Membrane Targeting of L-type Calcium Channels

ROLE OF PALMITOYLATION IN THE SUBCELLULAR LOCALIZATION OF THE β2a SUBUNIT

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Voltage-dependent calcium channels are heteromultimeric proteins composed of a pore-forming α subunit, which determines many of the biophysical and pharmacological properties of the channel, and at least two other modulatory subunits, termed αδ and β (1, 2). Although these channels have been extensively studied electrophysiologically, less is known about the biochemical properties of these proteins due to their rarity in native tissues. The αδ and β subunits contain no homology to any known proteins and are involved in the modulation of channel properties. To date, four separate β isoforms have been identified, each of which contains a central conserved core flanked by unique N- and C-terminal regions specific to each isoform (1). Although the β subunits are highly hydrophilic proteins with no predicted membrane-spanning domains, we recently demonstrated that the cardiac β2a isoform was localized to the plasma membrane even in the absence of an α1 subunit (3).

Co-expression of an accessory β subunit with an α1 subunit in heterologous mammalian cell systems results in an increase in the number of drug/toxin binding sites (3–7), an increase in peak current amplitude (3, 8–11), and an increase in the number of channels at the cell surface (3, 8, 10, 11). The increase in channels at the plasma membrane has been demonstrated both biochemically (3) and electrophysiologically (8, 10, 11), and probably accounts for the increased drug/toxin binding sites and the increase in peak current amplitude observed upon β subunit co-expression. In addition, it has been demonstrated recently that calcium currents, charge movements, and the number of dihydropyridine receptors were largely reduced in skeletal muscle myotubes of β1 subunit null mice (12) but could be restored by transfection of the β1 subunit, suggesting that the β subunit played important roles in maintaining the expression of the α1 subunits. The major identified α1–β interaction site involved regions conserved among all known α1 and β subunit isoforms (13, 14), and further characterization of this domain revealed that interactions between different β subunits and a specific α1 subunit were fairly similar in affinity (15). Likewise, it has been shown that all four known β subunit isoforms were capable of modulating currents from channels containing the cardiac α1c subunit (6, 15–19).

Recently, we identified sites of palmitoylation in the rat β2a subunit of voltage-dependent calcium channels (8). Palmitoylation is a post-translational modification involving the reversible addition of a 16-carbon palmitic acid group to the cysteine residues of proteins through a labile thioester linkage (20). The mechanisms involved in the addition and removal of palmitic acid are still unclear, although palmitoyl transferases that exhibit catalytic selectivity for palmitic acid have been recently purified (21, 22). Palmitoylation can be dynamically regulated due to the labile nature of the thioester bond, and studies have demonstrated the receptor-regulated depalmitoylation of certain proteins (23, 24). Three other known β subunit isoforms (β1, β3, and β4) were found not to be palmitoylated (8), making palmitoylation the first identified biochemical modification unique to a specific β subunit isoform. Consequently, palmitoylation could provide a mechanism for the selective regulation of voltage-dependent calcium channels containing a β2a subunit.

Palmitoylation of β2a required both the Cysβ and Cysα residues in the N terminus, since site-directed substitution of either of these residues resulted in the loss of palmitate incorporation (8). The mutation of these residues in the palmitoylation-deficient β2a(Cys/ Cys) protein resulted in dramatic changes in whole-cell ionic con-
ductance without affecting the number of functional channels at the cell surface (8). The studies presented herein describe the effects of palmitoylation on the subcellular localization of the β2α subunit and further define the determinants of palmitoylation in the β2α sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**—The large T-antigen-transformed human embryonic kidney cells (tsA201) were the generous gift of Dr. Richard Horn (Thomas Jefferson University). The Card C antisera that detects the αt subunit, the β3 antisera that is specific for β3, and the “β-general” (βgen) antisera that detects all β subunit isofoms were previously described (3, 8). Protein G-Ultralink resin and the Supersignal enhanced chemiluminescence detection kit were purchased from Pierce. [3H]Palmitic acid was purchased from American Radiolabeled Chemicals (St. Louis, MO). Fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-coupled secondary antibodies for immunohistochemical studies were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). All other reagents were from standard sources.

