Role of the C terminus of the \( \alpha_{1C} \) (Ca\( V_{1.2} \)) Subunit in Membrane Targeting of Cardiac L-type Calcium Channels*

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We have previously demonstrated that formation of a complex between L-type calcium (Ca\(^{2+} \)) channel \( \alpha_{1C} \) (Ca\( V_{1.2} \)) and \( \beta \) subunits was necessary to target the channels to the plasma membrane when expressed in tsA201 cells. In the present study, we identified a region in the \( \alpha_{1C} \) subunit that was required for membrane targeting. Using a series of C-terminal deletion mutants of the \( \alpha_{1C} \) subunit, a domain consisting of amino acid residues 1623–1666 (“targeting domain”) in the C terminus of the \( \alpha_{1C} \) subunit has been identified to be important for correct targeting of L-type Ca\(^{2+} \) channel complexes to the plasma membrane. Although cells expressing the wild-type \( \alpha_{1C} \) and \( \beta_{2a} \) subunits exhibited punctate clusters of channel complexes along the plasma membrane with little intracellular staining, co-expression of deletion mutants of the \( \alpha_{1C} \) subunit that lack the targeting domain with the \( \beta_{2a} \) subunit resulted in an intracellular localization of the channels. In addition, three other regions in the C terminus of the \( \alpha_{1C} \) subunit that were downstream of residues 1623–1666 were found to contribute to membrane targeting of the L-type channels. Deletion of these domains in the \( \alpha_{1C} \) subunit resulted in a reduction of plasma membrane-localized channels, and a concomitant increase in channels localized intracellularly. Taken together, these results have demonstrated that a targeting domain in the C terminus of the \( \alpha_{1C} \) subunit was required for proper plasma membrane localization of the L-type Ca\(^{2+} \) channels.

Voltage-activated calcium channels are heteromeric complexes composed minimally of an \( \alpha_1 \) subunit together with accessory \( \beta \) and \( \alpha_2 \delta \) subunits. The \( \alpha_1 \) subunit is the channel pore-forming subunit, which contains binding sites for pharmacological agents or toxins and determines basic electrophysiological properties of different types of Ca\(^{2+} \) channels. The accessory \( \beta \) and \( \alpha_2 \delta \) subunits play important roles in modulating Ca\(^{2+} \) channel function, including modulation of voltage-dependent properties and membrane targeting of channel complexes (1–6). The roles of the \( \beta \) subunits are better understood than those of the \( \alpha_2 \delta \) subunits. For example, \( \beta_1 \) subunit null mice express greatly reduced Ca\(^{2+} \) currents and dihydropyridine binding sites in skeletal muscle myotubes, suggesting that the \( \beta \) subunit plays critical roles in maintaining the expression of the \( \alpha_1 \) subunits (7).

The molecular events responsible for targeting the subunits of voltage-activated Ca\(^{2+} \) channels to the plasma membrane are only beginning to be understood. There are at least 10 identified genes for different \( \alpha_1 \) subunits (8), and each is predicted to contain 24 membrane-spanning domains, with the N and C-terminal domains located intracellularly (6). In early studies with the L-type \( \alpha_{1C} \) subunit (Ca\( V_{1.2} \); Ref. 8), a surprising finding was that this protein was not targeted to the plasma membrane when it was expressed alone, but rather remained in a perinuclear location (4). All of the four known \( \beta \) subunits are very hydrophilic and have no known transmembrane domains (9, 10). With few exceptions, these subunits are localized cytoplasmically when expressed in the absence of the \( \alpha_1 \) subunits (11). One exception is the rat \( \beta_3 \) subunit that is dually palmitoylated in its N terminus (10); this modification serves as a membrane targeting signal for this subunit (11). In contrast to what is observed when the channel subunits are expressed alone, co-expression of the \( \alpha_{1C} \) subunit with any \( \beta \) subunit allows for membrane targeting of both proteins (4, 12).

All \( \beta \) subunits contain two conserved domains in the central region flanked by unique N and C termini (9, 10). A region in the second conserved domain of all \( \beta \) subunits has been identified to mediate interaction with the \( \alpha_1 \) subunits, and this region is termed the \( \beta \)-interaction domain (BID)\(^1\) (13, 14). In addition, an Src homology 3 (SH3) domain has been mapped to the first conserved domain of \( \beta \) subunits (11). Mutations of key residues in either the BID or the SH3 domain disrupt interaction between the \( \alpha_1 \) and \( \beta \) subunits (11, 13, 14). More recently, we have demonstrated that mutations in the BID or the SH3 domains of \( \beta \) subunits also disrupt membrane targeting of the channels (12). This finding, as well as the observation that membrane targeting of the \( \alpha_{1C} \) can occur with any \( \beta \) subunit (regardless of its state of palmitoylation), has led us to suggest that correct plasma membrane targeting of the calcium channel subunits requires complex formation between the \( \alpha_1 \) and \( \beta \) subunits rather than palmitoylation of the \( \beta \) subunit (12).

