{v}2.2 than that of Ca\textsubscript{v}1.2 (31). It appears that Ca\textsubscript{v}β is able to interact with an unfolded I-II loop via its AID, inducing a conformational change and extending the α-helical structure along the I-II linker toward the IS6 (28). The N terminus of the I-II loop contains a number of basic amino acids (see Fig. 4A). Fig. 9 shows two helical wheel diagrams of the region of the I-II linker upstream of the AID (left) and including the AID (right); formation of an α-helix would allow two clusters of basic amino acids to align along one side and form a polybasic region. This may act as a trafficking signal to direct the construct to the plasma membrane and promote stable dipalmitoylation. Interestingly, the Trp-391 residue, which is critical for the interaction between the I-II loop and the Ca\textsubscript{v}β subunit, is located along the same face of the helix although beyond the polybasic regions (Fig. 9).

Alternatively, formation of an α-helix may allow another binding partner to interact with the I-II loop and direct it to the plasma membrane. The I-II linkers of Ca\textsubscript{v}2.1 (32) and Ca\textsubscript{v}2.2 (33) were found to interact directly with Gβγ. We have previously shown that Ca\textsubscript{v}β and Gβγ subunits bind at the same time and that Ca\textsubscript{v}β must be present for the Gβγ subunit to induce voltage-dependent modulation of Ca\textsubscript{v}2.2 (5). Binding of Gβγ subunits was found to be dependent on the formation of a rigid IS6-AID linker induced by the binding of Ca\textsubscript{v}β (34). The G protein Goq, has been shown to require an association with Gβγ before it can be palmitoylated and stabilized at the plasma membrane (26). To examine whether Gβγ subunits were involved in the trafficking of the I-II loop constructs, we included a minigene derived from the C terminus of β-adrenergic receptor kinase (5) to bind and remove free Gβγ subunits. Confocal experiments showed that transfection of β-adrenergic receptor kinase had no effect on the trafficking of palm Ca\textsubscript{v}2.2 I-II in the presence of Ca\textsubscript{v}β1b,4 providing no evidence that Gβγ binding is involved.

In the absence of Ca\textsubscript{v}β, the I-II loop remains in a misfolded state. The Ca\textsubscript{v}β subunit is able to act as a molecular chaperone to induce correct folding of the I-II loop of the Ca\textsubscript{v}α1 subunit. Misfolded proteins are more likely to aggregate as they have exposed hydrophobic domains, and a key role of the chaperone is to interact co-translationally as the nascent protein emerges from the ribosome and prevent misfolding and aggregation (35). In the absence of the chaperone, misfolded proteins are rapidly targeted to the ubiquitin-proteasome system for degradation.

Substrates for the ubiquitin-proteasome system are tagged with ubiquitin; addition of ubiquitin moieties is thought to occur sequentially, one at a time, with the rate-limiting step being the addition of the first ubiquitin (36). It has previously been suggested that a chain of four ubiquitins is the minimum signal for targeting the substrate to the proteasome (37), but more recently it has been shown that only a single ubiquitin is needed for small proteins (20–150 amino acids) (38). Ubiquitination also has other functions, however, such as protein trafficking (14), endocytosis, and signaling (39), which are independent of the proteasome. Our data show that the palmitoylated I-II loop carries up to four ubiquitins (Fig. 3) even in the presence of the Ca\textsubscript{v}β subunit and the proteasomal inhibitor MG132; conditions in which the protein is not degraded. Relevant to this, a mass spectrometry study has shown that in almost 50% of cases, ubiquitination of proteins has a non-proteasomal function (40).

In the absence of Ca\textsubscript{v}β, the I-II loop was rapidly degraded as shown in Fig. 2. It has been shown that nascent proteins can be ubiquitinated co-translationally and targeted for degradation before translation has finished (41). Ubiquitination does not necessarily lead to degradation of newly synthesized ubiquitinated proteins; if correct folding does occur with the help of a chaperone, the ubiquitins may be removed by the action of deubiquitinating enzymes (42). In this regard, binding of the Ca\textsubscript{v}β subunit to the ubiquitinated I-II loop clearly rescues it from degradation, although our evidence does not indicate it promotes deubiquitination. By contrast, it is possible that oligoubiquitination is functionally important for the effect of Ca\textsubscript{v}β because the full-length Ca\textsubscript{v}2.2 K-R mutant showed reduced functional expression, possibly reflecting reduced interaction with the Ca\textsubscript{v}β subunit.