**Preparation of Expression Vectors for Mutant and Chimeric β Subunits**—The pCMV-β1b, pCMV-β2a, and pCR-β2 expression plasmids were previously described (8). The pCR-β2a and pCR-β2a(C3S/C4S) expression plasmids used for the studies described herein were generated by subcloning a 2.3-kilobase pair EcoRI-PstI fragment from the pRBC-β2a or pRBC-β2a(C3S/C4S) vector (3) into the EcoRI and PstI sites of the pCR3 vector (Invitrogen). Plasmids for the expression of the rabbit β2a and rabbit β3 subunits, pCDNA-β2a and pCDNA-β3, were the generous gift of Dr. Franz Hofmann (Technical University of Munich).

Site-directed mutagenesis of the β2a subunit was performed using a megaprimer mutagenesis protocol as described previously (8). Briefly, mutagenic oligonucleotide primers were used in combination with another oligonucleotide primer to the β2a sequence to amplify a 400–700-base pair megaprimer product by the polymerase chain reaction (PCR). This product was used in combination with another oligonucleotide primer for PCR, generating a product which contained unique restriction sites for subcloning into mammalian cell expression vectors. Sequences containing mutagenic sites were substituted into the pCR-β2a or the pRBC-β2a expression vectors as BglII/XhoI fragments. The mutagenic primers used to generate the various mutations described herein are as follows: β2a(P119A), 5'-GAAATCGGATTTATTGCTAGC-3'; β2a(P234R), 5'-GGTTGGTGCGGC-GATGCTGAGGGTGTAGGAG-3'; β2a(P1115A/F117A/P119L), 5'-G-GTAAAGAGGCGTGGCCAGCTGACTTCACACAGCGCGCCTG-3'. The N-terminal region of either β2a or β3(C3S/C4S) was amplified by PCR using the following oligonucleotide primers: plus strand, 5'-AAAGGCTACACGCTGCGTACCAG-3'; minus strand, 5'-GGATCCAGATCAGGAGTTGAGGACG-3'. The N-terminal deleted β2a and β3 sequences were generated by PCR using the following oligonucleotide primers: plus strand, 5'-GCGGGATCCGCCGACTCCTACACCAAGCCGCCG-3'; minus strand, 5'-CGGGATCCGCCGACTCCTACACCAAGCCGCCG-3'. The C-terminal region was amplified by PCR using the following oligonucleotide primers: plus strand, 5'-GCGGGATCCGCCGACTCCTACACCAAGCCGCCG-3'; minus strand, 5'-GGATCCAGATCAGGAGTTGAGGACG-3'. Chimeric fragments were subcloned into pCR3. Sequences of mutant β subunits were verified using dyeode sequencing.

**Cell Culture and Transfection Protocols**—Cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin at 37 °C in 5% CO2. Transfections were performed using the HEPES-buffered calcium phosphate method as described previously (8). A total of 30–40 μg of plasmid DNA/plate were used for each transfection. Briefly, plasmid DNA precipitates were layered on cells for 4–6 h, after which cells and plasmid precipitate were incubated in 10% MeSO4/Dulbecco’s modified Eagle’s medium for 6 min. Following this MeSO4 shock, cells were refed with fresh medium and analyzed 40–50 h later. For metabolic labeling experiments, two plates of transfected cells were used for each data point.

**Metabolic Labeling with [3H]Palmitic Acid**—Transfected cells were metabolized with 0.5 μCi/ml [3H]palmitic acid in DME/F-12 (Sigma) as described previously (8). Palmitoylation experiments were performed a minimum of two times with each mutant, with virtually identical results. Membrane particulate fractions from metabolically labeled cells were solubilized with 0.4 M NaCl and 1% digitonin and then immunoprecipitated with the β2α antisera. Gels for fluorography were treated with ENLIGHTNING (NEL Life Science Products) and then exposed to film for 2–4 weeks at −80 °C.

