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Journal of Biological Chemistry, 294(7):2569-2578

2019-02-15

Journal Article / 学術雑誌論文

This research was originally published in the Journal of Biological Chemistry. Adachi, Naoko / Hess, Douglas T. / Kaku, Mika / Ueda, Chie / Numa, Chisato / Saito, Naoaki. Differential S-palmitoylation of the human and rodent β3-adrenergic receptor. J. Biol. Chem. 2019; 294(7):2569-2578. © 2019 Adachi et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

10.1074/jbc.RA118.004978

http://www.lib.kobe-u.ac.jp/handle_kernel/90005833

PDF issue: 2020-05-07
Differential S-palmitoylation of the human and rodent β3-adrenergic receptors

With few reported exceptions, G protein–coupled receptors (GPCRs) are modified by Cys palmitoylation (S-palmitoylation). In multiple GPCRs, S-palmitoylation targets a canonical site within the C-terminal cytoplasmic tail adjacent to the C terminus of the seventh transmembrane domain, but modification of additional sites is exemplified by the β-adrenergic receptors (βARs). The β1AR is S-palmitoylated at a second, more distal site within the C-terminal tail, and the β2AR is modified at a second site within the third intracellular loop, neither of which is conserved in other βAR isoforms. The functional roles of S-palmitoylation of disparate sites are incompletely characterized for any GPCR family. Here, we describe S-palmitoylation of the β3AR. We compared mouse and human β3ARs and found that both were S-palmitoylated at the canonical site within the C-terminal tail, Cys-358 and Cys-361/363 in mouse and human β3ARs, respectively. Surprisingly, the human β1AR was S-palmitoylated at two additional sites, Cys-153 and Cys-292 within the second and third intracellular loops, respectively. Cys-153 is apparently unique to the human β1AR, and Cys-292 is conserved primarily in primates. Mutational substitution of C-terminal tail Cys in human but not mouse β3ARs resulted in diminished ligand-induced cAMP production. Substitution of Cys-153, Cys-292, or Cys-361/363 within the human β3AR diminished membrane-receptor abundance, but only Cys-361/363 substitution diminished membrane-receptor half-life. Thus, S-palmitoylation of different sites differentially regulates the human β1AR, and differential S-palmitoylation distinguishes human and rodent β3ARs, potentially contributing to species-specific differences in the clinical efficacy of β3AR-directed pharmacological approaches to disease.

It is estimated that >10% of mammalian proteins are subject to modification by long-chain fatty acid acylation, the predominant form of which is S-palmitoylation, the dynamic (reversible) ligation of a 16-carbon saturated fatty acid to a Cys residue via thioesterification (1). S-Palmitoylation operates throughout metazoan phylogeny. In mammalian cells, S-palmitoylation is mediated by the 23–24 members of the DHHC family of palmitoyltransferases (1), and the dynamic nature of S-palmitoylation reflects the operation of depalmitoylating enzymes, including the acyl-protein thioesterases APT1 and APT2 as well as other, less well-characterized members of the ABHD17 family of serine hydrolases/lysophospholipases (2). S-Palmitoylation was originally characterized as a mechanism for membrane localization of otherwise cytosolic proteins but was subsequently shown to modify multiple integral membrane proteins where insertion of the palmitate moiety into the membrane would alter protein configuration and thereby function (3). G protein–coupled receptors (GPCRs), a eukaryotic superfamily that comprises about 800 members and that represents the largest family of membrane receptors, are seven-transmembrane–spanning integral membrane proteins. The first example of S-palmitoylation of a ligand-activated GPCR was provided by the prototypical GPCR, the β2-adrenergic receptor (β2AR). S-Palmitoylation was shown to target Cys-341 of the human β2AR, located within the C-terminal tail in a juxtapositional position adjacent to the cytosolic terminus of the seventh transmembrane segment (4) (unless indicated, all residue numbering here and below is in accordance with human sequences). Where reported, almost all GPCRs examined subsequently were shown to be subject to S-palmitoylation, including S-palmitoylation of one to three Cys located within the C-terminal tail adjacent to the cytosolic terminus of the seventh transmembrane segment (5). However, not all GPCRs possess Cys within the C-terminal tail; S-palmitoylation of sites in addition to or other than this canonical site was shown to occur in a number of GPCRs more distally within the C-terminal tail (6–14), and S-palmitoylation of a site within either the first, second, or third intracellular loop has been demonstrated directly in a number of cases (15–17). Understanding of the different functional roles of S-palmitoylation of disparate sites within a given GPCR or across members of a GPCR family remains incomplete.

