Identification of Palmitoylation Sites within the L-type Calcium Channel β2a Subunit and Effects on Channel Function

(Received for publication, August 5, 1996, and in revised form, August 30, 1996)

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The hydrophobic β2a subunit of the L-type calcium channel was recently shown to be a membrane-localized, post-translationally modified protein (Chien, A. J., Zhao, X. L., Shirokov, R. E., Puri, T. S., Chang, C. F., Sun, D. D., Rios, E., and Hosey, M. M. (1995) J. Biol. Chem. 270, 30036–30044). In this study, we demonstrate that the rat β2a subunit was palmitoylated through a hydroxylation-sensitive thioester linkage. Palmitoylation required a pair of cysteines in the N terminus, Cys2 and Cys4; mutation of these residues to serines resulted in the absence of palmitoylation, suggesting that palmitoylation could provide a basis for the regulation of L-type channels through modification of a specific β isoform. Three other known β subunits were also analyzed and found not to undergo palmitoylation.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Palmitoylation Mutants—The CDNA for the different β subunit isoforms were generously donated by Dr. Ed Perez-Reyes (Loyola University, Chicago IL). The rat β1a, β2a, and β3 subunits were each expressed in tsA201 cells (HEK293 cells transformed with SV40 large T antigen) using methods previously described (2). The β3NKT3 mutant was constructed by ligation of two PCR products synthesized with the following primers: 5′-GGCGATGTACTAGTCCGCTGCACTGCATGAAGAGG-3′ (plus strand #1); 5′-GGATCCCATGAAGGAGG-GAGCCAGGC-3′ (minus strand). For all mutants, PCR fragments were then sublimated as BglII–XhoI fragments into the pRBGβ2a-KT3 plasmid (2). Three other confirmed mutations were used as a template. A BamIII site was inserted to encode for residues 17 and 18, resulting in a DNA sequence encoding for the β2a subunit residues 2–16. The β3CS, β3C4S, β3C3S/CS4S mutants were also analyzed and found not to undergo palmitoylation. Two known β subunits were analyzed and found not to undergo palmitoylation.

The L-type calcium channel β2a subunit is a highly hydrophobic protein with no predicted membrane-spanning regions (1). Nevertheless, we previously demonstrated that this protein was membrane-localized when expressed in human embryonic kidney tsA201 cells (2). In addition, pulse-chase analysis suggested that the β2a subunit was post-translationally modified, resulting in an increase in apparent molecular mass from 68 kDa for the nascent protein to proteins of 70–72 kDa. Here we demonstrate that one modification of the β2a subunit involves palmitoylation, a post-translational modification that has been shown to facilitate the membrane localization of other hydrophobic proteins. Palmitoylation involves the addition of palmitic acid to cysteine residues through a thioester linkage (3, 4). This modification is thought to be dynamic due to the reversible nature of the thioester bond (3, 4). However, despite the increasing number of palmitoylation sites identified, there appears to be no consensus motif for predicting candidate cysteine residues, which may be acylated. The residues involved in palmitoylation of the β2a subunit were identified, and the functional roles of these amino acids were investigated in biochemical and electrophysiological studies using mutant proteins. Three other known β subunits were also analyzed and found not to undergo palmitoylation.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 43, Issue of October 25, pp. 26465–26468, 1996 © 1996 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
RESULTS AND DISCUSSION

Palmitoylation of Different β Subunit Isoforms—An epitope common to the four known β subunit isoforms (1, 7–10) was used to generate a generic β antiseraum (βGEN). The βGEN antiseraum was able to specifically recognize β1b (72–75 kDa), β2a (68–72 kDa), β3 (58 kDa), and β4 (58 kDa) in tsA201 cells transiently transfected with cDNAs for each isoform (Fig. 1A). In order to investigate the possibility that β subunits may be palmitoylated, transiently transfected tsA201 cells expressing different β isoforms were metabolically labeled with [3H]palmitic acid, immunoprecipitated with the βGEN antiseraum, and subsequently analyzed for acylation of β proteins. The resulting fluorogram (Fig. 1B) indicated incorporation of 3H-radiolabel in a ~70-kDa protein in β2a cells (Fig. 1A, second lane), suggesting the addition of an acyl group. However, no specific radiolabeling of the β1b, β3, and β4 subunits was observed. The presence of the different β subunits in the immunoprecipitates was confirmed by immunoblotting (data not shown). Experiments were performed three times with identical results. Acylation of the β2a protein also occurred in Sf9 insect cells, indicating that this phenomenon was not cell-specific (data not shown).