**Membrane Isolation, Immunoprecipitation, and Immunoblotting**—Membrane particulate fractions were isolated from transfected cells as described previously (3). For co-immunoprecipitation of αt and β subunits, membrane particulate fractions were solubilized with a combination of 0.4 M NaCl, 1% Triton X-100, and 0.1% SDS. Cells were solubilized on ice for 30 min and then centrifuged in a Beckman Ty65 rotor at 45,000 rpm for 1–2 h. Soluble fractions were incubated with 40 μl of αt and 40 μl of protein G-Ultralink resin (50% slurry) overnight with agitation at 4 °C. Immunoprecipitates were washed with homogenization buffer 3–5 times and eluted with SDS sample buffer. Immunoblotting procedures were performed as described previously (3). Detection of immunoreactive bands was performed using either enhanced chemiluminescence (Pierce) or colorimetric enhanced diaminobenzidine substrate reaction (Pierce).

**Immunofluorescence and Confocal Microscopy**—For immunohistochemical studies, transfected cells were transferred to coverslips coated with 20 μg poly-1-lysine immediately following MeSO4 shock. At 40–50 h after MeSO4 shock, cells were fixed and permeabilized using methanol/acetic (1:1, v/v) at 4 °C for 10 min. Cells were then incubated for 6–12 h with the primary antisera in the presence of 0.1% bovine serum albumin (Bsa) in PBS. After washing PBS, secondary fluorescent isothiocyanate- and tetramethylrhodamine isothiocyanate-coupled antibodies were subsequently added and incubated for 2–4 h in the presence of 0.1% BSA serum albumin (w/v). Confocal immunofluorescence microscopy was performed in the Northwestern University Cell Imaging Facility using a Zeiss LSM-10 laser-scanning microscope.

**RESULTS AND DISCUSSION**

The Loss of Palmitoylation Affected the Subcellular Localization of the β2a Protein—Previously, we had performed biochemical studies demonstrating that the expressed rat β2β subunit fractionated to crude particulate fractions (3). In parallel, results of immunohistochemical studies demonstrated the localization of the β2a subunit to the plasma membrane even in the absence of a co-expressed αt subunit (3). Mutation of Cys2 and Cys3 in the β2a(C3S/C4S) mutant eliminated palmitoylation of the β2a subunit (8), while this mutant protein still fractionated almost exclusively to crude particulate fractions (8), so we did not examine the subcellular distribution of the mutant protein by confocal microscopy. Here we report the results of immunohistochemical studies that were performed to address further the subcellular distribution of the β2a(C3S/C4S) protein in transiently transfected tsA201 cells. Transfected cells expressing either the wild-type rat β2a subunit or the palmitoylation-deficient β2a(C3S/C4S) subunit were fixed with methanol/acetone as described under “Experimental Procedures,” immunohistochemically stained with the β2a antibody, and visualized using confocal immunofluorescence microscopy. Cells expressing the wild-type β2a subunit exhibited continuous staining along the plasma membrane and/or immediately adjacent to the plasma membrane (Fig. 1A), consistent with our previously reported results (3). In marked contrast, the palmitoylation-deficient β2a(C3S/C4S) subunit displayed a diffuse intracellular staining pattern (Fig. 1B). The staining pattern did not noticeably change following treatment of these cells with the protein synthesis inhibitor cycloheximide (Fig. 1B, right), which was previously shown to enhance visualization of plasma membrane staining of the β2a subunit in transfected cells. These results suggested that palmitoylation of the β2a subunit was critical for the plasma membrane localization of this subunit when it was expressed in the absence of an αt subunit.

The β1b and β3 Subunits Exhibited a Diffuse Intracellular Localization—We previously reported that two other β subunit isofoms, β1b and β3, were not palmitoylated (8). In order to assess their cellular localization, transiently transfected tsA201 cells expressing either the β1b or β3 subunit were frac-

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1 The abbreviations used are: PCR, polymerase chain reaction; SH3, src homology 3; BID, β interaction domain.
geting of different cell line, it is possible that interaction with cell type—which asserted a membrane localization for this protein in reported here differs from that reported in another study, of the cells with the protein synthesis inhibitor, cycloheximide (100 μM; right).