Although recent studies have addressed functional domains in the \( \beta \) subunit that are required for targeting calcium channel complexes to the plasma membrane (11, 12), less is known about the role of the \( \alpha_1 \) subunit in the membrane targeting process. Recently, an endoplasmic reticulum retention signal in the I-II loop of the \( \alpha_1 \) subunit was identified and shown to be neutralized by interaction with a \( \beta \) subunit (15). Other studies have focused on the C terminus of the \( \alpha_{1C} \) subunit and shown

\(^1\) The abbreviations used are: BID, \( \beta \)-interaction domain; SH3, Src homology 3; WT, wild-type; DHP, dihydropyridine; PRD, proline-rich domain; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
that it is involved in multiple events regulating calcium channel functions. For example, a protein kinase A-mediated protein phosphorylation site that can modulate channel function has been mapped to residue serine 1928 in the C terminus of the α1C subunit (16–18). In addition, the C terminus of the α1C subunit contains interaction sites for the Ca2+-binding proteins sorcin (19) and calmodulin (20–23). Ca2+-dependent inactivation and facilitation of cardiac L-type Ca2+ channels may be mediated in part by association of calmodulin with the α1C subunit (20–23). Furthermore, it has been suggested that the C terminus of the α1C subunit contains elements that are inhibitory to channel activity, as deletion of parts of the C terminus resulted in increased Ca2+ current in Xenopus oocytes expressing the L-type calcium channels (24).

In the present study, we investigated the role of the C terminus of the α1C subunit in the membrane targeting of cardiac L-type Ca2+ channels. By using a series of C-terminal deletion mutants of the α1C subunit, we identified a region in the C terminus of the α1C subunit that was required for membrane targeting of the L-type Ca2+ channels.

EXPERIMENTAL PROCEDURES

Materials—All reagents were obtained from general sources unless otherwise stated. The large T-antigen transformed human embryonic kidney cells (tsA201) were the generous gift of Dr. Richard Horn (Thomas Jefferson University, Philadelphia, PA). The Ca2+ channel subunit-specific antibodies used in this study including Card C, Card I, β2a, and anti-β2a antibodies were described previously (4, 25). The pCR3α1CΔ1733 expression vector was the generous gift of Dr. Roman Shirokov (Rush University, Chicago, IL). The expression plasmid of the rabbit β2a, pCDNAβ2a, was the gift of Dr. Franz Hofmann (Technical University of Munich, Munich, Germany). The expression plasmid pCR3α1CΔPRD was described previously (26).

Generation of Expression Constructs—A Transformer site-directed mutagenesis kit from CLONTECH was used to create C-terminal deletion mutants of the α1C subunit, including pCR3α1CΔ2024, pCR3α1CΔ1905, pCR3α1CΔ1463, pCR3α1CΔ1900–2026 (ΔI), pCR3α1CΔ1733–1905 (ΔII), pCR3α1CΔ1733–1733 (ΔIII), pCR3α1CΔ1668–1733 (ΔIV), and pCR3α1CΔ1663–1666 (ΔV). The experimental procedures were carried out following the manufacturer's protocol. The truncation mutants in plasmids pCR3α1CΔ2024, pCR3α1CΔ1905, and pCR3α1CΔ1663 were generated by introducing stop codons at amino acid residue 2024, 1905, and 1663 in the C terminus of α1C, respectively. The internal deletion mutant constructs pCR3α1CΔI, pCR3α1CΔII, pCR3α1CΔIII, pCR3α1CΔIV, and pCR3α1CΔV were created by deleting amino acid residues between the two indicated residues in the C terminus of α1C subunit.

Antibody Preparation and Purification—To generate an additional α1C subunit-specific antibody, a fusion protein encoding the second intracellular loop linking the transmembrane domains II and III (amino acid residues 738–940, termed α12) of the α1C subunit was produced. The sequence in the C2 region was amplified using PCR and subcloned into an expression vector pGEX-4T-1 (Amersham Pharmacia Biotech), resulting in an in-frame fusion of the C2 residues to glutathione S-transferase (GST). The GST-C12 fusion proteins were expressed in Escherichia coli, and purified following standard procedures. Purified GST-C12 fusion proteins were injected into a rabbit and polyclonal antibodies were prepared at Bethyl Laboratories (Montgomery, TX).