These generalities are well instantiated in the case of the βARs. The β1AR is S-palmitoylated at an additional site within the C-terminal tail distal to the canonical site, comprising two Cys that are not conserved in the β2AR or β3AR (9), whereas the...
β₂AR is S-palmitoylated at an additional site within the third intracellular loop that is not conserved within the β₁AR or β₃AR (15).

We describe here S-palmitoylation of the β₂AR. We examined mouse and human β₂ARs and found that both are modified at the canonical C-tail site (Cys-361/363 and Cys-358 in human and mouse, respectively). Surprisingly, however, the human β₂AR was modified at two additional sites, Cys-153 and Cys-292 within the second and third intracellular loops, respectively, which are not conserved in the β₁AR or β₃AR. Furthermore, Cys-153 is apparently unique in phylogeny to the human receptor, and Cys-292 is conserved primarily among primates. S-Palmitoylation of the canonical site within the β₂AR facilitated receptor-effector coupling in the human but not in the mouse β₂AR. S-Palmitoylation of the additional sites within the human β₂AR increased receptor membrane abundance, but only S-palmitoylation of Cys-361/363 enhanced the half-life of the receptor at the membrane. Thus, S-palmitoylation of different sites within the human β₂AR differentially regulates receptor disposition. The unique pattern of S-palmitoylation of the human β₂AR provides a molecular distinction between human and rodent β₂ARs, which may be associated with the differential response of humans and rodents to β₂AR agonists as therapeutic agents.

Results

The mouse β₂AR (mβ₂AR) is S-palmitoylated exclusively at the canonical site

To examine S-palmitoylation of the β₂AR, we used the acyl-RAC method (resin-assisted capture of fatty-acylated proteins) in which the thioester bond linking palmitate to Cys is cleaved with neutral hydroxylamine and the resultant free thiol is coupled to thiopropyl-Sepharose for pulldown and subsequent Western blot analysis (18). This method directly assesses the presence of palmitate-modified Cys, and omission of hydroxylamine provides a rigorous control for false positives. Human embryonic kidney (HEK) 293 cells were used in all experiments. We have shown previously that 19 of the 23 mammalian DHHC palmitoyltransferases as well as the protein depalmitoylases APT1/2 are expressed in HEK293 cells (15). We first used acyl-RAC with HEK293 cells stably expressing wildtype (WT) or Cys→Ala mutant β₂ARs to assess the occurrence of S-palmitoylation and to locate the sites of modification. Analysis was restricted to clones in which levels of receptor expression varied by <10%.

Analysis of HEK293 cells expressing FLAG-tagged WT mβ₂AR revealed that the mβ₂AR is basally S-palmitoylated (Fig. 1A). The mβ₂AR contains 10 Cys, two of which are predicted to be cytoplasmic: Cys-272 and Cys-358. One of these, Cys-358, is predicted to have a juxtamembrane location adjacent to the seventh transmembrane domain and thus represents the canonical site of GPCR S-palmitoylation (Fig. 1B and see also Fig. 2, D and E). Mutation of Cys-358 to Ala eliminated S-palmitoylation of the mβ₂AR (Fig. 1, C and D). Thus, the mβ₂AR is S-palmitoylated solely at the canonical C-tail site. Cys-358 within the mβ₂AR was identified previously as a site of S-palmitoylation in a global survey of the mouse brain palmitoyl proteome (19).

Alternative splicing generates a “b” isoform of the mβ₂AR (expressed in parallel but at substantially lower levels in multiple tissues) that contains an alternative stretch of residues near the C terminus, one of which is a Cys (Cys-400) (20). Cys-400 is conserved in the mouse β₁AR and β₃AR and in the human β₂AR but apparently is not S-palmitoylated (9).

Multiple S-palmitoylation of the human β₂AR (hβ₂AR) Analysis by acyl-RAC of FLAG-tagged hβ₂AR stably expressed in HEK293 cells demonstrated S-palmitoylation (Fig. 2A). However, unlike the mβ₂AR, mutation of either or both of the Cys residues comprising the canonical site, Cys-361/363, did not significantly diminish S-palmitoylation as detected with acyl-RAC (Fig. 2B), indicating the existence of additional sites of S-palmitoylation.