The palmitoylation-deficient β2a subunit does not localize to the plasma membrane. Cells expressing either the wild-type β2a subunit or the palmitoylation-deficient β2a(C3S/C4S) mutant were fixed, immunohistochemically stained with the β2a antibody, and analyzed by confocal immunofluorescent microscopy. A, the wild-type β2a subunit exhibited a plasma membrane staining pattern in transiently transfected human embryonic kidney-tsA201 cells, similar to previously described results (3). B, by contrast, the palmitoylation-deficient mutant exhibited an intracellular staining pattern, with little specific staining discernable at the plasma membrane. The staining pattern of the β2a(C3S/C4S) mutant did not change following a 2-h pretreatment of the cells with the protein synthesis inhibitor, cycloheximide (CHX; 100 μg/ml; right).

A
[Image 69x487 to 277x729]

B
β2a(C3S/C4S) β2a(C3S/C4S) + CHX

FIG. 1. The palmitoylation-deficient β2a subunit does not localize to the plasma membrane. Cells expressing either the wild-type β2a subunit or the palmitoylation-deficient β2a(C3S/C4S) mutant were fixed, immunohistochemically stained with the β2a antibody, and analyzed by confocal immunofluorescent microscopy. A, the wild-type β2a subunit exhibited a plasma membrane staining pattern in transiently transfected human embryonic kidney-tsA201 cells, similar to previously described results (3). B, by contrast, the palmitoylation-deficient mutant exhibited an intracellular staining pattern, with little specific staining discernable at the plasma membrane. The staining pattern of the β2a(C3S/C4S) mutant did not change following a 2-h pretreatment of the cells with the protein synthesis inhibitor, cycloheximide (CHX; 100 μg/ml; right).

A
[Image 327x446 to 535x729]

B
β1b β2a(P119A) β3

FIG. 2. The β1b and β3 subunits fractionate with crude membranes and exhibit a diffuse intracellular localization. A, transiently transfected cells expressing the β1b or β3 subunits were fractionated by high speed centrifugation into crude membrane particulate fractions (P) or cytosolic supernatants (S) and immunoblotted with the β2a SH3 domain antisera following SDS-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel. Equivalent proportions of crude membranes and supernatants were loaded for each sample, and the locations of the molecular mass markers are indicated on the right. Both the β1b and β3 subunits localized primarily to particulate fractions. B, transfected β1b and β3 cells were immunohistochemically stained with the β2a(C3S/C4S) antiserum and viewed by confocal immunofluorescence microscopy. Both the β1b and β3 subunits exhibited a diffuse pattern of intracellular staining, with no specific plasma membrane staining discernable. The staining patterns were unchanged following pretreatment of the cells with 100 μg/ml of cycloheximide (data not shown).

