Palmitoylation of the recombinant human A_1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors

Zhenhai GAO*, Yajun NI*, Gabor SZABO* and Joel LINDEN*†[1]

*Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A., and †Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

Palmitoylation of the recombinant human A_1 adenosine receptor (A_1AR) expressed in HEK-293 cells is demonstrated by showing that hexahistidine (His_6)/Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) (H/F) A_1ARs, purified to homogeneity from cells metabolically labelled with [^{3}H]palmitate, incorporate tritium into a 38–42 kDa receptor glycoprotein. The amount of palmitoylation is not affected by incubation of cells with the A_1AR-selective agonist N^6-cyclopentyladenosine (CPA). A_1AR palmitoylation is abolished by treatment with neutral hydroxylamine or by mutation of Cys-309 to Ala ($C^{309} \rightarrow A$). Based on Western blotting and pulse–chase experiments with [^{35}S]-methionine, at least 90 % of wild-type receptors are palmitoylated and turn over with a $t_{\frac{1}{2}}$ of 6.4 h. Of the $C^{309} \rightarrow A$ mutated receptors, 40 % appear to turn over like wild-type receptors, with a $t_{\frac{1}{2}}$ of 7.1 h, and 60 % appear to be rapidly cleaved to form a 25 kDa receptor fragment that turns over with a $t_{\frac{1}{2}}$ of 0.8 h. In HEK-293 cell lines expressing similar numbers of wild-type or $C^{309} \rightarrow A$ mutant A_1Rs, there is little difference in the kinetics of CPA-induced receptor internalization (1 h), down-regulation (24 h), inhibition of forskolin-stimulated cAMP accumulation, or activation of co-transfected G-protein-activated inward rectifier K^+/cardiac inward rectifying K^+ (GIRK1/CIR K^+) channels. Also unaffected by palmitoylation is guanosine 5′-[γ-thio]-triphosphate ([S]GTP)-sensitive binding to membranes by the agonist ^{125}I-labelled aminobenzyladenosine. The results suggest that palmitoylation has little effect on receptor–effector coupling, agonist-induced internalization or down-regulation. We speculate that palmitoylation may divert newly synthesized A_1ARs from a pathway leading to rapid degradation.

Key words: desensitization, down-regulation, GIRK1/CIR, G-protein, palmitoylation.

INTRODUCTION

Adenosine receptors (ARs) belong to a superfamily of the G-protein-coupled receptors that possess seven-transmembrane domains. To date, four subtypes of ARs have been cloned and designated A_1, A_{2A}, A_{2B} and A_3 [1]. Palmitoylation has been demonstrated to occur on one or more cysteines located in the C-terminal region of several G-protein-coupled receptors, including bovine rhodopsin [2,3], β_2 and α_{2A} adrenergic [4,5], D_1 and D_2 dopamine [6,7], 5-hydroxytryptamine 1B [8], metabotropic mGLUR4 glutamate [9], luteinizing hormone/human choriogonadotropin [10] and endothelin A and B [11,12]. All of the AR subtypes, except the A_{2A}, have one or more consensus palmitoylation sites [13]. Reports of the functional impact of palmitoylation on G-protein-coupled receptors have been inconsistent and contradictory (see Discussion section). In several instances, palmitoylation has been noted to influence the cell surface expression of transiently expressed G-protein-coupled receptors, albeit to a variable extent. In this study we demonstrate that the recombinant hexahistidine (His_6)/Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) (H/F)-A_1AR is palmitoylated on Cys^{309} and we show that a substantial fraction of receptors (60 %) that cannot be palmitoylated are, apparently, rapidly degraded. However, the fraction of non-palmitoylated receptors that escape rapid degradation appear to have a normal $t_{1/2}$ and to function normally. Palmitoylation has no effect on the number or distribution of H/F-A_1ARs expressed in transiently transfected cells.

A_1ARs couple to inhibition of adenylate cyclase and, in atrial myocardium and neuronal tissues, to activation of K^+-channels [14,15]. In order to examine the effects of A_1AR palmitoylation on K^+ channel conductance in this study, HEK-293 cells, stably transfected with cardiac inward rectifying K^+ channel (CIR), were co-transfected with A_1ARs, G-protein-activated inward rectifier K^+ channel (GIRK1) and m2 muscarinic receptors. We demonstrate, for the first time, coupling between recombinant A_1ARs and co-transfected GIRK1/CIR channels. Palmitoylation has no effect on A_1AR coupling to adenylate cyclase or GIRK1/CIR.

