DIFFERENTIAL REGULATION OF TWO PALMITOYLATION SITES IN THE CYTOPLASMIC TAIL OF THE β1-ADRENERGIC RECEPTOR

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β1-adrenergic receptor (AR) is a G protein-coupled receptor (GPCR) critical to proper heart function and memory formation (1-3), and is the major cardiac target of beta-blocker therapy for patients of chronic heart failure (4). Increasingly detailed molecular characterization of AR structure and signaling has lead to novel treatment strategies, including rational drug design (5), targeting multiple components of the signaling complex (6,7) and the potential to personalize treatment for patients based on genetic background (8).

The covalent addition of palmitic acid to cytoplasmic cysteine residues via a thioester bond is a prevalent modification of GPCRs. Unlike other acyl modifications, S-palmitoylation is reversible, and many proteins have regulated cycles of palmitoylation and depalmitoylation (9). Unlike soluble substrates, S-palmitoylation of integral membrane GPCRs is not required for membrane association, but instead changes their structure and contributes variably to receptor function (10,11).

The palmitoylation status of β1AR has not yet been determined. Recently the crystal structure of turkey β1AR was solved, providing insight into the organization of the transmembrane domains, and the ligand binding pocket (12). This structure, however, lacked a large portion of the C-terminal tail and had a mutated putative palmitoylation site. Thus, unlike for the crystal structures of rhodopsin, which included palmitoylated cysteines (13,14), no information on β1AR palmitoylation was gained.

We recently discovered that efficient delivery of β1AR to the cell surface required expression of a Golgi resident protein, golgin-160 (15). S-palmitoylation is known to influence the trafficking and the specific subcellular localization of many substrates (16,17). It has also been reported that golgin-160 interacts with GCP16 (18), which is a subunit of the Ras palmitoyltransferase (19). Thus, we reasoned that golgin-160 might influence the surface expression of β1AR by promoting proper palmitoylation at the Golgi. To test this hypothesis, we first investigated the palmitoylation of β1AR.

S-palmitoylation of G protein-coupled receptors (GPCRs) is a prevalent modification, contributing to the regulation of receptor function. Despite its importance, the palmitoylation status of the β1-adrenergic receptor, a GPCR critical for heart function, has never been determined. We report here that the β1-adrenergic receptor is palmitoylated on three cysteine residues at two sites in the C-terminal tail. One site (proximal) is adjacent to the seventh transmembrane domain and is a consensus site for GPCRs, and the other (distal) is downstream. These sites are modified in different cellular compartments, and the distal palmitoylation site contributes to efficient internalization of the receptor following agonist stimulation. Using a bioorthogonal palmitate reporter to accurately quantify palmitoylation, we found that the rates of palmitate turnover at each site are dramatically different. While palmitoylation at the proximal site is remarkably stable, palmitoylation at the distal site is rapidly turned over. This is the first report documenting differential dynamics of palmitoylation sites in a GPCR. Our results have important implications for function and regulation of the clinically important β1-adrenergic receptor.

β1-adrenergic receptor (AR) is a G protein-coupled receptor (GPCR) critical to proper heart function and memory formation (1-3), and is the major cardiac target of beta-blocker therapy for patients of chronic heart failure (4). Increasingly detailed molecular characterization of AR structure and signaling has lead to novel treatment strategies, including rational drug design (5), targeting multiple components of the signaling complex (6,7) and the potential to personalize treatment for patients based on genetic background (8).
We report here that β1AR is S-palmitoylated on its C-terminal tail proximal to the seventh transmembrane domain at residues Cys392 and/or Cys393, which comprise a de facto consensus site for GPCR palmitoylation. Unexpectedly, we identified a second site of palmitoylation, further downstream on the tail at residue Cys414. These sites are modified in different subcellular compartments, and mutation of Cys414 but not Cys392 or Cys393 affects agonist-mediated internalization of β1AR. Interestingly, while the palmitate modification at the proximal site is quite stable, modification at the distal site is rapidly turned over. These results provide new information on β1AR modification, and will inform future experiments that rely on an accurate structural understanding of this receptor.

**Experimental Procedures**

**Expression constructs**- A plasmid encoding human FLAG-tagged β1AR in pcDNA3 was provided by Randy Hall (Emory University, Atlanta, GA). Mutations were introduced using polymerase chain reaction-based QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). Nucleotide (nt) mutations introduced (individually, or in combination) were for Cys392 to Ser (nt 1175 G to C); Cys393 to Ser (nt 1178 G to C) and Cys414 to Ser (nt 1241 G to C).

**Cell culture and transfection**- HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) and 0.1 mg/mL normocin (InvivoGen, San Diego, CA) (or lacking normocin for alkynyl-16 labeling experiments) at 37°C in 5% CO2. For transient overexpression assays, cells were transfected with 3 µL of FuGENE6 (Roche, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen) per 1 µg of cDNA.