The hβ₂AR contains 16 Cys, eight of which are predicted to be cytoplasmic. Of those, four are predicted to be juxtamembrane, including, in addition to the canonical Cys-361/363, Cys-153 located adjacent to the N terminus of the fourth transmembrane domain and Cys-292 located adjacent to the N terminus of the sixth transmembrane domain (see Fig. 2, D and E). Mutation of all four of these Cys (designated C4A) effectively eliminated S-palmitoylation (Fig. 2C). Because the acyl-RAC assay cannot distinguish between one or more sites of S-palmitoylation within a given substrate, we stably expressed hβ₂AR
in which three of the four Cys that were potential sites of S-palmitoylation were mutated to Ala. The hβ3AR containing either Cys-153, Cys-292, Cys-361, or Cys-363 was S-palmitoylated, demonstrating that all four Cys were sites of S-palmitoylation (Fig. 2C). Note that the populations of S-palmitoylated receptors as revealed by acyl-RAC were diminished relative to WT receptor when only a single site of S-palmitoylation was present (Fig. 2C), suggesting that individual receptors may be differentially S-palmitoylated at one or more sites under these conditions. Notably, on the basis of comparison of available sequences of the β3AR, Cys-153 is apparently unique to the humans, and Cys-292 is restricted largely to primates.

**Figure 2. Multiple sites of S-palmitoylation within the hβ3AR.** A, as shown by acyl-RAC, FLAG-hβ3AR is S-palmitoylated (Palm) under basal conditions. B, unlike the mβ3AR (see Fig. 1), mutation of either or both Cys comprising the canonical C-terminal tail site (Cys-361/363) did not diminish S-palmitoylation as detected by acyl-RAC. Error bars represent S.D.; n = 3. *p < 0.05. Representative Western blots are shown below. Dotted line, within-blot cut. C, combined mutation of Cys-153, Cys-292, Cys-361, and Cys-363 (C4A) eliminated S-palmitoylation of the hβ3AR. The hβ3AR containing only Cys-153, Cys-292, Cys-361, or Cys-363 was S-palmitoylated, indicating that all four Cys are sites of S-palmitoylation. Representative Western blots are shown below. Error bars represent S.D.; n = 4–11. **p < 0.0001 with respect to WT by ANOVA. D, Cys-153 is conserved primarily in primates, whereas Cys comprising the canonical site of S-palmitoylation, Cys-361/363, are conserved across vertebrate phylogeny. E, a schematic summary comparing localization of S-palmitoylation within the mβ3AR and hβ3AR. Within the hβ3AR, Cys-153 and Cys-292 are located within the second and third intracellular loops, respectively, and are predicted to have a juxtamembrane location. Cys shown to be S-palmitoylated in the present study are indicated in blue and red; cytoplasmic Cys not subject to S-palmitoylation are indicated in green and white.
Figure 3. Diminished S-palmitoylation of the hβ3AR but not of the mβ3AR diminishes ligand-induced, G protein–coupled cAMP production. A, native HEK293 cells or HEK293 cells transiently expressing WT mouse FLAG-β3AR (mWT) or palmitoylation-deficient mutant FLAG-mβ3AR (mC358A) were treated with mirabegron at the indicated concentrations for 5 min, and cAMP accumulation was assessed by ELISA. Stimulation with mirabegron of native HEK293 cells (in the absence of transfected receptor) did not result in cAMP production. B, HEK293 cells transiently expressing WT human FLAG-β3AR (hWT), human WT receptor with Cys → Ala mutation of all four sites of S-palmitoylation (hC4A), or human WT receptor with individual mutation of Cys-153, Cys-292, or Cys-361/363 were treated with mirabegron at the indicated concentrations for 5 min, and cAMP accumulation was assessed by ELISA. C, EC50 values were calculated from dose-response curves shown in A and B. Within each individual experimental run, the significance of the variance between the EC50 value of WT and palmitoylation-deficient mutants was tested. EC50 values are presented as means with 95% confidence intervals (CI). Mirabegron-induced production of cAMP was not significantly diminished by C358A mutation of the mβ3AR (two-tailed unpaired Student’s t test), whereas cAMP production was significantly diminished (>2.2-fold) by C4A mutation and by C153A mutation of the hβ3AR (one-way ANOVA, post hoc Dunnett). D, diminished cAMP production by C4A mutation of the hβ3AR was confirmed in HEK293 cells stably expressing either WT or C4A mutant hβ3AR (two-tailed unpaired Student’s t test; n = 4–5). In A, B, and D, error bars represent S.D.

