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Posttranslational modification of voltage-dependent potassium channel Kv1.5: COOH-terminal palmitoylation modulates its biological properties

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Jindal HK, Folco EJ, Liu GX, Koren G. Posttranslational modification of voltage-dependent potassium channel Kv1.5: COOH-terminal palmitoylation modulates its biological properties. Am J Physiol Heart Circ Physiol 294: H2012–H2021, 2008. First published March 14, 2008; doi:10.1152/ajpheart.01374.2007.—The physiological function of ion channels is affected by protein-protein and protein-membrane interactions that modulate their activity and/or localization. Palmitoylation modulates protein function by facilitating targeted membrane association, interaction with other proteins, and determining subcellular localization. In this study, we demonstrate that the voltage-dependent potassium (Kv) channel Kv1.5 is palmitoylated and that the mutation of COOH-terminal cysteines is sufficient to abolish the palmitoylation of the Kv1.5 polypeptide in Chinese hamster ovary (CHO) cells. The labeling represented the thioester linkage of the labeled palmitic acid to cysteine rather than amide and oxygen ester linkages as judged by the release of the palmitic acid upon the treatment of the gel with hydroxylamine at a neutral pH. Site-directed mutagenesis and radiolabeling studies revealed that C593 was the sole site of palmitoylation. The elucidation of the biological function of palmitoylation revealed that the expression of the FLAG-Kv1.5 palmitoylation-deficient mutant (FL-Kv1.5Palm−) in stable CHO cells increased membrane expression as determined by the biotinylation of surface proteins and quantitative immunofluorescence analyses of these cells, in turn enhancing the outward potassium current. This enhanced surface expression and the currents were consequent to the slower rate of internalization, causing an increased localization of FL-Kv1.5Palm− in the plasma membrane compared with the wild-type FL-Kv1.5 channels. We conclude that the Kv1.5 channel is palmitoylated and that its palmitoylation modulates its biological functions and, therefore, might provide a physiological link between the metabolic state and the expression of Kv1.5 on the plasma membrane.

internalization; trafficking

VOLTAGE-DEPENDENT potassium (Kv) channels are involved in establishing the resting potential, shaping action potentials, and cellular repolarization in the heart and peripheral vascular tissues (21, 33) by regulating potassium ion fluxes in response to alterations of membrane potentials. Among the Shaker family of Kv channels, Kv1.5 is widely represented in the cardiovascular system (37). In the human heart, the Kv1.5 channel is selectively expressed in atrial myocytes (32), and a familial form of atrial fibrillation has been attributed to a loss-of-function mutation in Kv1.5 (36). The overexpression of Kv1.5 in rat cardiomyocytes has been shown to significantly shorten the duration of the action potential, producing a phenotype similar to that observed in short QT syndrome (45). Together, these observations thus render Kv1.5 a potential pharmacological target for cardiovascular diseases.

The function of cellular proteins, including ion channels, is often regulated by some form of posttranslational modification. Great progress has been made in elucidating the structural and functional properties of Kv channels (26), but the effect of posttranslational modifications on channel function is still not well understood. The role of posttranslational modifications such as phosphorylation (18, 23), glycosylation (24), and sumoylation of a subset of Kv channels has been reported (2). Palmitoylation is a reversible posttranslational lipid modification that involves palmitic acid thioesterification to cysteine residues on proteins and allows regulated membrane tethering for key proteins that are involved in neuronal transmission, membrane trafficking, and cell signaling (20, 30, 44). Substrates for palmitoylation include GTP-binding proteins, cytoskeletal proteins, neurotransmitter receptors, and synaptic scaffolding proteins (15, 16, 46). Palmitoylation can affect the affinity of a protein for membranes, subcellular localization, interaction with other proteins, and targeted membrane association and can also modulate protein conformation, dynamics, and functions (1, 13, 17). The palmitoylation of transmembrane proteins likely directs the protein partitioning into lipid-ordered microdomains such as lipid rafts and caveolae (6, 50). Palmitoylation in conjunction with a second lipid modification, such as prenylation or myristoylation, targets proteins to subdomains enriched in signaling molecules (41, 43).

Several receptors (11, 34, 38, 39), G protein-coupled receptors (40), regulator of G protein-signaling proteins (3), ion channels such as the nicotine acetylcholine receptor channel, a subset of voltage-gated ion channels such as sodium channels, and calcium channels have been reported to be covalently fatty acylated through posttranslational modification (19, 35, 42). Recently, the posttranslational modification of Kv1.1 in the form of palmitoylation (14) and S-acylation of the Kv1.5 channel (51) has been reported.