Mutation of the 12 lysine residues in the I-II loop of the full-length Ca\textsubscript{v}2.2 channel protected the channel from degradation. The full-length K-R mutant channel still contains multiple internal lysine residues that were not mutated and were therefore available as ubiquitination sites. However, the fact that the K-R channel was less degraded than WT shows that the 12 lysines within the I-II loop have a critical role in ubiquitination and degradation.

Trafficking of the palmitoylated Ca\textsubscript{v}2.2 I-II loop to the plasma membrane required the presence of the palmitoylation signal, the I-II loop, and the Ca\textsubscript{v}β subunit. Although the palmitoylated K-R mutant I-II loop was no longer ubiquitinated and degraded, it also was not trafficked to the plasma membrane in the absence of the Ca\textsubscript{v}β subunit; trafficking was restored when Ca\textsubscript{v}β was also co-expressed. It is possible that correct folding, which is only achieved in the presence of the Ca\textsubscript{v}β subunit, exposes a trafficking signal. Typical trafficking signals have been identified as short linear amino acid sequences, but it has been shown that export of the Kir2.1 channel from the Golgi to the plasma membrane requires the correctly folded N and C termini of the channel (43). The trafficking signal was only exposed when two separate domains were folded correctly in the tertiary structure of the channel.

In conclusion, our experiments indicate that, in the absence of Ca\textsubscript{v}β subunit, the I-II loop of Ca\textsubscript{v}2.2 is directly ubiquitinated and rapidly degraded by the proteasome system. When Ca\textsubscript{v}β is present, it is able to bind to the I-II loop even if the loop is already ubiquitinated. Binding appears to induce a conforma-

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4 K. M. Page, unpublished observations.
FIGURE 8. Full-length GFP-Ca\(_{\text{v}}\)2.2-HA containing 12 lysine-to-arginine mutations in the I-II loop is still trafficked and functionally active in the presence of Ca\(_{\text{v}}\)\(\beta\)1b. A, representative confocal images of tsA-201 cells expressing full-length GFP-Ca\(_{\text{v}}\)2.2-HA with Ca\(_{\text{v}}\)\(\beta\)1b and Ca\(_{\text{v}}\)\(\alpha\)\(\delta\)-1 and showing WT (top) and K-R mutant (bottom). Cells were non-permeabilized and incubated with rat anti-HA antibody overnight to show extracellular HA staining on the plasma membrane (left panels; grayscale) to be compared with intracellular GFP fluorescence (middle panels; grayscale). Merged images are shown on the right; DAPI was used to stain the nuclei (blue). Scale bars are 20 μm, and the grayscale calibration bar is shown below. B, box and whisker plots showing normalized fluorescence intensities of the images represented in B with intracellular GFP (left) for WT GFP-Ca\(_{\text{v}}\)2.2-HA (light blue; \(n = 1048\) cells) and GFP-Ca\(_{\text{v}}\)2.2 K-R-HA (orange; \(n = 998\) cells) and extracellular HA staining at the plasma membrane (right). GFP fluorescence at the plasma membrane (overlapping with membrane HA labeling) was excluded from the analysis to give intracellular GFP rather than total GFP. Data have been taken from three separate experiments involving three transfections. Although intracellular GFP fluorescence may be slightly increased for the K-R mutant compared with WT (mean fluorescent values S.E. of 1.000 ± 0.069 for WT and 1.000 ± 0.076 for K-R; *, \(p = 0.022\), Student's t test), extracellular HA staining is unchanged (mean values, 1.000 ± 0.025 for WT and 1.003 ± 0.029 for K-R). C, current-voltage relationships of full-length WT and K-R mutant Ca\(_{\text{v}}\)2.2 channels co-expressed with Ca\(_{\text{v}}\)\(\beta\)1b-GFP and Ca\(_{\text{v}}\)\(\alpha\)\(\delta\)-1 in tsA-201 cells. Top, example traces of WT Ca\(_{\text{v}}\)2.2/β1b-GFP/α\(\delta\)-1 (−40 to +10 mV in 5-mV increments; black traces) and K-R Ca\(_{\text{v}}\)2.2/β1b-GFP/α\(\delta\)-1 currents (−40 to +15 mV in 5-mV increments; red traces) recorded from a holding potential of −80 mV. The charge carrier was 1 mM Ba\(^{2+}\). Cells expressing wild-type or the K-R mutant channel had the same holding currents of 3 ± 4 pA (\(n = 12\)) and 3 ± 4 pA (\(n = 13\)), respectively. The scale bars refer to both traces. Bottom, mean current density-voltage relationships (pA/pF) were calculated from current amplitude (pA) measurements taken 15 ms into a 50-s depolarizing voltage pulse. Error bars represent S.E. \(I_{\text{max}}\) at −5 mV was 277 ± 36 pA/pF (\(n = 12\)) and 148 ± 27 pA/pF (\(n = 13\)) for WT and K-R Ca\(_{\text{v}}\)2.2, respectively (p = 0.0081, Student's t test). D, characteristic properties of the current density-voltage relationships of WT Ca\(_{\text{v}}\)2.2 and the K-R mutant channel. Values were derived from modified Boltzmann fits of individual current density-voltage plots describing WT and K-R Ca\(_{\text{v}}\)2.2 currents. Left, \(G_{\text{mmax}}\) was 7.7 ± 0.9 nS/pF (mean (solid line) ± S.E.; \(n = 12\)) and 4.5 ± 0.7 nS/pF (\(n = 13\)) for WT and K-R Ca\(_{\text{v}}\)2.2, respectively (**, \(p = 0.0079\), Student's t test). Right, \(V_{50,\text{act}}\) values were −4.7 ± 1.6 (\(n = 12\)) and 1.3 ± 1.3 mV (\(n = 13\)) for WT and K-R Ca\(_{\text{v}}\)2.2, respectively (**, \(p = 0.0071\), Student’s t test). ns, non-significant.
Ubiquitination and Degradation of the CaV Channel I-II Loop