Characterization of the Acyl Moiety on β2a—Acylation with palmitic acid occurs most frequently through a thioester linkage, which is sensitive to base hydrolysis (3, 4). To assess the nature of the acylation linkage on β2a, immunoprecipitated β2a from metabolically labeled cells was split into two equal fractions and treated with either 1 M hydroxylamine (pH 7.5) or 1 M Tris-HCl (pH 7.5) and analyzed by subsequent SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 1C). Immunoblotting was used to confirm that equal amounts of β2a protein were loaded in each lane (Fig. 1C, upper panel). Treatment with hydroxylamine led to a clear loss of [3H]signal on the fluorogram (Fig. 1C, lower panel), indicating that the acyl moiety on the β2a protein was attached via a base-sensitive

transfected cells expressing the different β subunit isoforms that were metabolically labeled with [3H]palmitic acid. The electrophoretic migrations of each β subunit, as determined by immunoblot, are indicated by arrows on the right. Numbers on the left indicate the migration of molecular mass standards. C, palmitoylated β2a from metabolically labeled cells was immunoprecipitated and assessed for sensitivity to either 1 M hydroxylamine (pH 7.5) or 1 M Tris-HCl (pH 7.5). Shown are the immunoblot (upper panel) and the corresponding [3H]-fluorogram of the same blot (lower panel). D, base-hydrolyzed extracts from β2a were analyzed by reverse phase HPLC. The arrowheads on top indicate the migration of [3H]-radiolabeled lipid standards: myristate (C14:0), palmitate (C16:0), and stearate (C18:0). Counts from the β2a base-hydrolyzed extract migrated as a single peak corresponding to the same elution fraction as the palmitic acid standard.

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American Radiolabeled Chemicals.

Immunoprecipitations and SDS-Polyacrylamide Gel Electrophoresis Analysis—Membrane preparations, immunoprecipitations, and immunoblots were performed as described previously (2). For fluorography, acrylamide gels were stained, fixed, treated with Amplify (Amerham Corp.) or ENHANCE (DuPont NEN), and then exposed to film (DuPont NEN) for 1–4 weeks.

Whole-cell Patch-clamp Analysis—Cells were analyzed by whole-cell patch-clamp using data acquisition and pulse generation protocols similar to those previously described (2). Voltage pulse duration was 300 ms. The pipette solution contained (in mM): 110 cesium aspartate, 20 CaCl2, 10 EGTA, 10 HEPES, 5 Mg-ATP. The extracellular solution contained (in mM): 150 tetraethylammonium chloride, 10 CaCl2, 10 HEPES. To record intramembrane charge movement, ionic currents were blocked by the addition of 10 μM GdCl3 in the bath (6). Holding potential was ~90 mV. Asymmetric currents were obtained by subtracting control transients obtained by pulsing between −150 and −90 mV. Maximal amplitude of the ionic current and maximal intramembrane charge movement were related to cell capacitance in order to compare their densities.

FIG. 1. Characterization of four β subunits from [3H]palmitate-labeled cells. A, a linear model of the β2a protein depicts the two domains conserved among all β subunits, as well as the epitope against which the βGEN antiseraum was generated. Immunoblots show that the βGEN antiseraum was capable of recognizing all four known β subunit isoforms expressed in transfected tsA201 cells. Each lane contained 50 μg of membrane particulate fractions. B, immunoprecipitates from
linkage consistent with palmitoylation.

Because palmitic acid can be naturally metabolized to myristic acid, reverse phase HPLC was used to identify the base-sensitive radioactive label. After labeling Sf9 cells expressing the β2a protein with [3H]palmitic acid, base hydrolysis and reverse phase HPLC were performed as described in Linder et al. (4). Fractions eluted off the column were assayed using liquid scintillation counting, which confirmed the results of the fluorogram; values obtained in this experiment are shown at the bottom of Fig. 2B. These results indicated that both Cys3 and Cys4 were necessary for palmitoylation of β2a.