Kv channels have been found to be differentially targeted to distinct microdomains on the plasma membrane such as lipid rafts or caveolae, e.g., in mouse L-cells, the Kv2.1 channel is...
targeted to noncaveolar lipid rafts, whereas the Kv1.5 channel is targeted to caveolae (27, 28). There is also compelling evidence that palmitoylated proteins are often targeted to caveolae; however, little is known about the regulation of this process (41, 43, 47). Furthermore, observations from our laboratory have suggested that Kv1.5 forms a tripartite complex with caveolin-3 (an important protein component of caveolae) and SAP97 and consequently recruited to the plasma membrane (12). These observations led us to hypothesize that Kv1.5 is palmitoylated and that the palmitoylation of Kv1.5 plays an important role in modulating its trafficking and function. Herein, we report that Kv1.5 is palmitoylated at CS93 and demonstrate that palmitoylation modulates its expression, trafficking, and physiological functions.

MATERIALS AND METHODS

Materials. We used the following antibodies: anti-FLAG epitope (M2, mouse monoclonal antibody) from Sigma and anti-Kv1.5 rabbit polyclonal antibody from Millipore/Chemicon. The secondary antibodies for immunoblotting experiments were from Zymed, and those for immunofluorescence labeling were from Invitrogen. Protein A/G agarose was from Santa Cruz Biotechnology, streptavidin-agarose, sodium periodate, and biotin-long chain (LC)-hydrazide were from Pierce, normal goat and donkey sera were from Sigma, and 9,10(n)-[3H]palmitic acid was from Amersham Biosciences.

DNA cloning/PCR and site-directed mutagenesis of Kv1.5. We amplified the mutant COOH-terminal fragment by PCR-based mutagenesis using FLAG-tagged Kv1.5 as a template. We used a synthetic forward oligonucleotide primer of 130 bases that incorporates the codons for the cysteine-to-alanine residues as well as an AgeI restriction site and a reverse oligonucleotide that incorporates the codons for the cysteine-to-alanine mutation containing the BamHI restriction site. The newly synthesized AgeI/BamHI PCR fragment 250 base pairs in length were finally subcloned into the FLAG-tagged Kv1.5 plasmid, and the mutations were confirmed by sequence analysis. The NH2 terminus of FLAG-Kv1.5 (FL-Kv1.5) with a length of 870 base pairs was amplified by PCR that used a forward PCR primer containing restriction-site HindIII before the start codon and a reverse primer containing the BamHI restriction site before an additional added stop codon. The amplified HindIII/BamHI-digested fragment was subcloned into the HindIII/BamHI-digested pcDNA cloning vector that had been restricted previously with HindIII/BamHI. The COOH terminus of Kv1.5 was removed by the restriction of the entire COOH terminus with AgeI and BamHI enzymes and cloned into the original FL-Kv1.5 pcDNA plasmid.

Cell lines and transfection. Chinese hamster ovary (CHO) cells were transfected with FuGENE-6 (Roche) both for transient transfection experiments and for generating stable cell lines. We chose to use CHO cells rather than COS-7 cells for our studies because variable fractions of the transiently transfected COS-7 cells expressed Kv1.5-encoded potassium currents (12, 31).

Generation of stable transfectants. Wild-type FL-Kv1.5 (FL-Kv1.5 WT) or FL-Kv1.5 palmitoylation-deficient mutant (FL-Kv1.5Palm−/−) cDNAs were subcloned as EcoRI or Clal/BamHI fragments in pcDNA3.1 with a FLAG tag at the NH2 terminus. The cDNA constructs were transiently transfected into CHO cells using FuGENE-6 transfection reagent (Roche) according to the manufacturer’s protocol. Forty-eight hours after transfection, 800 μg/ml geneticin (G418; Invitrogen) were used to select stable clones. Resistant colonies were pooled and serially diluted into 96-well plates. The clones arising from a single cell were expanded, and the expression of protein and surface currents were determined. The clones in which >90% of the cells exhibited a high surface current were selected for future studies.

Electrophysiological studies. Recordings were made with an Axopatch-200B amplifier (Axon Instruments) using a standard whole cell configuration of the patch-clamp technique as previously described (7, 31, 32). Briefly, the pipette resistances were 2–4 MΩ when they were filled with (in mM) 50 KCl, 65 K-glutamate, 5 MgCl2, 5 EGTA, 10 HEPES, 5 K2-ATP, and 0.2 Tris-GTP (pH 7.2). The extracellular bath solution contained (in mM) 140 NaCl, 5.4 KCl, 1 CaCl2, 1 MgCl2, 0.33 NaH2PO4, 7.5 glucose, and 5 HEPES (pH 7.4). The currents were recorded at room temperature (21–23°C). The holding potential was −70 mV; the test potential ranged from −60 to +60 mV, lasting 400 ms, and the tail currents were recorded at −30 mV. The data are expressed as means ± SE. ANOVA was applied to the multigroup data. Student’s t-test was used to compare unpaired data between two groups, and a two-tailed P < 0.05 was taken to indicate statistical significance.