EXPERIMENTAL PROCEDURES

Materials

N^6-Cyclopentyladenosine (CPA) and 8-sulphophenyltheophylline (8-SPT) were purchased from Research Biochemicals International (Natick, MA, U.S.A.); 8-Cyclopentyl-1,3-dipropylxanthine (CPX) and ^{125}I-labelled N^6-(4-aminobenzyl)adenosine (^{125}I-ABA) were gifts from Susan Daluge of Glaxo Wellcome (Research Triangle Park, NC, U.S.A.). [^{3}H]CPX was from New England Nuclear (Boston, MA, U.S.A.); ^{125}I-ABA was synthesized as described [16]. Tissue-culture reagents were from Gibco (Grand Island, NY, U.S.A.); restriction enzymes, competent JM109 *Escherichia coli* and Wizard mini- and mega-prep DNA purification systems were purchased from Promega Co-operation (Madison, WI, U.S.A.); adenosine deaminase and *N*-glycosidase F were from Boehringer Mannheim Biochemicals

Abbreviations used: AR, adenosine receptor; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; DMEM, Dulbecco's modified Eagle's medium; H/F, His_6/FLAG; hA_1AR, human A_1AR; [S]GTP, 5′-[γ-thio]triphosphate; Ni-NTA, nickel-nitrilotriacetic acid; ABA, N^6-(4-aminobenzyl)adenosine; A_Z-BW-A844, 8-cyclopentyl-3-azidophenethyl-1-propylxanthine; CPA, N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; ACh, acetylcholine; SPT, 8-sulphophenyltheophylline.

[1] To whom correspondence should be addressed (e-mail jlinden@virginia.edu).

(Indianapolis, IN, U.S.A.); anti-FLAG m2 affinity gel from Kodak IBI (New Haven, CT, U.S.A.); Amplify fluorographic, enhanced chemiluminescence reagents and hyper-film MP from Amersham (Arlington Heights, IL, U.S.A.); Centricon-30 concentrators from Amicon, Inc (Beverly, MA, U.S.A.); Westran PVDF membranes from Schleicher and Schuell (Keene, NH, U.S.A.); FLAG peptide and oligonucleotides used for mutagenesis were synthesized in the Biomolecular Research Facility of the University of Virginia; Unique site elimination (U.S.E.) mutagenesis kits from Pharmacia (Piscataway, NJ, U.S.A.); Hoefer semi-phor was from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Expression of H/F-A_1AR

The expression vector H/F-CLDN10B (pDoubletrouble: pDT) described previously [17] was used to prepare cDNA encoding the human A_1AR (hA_1AR) extended on the N-terminus with His_6 and the FLAG epitope (H/F-A_1AR). To generate a mutated H/F-A_1AR receptor lacking the palmitoylated cysteine, a mutagenic primer was prepared to change Cys^{309} to Ala: (5′-pdG AAT GAC CAT TTC CGC GCC CAG CCT GCA CCT CCC-3′). A selection primer (5′-pdCTC TTC CTT TTT CAG GCC TAT TGA AGC ATT TAT CAG G-3′) was prepared to eliminate a unique *Ssp*1 restriction site in the pDT vector and subsequently for selection of mutated plasmids. After annealing of both primers to the heat-denatured plasmid, primer-directed DNA polymerization was carried out using T4 DNA polymerase. The newly synthesized DNA was then digested with *Ssp*1 and the digested mix was used to transform repair-defective *E. coli* strain BMH71-18 mutants. After a second round of restriction enzyme selection, the *E. coli* host JM109 was transformed with the *Ssp*1-digested plasmid preparation. DNA products from individual colonies were isolated, and a $C^{309} \rightarrow A$ mutant was selected and sequenced on both strands (University of Virginia Biomolecular Research Facility).

Cell culture and transfection

Native, H/F-tagged hA_1AR or a $C^{309} \rightarrow A$ palmitoylation-deficient mutant was transfected into CHO-K1 or HEK-293 cells by means of Lipofectin (stable expression) or Lipofectamine (transient expression). Stably transfected CHO-K1 or HEK-293 cells were maintained in Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.5 mg/ml G418 respectively, in an incubator of 5% CO_2/95% air at 37 °C.