**3H-Palmitic acid labeling**- Transiently transfected cells were labeled for 30 min with 0.5 mL of 0.5 mCi/mL 3H-palmitic acid (Perkin Elmer, Boston, MA) in DMEM with 1% dimethyl sulfoxide (DMSO), 2.5% FCS, 1x non-essential amino acids and 1x sodium pyruvate (Invitrogen). Cells were lysed in detergent solution (50 mM Tris pH 8.0, 62.5 mM EDTA, 1% NP-40, 0.4% deoxycholic acid (DOC)) for 20 min at 0°C, and debris was removed by centrifugation at 16,000 x g for 15 min. FLAG-β1AR was immunoprecipitated with FLAG-M2 beads (Sigma, Milwaukee, WI) as described (15). Protein was eluted with 2 x SDS-PAGE buffer (100 mM Tris pH 6.8, 4% SDS, 30% glycerol, 0.1% bromophenol blue, 2% β-mercaptoethanol) for 20 min at room temperature (RT). The sample was resolved by SDS-PAGE. The gel was equilibrated in DMSO and incubated for 2 h in 2.5 diphenyloxazole, washed with water, dried and exposed to film at −80°C.

**Metabolic labeling with bioorthogonal palmitate reporter**- Transiently transfected cells were labeled for 30 min with 0.5 mL of 50 µM alkynyl-16 (alk-16) in DMEM with 10% FCS. To determine palmitate turnover, labeled cells were chased in normal growth medium for the indicated times. Cells were lysed in Brij lysis buffer (1% Brij-97, 150 mM NaCl, 50 mM triethanolamine pH 7.4) with EDTA-free protease inhibitor cocktail (Roche) on ice. Cell lysates were collected following centrifuging at 16,000 x g for 20 min at 4°C to remove cell debris. Immunoprecipitations were performed using anti-FLAG-M2 affinity resin as above. The beads were resuspended in 40 µL of SDS buffer (4% SDS, 50 mM triethanolamine pH 7.4, 150 mM NaCl) and 3 µL freshly prepared click-chemistry reaction mixture [azide-rhodamine (100 µM, 10 mM stock solution in DMSO), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in deionized water), Tris[(1-benzyl-1H,1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 µM, 10 mM stock solution in DMSO) and CuSO4·5H2O (1 mM, 50 mM freshly prepared stock solution in deionized water)]. Reactions were incubated with shaking for 1 h at 30°C. The reactions were diluted with 5 x SDS-PAGE buffer (250 mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.5% bromophenol blue) and 0.5% β-mercaptoethanol, incubated for 20 min at 37°C. Reactions were resolved by SDS-PAGE.

**In-gel fluorescence imaging and immunoblotting**- After proteins were separated by SDS-PAGE, the gel was washed twice with deionized water for a total of 20 min. Palmitoylated β1AR was visualized by directly scanning the gel (excitation 532 nm, 580 nm filter, 30 nm band-pass) on a Typhoon 9400 imager (GE Healthcare, Pittsburgh, PA) and the gels were scanned on the Typhoon 9400 imager (GE Healthcare, Pittsburgh, PA).
Healthcare, Sweden). No signal saturation was observed. Images were processed and analyzed using the ImageQuant TL software (GE Healthcare). Following in-gel fluorescence imaging, total β₁AR was detected by either in-gel immunoblotting or traditional immunoblotting as previously described (15).

For in-gel immunoblotting, gels were washed in PBS with 0.1% Tween-20 (PBST) for 10 min at RT. Gels were incubated with anti-FLAG M2 antibody (Sigma) in PBST followed by IRDye800 conjugated anti-mouse IgG secondary antibody (Rockland, Gilbertsville, PA) in PBST. In all cases immunoblot images were collected using the Odyssey infrared imaging system (Licor, Lincoln, NE). For data analysis, the alk-16 signal was normalized to the relative amount of total β₁AR detected by immunoblot. For Fig. 2B, normalized signal for the wildtype protein was set to 100% for each experiment, and normalized signal from all mutants were compared to this signal. For Fig. 2C, total signal for each β₁AR construct in each experiment was set to 100%, and the contributions of mature and immature bands were calculated. For Fig. 3B, the 0 chase time point was set to 100%, and subsequent signals (normalized based on expression level) for each mutant from each experiment were compared. Variance was determined by one-way ANOVA and P values were calculated with the Tukey test.

**Measurement of β₁AR half-life**: HEK293 cells grown in 35 mm dishes were transfected with 0.5 µg each of the indicated construct. 16 h later, the cells were starved for 15 min with DMEM lacking Met and Cys, and labeled for 15 min in fresh Met/Cys free DMEM with 0.2 mCi/ml Expre³⁵S³²P labeling mix (Perkin-Elmer). Medium was replaced with normal growth medium for the indicated times. Cells were lysed with detergent solution and immunoprecipitated as described above. SDS/PAGE gels were dried, and radiolabeled proteins were detected by phosphorimaging (Molecular Imager FX, BioRad). Bands were quantified using Quantity One software (Bio-Rad), analysis performed with Microsoft Excel.