Incorporation of palmitic acid. CHO cells were transiently transfected with FL-Kv1.5 (WT) or FL-Kv1.5Palm−/− expression vectors. Forty-eight hours after transfection, the cells were washed twice with F-12 medium without FBS, followed by an incubation for 45 min in F-12 medium containing 5% dialyzed fetal calf serum (FCS). The cells were incubated with 1 μCi of [9,10-3H]palmitic acid for 2 h at 37°C in 2 ml of F-12 medium containing 10% dialyzed FCS. After incubation, the cells were washed extensively with PBS containing (in mM) 137 NaCl, 2.6 KCl, 10 Na2HPO4, and 1.8 KH2PO4 and lysed in radioimmunoprecipitation assay (RIPA) buffer of 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitors cocktail (Roche). The lysates were rocked end over end for 15 min at 4°C and centrifuged at 15,000 rpm for 15 min at 4°C. Clear lysates were incubated overnight with anti-FLAG monoclonal antibody at 4°C, incubated with the protein A/G Sepharose beads for 4–6 h at 4°C, and pelleted by centrifugation at 3,000 g for 20 s with three washes of RIPA buffer. A 2× SDS sample buffer was added, and the samples were boiled for 2 to 3 min before SDS-PAGE. The SDS samples were divided into two portions: one was resolved by SDS-PAGE and analyzed by autoradiography for the incorporation of palmitic acid, and the other was separated by SDS-PAGE and analyzed by immunoblotting using anti-FLAG antibody to check the protein expression. Before autoradiography, the gels were fixed for 1 h, stained with Coomassie blue, destained, incubated with autoradiography enhancer solution (PerkinElmer) for 30 min, dried, and exposed to Kodak X-AR film for 2 to 3 wk to detect the incorporation of [3H]palmitic acid.

Determination of hydroxylamine sensitivity. To verify that the radiolabeled palmitic acid was linked by a thioester bond, we tested for susceptibility to hydroxylamine treatment as described (14). After the immunoprecipitation of FL-Kv1.5 (WT) or FL-Kv1.5Palm−/− polypeptides, the protein A/G Sepharose beads/antibody-antigen complexes were first washed with RIPA and then with PBS and resuspended in 25–50 μl PBS containing 1 M hydroxylamine (pH 7.0) or as control in PBS containing 1 M Tris (pH 7.0) for 2 h at room temperature. Following incubation, 2× SDS sample buffer was added and samples were boiled for 2–4 min and then analyzed by SDS-PAGE.

Biotinylation of Kv1.5 channels. Cell surface labeling of glycoproteins was performed with the periodate-biotinylation assay as described (22). Briefly, CHO (80–95% confluent) stable cells overexpressing either FL-Kv1.5 (WT) or FL-Kv1.5Palm−/− channels were chilled to 4°C, washed with ice-cold PBS containing (in mM) 0.1 NaCl and 1 MgCl2 (PBS-CM), and incubated with 2 mM sodium periodate (NaIO4) in PBS-CM for 30 min at 4°C in the dark to oxidize the cell surface carbohydrate residues. After oxidation and extensive washing with PBS-CM, fresh prewarmed F-12 medium containing 10% FBS was added, and the cells were returned to the incubator to proceed with the internalization process. At specified times (t = 0–300 min), the cells were washed three times with PBS-CM and...
labeled with 2 mM biotin-LC-hydrazide in 100 mM sodium acetate (pH 5.5) for 30 min at 4°C in the dark. Following biotinylation, the cells were washed extensively with PBS-CM and lysed in RIPA buffer containing 1% Triton X-100, 1 mM PMSF, and a protease inhibitors cocktail (Roche). Biotinylated proteins were isolated by incubating with streptavidin-agarose beads for 12–16 h at 4°C. After an end-over-end mixing of the mixture overnight at 4°C, biotin-streptavidin agarose complexes were harvested by centrifugation at 3,000 \( g \) for 1 to 2 min and were washed three times with RIPA buffer. The beads were resuspended in 2\%/11003 \( SDS \) sample buffer and boiled for 3–5 min at 95–100°C; biotinylated proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The proteins were analyzed by immunoblotting with anti-FLAG.

**Immunoprecipitation and Western blot analysis of Kv1.5.** The immunoprecipitation experiments were carried out as described previously (29, 31). Protein samples were heated in SDS sample buffer, and the proteins were resolved by electrophoresis on 8% or 10% SDS-PAGE gels. The proteins were transferred electrophoretically onto a nitrocellulose membrane and immunoblotted with affinity-purified anti-FLAG monoclonal antibodies (2–5 \%/H9262 \( g \)/ml) for 1 h. The blots were incubated with horseradish peroxidase-conjugated secondary antibody at 1:10,000 for 1 h. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Pierce).