Cell Culture and Transfection—HEK tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.) and 1% penicillin/streptomycin at 37 °C in 5% CO2. Transient expression of different Ca2+ channel subunits in tsA201 cells was carried out using the calcium phosphate precipitation method (4).

Immunofluorescence Staining—Different combinations of Ca2+ channel subunits were transfected into tsA201 cells, and immunofluorescence staining was performed 36–48 h after transfection. Prior to staining, cells were washed twice with PBS, followed by fixing in pre-cooled (–20 °C) methanol/aceton (1:1) for 5–10 min at 4 °C. After washing with PBS, cells were incubated in labeling buffer (1% bovine serum albumin and 2% normal goat serum in PBS) at room temperature for 1 h to block nonspecific binding. Different primary antibodies were diluted into the labeling buffer and incubated with cells for 1–2 h at room temperature. A secondary antibody, Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes), was used subsequently. Coverslips were mounted onto slides and viewed on a laser-scanning confocal microscope (Zeiss LSM-510).

Immunoprecipitation and Immunoblotting—For co-immunoprecipitation of α1C and β2a subunits, whole cell lysates were prepared from tsA201 cells transfected with pCDNAβ2a and pCR3α1CΔ2024 in 50 mM Tris-HCl, 7.4 mM EDTA, 5 mM EGTA, 0.4 mM NaCl, 1% Triton X-100, and 0.1% SDS containing protease inhibitors (Ref. 18). The cell lysates were immunoprecipitated overnight with agitation at 4 °C using the CI2 antibody coupled to Protein A-Ultralink resin (Pierce). Immunoprecipitates were washed with the lysis buffer three to five times and eluted with SDS sample buffer. The Card I antibody was used to detect the wild-type (WT) and β2a subunits on immunoblots, and detection was with horseradish peroxidase-conjugated anti-goat IgG and enhanced chemiluminescence (ECL, Pierce). The β2a subunits that were co-immunoprecipitated with the α1C subunits were detected by the anti-β2a antibody visualized using horseradish peroxidase-conjugated anti-goat IgG and ECL (Pierce).

Intact-cell Radioligand Binding—To detect expression of functional L-type Ca2+ channels, ligand binding experiments were performed using the dihydropyridine (DHP) radioligand [3H]PN200-110 with intact cells transfected with different combinations of the channel subunits. The experimental procedures were as described previously (4). Scatchard analyses were performed to assess Bmax and Kd values.

Electrophysiology—Transiently transfected tsA201 cells expressing different combinations of the Ca2+ channel subunits were measured for electrophysiological studies. Approximately 36–40 h after transfection, Ba2+ currents through L-type calcium channels were measured in the whole cell configuration using the patch clamp technique as described previously (26, 27).

RESULTS

Expression of the C-terminal Mutants of the α1C Subunit and Their Interaction with the β2a Subunit—To assess the role of different regions in the C terminus of the α1C subunit in membrane targeting of L-type Ca2+ channel complexes, several C-terminal deletion mutants of the α1C subunit were created by site-directed mutagenesis as described under “Experimental Procedures.” Two types of deletion mutants were generated. In one group of the mutants, including α1CΔ2024, α1CΔ1905, α1CΔ1733, and α1CΔ1663, different sized truncations of the C terminus were constructed such that new C termini were created (Fig. 1A). In the other group of mutants, including α1CΔ1900–2026 (ΔI), α1CΔ1733–1905 (ΔII), α1CΔ1733–1733 (ΔIII), α1CΔ1668–1733 (ΔIV), and α1CΔ1663–1666 (ΔV), different regions within the C terminus of α1C were deleted intracellularly (Fig. 1A). In addition, a proline-rich domain (PRD) in the C terminus of the α1C subunit has been identified that may interact with SH3 domain-containing proteins (26). Thus a PRD-deletion mutant, α1CΔPRD (α1CΔ1966–2004; Ref. 26), was included in this study as well (Fig. 1A).