**Immunoblotting**- HEK293 cells grown on poly-L-lysine coated glass coverslips were fixed and permeabilized as described (20). Antibodies used were anti-FLAG M2 (Sigma), sheep anti-TGN46 (Srotec Inc., Raleigh, NC), Alexa₄₈₈ anti-mouse IgG (Molecular Probes, Eugene, OR) and Texas Red anti-sheep IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in 1% fish skin gelatin. Images were collected on an Axioskop microscope (Zeiss, Thornwood, NY) equipped with epifluorescence and a Sensys CCD camera (Photometrics, Tucson, AZ) using IP Lab software (Signal Analytics, Vienna, VA).

**Measurement of β₁AR surface levels**: HEK293 cells were grown on poly-L-lysine coated wells in 12-well dishes, and in 35 mm dishes for expression control. Each construct was transfected in triplicate in the 12-well dishes, plus one 35 mm dish, and one untransfected control to determine background binding. At 16 h post-transfection, the cells in the 35 mm dishes were lysed as described (15) for analysis by Western Blot. Cells in the 12-well dishes were rinsed 3 times on ice with cold PBS, and incubated with 10 nM ^3^H CGP-12177 (Perkin Elmer) in KRH buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄, 1.25 mM CaCl₂, 20 mM HEPES pH 7.4, 2 mg/ml BSA) for 3 h at 4°C. Cells were then rinsed 3 times on ice with cold PBS, and lysed with detergent solution. Lysate was added to scintillation fluid and counted. For analysis, values were from samples where the maximum ligand binding was < 30% of the input. Binding of ligand to non-transfected cells was less than 5% of that for cells expressing wildtype β₁AR.

**Internalization assay**: HEK293 cells grown on poly-L-lysine coated glass coverslips transiently expressing the indicated FLAG-β₁AR construct were fed anti-FLAG M2 antibody at 1 µg/ml dilution and treated with or without 10 µM isoproterenol at 37°C (Sigma) for the times indicated. Following treatment, cells were washed with PBS, and were untreated or surface antibody was removed by an acid wash (0.5 M NaCl, 0.5% HOAc, pH 1) for 1 min at room temperature. Cells were washed with PBS, fixed and permeabilized as above. Fixed cells were probed with rabbit anti-β₁AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with 1% bovine serum albumin (BSA), followed by incubation with Alexa₄₈₈ anti-mouse and Texas Red anti-sheep IgGs. All fields were selected for similar expression levels and expression profile in the anti-β₁AR field before
viewing in the anti-FLAG field. For each experiment, images were taken on the same day at the same shutter speed, and all manipulations of image intensity were applied consistently to all images. Average pixel intensity of internalized antibody was determined using Image J software (NIH). Variance was determined by one-way ANOVA and P values were calculated with the Tukey test.

RESULTS

β1AR is palmitoylated on cysteines 392, 393 and 414. Many GPCRs are S-palmitoylated on their C-terminal tails, downstream of the seventh transmembrane domain (21). We used two programs to predict palmitoylation sites on β1AR, NBA-Palm and CSS-Palm 2.0 (22,23). Both programs predicted palmitoylation on cysteines 392 and 393, which reside at this position and are highly conserved across species (Fig. 1A). This position is also analogous to the palmitoylation site of the closely related β2AR, which has a single modified cysteine (24). To experimentally examine the palmitoylation state of β1AR, we incubated HEK293 cells transiently expressing FLAG-tagged β1AR with 3H-palmitic acid. Parallel dishes were incubated with [35S]-methionine/cysteine, to monitor protein expression levels. Lysates were immunoprecipitated with an anti-FLAG M2 antibody and examined by fluorography. As previously described (15), two major bands were observed for β1AR; a faster migrating immature band (~56 kDa), and a slower migrating mature band (~64 kDa), representing the O-glycosylated mature form of β1AR (Fig. 1B, left panel)(25). Radiolabeled palmitic acid was incorporated into β1AR (Fig. 1B, lane 1 right panel), predominantly in the mature band. To confirm that the labeled palmitate was incorporated via a thioester bond, labeled β1AR was incubated with 1M hydroxylamine. In-gel hydroxylamine treatment resulted in the loss of 3H signal, compared to a parallel gel treated with 1M Tris (data not shown). These data indicate that, as expected, β1AR is modified with palmitic acid via a thioester bond. We next sought to identify the specific residues modified by palmitic acid. We expressed a construct with Cys392 and Cys393 mutated to serines (β1AR C392S/C393S) and found that while incorporation of 3H-palmitic acid was reduced, it was not eliminated (Fig. 1B, lane 2), suggesting additional sites of palmitoylation. We therefore introduced mutations at each of the other cytoplasmic facing cysteine residues (cysteines 261, 378, 414, 451, 467) in combination with C392S/C393S. Only when Cys414 was mutated together with residues Cys392 and Cys393 was incorporation of 3H-palmitic acid abolished (Fig. 1B, lane 4, and data not shown). This site is highly, although not universally, conserved across species (Fig. 1A). Because of their relative positions on the C-terminal tail, we refer to residues 392 and 393 as the proximal palmitoylation site and amino acid 414 as the distal palmitoylation site. The triple cysteine mutant (C392S/C393S/C414S) is referred to as palmitoylation null (PN). Interestingly, the immature form of β1AR was labeled only when the proximal site cysteines were present (Fig. 1B), indicating that the proximal site is modified earlier in the secretory pathway than the distal site.