**Immunofluorescence microscopy.** FL-Kv1.5 (WT) or FL-Kv1.5\%/H11002-stable CHO cells were grown to 50–60% confluence on glass cover-slips in six-well plates, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and blocked with 5% donkey serum in PBS for 1 h at room temperature (29, 31). The cells were then incubated with rabbit polyclonal or mouse monoclonal antibodies at 1:100–250 in 5% donkey serum for 1 to 2 h at room temperature. After three washes with PBS, the cells were incubated with corresponding Alexa Fluor-488-conjugated donkey anti-rabbit/mouse IgG (Invitrogen) at 1:400–500 dilutions for 1 h at room temperature.

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**Fig. 1.** Fluorography of \( [3H] \)palmitate incorporated into voltage-dependent potassium (Kv)1.5 channels expressed in Chinese hamster ovary (CHO) cells. A: palmitoylation of wild-type FLAG-Kv1.5 [FL-Kv1.5 (WT)] and truncation mutant. CHO cells were transiently transfected with vector alone (lane 1), FL-Kv1.5 (WT) (lanes 2 and 4), or COOH-terminal truncation mutant (TrFL-Kv1.5; lanes 3 and 5) for 48 h and labeled with 1 mCi \( [3H] \)palmitate for 2 h. Equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. Lanes 1–3 represent the immunoprecipitate pellets obtained by incubating the lysates with anti-FLAG, whereas lanes 4 and 5 represent equal amounts of protein from total cell lysates. The results shown are representative of 4 independent experiments. B: CHO cells were transiently transfected with FL-Kv1.5 (WT) vector for 48 h and labeled with 1 mCi \( [3H] \)palmitate for 2 h in the absence (lane 1; control) or presence of 30 \%/H9262 \( \mu \)M 2-bromopalmitate (lane 2) or 40 \%/H9262 \( \mu \)g/ml cycloheximide (lane 3). Equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. The results are representative of 3 independent experiments. C: CHO cells were transiently transfected with FL-Kv1.5 (WT) vector and labeled with 1 mCi \( [3H] \)palmitate for 2 h. Equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were treated with 1 M Tris at pH 7.0 (lane 1) or 1 M hydroxylamine at pH 7.0 (lane 2) before SDS-PAGE and fluorography. The results are representative of 3 independent experiments. D: CHO cells were transfected with FL-Kv1.5 (WT) vector for 48 h and labeled with 1 mCi \( [3H] \)palmitate for 2 h followed by a chase with an excess of unlabeled palmitate (100 \%/H9262 \( \mu \)M) for the indicated time intervals. Equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. E: palmitoylation turnover data from experiments in D. Densitometry measurements \( (n = 2) \) were used at each indicated time point; the values at the beginning of the chase experiment (i.e., \( t = 0 \)) were taken as 100%.
three washes with PBS, the coverslips were mounted on glass slides with a drop of antifade agent (Vectorshield; Vector Laboratories) and visualized with a fluorescence microscope. Multiple control experiments that used either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody showed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in stable CHO cells was specific. For the purpose of quantification, fluorescence intensities in the cytosol and on the membrane area were calculated by digital image analysis routines with Metamorph version 7 (Molecular Devices, Sunnyvale, CA) to automatically detect the cell boundary. Fluorescence images were converted into a binary image by setting a threshold. After binary conversion, contiguous regions were identified using the “create region around object” tool from the Metamorph software. Smaller regions (100 pixels) most likely from background noise were removed, and only the regions of cells were isolated. From the isolated cell image, outlines of individual cells were identified using binary—outline commands from Metamorph. The thickness of the outline was increased to 20 pixels with the dilate operation with a 20-pixel circular kernel, and the average of intensity from isolated outline pixels and cytosol regions was calculated to compare fluorescence intensity in the cytosol and cell membrane.

RESULTS

Kv1.5 is palmitoylated. To determine whether the Kv1.5 channel is a substrate for palmitoylation, we transiently transfected FL-Kv1.5 (WT) and a COOH-terminus-truncated form of rat Kv1.5 (i.e., Kv1.5 truncated after transmembrane domain 6 from amino acids 517–602) into CHO cells, labeled the cells with 1 mCi [3H]palmitate, and immunoprecipitated channel polypeptides with anti-FLAG. The antibodies precipitated a labeled polypeptide with an apparent molecular mass of ~75 kDa corresponding to the Kv1.5 channel (Fig. 1A, lane 2). The truncation of the COOH terminus of FL-Kv1.5 abolished palmitoylation (Fig. 1A, lane 3), indicating that the COOH terminus of Kv1.5 contains the potential site/sites for palmitoylation of Kv1.5. The palmitoylated complex with an apparent high molecular mass likely represents tetrameric channel complex (lane 2). It is obvious from our results (Figs. 2C and 4A) that Kv1.5 is immunodetectable at two different molecular masses, corresponding to a core-glycosylated protein of ~68 kDa and a fully glycosylated protein of ~75 kDa which most likely represents the cell surface form of the channel. Our palmitoylation results revealed that it was the fully glycosylated, 75-kDa polypeptide that was palmitoylated. We further characterized the palmitoylation of Kv1.5 channels. To determine whether Kv1.5 palmitoylation is driven by palmitoyl acyltransferase (PAT) activity, we transiently transfected CHO cells with FL-Kv1.5 (WT) and examined palmitoylation in the presence of the specific PAT inhibitor 2-bromopalmitate. We observed that the addition of 30 μM 2-bromopalmitate completely inhibited Kv1.5 palmitoylation (Fig. 1B, lane 2), indicating the specificity of the palmitoylation reaction. Figure 1B also shows that Kv1.5 palmitoylation is posttranslational, because it was not inhibited by the addition of cycloheximide at 40 μg/ml (lane 3). Treatment with 1 M hydroxyamine but not treatment with 1 M Tris at pH 7.0 resulted in a complete removal of palmitic acid (Fig. 1C), indicating a thioester linkage between palmitic acid and cysteine rather than amide or oxygen ester linkages.