Metabolic labelling of cells with [^{3}H]palmitic acid and affinity purification of H/F-A_1AR

Ninety percent confluent CHO-K1 or HEK-293 cells stably expressing the H/F-A_1AR or the $C^{309} \rightarrow A$ mutant were washed with ice-cold PBS and detached from tissue culture plates with the same buffer (for CHO-K1 cells, 5 mM EDTA was added). After washing three times with DMEM the cells were resuspended in DMEM (for CHO-K1) or DMEM/F12 (for HEK-293) supplemented with 25 mM Hepes, 5% fetal calf serum and 0.5 mCi/ml [^{3}H]palmitic acid, which had been dried under nitrogen, and redissolved in DMSO. After incubation at 37 °C in 5% CO_2/95% air for 2 h, intact cells were pelleted by centrifugation at 4 °C, washed three times with ice-cold PBS and receptors were purified by anti-FLAG affinity chromatography as described in [18].

SDS/PAGE, Western blot analysis and autoradiography

Electrophoresis was conducted according to the method of Laemmli [19] using 12% polyacrylamide gels. Autoradiography of tritiated proteins was performed with Amplify™ according to the manufacture's protocol. The gels were dried at 80 °C for 2 h and exposed to Hyperfilm-MP at −80 °C for 7–30 days (^{3}H). For Western analyses H/F-A_1ARs were transferred onto Westran PVDF membranes and incubated with anti-FLAG m2 antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody.

Pulse–chase experiments

To determine the half-life of wild-type and palmitoylation-deficient hA_1AR, 90% confluent cells grown on 60-mm dishes were metabolically labelled for 2–5 h with 50–100 μCi/ml [^{35}S]-methionine in a total volume of 2 ml/dish in methionine-free DMEM. At time 0, cells were quickly washed with 10% fetal calf serum/DMEM supplemented with 1 mM methionine and further incubated in the same medium. At various times, cells were washed with cold PBS and lysed in hypotonic buffer A (10 mM Hepes/20 mM EDTA, pH 7.4). Receptors were then purified by anti-FLAG affinity chromatography as described above and resolved by SDS/PAGE followed by Western transfer to nitrocellulose membranes. [^{35}S]-Labelled hA_1ARs were detected with a PhosphoImager (Molecular Dynamics).

cAMP assays

Weakly adherent intact HEK-293 cells expressing wild-type or mutant A_1ARs were detached from tissue culture plates and washed by centrifugation in PBS. After two additional washes with serum-free DMEM supplemented with 20 mM Hepes, the cells were resuspended in the same medium containing 1 unit/ml adenosine deaminase. Aliquots of cells ($\sim$ 40000/200 μl) were transferred to polypropylene test tubes and maintained for 1 h at room temperature. The cells were then transferred to a 37 °C shaker bath and various amounts of CPA and 10 μM (final) forskolin were added. After 15 min, 500 μl of 0.15 M HCl was added and lysed cells were pelleted by centrifugation at 1700 ***g*** for 10 min. Supernatant (500 μl) was removed, acetylated and assayed for acetyl cAMP by automated RIA [20].

Activation of K^+ channels by H/F-A_1ARs

HEK-293 cells stably expressing CIR (Kir3.4, a gift from Dr. L. Y. Jan, University of California, San Francisco, CA, U.S.A.) and transiently transfected with either wild-type or palmitoylation-deficient mutant H/F-A_1ARs, m2 muscarinic receptor (a gift from Dr. E. Peralta, Harvard University, Cambridge, MA, U.S.A.), GIRK1 (Kir3.1, a gift from Dr. D. Clapham, Mayo Clinic, Rochester, MN, U.S.A.) and green fluorescent protein were placed in a chamber with Tyrode's solution containing (in mM) 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 5 Hepes, 0.5 $MgCl_2$, 1.1 glucose, pH 7.4, and were superfused with Tyrode's solution to wash out the tissue culture medium. Individual cells, which appeared green under fluorescent illumination, were patch-clamped with a pipette containing (in mM) 20 KCl, 130 potassium aspartate, 1 KH_2PO_4, 5 Hepes, 5 EGTA, 3 $MgCl_2$, 3 Mg^{2+}-ATP, 0.5 GTP, pH 7.4. Microelectrodes (3–6 MΩ) were fabricated from square-bore borosilicate capillaries using a P-80 puller (Sutter Instruments, Novato, CA, U.S.A.). Currents were recorded using an Axopatch 200A current amplifier (Axon Instruments, Foster City, CA, U.S.A.), filtered at 10 kHz, stored on videotape using a VR-10B converter (Instrutech, Great Neck,

NY, U.S.A.) and digitized at 3.0 kHz with a DAP 800 processor. The processor was also used to generate command potentials. Cells were held at a resting potential of −85 mV. Test potentials of 250 ms, incremented in steps of 10 mV from −130 mV to 40 mV, were separated by 250 ms periods at the resting potential. Currents were measured at the end of each test pulse in the absence and presence of agonists [1 μM CPA or 1 μM acetylcholine (ACh), a muscarinic receptor agonist] and plotted against the potential. The bath chamber was washed thoroughly with Tyrode's solution for 8 min between the applications of CPA and ACh.