Mutation of the palmitoylation sites does not destabilize β1AR or affect its steady-state localization. Mutation of the palmitoylation sites of several GPCRs leads to destabilization of the receptors, most likely due to misfolding. We found no significant difference in the half-lives or extent of maturation through the medial-Golgi for any of the mutant proteins (Fig. 2).

Since several GPCRs with mutated palmitoylation sites are trafficked inefficiently (26-29), we examined the steady-state distribution of β1AR palmitoylation mutants by indirect immunofluorescence microscopy using an antibody recognizing the N-terminal FLAG epitope. All mutant proteins were expressed at the plasma membrane, similar to the wildtype protein (Fig. 3A). The internal juxtanuclear staining co-localized with TGN46, a marker of the trans-Golgi network (Fig. 3A and Supplementary Fig. 1), most likely representing β1AR en route to the plasma membrane. None of the mutant proteins accumulated in the endoplasmic reticulum, which together with the similar half-lives of the proteins, suggest that the mutant proteins were not misfolded.

To quantify the surface levels of β1AR, we assayed the binding of a radiolabeled ligand to the surfaces of cells expressing each of our constructs.
We found no substantial difference in surface levels in cells expressing any of the mutants, compared to wildtype (Fig. 3B). Nearly all binding was due to expression of the transfected β₁AR constructs, since untransfected controls bound less than 5% of ligand, relative to the wildtype β₁AR expressing samples (data not shown). Taken together, these data indicate that mutation of palmitoylated cysteines of β₁AR does not disrupt the stability or steady state distribution of the receptor. Thus, preventing palmitoylation of β₁AR did not mimic the phenotype of reduced delivery to the cell surface observed in cells lacking golgin-160 (15). This observation along with the finding that overexpression of golgin-160 promotes PN β₁AR surface expression similar to wildtype β₁AR (data not shown), suggests that golgin-160 does not have a role in palmitoylation of β₁AR.

Agonist stimulated internalization is impaired in mutants lacking a distal palmitoylation site. To evaluate the effect of β₁AR palmitoylation on receptor internalization following agonist stimulation, we measured the surface levels of β₁AR by binding radiolabeled ligand following stimulation with 10 μM isoproterenol (Iso) or vehicle control. However, we saw no significant change in surface levels (data not shown), consistent with previously published reports that β₁AR has low levels of internalization following agonist stimulation in HEK293 cells (e.g.,(30)). To detect the low level of receptor internalized in these cells in a highly sensitive assay, we fed anti-FLAG M2 antibody to live cells in the absence or presence of 10 μM Iso and visualized internalized antibody after removing surface antibody with an acid wash. Without an acid wash, the signal was primarily at the cell surface (Fig. 4A). However, when acid washed, only weak, punctate staining representing internalized receptor was observed, indicating very low levels of basal internalization. This signal increased significantly for cells treated with Iso, although still represented only ~6% of total fluorescent labeling (Fig. 4A and data not shown). Additionally, agonist stimulation did not lead to a significant loss of fluorescence signal in the absence of an acid wash, consistent with the results of the radioactive ligand binding experiment. Therefore, we examined the agonist stimulated internalization of β₁AR by measuring the signal from internalized wildtype FLAG-β₁AR or the indicated FLAG-β₁AR mutants. We consistently observed that cells expressing FLAG-β₁AR lacking the distal palmitoylation site internalized less antibody than when the distal site was intact (Fig. 4B). A quantification of the intensity of signal revealed that mutation of the distal site alone, or in combination with the proximal site, reduced internalization of β₁AR by approximately half, while mutation of the proximal site alone caused no defect (Fig. 4C).