Fig. 2. Palmitoylation of Kv1.5 channels at COOH terminus in CHO cells. A: schematic representation of the various palmitoylation sites present on rat Kv1.5 channel as indicated by cysteine residues. C, cysteine residues at positions 26, 34, 36, 337, 547, 553, 570, 575, and 593; TM, transmembrane domain; P, pore. B: CHO cells were transfected with FL-Kv1.5 (lane 1), all COOH-terminal cysteines mutated to alanines (FL-Kv1.5C; lane 2), COOH-terminal plus NH2-terminal cysteines mutated to alanines (FL-Kv1.5N+C; lane 3), and NH2-terminal cysteines mutated to alanines (FL-Kv1.5N; lane 4) for 48 h and labeled with 1 mCi [3H]palmitate for 2 h. Equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. C, top: CHO cells were transfected with FL-Kv1.5 (WT) (lane 1), cysteine 593 mutated to alanine (lane 2), cysteines 570 and 575 mutated to alanines (lane 3), and cysteines 547 and 553 mutated to alanine (lane 4) for 48 h and labeled with 1 mCi [3H]palmitate for 2 h. Equal amount of proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. C, bottom: equal amounts of FLAG-tagged proteins from cellular lysates were immunoprecipitated with anti-FLAG, and immunoprecipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-FLAG. D: the amino acid sequence of the COOH terminus of rat Kv1.5 (R532 to L602). The sole palmitoylation site, C593, as determined in our studies is indicated by the black box. S* depicts putative PKA phosphorylation sites.
To further characterize Kv1.5 palmitoylation, we examined the palmitate turnover on Kv1.5 polypeptide by labeling transiently transfected CHO cells with [3H]palmitate followed by a chase with an excess of unlabeled palmitate (100 μM). Our results revealed that the palmitoylated Kv1.5 polypeptides were no longer detectable after 60 min (Fig. 1D), and the densitometric analysis showed that the decay of the palmitoylated Kv1.5 polypeptide followed a monoexponential pattern with an apparent half-life of ~10 min (Fig. 1E). It is important to state that the half-life of 10 min represents the turnover rate of the palmitoylated Kv1.5 channel. The half-life of the FL-Kv1.5 (WT) polypeptide as such.

**Mapping of the site/sites of palmitoylation in the Kv1.5 channel.** In transmembrane proteins, palmitoylation generally takes place on intracellular cysteines that are proximal to transmembrane domains (47), and the potential palmitoylated cysteines are often surrounded by positively charged or hydrophobic amino acids (4). The analysis of the amino acid sequence of the NH2 terminus of the Kv1.5 channel revealed the presence of several cysteine residues (C26, C34, and C36) surrounded by positively charged amino acids; one cysteine is located at position 337 in the linker between transmembrane domain M2 and transmembrane domain M3, and five additional cysteines are located near the COOH terminus at positions 547, 553, 570, 575, and 593 (Fig. 2A). To map the palmitoylation sites, we divided the cysteine residues into two groups, the NH2-terminal and the COOH-terminal cysteines (Fig. 2A), mutated them to alanines, transfected the constructs into CHO cells, and performed the palmitic acid labeling. We observed that the mutation of all NH2-terminal cysteines to alanines resulted in a polypeptide palmitoylated to the same extent as FL-Kv1.5 (Fig. 2B, lanes 4 and 1, respectively). In contrast, the mutation of the COOH-terminal cysteines abolished the FL-Kv1.5 palmitoylation (Fig. 2B, lanes 2 and 3). To further determine which of the five COOH-terminal cysteine residues (i.e., C547, C553, C570, C575, and C593) is/are potential palmitoylation site(s), we mutated them in-group of two cysteine residues to alanines or individual cysteine residues to alanines. The analysis of the anti-FLAG immunoprecipitates revealed that the mutation of the last cysteine residue at 593 to alanine (C593A) alone was sufficient to completely abrogate the palmitoylation of rat Kv1.5 polypeptide (Fig. 2C, top). The expression of this palmitoylation-deficient mutant, which we have designated FL-Kv1.5Palm−, was comparable with that of FL-Kv1.5 (WT) (Fig. 2C, bottom). Thus our results indicate that the C593 is the sole palmitoylation site on rat Kv1.5 channels. This site is surrounded by hydrophobic amino acids (Fig. 2D), which might modulate the palmitoylation at C593.