S-Palmitoylation of the hβ3AR but not the mβ3AR facilitates receptor-effector coupling

It was reported that mutation of the canonical C-tail target of S-palmitoylation within the human β3AR (Cys-341) diminished ligand-induced production of cAMP, interpreted as disrupted coupling of the receptor via Gs to G protein–activated adenyl cyclase (4). To examine the role of S-palmitoylation on receptor-effector coupling of the β3AR, we transiently expressed, in HEK293 cells, WT or Cys-mutant hβ3AR or mβ3AR and, 24 h after transfection, assessed by ELISA cAMP production elicited by exposure for 5 min to the β3AR-specific synthetic ligand mirabegron (21).

To avoid interpretive difficulty resulting from different levels of expression of different constructs, we calculated the EC50 following exposure to mirabegron over the effective concentra-

(human, ape, and Old World monkey) (Fig. 2D). Fig. 2E summarizes the differences between S-palmitoylation of the mβ3AR and hβ3AR.

S-Palmitoylation of the hβ3AR but not the mβ3AR mediates different functions, and S-palmitoylation of
Figure 4. S-Palmitoylation of the β3-AR regulates plasma-membrane receptor abundance. A, WT or palmitoylation-deficient FLAG-mβ3-AR (Mouse; at left) or FLAG-hβ3-AR (Human; at right) were transiently expressed in HEK293 cells. Plasma-membrane receptor abundance was assessed by extracellular labeling with anti-FLAG Ab followed by FACS. Mutation of Cys-358 within the mβ3-AR resulted in a small (−8%) decrease in membrane-receptor abundance; n = 3, *p < 0.005 by two-tailed unpaired Student’s t test. Mutation of all four S-palmitoylated Cys (C4A) within the hβ3-AR or separate mutation of Cys-153, Cys-292, or Cys-361/363 resulted in a larger (−20–32%) decrease in membrane-receptor abundance; n = 4, *p < 0.006 by one-way ANOVA, post hoc Dunnett. B, to assess total receptor abundance, cells were fixed, permeabilized and exposed to anti-FLAG Ab conjugated with Alexa Fluor 488 followed by FACS. C358A mutation of the mβ3-AR had no effect on receptor abundance (mouse, at left). C4A or C292A mutation of the hβ3-AR significantly decreased total receptor abundance (human, at right); n = 4, *p < 0.004 by one-way ANOVA, post hoc Dunnett. C, none of the mutations examined in either the mβ3-AR or hβ3-AR resulted in a change in β3-AR mRNA as assessed by quantitative PCR; n = 4, ns, not significant (p > 0.05). In A–C, error bars represent S.D. D, HEK293 cells transiently expressing WT or mutant hβ3-AR were labeled with anti-FLAG β3-AR Ab conjugated to fluorescent dye and visualized by confocal fluorescence microscopy. In the left column, cells were exposed to anti-FLAG Ab conjugated to Alexa Fluor 488 (green) prior to fixation (selective staining of plasma-membrane receptor). Note that exposure of cells to Ab without permeabilization did not result in staining other than that at the plasma membrane. In the middle column, cells were then fixed, permeabilized, and exposed to anti-FLAG Ab conjugated to Alexa Fluor 594 (red) to visualize total hβ3-AR. In the right column, merged images are shown with filter settings that also allow visualization of nuclear staining (Hoechst 33342; blue). Scale bars, 10 μm.

**S-Palmitoylation of the β3-AR influences receptor disposition**

S-Palmitoylation of GPCRs (which may be co- and/or post-translational) has been implicated in the governance of receptor plasma-membrane abundance that may reflect receptor processing/membrane targeting as well as plasma-membrane stability (turnover) (5). We first assessed steady-state plasma-membrane abundance of WT versus Cys-mutant mβ3-AR or hβ3-AR in HEK293 cells transiently expressing FLAG-tagged receptor. The FLAG tag in all constructs was localized to the N terminus of the β3-AR and was therefore extracellular in plasma-membrane–localized receptors. Twenty-four hours following transfection, cells were labeled with anti-FLAG Ab without membrane permeabilization followed by a fluorescently labeled secondary Ab and analysis by fluorescence-activated cell sorting (FACS). Fluorescence immunocytochemistry confirmed that very little intracellular labeling occurred with this protocol (see Fig. 4D).