Use of novel palmitoylation reporter to accurately quantify extent of palmitoylation. To investigate the level of labeling at each site and to characterize the dynamics of palmitoylation, we used a recently developed labeling method that could be easily and accurately quantified. Proteins labeled with 3H-palmitate must be detected by fluororography, and it is difficult to obtain an accurate quantitative signal on X-ray film due to the non-linear exposure of silver grains by photons (31). We thus used bioorthogonal labeling and in-gel fluorescence for quantification (32). Transiently transfected HEK293 cells were incubated with medium containing the bioorthogonal palmitic acid reporter (alkynyl-16, alk-16) for 30 min. After lysis and immunoprecipitation, samples were reacted with azide-rhodamine (on-bead Cu-catalyzed azide-alkyne cycloaddition). This labeling method has been shown to be more specific, sensitive and efficient than radioactive methods (32,33). Following SDS-PAGE, in-gel fluorescence analysis provided a linear, quantitative signal. The labeling pattern was similar to what we observed after ³H-palmitic acid labeling (compare Fig. 1B and Fig. 5A). To quantify the labeling, the fluorescent signals from five independent experiments were normalized to overall β₁AR expression level as determined by Western blotting of the same gel (Fig. 5B). When Cys³⁹² and Cys³⁹⁵ were mutated to serines, the label was 44% +/- 11% of that obtained for the wildtype protein. When Cys¹⁴ was mutated to serine, the label was 64% +/- 21% of that for the wildtype protein. When both sites were mutated, a low level (7% +/- 2%) of labeling was observed. It is possible that when the normally palmitoylated cysteines are absent, additional cysteines can be S-palmitoylated to a minor extent. To examine the
usage of each of the cysteines in the proximal site, we expressed β1AR with Cys392 and Cys414 mutated to serine, and β1AR with Cys393 and Cys414 mutated to serine. No significant difference (45% +/- 15% and 50% +/- 24% of wildtype, respectively) was observed compared to the distal site mutant with both proximal cysteines available. This most likely indicates that most β1AR molecules expressed in HEK293 cells are palmitoylated on only one of the two cysteines in the proximal site, with only a small percentage of receptor, if any, modified simultaneously on both cysteines.

We also observed that the immature form of β1AR was labeled in all cases where a proximal site cysteine was available, but not when both were mutated to serine (Fig. 5A). We quantified the contribution of signal from mature and immature bands for each construct (Fig. 5C). While labeling of the immature form accounted for 23% +/- 8% of the wildtype signal, it contributed only 9% +/- 4% of the signal in the C392S/C393S mutant, an amount that is similar to the labeling of PN described above. This indicates that the proximal site can palmitoylated before the protein is processed in the medial Golgi, but the distal site is primarily or exclusively modified later in the secretory pathway.

**Turnover at the distal site is highly dynamic.** The previous experiments measured the steady-state levels of palmitoylation at each site of β1AR. The intensity of labeling is determined by both the extent of the incorporation of alk-16 as well as the rates of turnover. Palmitoylation sites with high rates of turnover will have increased signal, due to replacement of non-labeled palmitic acid with the labeled analog. To study the dynamics of S-palmitoylation at each site, we examined the rates of turnover of palmitate at the proximal and distal sites using pulse-chase labeling. Cells expressing wildtype β1AR, C392S/C393S or C414S were labeled with alk-16 for 30 min, followed by chase in medium lacking alk-16 for various times. While signal from the proximal site showed no reduction after 90 minutes of chase, the label incorporated at the distal site was rapidly turned over, with very little palmitoylated protein left at 15 min of chase (Fig. 6). Since the half-lives of the proteins were all much longer than the loss of signal at the distal site (Fig. 2), the loss of signal is most likely due to palmitic acid turnover, and not protein degradation. Taken together, these data indicate that the proximal site is modified early in the secretory pathway and turns over slowly, while the distal site is modified after trafficking through the medial Golgi and has a high rate of turnover. The surprising difference in turnover at the proximal and distal sites makes comparison of steady-state palmitoylation at each site difficult, since only newly synthesized β1AR appears to be palmitoylated at the proximal site, while a larger pool of mature β1AR is likely available for modification at the distal site.

**DISCUSSION**

β1AR is S-palmitoylated at two sites in its cytoplasmic tail. We report here that β1AR is palmitoylated at the two cysteines residing on the C-terminal tail proximal to the membrane (Cys392 and Cys393), and also further downstream at Cys414. By primary sequence, β1AR is most closely related to β2AR (52% amino acid identity), which has a single palmitoylated cysteine, equivalent to the proximal site of β1AR (24). Based on this homology and the lack of a known sequence requirement for palmitoyltransferases, it has been assumed that β1AR is S-palmitoylated only at the cysteines residing at this proximal site (12,34,35). Our findings underscore the necessity to experimentally determine all of the residues that are modified on GPCRs to provide a complete understanding of receptor regulation and function. Making conserved mutations to a protein of interest is also the most direct way to examine the contribution of palmitoylation to that protein’s function, since treatment with an inhibitor (such as 2-bromopalmitate) globally prevents palmitoylation, and may indirectly impact the function of a protein of interest.

The finding that β1AR has an additional palmitoylation site relative to β2AR is surprising given the similarities regarding ligand binding and tissue distribution. However, these receptors have distinct activities. While β2AR localizes to caveolae in unstimulated cardiomyocytes and relocates following ligand binding, β1AR is distributed throughout the plasma membrane, and does not relocalize after ligand binding (36).
Similarly, within cardiomyocytes co-cultured with sympathetic ganglion neurons (SGNs), β1- and β2AR localize to contact sites, but only β2AR relocates away from the contact sites following SGN stimulation, revealing differences in the spatial-temporal regulation of the receptors (37). The two receptors have unique binding partners (38), and form distinct signaling complexes through varied interactions with cAMP phosphodiesterases, which are differently regulated in response to agonist signaling (39). β1- and β2AR also promote distinct downstream signaling events. β1AR couples only to Gαs, and excessive stimulation leads to apoptosis of cardiomyocytes. On the other hand, β2AR can couple to either Gαs or Gαt, and stimulation was found to protect cardiomyocytes against apoptosis (40). S-palmitoylation has been shown for other proteins to regulate behavior such as protein localization (particularly regarding cholesterol-rich domains) and protein-protein interactions (11). It is possible that associated proteins regulate the function of these receptors by modulating the palmitoylation state at each site, allowing for a “fine-tuned” response.