Since a functional interaction between the α1C and β2a subunits is required for correct membrane targeting of the Ca2+ channel complexes (12), we first tested whether these deletion mutants of the α1C subunit were able to associate with the β2a subunits. The wild-type and the mutant α1C subunits were co-transfected with the rabbit β2a subunit into tsA cells, and the channel proteins were immunoprecipitated from the transfected cells using an α1C subunit-specific antibody, CI2. The immunoprecipitated proteins were analyzed using SDS-polyacrylamide gel electrophoresis and immunoblotting. The different α1C subunits were immunoprecipitated from the transfected cells using an α1C subunit-specific antibody, CI2. The immunoprecipitated proteins were analyzed using SDS-polyacrylamide gel electrophoresis and immunoblotting. The different α1C subunits were immunoprecipitated with all different mutant α1C subunits from co-transfected cells, and detected on the bottom half of the blot with the anti-β2a antibody (Fig. 1B, lower panel). The expression level of different combinations of channel subunits varied somewhat among individual transfections; however, the amount of β2a subunit present in the
immunoprecipitates appeared to roughly correlate with the amount of α1C subunit in the same immunoprecipitate (Fig. 1B). This result indicated that deletion of the C-terminal regions did not interfere with α1Cβ2 subunit interaction, suggesting that the overall conformation of the mutant α1C subunits was not drastically altered by the mutations. In addition, all mutants, with the exception of the α1CΔ1623, α1CΔIII, and α1CΔIV, exhibited voltage-dependent barium currents that were equivalent to, or greater than, those recorded from the WT α1C subunits. Different mutant constructs of the α1C subunit were co-transfected with the rabbit β2a subunit into tsA201 cells. Whole cell lysates were prepared from the transfected cells. The channel proteins were immunoprecipitated with the α1C subunit-specific antibody, CI2, and the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting. The wild-type and mutant α1C subunits were detected using the anti-β2aRab antibody, whereas the co-immunoprecipitated β2a subunits were detected using the anti-β2aRab antibody.

The C Terminus of the α1C Subunit Played a Critical Role in the Plasma Membrane Targeting of Channel Complexes—To examine whether the C-terminal region of the α1C subunit was involved in targeting of the channel to the plasma membrane, we first asked if the C-terminal truncation mutants of the α1C subunit were impaired in membrane targeting. We used the rabbit β2a subunit in membrane targeting studies, as this subunit does not undergo palmitoylation like the rat β2a subunit and consequently cannot target to the plasma membrane unless it is complexed with an α1C subunit (11). Different combinations of the mutant α1C subunits and the rabbit β2a subunit were co-expressed in tsA201 cells, and the transfected cells were immunostained with the α1C subunit-specific antibody, CI2, to reveal the expression pattern of the channel. Consistent with the results that we reported previously (4), the WT α1C subunits were localized almost exclusively at the cell surface when co-expressed with the β subunits in tsA cells (Fig. 2A), indicating that these proteins were targeted to the plasma membrane. Punctate clusters of channel complexes as detected by the CI2 antibody were observed along the cell surface (Fig. 2A, indicated by arrows). The localization of the β2a subunits was examined using the β2aRab specific antibody in co-transfected cells, and the expression pattern was similar to that observed with the co-expressed WT α1C subunits (Fig. 2B) (13). Similarly, confocal images taken from cells expressing the C-terminal truncation mutant α1CΔ2024 and β2a subunits demonstrated that the α1CΔ2024 mutant subunits, together with the co-expressed β2a subunits, were targeted to the plasma membrane and formed punctate clusters (as indicated by arrows in Fig. 2C and D). Among the WT α1Cβ2a-transfected cells, membrane staining of channel clusters was observed in 90 ± 2% of the cells (Table I). A similar percentage of cells showed punctate membrane staining in the α1CΔ2024β2a-transfected cells (88 ± 2%, Table I). In addition to the plasma membrane staining as observed in Fig. 2, certain cells expressing the WT α1Cβ2a or α1CΔ2024 subunits exhibited a small amount of intracellular staining of the channels, which might have been due to overexpression of the channel proteins in...
These cells, however, the total percentage of cells with plasma membrane staining was consistent for each of the individual WT or mutant α1C subunits analyzed (Table I). These results suggested that deletion of amino acid residues 2025–2171 from the C terminus of the α1C subunit did not affect membrane targeting of the channel complexes in tsA cells.

To further analyze a potential role for the C terminus, two other deletion mutants of the α1C subunit, α1CΔ1733 and α1CΔ1905, were co-expressed with the β2a subunit in tsA201 cells. Interestingly, the confocal images obtained from the α1CΔ1905β2a- or α1CΔ1733β2a-transfected cells demonstrated that these more substantial truncations of the C terminus of the α1C subunit resulted in an altered subcellular distribution pattern (Fig. 3). In contrast to the WT α1C subunit, significant amounts of the α1CΔ1905 and α1CΔ1733 mutant subunits were localized intracellularly, as detected by the CI2 antibody, and a smaller number of cells exhibited plasma membrane staining of the mutant α1C subunits (Fig. 3, A and C). Consistently, the co-expressed β2a subunits, detected with the anti-β2 antibody, exhibited a localization pattern similar to that observed for the α1CΔ1905 and α1CΔ1733 mutant subunits (Fig. 3, B and D). The percentages of cells with plasma membrane staining in the α1CΔ1905β2a- and α1CΔ1733β2a-transfected cells were 69 ± 2% and 40 ± 5%, respectively, both of which were significantly lower than that of the wild-type channels (Table I). A decrease in the number of cells with membrane staining observed with these other mutant α1C subunits reflected an impairment of membrane targeting of the channels (Table I and see below).