C358A mutation of the mβ3-AR resulted in a small (−8%) but consistent decrease in steady-state levels of plasma-membrane–localized receptor (Fig. 4A). C4A mutation of the hβ3-AR or mutation of either Cys-153, Cys-292, or Cys-361/363 resulted in a larger decrease that was of similar magnitude for each mutation (−20–32% decrease) (Fig. 4A). We asked whether diminished levels of plasma-membrane–localized hβ3-AR might reflect a decrease in overall receptor abundance. We transiently expressed FLAG-tagged WT or mutant receptor, and 24 h following transfection, cells were fixed, permeabilized to allow intracellular access of antibody, and labeled with anti-FLAG Ab conjugated with fluorescent dye followed by FACS. C4A mutation significantly diminished hβ3-AR abundance, and mutation of individual Cys revealed that this decrease was due to abrogated S-palmitoylation of Cys-292
The abundances of mβ3AR and hβ3AR mRNAs were unaltered by any mutation as assessed by quantitative PCR (Fig. 4C). Diminished levels of C292A hβ3AR would be consistent with deficient processing resulting from abrogation of cotranslational S-palmitoylation of Cys-292 that triggers (endoplasmic reticulum–based) proteostatic degradation and/or with post-translational destabilization.

We then further examined the influence of S-palmitoylation on receptor disposition with fluorescence immunocytochemistry, comparing transiently expressed WT FLAG-mβ3AR with C4A receptor as well as with receptors with individual Cys mutation (Fig. 4D). In cells that were permeabilized before staining (as above), we observed consistently that the C4A receptor accumulated in the cytoplasm where it exhibited predominantly a punctate distribution (Fig. 4D). Mutation of Cys-292 resulted in a diffuse intracellular distribution consistent with receptor degradation. Thus, the decrease in plasma-membrane–localized C153A receptor, demonstrated by FACS (Fig. 4A), is due at least in part to deficient receptor targeting to the plasma membrane, consistent with a requirement for Cys-153 S-palmitoylation in efficiently engaging the transport mechanism that conveys receptors from the Golgi to the plasma membrane (22).

Our results therefore indicate that the diminished plasma-membrane abundance of the hβ3AR resulting from mutation of Cys-153 and Cys-292 (Fig. 4A) reflects at least in part different mechanisms.

On the basis of mutational analysis, S-palmitoylation of Cys-361/363 within the hβ3AR also diminishes plasma-membrane abundance (Fig. 4B) but does not result in enhanced receptor degradation (diminished total levels of receptor) (Fig. 4B) or diminished transport of receptor to the plasma membrane (signified by cytoplasmic accumulation of receptor) (Fig. 4D). Membrane-receptor abundance will be determined at least in part by membrane-receptor turnover (membrane stability). To examine directly a possible role for S-palmitoylation in membrane-receptor stability, we labeled cells transiently expressing WT or Cys-mutant mβ3AR or hβ3AR with anti-FLAG Ab and, at subsequent intervals over 25 h, labeled unpermeabilized cells with fluorescent secondary Ab followed by FACS.

We observed that the half-life of the plasma-membrane–localized mβ3AR was about 4 times greater than that of the
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hβ3AR (29.3 versus 7.82 h) (Fig. 5). Mutation of Cys-358 did not affect the membrane half-life of the mβ3AR (Fig. 5, A and C). Thus, the small diminishment of plasma-membrane receptor abundance associated with mutation of Cys-358 within the mβ3AR (Fig. 4A) is likely to reflect deficient receptor processing/targeting. In contrast, transient expression of C4A mutant hβ3AR substantially diminished membrane-receptor half-life (55% decrease), which we confirmed in cells stably expressing C4A mutant hβ3AR (Fig. 5B). Analysis of the effects of individual mutations demonstrated that diminished membrane-receptor half-life could be ascribed to Cys-361/363 (Fig. 5, C and D). Thus, S-palmitoylation of different sites within the hβ3AR regulates membrane-receptor abundance differentially: S-palmitoylation of Cys-153 and Cys-292 within the second and third intracellular loops, respectively, is required for efficient receptor processing/targeting, whereas S-palmitoylation of Cys-361/363 stabilizes the receptor at the plasma membrane.