Some GPCRs are not S-palmitoylated, and the majority of those that are S-palmitoylated are modified only at the consensus site. The presence of an additional distal palmitoylation site on the tail of β1AR places it in a third group of GPCRs. The 5-hydroxytryptamine (HT) 4(a), 5-HT7(a), the TPβ isoform of thromboxane A2 (TPβ) and the follicle-stimulating hormone (FSH) receptors have all recently been reported to have distal palmitoylation sites, in addition to palmitoylation at the proximal site (41-44). Functionally, there is no obvious connection between these receptors, which have varied tissue distribution, signaling pathways and are coupled to different G proteins. But they all likely adopt a conformation consisting of five intracellular loops when fully S-palmitoylated (Fig. 7), and the distal site may regulate receptor internalization similarly for all receptors (see below).

The distal palmitoylation site contributes to internalization following agonist stimulation. Following agonist binding and second messenger transduction, many GPCRs are desensitized, turning off signaling. The common route of desensitization involves phosphorylation by the GPCR kinase (GRK) family and/or second messenger regulated kinases PKA or PKC. Many receptors are then internalized and sequestered within the cell, destined for recycling to the surface, or downregulation (reviewed in (45)). We observed a low level of β1AR internalization following agonist stimulation. This is consistent with several previous studies of β1AR internalization in HEK293 cells (e.g.(30)), though not all (e.g. (46)). In cell culture studies more closely resembling physiological conditions, isoproterenol treatment of rat cardiac myocytes was found to cause internalization and down-regulation of β1AR. Interference with the endocytosis machinery caused surface retention of β1AR and deficient downstream signaling through Akt (47). In light of cell type differences, trafficking events other than internalization may be used to spatially regulate β1AR function (e.g. relocalization within the membrane (see above)). We consider the internalization defect we observed for distal site mutants to be an intriguing preliminary observation, which may reflect a more specific paradigm of regulated relocalization that may only be observed in a relevant culture system, and may be different for different cell types.

The involvement of a distal site in efficient internalization is analogous to observations made for the other GPCRs with acylation at distal sites. Mutation of the distal site reduced the rates of internalization of the FSH receptor (44), and while all three palmitoylation sites of TPβ contributed to ligand-induced internalization, only the distal site mutants were deficient in tonic internalization. By contrast, the proximal site alone was found to promote maximal coupling to Gαq (42). Studies also show a contribution of palmitoylation to internalization of 5-HT4(a). Unlike β1AR, FSH or TPβ receptors, mutation of the 5-HT4(a) distal sites did not inhibit internalization. However, when the proximal site was mutated, there was a pronounced increase in ligand-stimulated internalization that was lost when the distal sites were mutated in combination (48), indicating the need for an intact distal site for the hyper-internalization phenotype. The consequence of mutation of the distal site to HT7(a) internalization was not reported (43). It is interesting to note that such a diverse group of GPCRs appear to have a similar use for a distal
palmitoylation site, although the significance and universality of this feature is currently unclear. Characterization of additional GPCRs, as well as a better mechanistic understanding of the distal site usage may reveal a common route.

**Differences in regulation at two palmitoylation sites suggest unique functions.** In order to accurately measure the relative levels of S-palmitoylation and the dynamics of turnover we needed a quantitative measure of palmitoylation. Fluorography is required to detect tritium on X-ray films, and densitometry of these films is imprecise due to the inherent nonlinearity of exposure of the silver grains by photons (31). To accurately quantify S-palmitoylation, we used a newly developed method of bioorthogonal labeling and in-gel fluorescence. This method allowed us to accurately quantify levels of incorporation of the palmitic acid analog, and to normalize the signal to overall β1AR expression levels by immunoblot of the same gel. This method provided a linear fluorescent signal that could be easily quantified, and was more practical than the acyl-biotin switch assay or mass spectrometry (49). We observed that the sums of the signal obtained from mutations at the proximal and distal site (43% and 61%, respectively) nearly equaled the signal from the wildtype protein. This further suggests that the sites are independently modified, and attests to the accuracy of the labeling method.