Experimental Procedures

Molecular Biology—The calcium channels used were rabbit CaV2.2 (GenBank Accession number D14157), rat CaVα2βδ-1 (M86621), and rat CaVβ1b (X61394), all expressed in the pMT2 vector. The green fluorescent protein mut3bGFP (GFP) (44) or mCherry was fused to the C terminus of CaVβ1b. GFP-Ub and GFP-UbKO (18) were obtained from Addgene. The first intracellular loop of CaV2.2 (amino acids 356–483, beginning ESGEF... and ending ... KAQ) had a palmitoylation sequence, MTLESIMACCL, added to the N terminus and an HA tag (TSYPYDVPDYA) added to the C terminus to give a construct termed palm CaV2.2 I-II-HA in this study. Further constructs were made where the HA tag was substituted with mCherry. The mutant palm CaV2.2 I-II K-R-HA was made by mutating all 12 lysine residues in the I-II loop to arginines, and two further constructs were made by mutating either the first four (palm CaV2.2 I-II 5′ K-R-HA) or the last eight (palm CaV2.2 I-II 3′ K-R-HA) lysines to arginines. The I-II loop containing the 12 lysine-to-arginine substitutions was inserted into the full-length CaV2.2, which also contained an extracellular HA tag (6) and GFP fused to the N terminus to give GFP-CaV2.2 K-R-HA. The sequences of all constructs were confirmed by DNA sequencing.

Cell Culture and Transfection—tsA-201 cells (European Collection of Authenticated Cell Cultures (ECACC)) were cultured in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum, penicillin, streptomycin, and 2% GlutaMAX (Invitrogen). For immunocytochemistry and co-immunoprecipitation experiments, transfections were carried out using PolyJet (SignaGen) according to the manufacturer’s instructions using equal ratios of constructs unless otherwise stated. When included, the protease inhibitor MG132 (Calbiochem) was added at a concentration of 4 μM 24 h after transfection, and the cells were harvested or fixed a further 16–18 h afterward. At the concentration of MG132 used (4 μM) and the time of incubation (16–18 h), MG132 was found to be effective at inhibiting proteasomal degradation without being detrimental to cell survival. Total protein yields from transfections in the absence and presence of MG132 were measured (for Western blotting and co-immunoprecipitation analysis) and found to be similar. For electrophysiological experiments, transfections were performed using FuGENE 6 (Promega) with GFP-CaV2.2-HA, CaVα2-δ-1, and CaVβ1b subunits in a ratio of 3:2:2.