However, the mutant channels that were localized to the plasma membrane were able to form punctate clusters (Fig. 3, indicated by arrows), similar to those observed in the WT α1Cβ2a-transfected cells. In contrast to these C-terminal deletion mutants, further deletion of the C terminus to residue 1623 resulted in a total loss of membrane localization of the channels (Fig. 4). In cells expressing α1CΔ1623β2a, no plasma membrane staining of the mutant α1CΔ1623 subunits was observed (Fig. 4A). A membrane outline of the cells was drawn on the phase image, and the same line was superimposed onto the immunostaining image to show lack of plasma membrane staining of the α1CΔ1623 subunits (Fig. 4, A and B). The β2a subunit, when co-expressed with the mutant α1CΔ1623 subunit, also failed to target to the plasma membrane (Fig. 4C). The intracellular staining pattern of the α1CΔ1623 subunit resembled an endoplasmic reticulum-like network. Taken together, these images indicated that the distribution pattern of the channel complexes was drastically altered upon deletion of additional residues between amino acids 1623–2024 in the C terminus of the α1C subunit, suggesting that this region played an important role in membrane targeting of the Ca2+ channels.

**Functional Effect of a Proline-rich Domain of the α1C Subunit in Membrane Targeting of the Channels—**Based on the above results, it was of particular interest to further narrow down the region within 1623–2024 that was required for membrane targeting of the channels. We divided the region between 1623 and 2024 into three separate domains: domain I, residues 1905–2024; domain II, residues 1733–1905; and domain III, residues 1623–1733. In addition, a proline-rich domain has previously been mapped to residues 1974–2000 in the C terminus of the α1C subunit within domain I (26). PRDs have been implicated in mediating protein-protein interactions through binding to proteins containing SH3 domains (28), and the PRD of the α1C subunit binds to SH3 domains of c-Src, Grb, Hck, and the channel β subunit (26). The functional impact of domains I–III and the PRD in membrane targeting was analyzed in detail as follows.

We first examined whether the PRD of α1C played any role in membrane targeting of the channels. The PRD-deletion mutant α1CΔPRD and β2a subunits were co-transfected into tsA201 cells, and immunofluorescence staining obtained with both Card C and CI2 antibodies was analyzed to reveal the subcellular distribution of α1CΔPRD. The α1CΔPRD mutant subunits were localized to the plasma membrane as the Card C and CI2 antibodies detected clear membrane staining (Fig. 5, A and B). A similar localization pattern was observed for the co-expressed β2a subunits (data not shown). The subcellular localization and the percentage of cells with membrane staining of α1CΔPRD (87 ± 1%) were similar to that obtained for WT α1C mutants (Table I). Thus, deletion of only the PRD region did not affect targeting of the channels to the plasma membrane. We next examined a larger deletion mutant α1CΔ1900–2026 (ΔI) around the PRD region. The cells expressing the α1CΔI and β2a subunits were immunostained with both the Card C and CI2 antibodies to detect the distribution pattern of the α1CΔI mutant subunits (Fig. 5, C and D). Confocal images indicated that the α1CΔI mutant subunits were partially localized to the plasma membrane and formed punctate clusters (as marked by arrows; Fig. 5, C and D). However, substantial intracellular staining was observed as well. The percentage cells with membrane staining was 69 ± 2%, which was significantly lower than that of the wild-type channel (Table I). The staining pattern of α1CΔI was similar to that of α1CΔ1905 (Fig. 3). These results suggested that the region surrounding the PRD contributed partially to membrane targeting of the channels.

**Identification of a Critical Targeting Domain in the C Terminus of the α1C Subunit—**We next analyzed two other internal deletion mutants of the α1C subunit. TsA201 cells were transfected with either α1CΔ1733–1905 (ΔII) or α1CΔ1623–1733 (ΔIII) and the β2a subunit. Subcellular distribution of the α1CΔII subunits was revealed by immunostaining with the Card C and CI2 antibodies (Fig. 6, A and B). Plasma membrane