Additionally, we were able to use the bioorthogonal palmitate reporter in pulse-chase experiments to compare the rates of turnover for palmitic acid at each site. Unlike other acyl modifications, S-palmitoylation is reversible, and many proteins have regulated cycles of palmitoylation and depalmitoylation (9). We found palmitoylation of β1AR at the proximal site to be relatively stable. By contrast, palmitate at the distal site was rapidly turned over, with nearly all signal chased within 15 min. Because of the stable modification at the proximal site, it was not possible to accurately calculate a half-life for the palmitoylation with the chase times we used. However, since there was no loss of signal by 90 minutes, it is possible that the proximal site is modified once and only once during the course of the life of the protein. Thus, S-palmitoylation of the proximal site could cause a stable structural modification, as opposed to that at the distal site, which is more dynamic and therefore may function as a “switch” (Fig. 7). This is the first report of differential S-palmitoylation dynamics in a GPCR, and predicts important functional consequences.

**Consequences of β1AR palmitoylation.** We observed that S-palmitoylation at the two sites in newly synthesized β1AR likely occurs in different cellular compartments. The immature form of β1AR was palmitoylated only when the proximal site was intact. This indicates that the proximal site can be palmitoylated prior to trafficking through the Golgi. Because of the relatively long labeling time (compared to the rate of trafficking), we cannot currently determine if the proximal site can also be palmitoylated in a later compartment, or if the mature signal represents β1ARs that were palmitoylated pre-Golgi or in the early Golgi and chased into the mature fraction. Because of the long half-life of the proximal modification, it does not appear likely that this signal comes from dynamic turnover of palmitic acid. This suggests that the two sites in β1AR may be modified by different protein palmitoyltransferases, allowing for a greater flexibility of receptor activity and additional pathways of regulation.

Due to early acquisition of palmitate at the proximal site of β1AR and its low rate of turnover, this modification may play a structural role contributing to the proper folding of β1AR. The proximal palmitoylation site is directly downstream of the eighth helix (H8), a structural component known to contribute to G protein coupling of many GPCRs, including rhodopsin (50,51) and β1AR (52). We observed that the proximal site is palmitoylated early in the secretory pathway. It is possible that palmitoylation contributes to the structural stability of H8, strongly anchoring it to the membrane, thus contributing to G protein coupling. While proteins mutated at this site do not have a shorter half-life in our cell culture system, it is possible that there is a long-term consequence to an animal expressing β1AR mutated at this site, particularly under stress conditions. For example, a recent report describes the photoreceptor cell degeneration of mice expressing a palmitoylation-null rhodopsin, but only when the mice were exposed to bright light. Under normal laboratory lighting conditions, no defect was noted (53). It is possible that β1AR
mutated at the proximal site would similarly have a dramatic defect in experiments testing stress conditions over a long-term experiment in animal models.

We found that palmitoylation at the distal site of β1AR was highly dynamic, and mutation of this site impaired agonist stimulated internalization. It is therefore possible that regulation of this site contributes to desensitization following signaling. Despite their sequence similarities, β1AR does not behave like β2AR following ligand binding. β1AR internalizes at a higher rate in cardiomyocytes, and does not relocalize away from contact sites with SGNs or out of caveolar fractions following ligand binding (36,37). Therefore, regulation of S-palmitoylation at the distal site by a specific subset of palmitoyltransferases could contribute to discrimination between these receptors. This would provide additional control of the signaling response. β1AR could be desensitized by increased phosphorylation, arrestin binding and/or decreased coupling to G proteins. It is possible that modulation of distal site palmitoylation can provide a rapid mechanism for control of these phenomena, contributing to desensitization, downregulation or recycling.

The prediction of palmitoylation sites is inexact, and can only be conclusively demonstrated experimentally. We have identified three cysteines at two sites on β1AR that are palmitoylated. S-palmitoylation at the two sites is apparently regulated independently, since modification occurs in different compartments and is turned over at different rates. This study provides information necessary to further investigate the contribution of each palmitoylation site to the function of β1AR in specific contexts, cell culture and animal systems.

REFERENCES


**FOOTNOTES**

We thank members of the Machamer laboratory and Egbert Hoiczyk for helpful discussions and comments on the manuscript. This work was supported by National Institutes of Health Grant GM42522 to C.E.M, and 1R01GM087544 to HCH. S.W.H. was supported by National Research Service Award postdoctoral fellowship AI069704 from the National Institutes of Health (NIH).

The abbreviations used are: AR, adrenergic receptor; GPCR, G protein-coupled receptor; PN, palmitoylation null; alk-16, alkynyl-16; Iso, isoproterenol; SGN, sympathetic ganglion neurons; HT, hydroxytryptamine; TPβ, TPβ isoform of thromboxane A₂; FSH, follicle stimulating hormone.

**FIGURE LEGENDS**

**Fig. 1.** β1AR is palmitoylated at cysteines 392, 393 and 414. *A*, BLAST alignment of residues 385 to 417 of the human β1AR with β1AR from the indicated species. *B*, 1H-palmitic acid signal revealed by fluorography (right panel), and similar protein expression demonstrated by 35S-methionine/cysteine label (left panel). The amino acids mutated from cysteine to serine are indicated.