Identification of Sites Important for Palmitoylation of β2a—The addition of palmitic acid usually occurs through a thioester linkage on cysteine residues, although no consensus sequence has been identified. To further investigate the site or sites of palmitoylation, site-directed mutants at Cys3 and Cys4 were expressed in metabolically labeled transfected tsA201 cells expressing either the wild-type β2a or the palmitoylation-deficient mutants were fractionated by centrifugation into membrane particulate fractions and soluble “cytosolic” fractions as described previously (2). The immunoreactive bands were excised from the nitrocellulose and analyzed with liquid scintillation counting, which confirmed the results of the fluorogram; values obtained in this experiment are shown at the bottom of Fig. 2B. These results indicated that both Cys3 and Cys4 were necessary for palmitoylation of β2a.
Palmitoylation of Calcium Channel β Subunit

Fig. 4. Electrophysiological analysis of channels containing wild-type or palmitoylation-deficient β_2a subunits. A, traces of whole-cell calcium current (left) and whole-cell charge movement (right) are shown from representative cells expressing α_1C alone and α_1C with either β_2a or the β_2a(C3S/C4S) mutant. Note the difference in vertical scale for α_1C charge movement measurements. B, results from several cells are plotted to allow comparison of current density (I_Ca) or β_2a(C3S/C4S) (Δ, n = 8) subunits. The inset contains data from cells expressing α_1C alone (C, n = 12), which had very low whole-cell calcium current as well as charge movement, which was at the limits of detection.

either salt- or detergent-solubilized fractions of transfected cells metabolically labeled with [3H]palmitic acid (Fig. 3B, top). However, only β_2a immunoprecipitated from the detergent-solubilized fraction contained [3H]palmitate-labeled β_2a (Fig. 3B, bottom). This result demonstrates that the palmitoylation of β_2a modifies the nature of its association with the membrane, confirming our previous hypothesis, which predicted distinct salt- and detergent-soluble populations of the β_2a subunit (2).

Effects of Palmitoylation on Channel Targeting and Channel Function—To address potential roles of palmitoylation on channel function and/or protein multimerization, cells expressing both the cardiac α_1C subunit (11) as well as either β_2a or β_2a(C3S/C4S) were analyzed. Both β_2a and β_2a(C3S/C4S) could be co-immunoprecipitated with α_1C, indicating that the subunits could be co-expressed and directly interact (data not shown). However, electrophysiological analyses demonstrated striking differences in the biophysical properties of channels with the β_2a(C3S/C4S) subunit (Fig. 4, A and B). Measurements of whole-cell calcium currents demonstrated that the expression of β_2a increased currents relative to α_1C alone; however, drastic reductions were seen in cells expressing α_1C and β_2a(C3S/C4S) (Fig. 4A, left panel). In contrast, charge movement was increased to similar values in either α_1C/β_2a or α_1C/β_2a(C3S/C4S) cells relative to α_1C cells, which exhibited charge movement that was at the lower limits of detection (Fig. 4A, right panel). This latter result indicated that similar numbers of functional channels were present in the plasma membrane of cells expressing either the wild-type or mutant β_2a subunits. Because previous studies have demonstrated a role for β subunits to recruit functional channels to the membrane (2, 12, 13), the present results demonstrate that wild-type and palmitoylation-deficient β_2a subunits are equivalent in this function. However, a plot of peak current amplitude versus charge movement demonstrated that the relationship of the amount of peak current to charge movement was dramatically decreased in channels with the β_2a(C3S/C4S) mutants compared with channels with wild-type β_2a (Fig. 4B), which indicated that less current was carried per functional channel in channels containing β_2a(C3S/C4S). These results suggested that the lack of palmitoylation and/or the lack of further post-translational modification that could require palmitoylation may have resulted in a β_2a subunit that was able to target channels to the membrane as described previously (2) but was altered in its ability to modify calcium channel currents.

Although it is difficult to attribute these functional changes directly to the loss of palmitic acid, it is clear that Cys²₈ and Cys⁴ were important determinants of palmitoylation and β_2a modulation of channel function. The molecular mechanisms underlying dynamic palmitoylation and de-palmitoylation remain unclear, although it has been suggested that agonists of certain receptors can stimulate the de-palmitoylation of proteins (14, 15). Intracellular enzymes involved in either the palmitoylation or de-palmitoylation of proteins have only recently been identified (16, 17). Continuing investigations on the pathways and enzymes involved in the regulation of palmitoylation and de-palmitoylation should facilitate studies on the role of this post-translational modification with respect to channel function.

Acknowledgments—The tsA201 cell lines were the generous gift of Dr. Richard J. Horn (Thomas Jefferson University, Philadelphia, PA). We thank Tipu S. Puri for assistance with the Sf9 cell culture and Dr. Maureen Linder for helpful advice concerning the metabolic [3H]palmitate labeling and the reverse phase HPLC analysis.

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J. Biol. Chem. 1996, 271:26465-26468.
doi: 10.1074/jbc.271.43.26465

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