Agonist-induced internalization of H/F-A_1ARs

HEK-293 cells expressing H/F-A_1ARs were pre-treated with 10 μM CPA or vehicle (0.1% DMSO) for various times as indicated in the Figures. Tissue culture plates were placed on ice and cells were washed and detached in ice-cold PBS. After an additional three washes with cold DMEM/F12 supplemented with 25 mM Hepes, cells were resuspended in wash buffer with adenosine deaminase (1 unit/ml). Total and internalized receptor-binding sites were measured by incubation at 4 °C for 3 h with [^{3}H]CPX (< 10 nM, a hydrophobic cell permeant antagonist) in the absence or presence of 8-SPT (a hydrophilic cell impermeant antagonist) respectively. Non-specific binding was determined in the presence of 1 μM CPX.

Other assays

Photoaffinity labelling, radioligand binding to receptors on membranes and down-regulation of H/F-A_1ARs were evaluated as described previously [18]

RESULTS

Palmitoylation of the H/F-A_1AR

Stably transfected CHO-K1 cells expressing a high number of H/F-hA_1ARs (~ 25 pmol/mg of membrane protein) were metabolically labelled with [^{3}H]palmitic acid. The receptors were purified on an anti-FLAG column and subjected to SDS/PAGE and autoradiography. As shown in Figure 1 (lane 1), a single tritiated band migrating with an apparent molecular mass of ~ 40 kDa was purified. After treatment with *N*-glycosidase F, the palmitoylated protein shifts to a new apparent molecular mass of

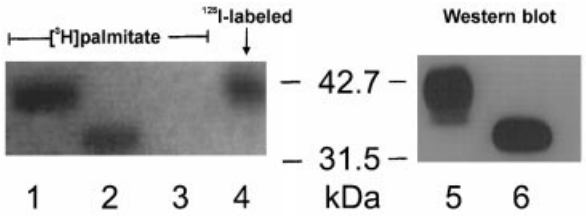

Figure 1 Metabolic labelling of the H/F-A_1AR with [^{3}H]palmitic acid

CHO-K1 cells stably transfected with H/F- A_1AR or native A_1AR were metabolically labelled with [^{3}H]palmitic acid (lanes 1–3) or covalently photoaffinity labelled with ^{125}I-Az-BW-A844 (lane 4) and affinity purified as described in the Experimental procedures section. The samples were analysed by SDS/PAGE and autoradiography (lanes 1–4) or Western blotted with anti-FLAG antibodies (lanes 5–6). Receptors purified from CHO-K1 cells expressing H/F-A_1AR were incubated with *N*-glycosidase F (lanes 2 and 6) or vehicle (lanes 1, 3, 4 and 5) prior to electrophoresis. The sample applied to lane 3 was derived from cells transfected with native-A_1ARs lacking the FLAG epitope. Portions of gels that are not shown are blank (see also Figures 2–4). The results are typical of three experiments.

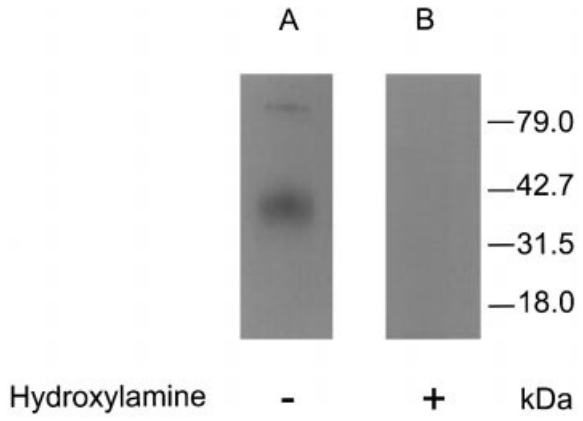

Figure 2 Effect of hydroxylamine on palmitoylation of A_1ARs

CHO-K1 cells stably transfected with H/F-A_1AR were purified from cells pre-incubated with [^{3}H]palmitic acid. After electrophoresis, the gels were placed in fixative solution (40% methanol/10% acetic acid/3% glycerol) for 4 h and then immersed in either 1 M Tris/HCl, pH 7.0 (**A**) or 1 M NH_2OH, pH 7.0 (**B**) for 12 h at room temperature. After washing with 2-propanol/water (1:1, v/v), the gels were exposed to film for 14 days at −80 °C. The results are typical of three experiments.