Discussion

We describe here S-palmitoylation of the mammalian β3AR. Both the mouse and human β3ARs are S-palmitoylated at the canonical site within the C-terminal cytoplasmic tail, but the hβ3AR is S-palmitoylated at two additional Cys within the second and third intracellular loops. The site within the second intracellular loop, Cys-153, appears to be unique to humans, and the site within the third intracellular loop, Cys-292, is conserved principally among primates. This observation provides, to the best of our knowledge, the first example of human/primate–specific modification of any GPCR.

It has been demonstrated previously that differential S-palmitoylation distinguishes βARs. Both the human β1AR and β2AR have been shown to be S-palmitoylated at the canonical C- and tail site (in general, other species have not been examined), but the β3AR is S-palmitoylated at an additional Cys more distal within the C-terminal tail (9) and the β3AR is S-palmitoylated at an additional Cys within the third intracellular loop (15), neither of which are conserved across the βARs. Our results indicate that S-palmitoylation of the C-tail versus intracellular loop sites within the hβ3AR subserve different functions: S-palmitoylation of the canonical-site Cys affects both receptor-effector coupling (as readout by ligand-induced cAMP production) and plasma-membrane receptor stability, whereas S-palmitoylation of the sites within the second and third intracellular loops affects receptor processing/targeting. The intracellular loop sites within the hβ3AR are S-palmitoylated under basal conditions, whereas S-palmitoylation of the targeted Cys within the third intracellular loop of the β3AR is strictly dependent upon ligand-induced activation of the receptor and subsequent phosphorylation, internalization, and trafficking to the Golgi (15). The possible functional roles(s) of changes in conformation of the plasma-membrane–localized receptor that would result from S-palmitoylation of Cys-153 and Cys-292 remains to be explored.

S-Palmitoylation of Cys-361/363 within the hβ3AR also affects plasma-membrane receptor stability and thereby provides a third S-palmitoylation–dependent mechanism for regulating plasma-membrane receptor abundance. The β3AR is well recognized as a tertium quid among βARs in that it lacks the sites of phosphorylation that regulate the protein–protein interactions mediating ligand-coupled receptor internalization and is not internalized following activation (23, 24). Generally, little if anything is known about the mechanisms that might influence β3AR plasma-membrane abundance. Our finding that S-palmitoylation of the canonical C-terminal site within the hβ3AR regulates plasma-membrane stability and thereby plasma-membrane abundance provides the first demonstration of which we are aware of regulation of β3AR disposition by post-translational modification.

Our finding that S-palmitoylation of the hβ3AR influences receptor processing, targeting, and plasma-membrane stability is broadly consistent with extensive prior literature describing the roles of S-palmitoylation of GPCRs (5, 25). Our discovery that S-palmitoylation of Cys-292 and Cys-153 is required for proper receptor processing and targeting, respectively, whereas S-palmitoylation of Cys-361/363 stabilizes the receptor at the plasma membrane provides a demonstration of different functionality of S-palmitoylation of different sites within the hβ3AR, which extends previous descriptions of different functionality of S-palmitoylation of different sites within the (human) β1AR (9) and β2AR (15).

Following the initial report that mutation of the Cys at the canonical C-tail site of S-palmitoylation within the (human) β3AR diminished ligand-induced and G protein–dependent cAMP production, multiple mutational analyses in different GPCRs have shown that S-palmitoylation of Cys comprising the canonical site either does or does not suppress ligand-effector coupling. In the case of βARs, mutation of S-palmitoylated Cys within the (human) β1AR, unlike the case in the (human) β2AR (4), had no effect on ligand-induced cAMP production (9). Our finding that mutation of Cys comprising the canonical site diminishes ligand-induced cAMP production via the human but not the mouse β3AR provides the first example of which we are aware in which S-palmitoylation of the canonical site in a particular GPCR differentially regulates receptor-effector coupling in different mammalian species.

The β3AR is expressed in multiple tissues, including adipocytes and the heart (26, 27). β3ARs are the principal effectors of the sympathetic influence on adipocytes, and in rodents, β3AR agonists increase energy expenditure and fatty acid oxidation, deplete fat stores, preserve lean body mass, and improve insulin sensitivity (28). β3AR agonists therefore represent promising therapeutic agents in obesity and in type 2 diabetes. However, human trials to date have failed (28), pointing to functional differences between the human and mouse β3ARs. In the myocardium, multiple studies in mouse models point to a potential beneficial role of β3AR stimulation in heart failure, and clinical trials designed to assess the efficacy of β3AR agonism in heart failure are underway (29). Our results demonstrate multiple differences between the hβ3AR and mβ3AR assessed in vitro. The half-life at the plasma membrane of the mβ3AR is greatly extended vis-à-vis the hβ3AR, and the half-life of plasma-membrane–localized hβ3AR but not the mβ3AR is determined at least in part by S-palmitoylation. More generally, the disposition of the hβ3AR but not the mβ3AR is substantially determined by S-palmitoylation. Furthermore, S-palmitoyla-
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ion affects receptor-effector coupling of the hβ₃AR but not of the mβ₃AR. Our demonstration that the hβ₃AR has a unique molecular signature among mammalian β₃ARs supports increased caution in the design of β₃AR-directed therapeutic approaches based largely on animal models.