**Expression of Kv1.5 Palm− channels enhances the expression of surface current in stable CHO cells.** To explore the role of palmitoylation on the electrophysiological properties of Kv1.5,
we examined the voltage profiles of the channel-encoded surface currents in FL-Kv1.5 (WT) or FL-Kv1.5Palm– stable CHO cell lines. Because of the large variations in the level of expression in transient transfections, we created stable cell lines expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels. For electrophysiological studies, we chose two clones each expressing equal levels of FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels, respectively. A measurement of the Kv1.5 current density by patch-clamp analysis showed a higher current density in stable cells overexpressing the FL-Kv1.5Palm– channels than in those expressing the FL-Kv1.5 (WT) channel (Fig. 3B), with a mean current density of 907.37 ± 67.88 pA/pF (n = 45; means ± SE) and 620.31 ± 53.75 pA/pF (n = 50; means ± SE) for FL-Kv1.5Palm– and FL-Kv1.5 (WT) channels, respectively (P = 0.0015), which reflects a ~40–45% increase in the surface current density in stable cells expressing FL-Kv1.5Palm– compared with that of the stable cells expressing FL-Kv1.5 (WT) channels. However, the results of the steady-state activation of FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels in stable CHO cells revealed that the expression of FL-Kv1.5Palm– channels did not affect the voltage dependence of steady-state activation (Fig. 3C). The voltage at half-maximal activation (V1/2) is −1.19 ± 0.65 mV, n = 22, for the FL-Kv1.5 (WT) channel; V1/2 is −2.96 ± 0.78 mV, n = 20, for the FL-Kv1.5Palm– channel. Our results demonstrated that the expression of FL-Kv1.5Palm– channels enhances the expression of the surface current in stable CHO cells.

The intracellular occurrence of multiple palmitoylated polypeptides precludes the use of pharmacological inhibitors of palmitoylation as tools for functional analysis, because they would likely result in the modulation of channel expression and activity at multiple levels. This is a potential limitation to directly demonstrate that palmitoylation is the cause of the observed electrophysiological phenotype.

**Palmitoylation-deficient Kv1.5 channels exhibit more surface expression in stable CHO cells.** In light of the above observations that the expression of FL-Kv1.5Palm– polypeptides exhibited a higher surface current in the stable CHO cells, we anticipated a possibly enhanced localization or trafficking of FL-Kv1.5Palm– channels to the plasma membrane, which in turn might increase the expression of the surface current on CHO cells. To address this phenomenon, we selected two

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**Fig. 4.** Differential expression of FL-Kv1.5 and FL-Kv1.5Palm– channels on plasma membrane. A: equal amounts of protein from cellular lysates from stable CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels were immunoprecipitated with anti-FLAG. The immunoprecipitated proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-FLAG. B: CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels were subjected to biotinylation (see MATERIALS AND METHODS). After biotinylation, equal amounts of lysate proteins were incubated with streptavidin-agarose beads for 4–6 h at 4°C to harvest biotinylated proteins. Biotin-streptavidin-agarose complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-FLAG. C: equal amounts of protein from supernatant harvested after streptavidin-agarose incubation from stable CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels were immunoprecipitated with anti-FLAG, analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-FLAG. D: densitometry measurements are summarized in bar graph of data for the total protein expressed in stable CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels, respectively. The values are normalized to the intensity of 1 of the nonspecific protein bands (control) present on the autoradiogram. The apparent molecular mass of the control protein was ~55 kDa, and the variation in the intensity of the control protein bands at various time points was <10%. The values from FL-Kv1.5 (WT) were taken as 100% (n = 4). E: densitometry measurements are summarized in bar graph of data for the biotinylated plasma membrane proteins from stable CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels, respectively. The values are normalized as in D. F: densitometry measurements are summarized in bar graph of data for the cellular protein from stable CHO cells expressing FL-Kv1.5 (WT) or FL-Kv1.5Palm– channels, respectively. The values are normalized as in D.
Expression of Kv1.5Palm channels exhibits enhanced expression on the membrane. We next investigated the expression of FL-Kv1.5 (WT) or FL-Kv1.5Palm channels in stable CHO cells by reacting stable cells with rabbit anti-Kv1.5 (1:500) followed by immunofluorescence microscopy. The expression of FL-Kv1.5Palm− was significantly greater than that of FL-Kv1.5 (WT) channels on the plasma membrane (Fig. 5A). The digital imaging analysis of the fluorescence intensities in the cytosol and on the membrane area in the stable CHO cells (Fig. 5B) revealed relative intensities of the FL-Kv1.5 (WT) and FL-Kv1.5Palm− expressed on the membrane of the stable CHO cells of 19 ± 5.3 units (n = 5; means ± SD) and 33 ± 6.5 units (n = 5; means ± SD), respectively, representing an enhancement by ~60–62% of the FL-Kv1.5Palm− channels on the membranes compared with that of the FL-Kv1.5 channels (P = 0.016). The intensities of the cytosolic FL-Kv1.5 (WT) and FL-Kv1.5Palm− channels were 51 ± 12 arbitrary units (n = 5; means ± SD) and 55 ± 11 (n = 5; means ± SD), respectively, suggesting a very imperceptible change in the expression of two polypeptides (P = 0.87) in the cytoplasm. Thus the ratio of the membrane/cytosolic expression of the FL-Kv1.5 (WT) channels was 0.37 ± 0.068 (n = 5; means ± SD) versus 0.61 ± 0.12 (n = 5; means ± SD) for FL-Kv1.5Palm− (P = 0.0046). Collectively, these results support the functional and biochemical observations suggesting the enhanced membrane expression of FL-Kv1.5Palm− compared with FL-Kv1.5 (WT) and indicate that COOH-terminal C593 is important for the palmitoylation-dependent regulation of cell surface expression.