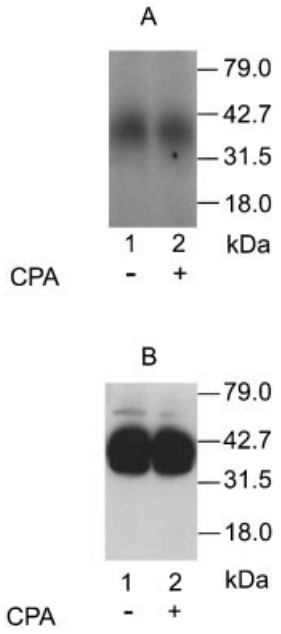

Figure 3 Effect of treatment with CPA on the incorporation of [^{3}H]palmitic acid into A_1ARs

CHO-K1 cells stably transfected with H/F-A_1ARs were metabolically labelled with [^{3}H]palmitic acid for 2 h and then stimulated with vehicle or 10 μM CPA for 30 min. H/F-A_1ARs purified from equal numbers of untreated cells (lane 1) or CPA-treated cells (lane 2) were subject to SDS/PAGE and autoradiography (**A**) or anti-FLAG Western blot analysis (**B**). The results shown are representative of triplicate experiments.

~ 32 kDa (lane 2). The same molecular masses of the purified glycosylated and deglycosylated silver-stained H/F-A_1AR proteins have been noted previously [17]. No [^{3}H]palmitate was detected in affinity purified samples derived from CHO-K1 cells transfected with native-A_1ARs (lane 3). This was to be expected, since native receptors lack the FLAG epitope required for

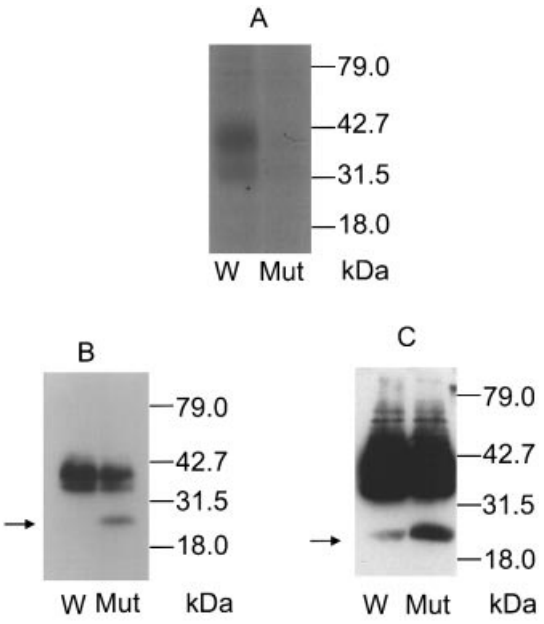

Figure 4 Effect of $C^{309} \rightarrow A$ mutation on palmitoylation and proteolysis of the H/F-A_1AR

HEK-293 cells stably transfected with wild-type (W) or mutant (Mut) H/F-A_1ARs were metabolically labelled with [^{3}H]palmitic acid. Purified wild-type or mutant receptors were subjected to SDS/PAGE and autoradiography by exposure to Hyperfilm-MP for 1 month at −80 °C (**A**) and immunoblotted with anti-FLAG m2 antibody (**B** and **C**). The arrow indicates the position of a putative receptor proteolytic fragment. In overloaded gels (**C**) the proteolytic receptor fragment could be detected with both wild-type and mutant receptors.

adherence to the anti-FLAG affinity column. The tritiated protein detected in lane 1 co-migrates with receptors photoaffinity labelled with ^{125}I-labelled 8-cyclopentyl-3-azidophenethyl-1-propylxanthine (A_z-BW-A844) (lane 4). In addition, tritium in glycosylated and deglycosylated receptors co-localizes with receptor proteins detected by Western analysis using anti-FLAG m2 monoclonal antibodies (lanes 5 and 6). Using the sensitive Western blotting techniques, the receptor appeared as a broad smear containing two or three distinct individual bands. These probably represent heterogeneously glycosylated forms of the receptors, since only a single band is detected upon deglycosylation. We conclude that the tritiated protein is the H/F-A_1AR. In several instances we saw evidence of high-molecular-mass receptor aggregates (Figures 2–4). It is not clear that such aggregates occur in membranes, since purified receptors spontaneously aggregate in a time- and concentration-dependent manner.