Experimental procedures

Reagents and plasmids

All reagents were from Sigma-Aldrich unless otherwise specified. Hydroxylamine was from Wako, thiopropyl-Sepharose was from GE Healthcare, and mirabegron was from Santa Cruz Biotechnology. Sources of primary antibodies were as follows: anti-DDDDK (FLAG)-HRP-DirecT antibody (M185-7), anti-DDDDK (FLAG) tag mAb–Alexa Fluor 488 (M185-A488), anti-DDDDK (FLAG) tag mAb–Alexa Fluor 594 (M185-A59), and mouse anti-DDDDK (FLAG) tag antibody (M185–3L) were from MBL (Nagoya, Japan), and anti-β₃AR (C-20) antibody (sc-1472) was from Santa Cruz Biotechnology. Alexa Fluor 488– and Alexa Fluor 594– conjugated secondary antibodies were from Invitrogen. Human ADRB3 cDNA ORF clone plasmid (RC210428) was purchased from Origene. The plasmid contained two mutations (N55D and P252S) that were corrected (RC210428) was purchased from Origene. The plasmid contained two mutations (N55D and P252S) that were corrected.

Cell culture and transfection

HEK293 cells were maintained in Eagle’s minimum essential medium (Wako) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Cells were transfected at ~70% confluence using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. To generate stable cell transfectants, G418 (Nacalai Tesque) was added to a final concentration of 150 µg/ml active antibiotic.

Detection of S-palmitoylation by acyl-RAC

The acyl-RAC method was applied essentially as described (18) with minor modifications. Cells in a 6-well plate were harvested and lysed in 400 µl of blocking buffer (100 mM HEPES, 1 mM EDTA, 2.5% SDS, 0.1% methyl methanethiosulfonate, pH 7.4), disrupted by sonication, and incubated at 50 °C for 10 min. Following two acetone precipitations, the pellets were washed with 70% acetone and resuspended in binding buffer (100 mM HEPES, 1 mM EDTA, 1% SDS, pH 7.4). Total protein was quantified with a bicinchoninic acid assay (BCA; Pierce) using BSA as the standard, and equal amounts of protein (50–200 µg) were rediluted in 150 µl of binding buffer. Approximately 60 µg of protein from each sample was retained to assess input. When used, an equal volume of freshly prepared 1 M NH₂OH, pH 7.2, was added followed by ~30 µl of prewashed thiopropyl-Sepharose. Binding was carried out on a rotator at room temperature for 2 h. The resin was washed four times with binding buffer and eluted in 25 µl of SDS sample buffer containing 1% 2-mercaptoethanol at 42 °C for 10 min prior to SDS-PAGE and Western blotting.

Western blot analysis and data presentation

Western blotting signals were detected using the ChemiDoc XRS system (Bio-Rad), and densitometric analysis was performed with Quantity One software. All comparisons were made between bands on a single blot. Densitometric values for S-palmitoylated β₃AR were normalized with respect to total β₃AR. However, we found that population stoichiometry (i.e., the proportion of β₃ARs that are S-palmitoylated) cannot be determined from our results. This is because Western blots of FLAG from samples derived from acyl-RAC and from starting material cannot be compared directly: we never saw a ratio of S-palmitoylated β₃AR/total β₃AR less than 1, but our analysis shows that the values for S-palmitoylated β₃AR following acyl-RAC are in fact always proportionately greater than those for total β₃AR. This is because multiple proteins in the range of ~40–70 kDa are present in the starting material samples (but not the acyl-RAC samples) and evidently (epitope-) mask the Western blotting signal from the β₃AR in starting material samples. All Western blotting data shown in the figures are from single blots. Within-blot cuts, for clarity of presentation, are indicated by dotted lines.