Mutations in a src Homology 3 (SH3) Motif Affected Subcellular Localization of the β2a Subunit—All four calcium channel β subunits contain a region resembling SH3 domains, which have been implicated in mediating protein-protein interactions through the binding of proline-rich sequences (26). The SH3-like motif is located in the first of two domains that are highly conserved among the four β subunit isoforms identified to date (Fig. 3A). Fig. 3A shows a Lipmann-Pearson alignment of the SH3 domain from β2a and the original SH3 domain from src (26). Also shown for comparison is an alignment of the src SH3 domain with the SH3 domain of spectrin (27). To assess whether the SH3 domain of β2a played a role in membrane localization, site-directed mutagenesis was used to target residues in the C-terminal region of the β2a SH3 domain corresponding to residues shown to be important for the binding of the src SH3 domain to proline-rich ligands (28). The β2a(I115A/F117A/P119L) and β2a(I115A/V117A/P119L) subunits were transiently transfected into tsA201 cells and analyzed by immunohistochemical staining. As seen in Fig. 3B, the β2a(P119A) mutant exhibited considerable staining at the plasma membrane as well as some diffuse intracellular distribution. The β2a(I115A/V117A/P119L) mutant appeared to be localized intracellularly, with less clear staining visible at the plasma membrane (Fig. 3C). These results initially suggested that mutations disrupting the β2a SH3 domain affected targeting of
FIG. 3. Mutations in the SH3 motif and BID affected subcellular localization of the β2 protein. A, a linear representation of the β subunit indicates the two domains conserved among the four different β subunit isoforms as well as the relative locations of the SH3 motif and the BID. Also shown is an alignment of the β2a SH3 motif with the SH3 domain of src; an alignment of the src SH3 domain with the well characterized spectrin SH3 domain is given for comparison. The arrowheads indicate mutated residues in the β2a subunit corresponding to residues in the src SH3 domain that were shown to be important for mediating protein-protein interactions. Cells expressing the β2a(P119A) mutation (B), the β2a(I115A/F117A/P119L) mutation (C), or the β2a(P234R) mutation in the BID (D) were immunohistochemically stained with the β2a antibody and analyzed by confocal immunofluorescence microscopy. Cells expressing the β2a(P119A) mutation typically revealed discernible plasma membrane staining in addition to some diffuse intracellular staining. Plasma membrane staining was not clearly visible in cells expressing the β2a(I115A/F117A/P119L) subunit or the β2a(P234R) subunit.
the β_{2a} protein to the plasma membrane, potentially through disruption of an SH3-mediated interaction. However, the possibility that these mutations may have disrupted the global structure of the β_{2a} protein cannot be discounted, although the ability of the mutants to be recognized by the β_{2} and β_{GEN} antibodies suggests that the mutations do not drastically alter the structure of the protein.

**Mutations of the β Interaction Domain (BID) Affected Subcellular Localization of the β_{2a} Subunit**—Site-directed mutagenesis was used to create a mutation at Pro^{234} in the previously identified BID, which has been suggested to provide the major site of interaction of β subunits with α_{1} subunits (14). Mutation of the corresponding Pro^{237} residue in the β_{1b} subunit to Arg appeared to eliminate completely α_{1}-β interaction (14). Transfected cells expressing the β_{2a}(P234R) mutant were immunohistochemically stained and analyzed by confocal immunofluorescence microscopy to assess subcellular distribution (Fig. 3D). Unexpectedly, staining with the β_{2a} antibody revealed that the β_{2a}(P234R) subunit exhibited a diffuse intracellular staining pattern (Fig. 3D) similar to that seen with the β_{2a}(C3S/C4S) mutant and the nonpalmitoylated β_{1b} and β_{3} subunits (see Figs. 1 and 2). Since the β_{2a}(P234R) mutation is expected to impair the BID, this result suggested the possibility that the plasma membrane staining normally seen with the wild-type β_{2a} subunit might be due to interaction with an endogenous α_{1} subunit. However, this possibility seemed unlikely given that the high levels of heterologous β_{2a} expression were most certainly higher than levels of endogenous channels. Additionally, electrophysiological measurements did not reveal the presence of measurable calcium currents or charge movement in cells transfected with only the β_{2a} subunit (data not shown). An alternative conclusion is that mutation of Pro^{234} may have affected subcellular localization through global disruption of the β_{2a} protein structure, although the mutant protein retained sufficient native structure to be identified by the anti-β antibodies.