Immunocytochemistry—Cells were transfected on polylysine-coated coverslips and fixed with 4% paraformaldehyde in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.4) for 5 min. 4′,6-Diamidino-2′-phenylindole dihydrochloride (DAPI) was used to stain the nuclei. When used, the plasma membrane stain CM-DiI (ThermoFisher Scientific) was used at a dilution of 1:200 for 20 min at room temperature. Coverslips were mounted in VectaShield (Vector Laboratories). When anti-HA staining was used, cells were incubated with blocking buffer (20% goat serum, 4% BSA in TBS) for 1 h at room temperature before being incubated with rat anti-HA (Roche Applied Science) diluted 1:200 in 0.5% blocking buffer for 1 h at room temperature before being stained with DAPI and mounted. Samples were viewed on an LSM 780 confocal microscope (Zeiss) using a 63×/1.4 numerical aperture oil immersion objective in 16-bit mode. The tile function (3 × 3 tiles; each tile consisting of 1024 × 1024 pixels) was used, and every transfected cell within the image was analyzed to remove collection bias. Confocal optical sections were 1 μm, and acquisition settings were kept constant. Images were analyzed using NIH ImageJ. Images that were analyzed were not saturated.

Western Blotting Analysis—Transfected tsA-201 cells were harvested in phosphate-buffered saline (PBS) containing protease inhibitors (Complete tablet from Roche Applied Science). Cells were lysed by sonication for 10 s with 1% Igepal in PBS in the presence of protease inhibitors followed by incubation on ice for 30 min, and whole cell lysates (WCLs) were collected after centrifugation (14,000 × g for 30 min at 4 °C). Samples were incubated for 15 min at 55 °C with 100 mM dithiothreitol and 2× Laemmli sample buffer, and proteins were separated by SDS-PAGE on 4–12% Bis-Tris gels and then transferred to polyvinylidene fluoride membranes. Membranes were incubated in blocking buffer (10 mM Tris, pH 7.4, 500 mM NaCl, 0.5% Igepal, 3% BSA) for 1 h followed by incubation with the primary antibody. The following primary antibodies were used: rat anti-HA (Roche Applied Science) at 1:1000 overnight, mouse anti-ubiquitin (P4D1, Santa Cruz Biotechnology) at 1:500 overnight, and mouse anti-GFP (Roche Applied Science) at 1:1000 for 1 h at all at 4 °C. The appropriate secondary antibodies coupled to horseradish peroxidase were incubated at a dilution of 1:2000 for 1 h at room temperature. Proteins were detected using the enhanced ECL Plus reagent (GE Healthcare) on a Typhoon 9410 scanner (GE Healthcare). Quantification of Western blotting was carried out using the gel analysis tool of ImageJ. A rectangular section was placed over the entire lane, and each selected lane was plotted as a line profile. The peak
band intensities above background were measured. This allows the changes in background along the length of the lane to be taken into account and accurately subtracted.

**Immunoprecipitation**—The total protein concentration was determined (Bradford assay, Bio-Rad) for each sample, and 1 mg of WCL was precleared on 50 μg of protein A-Sepharose (GE Healthcare) for 2 h at 4 °C. Sepharose beads were discarded, and supernatants were incubated with rabbit anti-HA (Sigma) or rabbit anti-GFP (Clontech) at a dilution of 1:200 overnight at 4 °C. Immunoprecipitated proteins were captured carded, and supernatants were incubated with rabbit anti-HA (GE Healthcare) for 2 ha t4 ° C. Sepharose beads were dis-

**Electrophysiology**—Whole cell voltage clamp recordings were performed on tsA-201 cells at room temperature (20–24 °C). Single cells were clamped using an Axopatch 200B patch clamp amplifier (Axon instruments). Borosilicate glass patch pipettes were filled with a solution containing 140 mm cesium aspartate, 5 mm EGTA, 2 mm MgCl₂, 0.1 mm CaCl₂, 2 mm K₂ATP, and 10 mm HEPES. CsOH was added to achieve pH 7.2. The external solution contained 150 mm tetraethylammonium chloride, 4 mM glucose, and 1 mM BaCl₂. pH was adjusted to 7.4 with Tris base. Current density-voltage relationships were fitted with a modified Boltzmann equation as follows: 

\[ I = G_{\text{max}} \times \left( V - V_{\text{rev}} \right) / \left(1 + \exp\left(-\left( V - V_{50, \text{act}} \right) / k \right) \right) \]

where \( I \) is the current (in pA/pF), \( G_{\text{max}} \) is the maximum conductance (in nS/pF), \( V_{\text{rev}} \) is the reversal potential, \( V_{50, \text{act}} \) is the midpoint voltage for current activation, and \( k \) is the slope factor.

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