Assay of cAMP

HEK293 cells transiently or stably overexpressing WT or mutant FLAG-β₃AR were stimulated with mirabegron in the presence of the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro 20-1724; 20 µM). Cells were washed and collected, and cAMP was assayed with a cAMP Parameter Assay kit (R&D Systems) according to the manufacturer’s instructions.

Immunofluorescence staining and confocal imaging

To visualize plasma-membrane receptors, cells in glass-bottom dishes were exposed to anti-DDDDK (FLAG) tag mAb–Alexa Fluor 488 (1:100) for 1 h on ice followed by fixation with 4% paraformaldehyde in PBS and incubation in 10% normal goat serum in PBS containing 0.1% Tween 20 for 30 min before exposure to anti-DDDDK (FLAG) tag mAb–Alexa Fluor 594 (1:100; 1 h). The total population of receptors was visualized identically except that cells were permeabilized with 10% normal goat serum in PBS containing 0.1% Tween 20 for 30 min before initial exposure to anti-DDDDK (FLAG) tag mAb–Alexa Fluor 594 (1:100; 1 h). Hoechst 33342 (0.2 µg/ml for 5 min) was used for nuclear staining. Immunostaining was assessed using a confocal immunofluorescence microscope (Carl Zeiss, LSM700).

FACS analysis of total or cell-surface receptor abundance

To examine total receptor abundance, WT or mutant FLAG-β₃AR was transiently expressed in HEK293 cells. One day after
transfection, cells were washed with PBS and harvested with 0.05% trypsin, EDTA. Cells were then fixed with 1% paraformaldehyde in PBS for 15 min and incubated in 10% normal goat serum in PBS containing 0.1% Tween 20 for 15 min on ice before exposure to anti-DDDDK (FLAG) tag mAb—Alexa Fluor 488 (1:150; 20 min). After washing and passing through a sieve (catalog number 38030, Falcon), FACS was carried out with a BD Accuri C6 flow cytometer (BD Biosciences). To examine cell-surface receptor abundance, HEK293 cells transiently expressing WT or mutant FLAG-β3AR in 6-well plates were exposed to anti-DDDDK (FLAG) tag Ab (1:500) for 20 min at 37 °C in culture medium. The cells were washed with ice-cold PBS and harvested with 0.05% trypsin, EDTA. The cells were resuspended in ice-cold stain buffer (2% fetal bovine serum and 0.09% NaCl in PBS) and exposed to anti-mouse IgGs conjugated with Alexa Fluor 488 (1:150) for 20 min. After washing and sieving, FACS was carried out with a BD Accuri C6 flow cytometer.

To examine plasma-membrane receptor stability (turnover), WT or mutant FLAG-β3AR was transiently or stably expressed in HEK293 cells. Cells were labeled extracellularly with DDDDK (FLAG) tag Ab (1:500) for 20 min at 37 °C. Excess Ab was removed by two washes with fresh medium, and cells were then incubated with mirabegron (10 μM) at 37 °C in a humidified 5% CO2 atmosphere. Cells were harvested with 0.05% trypsin, EDTA and incubated with anti-mouse IgG conjugated with Alexa Fluor 488 (1:150) for 20 min in ice-cold staining buffer. After washing and passing through a strainer, cell-surface fluorescence was detected with a BD Accuri C6 flow cytometer. Data were fitted to a one-phase decay curve and are presented as mean ± S.D.

Reverse transcription and real-time PCR

RNA was extracted using TRIzol RNA isolation reagents (Invitrogen), and 3 μg of RNA was treated with DNase I (catalog number 2270A, Takara Bio Inc.) and used to prepare cDNA with random hexamer oligonucleotide primers using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Gene-specific primers were used for real-time PCR in a LightCycler 480 System (Roche Applied Science) using iTaqrogen). Gene-specific primers were used for real-time PCR in a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Gene-specific primers were used for real-time PCR in a LightCycler 480 System (Roche Applied Science) using iTaqrogen). Gene-specific primers were used for real-time PCR in a LightCycler 480 System (Roche Applied Science) using iTaq

Data presentation and statistical analyses

All quantified data are presented as mean ± S.D. Comparisons between groups using Student’s t test or one-way ANOVA and Dunnett or Tukey post hoc test as appropriate were conducted using GraphPad Prism 7 (La Jolla, CA). p < 0.05 was considered significant.

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J. Biol. Chem. 2019, 294:2569-2578.
doi: 10.1074/jbc.RA118.004978 originally published online December 12, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.004978

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