**Mutations Distal to the N Terminus of β_{2a} Affected Palmitoylation**—Mutations in the BID and SH3 domain resulted in a diffuse intracellular localization of the β_{2a} protein that resembled the subcellular localization observed with the palmitoylation-deficient β_{2a}(C3S/C4S) mutant (Fig. 1B) and the nonpalmitoylated β_{1b} and β_{3} subunits (Fig. 2B). Metabolic labeling of transfected cells with [H]palmitic acid was performed to address whether the β_{2a}(P234R), β_{2a}(P119A), and β_{2a}(I115A/F117A/P119L) mutants were palmitoylated yet unable to target to the plasma membrane due to disruption of a protein-protein interaction. Following the metabolic labeling of transfected cells, proteins were immunoprecipitated from solubilized membrane fractions using the β_{GEN} antiserum as described under “Experimental Procedures.” Immunoprecipitated fractions were analyzed both by quantitative immunoblotting and by fluorography. Incorporation of [H]palmitic acid in the immunoreactive bands was quantified using liquid scintillation counting and normalized by protein amounts to values obtained for the wild-type β_{2a} subunit. A representative experiment is summarized in Fig. 4. Unexpectedly, the BID and SH3 mutations all resulted in decreased amounts of palmitate incorporation compared with wild-type β_{2a}, although the reductions in palmitoylation of the β_{2a}(P234R) and β_{2a}(I115A/F117A/P119L) mutants were much more extensive than the very modest reduction observed with the β_{2a}(P119A) mutant. Since metabolic labeling measures steady-state levels of [H]palmitate incorporation, it was unclear whether the decreased palmitoylation observed in the different mutants resulted from changes in the kinetics of either palmitate addition or removal.

These results suggested that the altered subcellular localization seen with the BID and SH3 domain mutants could be due solely to changes in the palmitoylation of β_{2a} rather than through the disruption of protein-targeting interactions mediated by these domains. In support of this hypothesis, the β_{2a}(P119A) mutant, which should disrupt SH3 ligand binding (28), exhibited only a small decrease in palmitoylation and was associated with the plasma membrane with only a small amount of intracellular localization. Potentially, mutation of the BID and SH3 domains affected protein interactions that were critical for palmitoylation of the β_{2a} protein. In the absence of a known tertiary structure for the β_{2a} protein, it is uncertain whether these domains may be in close topological proximity on the surface of the protein. Conceivably, the changes in subcellular localization of the SH3 and BID mutants, as well as any potential effects on channel function, could result primarily from a disruption of palmitoylation of the β_{2a} protein. These results caution that structure-function analysis of calcium channel β subunits through site-directed mutagenesis, in the absence of biochemical studies, could result in misleading interpretations. Elucidation of the three-dimensional structure of β subunits should facilitate the characterization of these proteins and reveal the mechanisms by which different mutations could affect protein structure and/or function. The present findings do not completely rule out a role for the BID and/or SH3 domain in β subunit localization. Further studies may further characterize protein interactions involving these domains and their potential role in channel regulation.

**The 16-Amino Acid N Terminal of the β_{2a} Protein Was Sufficient to Confer Palmitoylation**—The loss of palmitoylation in different β_{2a} mutants appeared to correlate with a loss of plasma membrane localization, indicating that palmitoylation could be the predominant mechanism regulating the subcellular localization of the β_{2a} protein. To define further the structural determinants necessary for palmitoylation, the N-terminal regions of the nonpalmitoylated β_{1b} and β_{3} subunits were
cDNA sequences (Fig. 5) exhibited a molecular mass higher than that predicted by previously published reports (8). Surprisingly, replacement of the 15-amino acid N terminus of \( \beta_3 \) with the larger 16-amino acid N terminus of \( \beta_{2a} \) resulted in a decreased apparent molecular mass of the \( \beta_{2a/3} \) chimera, suggesting that the increased electrophoretic mobility observed with the \( \beta_3 \) protein probably involved structural determinants and/or post-translational modifications in the \( \beta_3 \) N terminus. The \( \beta_{2a/1b} \) chimeras still exhibited molecular masses higher than those predicted by the cDNA sequences, suggesting that structural elements responsible for the aberrant electrophoretic mobility of this protein were located in the isoform-specific C-terminal region rather than the N terminus. Post-translational modifications unique to the \( \beta_1 \) and \( \beta_3 \) isofoms could be the cause of the higher molecular masses observed with these two proteins.