**Fig. 2.** The half-life of β1AR is unaffected by cysteine to serine mutations. *A*, autoradiographs are representative of 4-5 independent experiments. The immature (open arrowhead) and mature (closed arrowhead) forms of β1AR are indicated. *B*, data points on the graph represent the mean intensity of signal relative to the 0 chase time point. Error bars represent standard deviation. Half-lives: wildtype, 6.0 h; C392S/C393S, 6.2 h; C414S, 6.3 h; C392S/C393S/C414S, 6.2 h

**Fig. 3.** Mutation of β1AR palmitoylation sites does not affect steady-state surface levels. *A*, immunofluorescence micrograph showing indicated β1AR constructs at cell surface and co-localized with Golgi marker, TGN46. Bar, 10 µm. *B*, surface levels of β1AR measured by radioactive ligand binding. Data represent the mean of at least 3 independent experiments. Error bars represent standard deviation.

**Fig. 4.** Mutation of the distal palmitoylation site inhibits β1AR internalization following agonist stimulation. *A*, Immunofluorescence micrographs of cells expressing FLAG-β1AR incubated with anti-FLAG antibody for 15 min in the absence or presence of 10 µM Iso. Prior to permeabilization and incubation with fluorescent secondary antibody, a brief acid wash (as indicated) was used to remove anti-FLAG bound to surface molecules for visualization of internalized antibody. Bar, 10 µm. *B*,...
Representative immunofluorescence micrographs of acid washed cells expressing indicated constructs of FLAG-β₁AR after 30 min of antibody uptake in the presence of 10 µM Iso. Images are representative of multiple fields photographed from three separate experiments. Bar, 10 µm. C, the intensities of the signal from experiments above were quantified using Image J software. Error bars represent standard deviation. *, P < 0.01 relative to wildtype.

Fig. 5. Palmitoylation of the immature β₁AR takes place at the proximal site. A, representative gel showing incorporation of alkynyl-16 (Alk-16) into the indicated β₁AR construct (upper panel) and relative β₁AR levels by immunoblot with anti-FLAG antibody (lower panel). B, mean intensity of Alk-16 signal from 5 separate labeling experiments, normalized to β₁AR expression level and wildtype Alk-16 signal. Error bars represent the standard deviation. C, mean contribution of mature and immature bands to the total signal for each construct. Error bars represent the standard deviation.

Fig. 6. β₁AR palmitoylation sites are differentially regulated. A, representative gels for palmitoylation turnover experiments. Cells were pulse labeled with Alk-16 and chased for indicated times. B, data points on the graph represent the mean intensity of signal relative to the 0 chase time point from three independent experiments. Error bars represent the standard deviation. *, P < 0.05 relative to wildtype at that time point.

Fig. 7. Two potential cytoplasmic tail conformations dependent on palmitoylation at the distal site. Cartoon representation of β₁AR with oligosaccharide and lipid modifications. Prior to arriving in the medial Golgi, β₁AR is palmitoylated at the proximal site, and has one immature (endoglycosidase H sensitive) N-glycan. After trafficking through the medial Golgi, β₁AR acquires O-linked glycans and is additionally palmitoylated at the distal site, generating a fifth cytoplasmic loop. Dynamic depalmitoylation and repalmitoylation of the distal site could allow a switch in tail conformation that would likely affect function of the receptor.
A.  

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B.  

[Image of gel electrophoresis with bands at 66 kDa and 46 kDa marked with labels for 35S met/cys and 3H palmitic acid]
Figure 2

[Graph showing the percentage of wt, C392S/C393S, C414S, and C392S/C393S/C414S remaining at different chase times (0-8 hours).]

Chase (h)  | 0  | 2  | 4  | 6  | 8
---|---|---|---|---|---
w1t     |    |    |    |    |    
C392S/C393S |    |    |    |    |    
C414S    |    |    |    |    |    
C392S/C393S/C414S |    |    |    |    |    

Percent Remaining

Chase (h)  | 0  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9
---|---|---|---|---|---|---|---|---|---|---
1000 |    |    |    |    |    |    |    |    |    |    
100  |    |    |    |    |    |    |    |    |    |    
10   |    |    |    |    |    |    |    |    |    |    
1    |    |    |    |    |    |    |    |    |    |    

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B.

![Relative Surface Levels (%) vs. genotypes](image)
Figure 4

A.

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B.

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C.

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Figure 5

A.

B.

C.
Figure 6

A. Time Chase (min):
- 0
- 15
- 30
- 90

Wildtype
C392S/C393S
C414S

B. Relative Signal vs. Chase Time (min)

Chase Time (min)

Relative Signal

C414S
C392S/C393S
wt
Figure 7

pre-medial Golgi

post-medial Golgi
**Supplementary Fig. S1.** Internal juxta-nuclear FLAG-β₁AR signal co-localizes with Golgi marker TGN46. Merged image of fields from Fig. 5A. FLAG signal is red, TGN46 signal is green and overlap is yellow.
Differential regulation of two palmitoylation sites in the cytoplasmic tail of the β1-adrenergic receptor

David M. Zuckerman, Stuart W. Hicks, Guillaume Charron, Howard C. Hang and Carolyn E. Machamer

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