Palmitoylation-deficient Kv1.5 channels have slower rate of internalization in stable CHO cells. To explore the functional role of Kv1.5 palmitoylation on the biological properties and to further substantiate the consequences of palmitoylation on the electrophysiological properties of Kv1.5, we used the biotinylation of membrane proteins in FL-Kv1.5 (WT) or FL-Kv1.5Palm− stable cell lines (as described in MATERIALS AND METHODS) showing equivalent channel expression to examine whether palmitoylation modulates the internalization of the Kv1.5 channel polypeptides. The analysis of the biotinylated proteins by SDS-PAGE followed by immunoblotting with

Fig. 5. Fluorescence images of FL-Kv1.5 (WT) or FL-Kv1.5Palm− channels in stable CHO cells. A: stable CHO cells expressing FL-Kv1.5 (WT) (left) or FL-Kv1.5Palm− (right) channels were immunostained with Alexa Fluor-488-conjugated donkey anti-rabbit/mouse IgG (1:500). B: fluorescence intensities in the membrane and cytosol of cells expressing FL-Kv1.5 (WT) (n = 5) and cells expressing FL-Kv1.5Palm− (n = 5) channels, respectively. The bar depicts fluorescence intensity in arbitrary units. NS, nonsignificant. *P ≤ 0.01.
anti-FLAG revealed a substantially slower internalization rate in FL-Kv1.5Palm− compared to FL-Kv1.5 (WT) channels (Fig. 6A). The densitometric analysis from three independent experiments revealed a significantly faster rate of internalization of FL-Kv1.5 (WT) than of FL-Kv1.5Palm− channels (Fig. 6B) and that the internalization of FL-Kv1.5 (WT) and FL-Kv1.5Palm− channels was a biphasic phenomenon, with the first phase relatively faster than the second phase. Therefore, a separate curve fit was performed in each phase independently with the statistics package R (available from http://www.r-project.org), choosing the break point that separates two phases where it minimized the regression error in both phases. The slopes of the first and the second phases were calculated separately by using the linear regression in each phase and representing the confidence interval as (±SE). The calculated half-lives of the internalization of FL-Kv1.5 (WT) and FL-Kv1.5Palm− channels during the first phase were 112 ± 7 (SE) and 230 ± 12 (SE) min, respectively, whereas the half-lives of internalization during the second phase were 188 ± 12 (SE) and 630 ± 28 (SE) min, for FL-Kv1.5 (WT) and FL-Kv1.5Palm− channels, respectively. Thus the internalization rate of FL-Kv1.5 (WT) was approximately threefold faster (P < 0.01) than that of FL-Kv1.5Palm− channels in both phases. These results suggest that the slower internalization rate of the Kv1.5Palm− polypeptides results in higher levels of expression of Kv1.5Palm− channels on the plasma membrane and thus in higher surface currents.

**DISCUSSION**

Many cellular integral membrane proteins are palmitoylated on cysteines that are either close to transmembrane domain/cytoplasmic domain boundary or in their cytoplasmic domain (4), which in turn confers the ability of palmitoylated proteins to interact with the membrane bilayer, an interface that facilitates dynamic bidirectional membrane-protein interactions during the action potential (14). The observations that Kv1.5 is localized to and interacts with specific microdomains such as caveolae on the plasma membranes (27, 28), often a hallmark of the palmitoylated proteins, and our recent observations that Kv1.5 forms a tripartite complex with caveolin-3 and SAP97 and is in turn recruited to the plasma membrane suggested to us that Kv1.5 might be palmitoylated. Therefore, we undertook to investigate whether Kv1.5 is palmitoylated and, if so, whether palmitoylation modulates its biological properties.