Transiently transfected cells expressing the different chimeric \( \beta \) subunits were metabolically labeled with \(^{3}H\)palmitic acid. Subsequently, the chimeric \( \beta \) proteins were isolated by immunoprecipitation and analyzed both by immunoblotting and fluorography (Fig. 5B). For comparison, cells expressing the wild-type \( \beta_{2a} \) subunit were also metabolically labeled in parallel. The immunoblot stained with the \( \beta_{\text{GEN}} \) antiserum (Fig. 5B, top) indicated the expression and immunoprecipitation of each \( \beta \) subunit. The fluorogram (Fig. 5B, bottom) indicated the incorporation of \(^{3}H\)palmitic acid only in \( \beta \) subunits containing the wild-type \( \beta_{2a} \) N terminus. By contrast, chimeric \( \beta \) subunits containing the \( \beta_{2a}(\text{C3S/C4S}) \) mutation were not palmitoylated. These results demonstrated that the 16-amino acid N-terminal region of \( \beta_{2a} \) contained all of the structural determinants necessary to confer palmitoylation to nonpalmitoylated \( \beta_{2a} \) subunits.

**Palmitoylation by Itself Was Not Sufficient to Confer Plasma Membrane Localization**—Transiently transfected cells expressing the different chimeric \( \beta \) subunits were analyzed by immunohistochemical staining and confocal immunofluorescence microscopy (Fig. 6). Although the \( \beta_{2a/1b} \) and \( \beta_{2a/3} \) subunits were palmitoylated (Fig. 5B), the distribution of these proteins was intracellular (Fig. 6, left panels), resembling the distribution of...
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the nonpalmitoylated β11 and β2 subunits (Fig. 2) and the palmitoylation-deficient β2a(C3S/C4S) mutant (Fig. 1). The staining pattern of the palmitoylated chimeras (Fig. 6, left panels) was difficult to distinguish from that of the nonpalmitoylated β2a/1b(C3S/C4S) and β2a/2a(C3S/C4S) subunits (Fig. 6, right panels). These results demonstrated that palmitoylation by itself was insufficient to confer the plasma membrane localization typically seen with the β2a subunit. Potentially, other elements of the β2a sequence, most likely in the isoform-specific C-terminal region, are also involved in determining subcellular localization. However, the results with the different β2a subunits described above, which localize intracellularly despite the presence of intact C-terminal regions, suggest that membrane targeting of the β2a protein requires both palmitoylation and other structural elements in the β2a sequence.

**Brefeldin A Increased Palmitoylation of β2a—**To assess whether palmitoylation of the β2a protein occurs prior to transport to the plasma membrane, transfected cells were treated with the fungal metabolite brefeldin A, which prevents the trafficking of proteins to the plasma membrane through the trans-Golgi network. Cells were metabolically labeled with [3H]palmitic acid following treatment with either brefeldin A or Me2SO vehicle and then metabolically labeled with [3H]palmitic acid to address the amount of protein in the immunoprecipitated pellet. Treatment with brefeldin A increased palmitoylation of the β2a protein about 2–6-fold in two separate experiments. Brefeldin A has been previously shown to increase (29, 30) as well as decrease (31) the palmitoylation of other proteins. The apparent increase in the palmitoylation of β2a upon brefeldin treatment probably results from the retention of the protein in the endoplasmic reticulum, thus preventing dynamic depalmitoylation of β2a by enzymes localized at the plasma membrane. These results suggest that palmitoylation of the β2a protein occurs shortly after synthesis and prior to transport through the trans-Golgi network.