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**Table I**

| Membrane targeting ( % of cells showing plasma membrane staining, n = 3) | DHP binding ( n ≥ 2) | Functional L-type Ba\(^2+\) currents ( n ≥ 6) |
|---|---|---|
| WT α1C | 90 ± 2 | + | + |
| α1CΔ2024 | 88 ± 2 | + | + |
| α1CΔ1905 | 69 ± 2* | + | + |
| α1CΔ1733 | 40 ± 5* | + | + |
| α1CΔ1623 | No | No | No |
| α1CΔ1900–2025 (ΔI) | 69 ± 2* | + | + |
| α1CΔ1733–1905 (ΔII) | 79 ± 2* | + | + |
| α1CΔ1623–1733 (ΔIII) | No | No | No |
| α1CΔ1623–1666 (ΔIV) | 105 ± 13* | N.D. | No |
| α1CΔPRD | 87 ± 1 | + | + |
staining was observed in the \( \alpha_{1C}\text{II}\beta_{2a} \) cells (as indicated by arrows in Fig. 6, A and B); however, intracellular staining was seen as well. The localization pattern of the co-expressed \( \beta_{2a} \) subunits was similar as that of \( \alpha_{1C}\text{II} \) (data not shown). The percentage of cells with membrane staining was 79 \( \pm \) 2% (Table I). These results suggested that deletion of the region between residues 1734 and 1905 only partially affected membrane targeting of the channels. In contrast, deletion of residues 1623–1733 (domain III) drastically disrupted channel targeting (Fig. 6 C). Confocal images obtained from cells expressing \( \alpha_{1C}\text{II}\beta_{2a} \) showed a complete loss of cell surface staining of the mutant channels (Fig. 6, C and D). The localization pattern of \( \alpha_{1C}\Delta\text{III} \) was similar to that of \( \alpha_{1C}\Delta 1623 \); both mutants exhibited a total disruption of membrane targeting of the channels. Taken together, these results suggested that the region between 1623 and 1733 (domain III) of the \( \alpha_{1C} \) subunit was critical for targeting the channels to the plasma membrane.

Interestingly, the domain III identified here to be important for membrane targeting has previously been shown to contain an IQ-like calmodulin binding motif (amino acids 1654–1665) that has been implicated in Ca\(^{2+}\)-dependent inactivation and facilitation of cardiac L-type channels (20–23). To further narrow down the targeting domain, we created two additional...
mutants, \( \alpha_{1C} \Delta IV \) (1668–1733) and \( \alpha_{1C} \Delta V \) (1623–1666), to delete the C- or N-terminal portion of the domain III, respectively. The \( \alpha_{1C} \Delta IV \) mutant lacked the IQ motif and 31 amino acids upstream. These two deletion mutants co-immunoprecipitated with the \( \beta_{2a} \) subunit to a similar extent as the WT and all other mutant \( \alpha_{1C} \) subunits tested (Fig. 1B). To reveal the subcellular localization of the \( \alpha_{1C} \Delta IV \) and \( \alpha_{1C} \Delta V \) mutant subunits, the cells expressing \( \alpha_{1C} \Delta IV \beta_{2a} \) subunits (A and B) or \( \alpha_{1C} \Delta V \beta_{2a} \) subunits (C and D) were immunostained with either the Card C (A and C) or the CI2 antibodies (B and D). The subcellular distribution of different mutants were analyzed using confocal microscopy. Membrane-localized punctate clusters of the channels were marked by arrows.

FIG. 4. Deletion of the C terminus of the \( \alpha_{1C} \) subunit to residue 1623 resulted in a total loss of membrane targeting of the channels. Transiently transfected cells expressing the \( \alpha_{1C} \Delta 1623 \beta_{2a} \) subunits were immunostained with either the CI2 (A and B) or the anti-\( \beta_{2a} \) antibodies (C). In the phase contrast image (B), an outline of the cell plasma membrane was marked. Note no membrane staining of \( \alpha_{1C} \Delta 1623 \) was observed along the cell surface (A).

FIG. 5. Role of the C-terminal region domain I and a proline-rich domain of the \( \alpha_{1C} \) subunit in membrane targeting of the \( \text{Ca}^{2+} \) channel complexes. Transiently transfected tsA cells expressing \( \alpha_{1C} \Delta PRD \beta_{2a} \) subunits (A and B) or \( \alpha_{1C} \Delta 1900–2026(\Delta1) \beta_{2a} \) subunits (C and D) were immunostained with either the Card C (A and C) or the CI2 antibodies (B and D). The subcellular distribution of different mutants were analyzed using confocal microscopy. Membrane-localized punctate clusters of the channels were marked by arrows.
of cells that showed membrane staining for \( \alpha_{1C}\Delta1733 \) (lacking both domain I and II) was lower than that of \( \alpha_{1C}\Delta1905 \) (lacking only domain I) (Table I).