With the use of the heterologous expression system, the present report demonstrates that the Kv1.5 channel undergoes palmitoylation and establishes the role of this covalent thioesterification in modulating the electrophysiological and biological properties of the Kv1.5 channel protein. Palmitoylation has been demonstrated for a small subset of voltage-gated channel proteins such as the α-subunit of the sodium channel (42), the β-subunit of voltage-gated calcium channel (8), Kv1.1 (14), and recently for the acylation of Kv1.5 (51). Our results revealed that Kv1.5 was indeed a substrate for palmitoylation and that it was the mature form of Kv1.5 (~75 kDa polypep-
tide) that showed palmitoylation, which is consistent with the recent report on the acylation of the human Kv1.5 channel (51). The approach we used to study the palmitoylation was based on metabolic labeling with radiolabeled palmitate, which is time consuming and reported to be less sensitive than the method based on fatty acyl exchange chemistry (10) that had been employed in recent studies of S-acylation of the human Kv1.5 (51). However, metabolic labeling with [3H]palmitate enables the investigation of the specific thioester modification, whereas the method based on fatty acyl exchange chemistry (10) does not allow a determination of the molecular identity of the modification. Our results indicated that C593, which resembles the sole palmitoylation site of Kv1.5 (Fig. 2C), is an intracellular cysteine residue located in the cytoplasmic portion of Kv1.5 channel near the COOH terminus (Fig. 2A). This location, which is likely equivalent to the unidentified S-acylation of the C604 residue of the human Kv1.5 (51), is consistent with the occurrence of thioesterification of transmembrane proteins on intracellular cysteine residues proximal to transmembrane domains (47). In addition, the recent report (51) has put forth that Kv1.5 is posttranslationally modified on both the NH2 and COOH termini via hydroxylamine-sensitive thioester bonds, whereas our results have only demonstrated the posttranslational modification on the COOH terminus of Kv1.5, and we didn't observe any posttranslational modification on the NH2 terminus of Kv1.5.

Recently, Kv1.1, another member of the Kv family, has been reported to be palmitoylated in the cytosolic portion of the S2-S3 linker domain on residue Cys243 (14), and this modification modulates voltage sensing through protein-membrane interactions. This suggests that different channels may undergo palmitoylation in distinct regions of the molecules, differentially affecting their functions. Gubitosi-Klug et al. (14) also identified a consensus sequence for palmitoylation, such as ACP/RSKT, which is conserved among multiple Kv1 channel family members and several other regulatory proteins. However, the identification of the palmitoylation site in our studies revealed that it does not correspond to the aforementioned consensus sequence but rather that it is surrounded by hydrophobic amino acids (Fig. 2D). It is very likely that the presence of hydrophobic amino acids near the palmitoylation site influences Kv1.5 palmitoylation, such as in the endothelial form of nitric oxide synthase (25). It has also been reported that hydrophobic residues enhance the palmitoylation of PSD95, a scaffolding protein of the membrane-associated guanylate kinase (MAGUK) family that is important for the clustering of neuronal receptors at postsynaptic densities (25) and GAP43 (46). Furthermore, in G protein-coupled receptors, clusters of hydrophobic and positively charged amino acids often precede and follow, respectively, palmitoylated cysteines. Our results thus suggest that structural features rather than specific sequence requirements are likely to be the key factors that influence Kv1.5 palmitoylation.

Our results demonstrate that the cysteine substitution at position 593 of the Kv1.5 significantly slows the rate of channel internalization (Fig. 6), which leads to an increase in the numbers of channel molecules on the cell surface and concomitantly to higher potassium currents. These changes resemble those reported in the human Kv1.5 (51), which demonstrates that although intracellular cysteines were required for S-acylation, the mutation of these residues resulted in an increase in Kv1.5 cell surface expression and an increase in the current density, but that study did not explore the mechanism leading to increased potassium currents by COOH-terminal cysteine mutation. Thus our findings represent a novel role of protein palmitoylation and show that palmitoylation can modulate steady-state expression in addition to its previously recognized functions in targeting a variety of peripheral proteins to the plasma membrane (5, 48, 49). The elucidation of the mechanisms by which palmitoylation regulates the channel internalization rate needs to be the subject of future investigations.

Limitations

Our initial efforts to detect the palmitoylation of the Kv1.5 channel in native cells such as rat primary aortic vascular smooth muscle cells, A10 cells, rat primary neonatal, and mouse adult cardiomyocytes were not successful, likely due to the relative low expression level of Kv1.5 polypeptides. Hence, we could not use these cells to analyze the role of the palmitoylation of the Kv1.5 channel in native tissues, limiting the interpretation of our observations.

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REFERENCES