**Rabbit β2 Isomers Lacked Palmitoylation and Exhibited Diffuse Intracellular Localization—**The sequences of several β2 subunit clones isolated from rabbit cardiac tissue were identified as β2 splice variants (32) by virtue of the similarity of their C-terminal sequences to that of the rat β2a subunit, which was the first β2 subunit identified (6). The first rabbit β2 clone identified was given the designation of β2a (32), despite the fact that it contained an N terminus that differed significantly from that of the rat β2a subunit. The rabbit β2a also contains a distinct N-terminal domain (32). Transiently transfected tsA201 cells expressing the rabbit β2a and β2b subunits were metabolically labeled with [3H]palmitic acid to address
whether these β2 splice variants were palmitoylated. Proteins were immunoprecipitated with the β2GN antiserum and analyzed by immunoblotting and fluorography (Fig. 8A). Immunoblotting with the β2a antibody was used to visualize the immunoprecipitated proteins (Fig. 8A, top). The rabbit β2a and β2b subunits were detected as immunoreactive bands at ~69 and 72 kDa, respectively, as predicted by their cDNA sequences (32). As expected from the absence of Cysα and Cysβ in the rabbit β2a and β2b sequences (32), the fluorogram indicated that 3Hpalmitic acid was only incorporated into the rat β2a protein (Fig. 8A, bottom). The lack of palmitoylation of the rabbit β2a subunit confirmed the expectation from the primary sequences that this protein is biochemically distinct from the rat β2a isoform, despite having the same subunit designation.

Further studies were performed to assess the subcellular localization of the rabbit β2a and rabbit β2b subunits in transfected cells (Fig. 8B). Confocal microscopy of immunohistochemical staining with the β2a antibody revealed that both the rabbit β2a and β2b proteins were localized to intracellular structures (Fig. 5B). The subcellular staining pattern of the rabbit β2a and β2b subunits differed markedly from that of the rat β2a subunit (Fig. 1A), further demonstrating the biochemical distinction between the originally identified rat β2a subunit (6) and the subsequently identified rabbit β2a subunit (32). The biophysical properties of the rat versus the rabbit β2a subunits have not been systematically compared.

Palmitoylation Affects β2a Subcellular Localization and Channel Function—Mutations affecting palmitoylation, which include the β2a(C3S/C4S) mutant, the β2a(P234R) mutant, and the β2a(1115A/F117A/P119L) mutant, also resulted in the localization of the β2a protein to intracellular membrane systems. Likewise, nonpalmitoylated rabbit β2 isoforms localized intracellularly rather than to the plasma membrane. These results suggest that palmitoylation is pivotal for the plasma membrane localization observed with the wild-type rat β2a protein. However, studies on chimeric β1 and β3 subunits, which were still intracellularly localized despite being palmitoylated, also clearly indicated that palmitoylation was by itself insufficient to localize these β subunits to the plasma membrane. Taken together, it appears that the unique plasma membrane localization of the rat β2a subunit requires a combination of both palmitoylation as well as other structural determinants that may be unique to the β2a sequence.

The mechanism behind the regulation of channel function by palmitoylation is still unclear. The β2a(C3S/C4S) subunit, when co-expressed with the α1C subunit in Xenopus oocytes, supported increases in current density that were similar to or greater than those observed with wild type β2a.2 In contrast, a decrease in whole-cell calcium currents was seen with the β2a(C3S/C4S) subunit in mammalian HEK cells (8). An examination of electrophysiological data collected from a large population of α1Cβ2a cells revealed that for cells expressing a given number of channels, as measured by charge movement, there was a wide range in the corresponding amount of whole-cell calcium current (8). The diversity in the ratio of ionic current to charge movement from cell to cell may reflect variations in the population of palmitoylated channels between different cells and suggests that the palmitoylation of β2a may indeed have a dynamic component.

A recent study in Xenopus oocytes demonstrated that the electrophysiological properties unique to the rat β2a isoform could be attributed directly to palmitoylation of the β2a subunit (33), specifically the lack of prepulse facilitation seen upon co-expression with the α1C channel, the inhibition of voltage-induced inactivation of α1E channels, and the blockage of α1E channel inhibition by G protein-coupled receptors. Since palmitoylation is thought to be regulated through receptor-mediated processes (20), the dynamic palmitoylation and depalmitoylation of β2a could potentially allow humoral regulation of certain subsets of channels in different cells, resulting in modulation of channel activity and properties such as neuronal plasticity. Further studies involving the identification of biochemical pathways involved in the palmitoylation of β2a may lend insight into the mechanism by which this modification regulates channel function, as well as the role of channel palmitoylation in the regulation of calcium entry in different cells.

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