**Functional Effect of Membrane Targeting of the L-type Channels**—To examine the functional importance of membrane targeting of the L-type Ca\(^{2+}\) channels, we performed intact-cell radioligand binding experiments using \([3H]PN200-110\), a DHP antagonist. The mutant \( \alpha_{1C} \) subunits whose membrane targeting was completely disrupted, including \( \alpha_{1C}\Delta1623 \) and \( \alpha_{1C}\DeltaIII \), exhibited no saturable binding (Table I). In cells expressing other mutant \( \alpha_{1C} \) subunits and the \( \beta_{2a} \) subunit, DHP binding was observed and was comparable for the WT and mutant channels (Table I). Although some mutant \( \alpha_{1C} \) subunits showed decreased membrane staining compared with the WT channels, and concomitantly, increased intracellular staining (see above and Table I), similar \( B_{max} \) and \( K_d \) values were obtained for the WT and the other mutant channels. The \( B_{max} \) values varied between different experiments (due to the variability obtained in transient transfections); however, the values obtained within each group of transfections was consistent. The average values were between 0.5 and 1 pmol of DHP receptors/mg of total protein.

In parallel experiments, we performed whole cell patch-clamp recordings to determine whether the mutant \( \alpha_{1C} \) subunits could form functional channels. No L-type Ba\(^{2+}\) currents were recorded from cells expressing \( \alpha_{1C}\Delta1668-1733\beta_{2a} \), \( \alpha_{1C}\Delta1623-1666\beta_{2a} \), or \( \alpha_{1C}\DeltaIII\beta_{2a} \) mutant subunits, indicating that complete disruption of membrane targeting of the mutant channels...
resulted in loss of functional channels. For the WT and all other mutants, L-type Ba\(^{2+}\) currents through these channels were recorded (with a mean peak current amplitude \(\geq 5\) pA/pF, \(n \geq 6\)) from the transfected tsA cells (Table I). Taken together, these results indicated that membrane targeting was a necessary step for the formation of the functional channels at the cell surface, as currents were not observed with the \(\alpha_{1C}\) mutants that completely failed to target to the plasma membrane.

**DISCUSSION**

Correct targeting and translocation of multisubunit ion channels to the plasma membrane of cells is an important process that allows for the proper formation of functional channels at the cell surface. The mechanisms that allow for membrane targeting of ion channels have been investigated extensively in many recent studies (4, 12, 15, 29–32). The assembly of different channel subunits into complexes at the early stage of protein synthesis appears to be a necessary step for proper membrane targeting of each \(K\) channels, G-protein-activated inward rectifier \(K\) channels, and \(K_{ATP}\) channels (29, 30). This is also likely to be the case for L-type Ca\(^{2+}\) channels (4, 12), although this has not been explicitly demonstrated. However, we have previously demonstrated that a functional interaction between the \(\alpha\) and \(\beta\) subunits is a critical factor for membrane targeting of L-type Ca\(^{2+}\) channels (12), and it is likely that complex formation occurs at the early stage of protein synthesis. Recent studies have demonstrated that \(\beta\) subunits can neutralize an endoplasmic reticulum retention signal in the I-II loop of \(\alpha\) subunits (15). Nevertheless, signals other than complex formation are required for the correct targeting of the L-type Ca\(^{2+}\) channels, as several of the \(\alpha_{1C}\) mutants studied here were able to complex with \(\beta\) subunits, yet they were impaired in membrane targeting.

In the present study, we further analyzed the mechanism of membrane targeting of the L-type calcium channels. To address the question whether the C-terminal domain of the \(\alpha_{1C}\) subunit plays a role in membrane targeting, we created several C-terminal deletion mutants of the \(\alpha_{1C}\) subunit, and immunocytochemical experiments were performed to study the subcellular localization of the mutants. Based on the results described above, several regions in the C terminus of the \(\alpha_{1C}\) subunit appeared to be involved in membrane targeting of the channels to different degrees. The most important region has been mapped to residues 1623–1666, as deletion of this small domain of the \(\alpha_{1C}\) subunit contributed partially to membrane targeting of the channels, as deletion of either of these domains resulted in a reduction of membrane-localized channels and an accumulation of intracellular channels. The results shown here provided the first explanation for failure of the previously identified "dead" mutant of the \(\alpha_{1C}\) subunit, \(\alpha_{1C}A1623\) (24) to function as a Ca\(^{2+}\) channel, as we demonstrated here that this mutant subunit lacked the ability to target to the plasma membrane. In addition, two other dead mutants of the \(\alpha_{1C}\) subunit (\(\alpha_{1C}D\) and \(\alpha_{1C}DIII\)) identified here further demonstrated the importance of C-terminal domains for proper membrane targeting to the function of L-type Ca\(^{2+}\) channels.

There are several possible explanations for the requirement for the C-terminal domains of the \(\alpha_{1C}\) subunit in membrane targeting of the channels. First, the C-terminal targeting domains may be critical for protein folding and proper maturation of the \(\alpha_{1C}\) subunit. Previous studies have identified several deletion or truncation mutations of the cystic fibrosis transmembrane conductance regulator that lack the ability to target to the plasma membrane (33). It has been suggested that the mutant CFTRs are misfolded proteins that are degraded more rapidly than the WT proteins (33). Deletion of the C-terminal domains of the \(\alpha_{1C}\) subunit may alter protein conformation and result in unstable proteins that are unable to target to, or be maintained at, the plasma membrane. However, since all the \(\alpha_{1C}\) mutant subunits were able to associate with the \(\beta_{2a}\) subunit (Fig. 1B) and be recognized by several \(\alpha_{1C}\)-specific antibodies, the overall conformation of the mutant \(\alpha_{1C}\) subunits was maintained to a certain degree.

Interestingly, although the membrane targeting of some mutant \(\alpha_{1C}\) subunits was partially impaired (e.g. \(\alpha_{1C}A1905\) or \(\alpha_{1C}A1733\)), as less plasma membrane staining and significant intracellular staining of the channels was observed (Fig. 3), similar amounts of DHP binding were obtained for these mutants and the wild type \(\alpha_{1C}\) subunit (see above). This result suggested that the plasma membrane-localized, as well as the intracellularly localized, forms of these particular mutants were capable of binding the hydrophobic radioligand PN200-110. A clear parallel existed in that the mutants that could exhibit membrane targeting, also could bind DHPs and exhibit Ba\(^{2+}\) currents, whereas those mutants that were completely unable to target to the plasma membrane were also unable to bind DHPs and mediate Ba\(^{2+}\) currents. This suggested that there were fundamental differences in the properties of the mutants. A conceivable explanation for the significant intracellular staining observed for the “functional mutants,” such as \(\alpha_{1C}A1905\) or \(\alpha_{1C}A1733\) that were also able to exhibit significant punctate staining at the plasma membrane, is that these mutants were able to target to the plasma membrane but had a diminished ability to be retained at the plasma membrane. Thus, at any given time, these mutants might exhibit significant intracellular staining due to trafficking of the channels to or from intracellular compartments. Such mutant channels would be expected to exhibit full function (DHP binding and production of currents), whereas the mutant channels that were incapable of any membrane targeting also lacked all function. Thus, the C terminus of the \(\alpha_{1C}\) subunit may be involved in constitutive recycling of the protein between the plasma membrane and intracellular compartments. The WT channels may be more stable at the plasma membrane and recycle at a slower rate, whereas the mutant channels may have recycled more rapidly and more intracellular localized channels were observed at a given time point. These mutants were functionally capable of making L-type channels and binding DHPs, but may have differed from WT in membrane retention. It would be of interest to learn if the dead channels that were incapable of membrane targeting might actually form functional channels if they were able to target to the plasma membrane.

Finally, the C-terminal domain may be responsible for binding to other proteins, and this association may participate in the processing and the localization of the channels to the plasma membrane. Interestingly, the targeting domain V identified here contains the “IQ” calmodulin binding site, which is located between positions 1654 and 1665 (20–23). Thus, it is possible that an interaction between the \(\alpha_{1C}\) subunit and calmodulin may play a role in membrane targeting of the Ca\(^{2+}\) channels. However, deletion of the IQ motif in a splice variant of the \(\alpha_{1C}\) subunit had no deleterious effect on peak currents when expressed in Xenopus oocytes (20). In several such mutants lacking the IQ motif, peak currents were greater than or equivalent to currents from wild type channels in Xenopus oocytes (20), suggesting that these mutants did not have targeting defects. In addition, neutralization of the basic residues within the IQ motif led to a loss of calmodulin binding but did not result in reduced currents in Xenopus oocytes (20). Taken together, these results suggest that the IQ motif does not play
a role in membrane targeting. However, further studies are necessary to test the role of the IQ motif and upstream residues in domain V in channel targeting in mammalian cells, and to test whether the disruption of the interaction between the \( \alpha_{1C} \) subunit and calmodulin affects the localization of the L-type channels in mammalian cells. In addition, since the protein sequence within the targeting domain “V” of the \( \alpha_{1C} \) subunit is homologous among all other \( \alpha_{1} \) subunits, this targeting domain identified here may be important for membrane targeting of other types of Ca\(^{2+}\) channels as well.

In summary, we have identified domains in the C terminus of \( \alpha_{1C} \) subunit that are critical for membrane targeting of the L-type Ca\(^{2+}\) channels, and targeting of the channels to the plasma membrane is required to obtain functional Ca\(^{2+}\) channels.

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