Table 2 Binding of the antagonist [^{3}H]CPX to HEK-293 cells transiently expressing wild-type or $C^{309} \rightarrow A$ mutant receptors

HEK-293 cells were transiently transfected with either wild-type or mutant A_1AR and whole cell binding was performed in the absence or presence of 1 mM 8-SPT (a hydrophilic antagonist) to define cell surface receptors. The total binding was 35 000–50 000 c.p.m. and the non-specific binding was 700–1000 c.p.m.

Receptor	Total receptors (fmol/mg)	Cell surface receptors (fmol/mg)	Cell surface/total (%)
Wild type	7864 ± 66	6513 ± 89	82.9 ± 0 .5
$C^{309} \rightarrow A$ mutant	9348 ± 143	7816 ± 201	83.6 ± 0.9

Neutral hydroxylamine treatment (Figure 2) completely removed the [^{3}H]palmitic acid incorporated into H/F-A_1AR, suggesting that palmitoylation occurs on a cysteine residue as only the thioester of cysteine is labile to hydroxylamine [21,22]. Exposure of cells to the agonist CPA (10 μM, 30 min) had no effect on the amount of tritium incorporated into the receptor. The ratio of [^{3}H]plamitate incorporated into the purified H/F-A_1AR (+CPA/−CPA) as assessed by densitometry of autoradiographs was 1.01 ± 0.04 (mean ± S.E.M., $n = 3$).

Expression of wild-type and $C^{309} \rightarrow A$ palmitoylation-deficient mutant receptors

Wild-type and $C^{309} \rightarrow A$ mutant H/F-A_1ARs were either stably or transiently transfected into CHO-K1 or HEK-293 cells. As summarized in Table 1, the $C^{309} \rightarrow A$ mutation did not change the affinity of the H/F-A_1AR for the antagonist [^{3}H]CPX, or the agonist ^{125}I-ABA. The number of receptors expressed in individual cell lines was measured using [^{3}H]CPX. When wild-type or $C^{309} \rightarrow A$ mutant H/F-A_1ARs were transiently expressed in HEK-293 cells, the number of [^{3}H]CPX binding sites on whole cells, as well as the number of cell surface-expressed receptors, were similar (Table 2), indicating that receptor palmitoylation is not essential for proper insertion into membranes. However, stable clonal cell lines of palmitoylation-deficient mutant H/F-A_1ARs generally expressed fewer receptors than did wild-type clonal cell lines. The highest expression levels of palmitoylation-deficient mutant H/F-A_1ARs found among 57 CHO-K1 colonies and 20 HEK-293 cell colonies was < 1 and 4 pmol/mg of membrane protein respectively. The B_{max} of wild-type H/F-A_1ARs in clones derived from these cell lines was usually higher, and exceeded 10 pmol/mg of protein in over 25% of CHO-K1 and HEK-293 clones selected with G418. Maximal levels of wild-

Table 1 Summary of radioligand binding parameters to wild-type and Cys309 → Ala mutant H/F-A_1AR on membranes prepared from HEK-293 cells pretreated for 24 h with either vehicle (DMSO) or 10 μM CPA

		[^{3}H]CPX			^{125}I-ABA		
	Parameter	Control	10 μM CPA	Down regulation (%)*	Control	10 μM CPA	Down regulation (%)*
Wild-type	B_{max} (fmol/mg)	4139 ± 51	2301 ± 517	48.0 ± 8.0	2249 ± 56	558 ± 20	64.3 ± 5.4
	K_D (nM)	1.98 ± 0.09	2.03 ± 0.10	–	0.55 ± 0.04	0.57 ± 0.06	–
Mutant	B_{max} (fmol/mg)	3378 ± 198	2111 ± 109	37.3 ± 3.9	1492 ± 117	605 ± 29	60.0 ± 4.4
	K_D (nM)	1.81 ± 0.10	2.07 ± 0.17	–	0.47 ± 0.05	0.67 ± 0.11	–

* Values are means ± S.E.M. of pooled data from three independent experiments. There is no statistically significant difference between the values for the wild-type and mutant receptors.

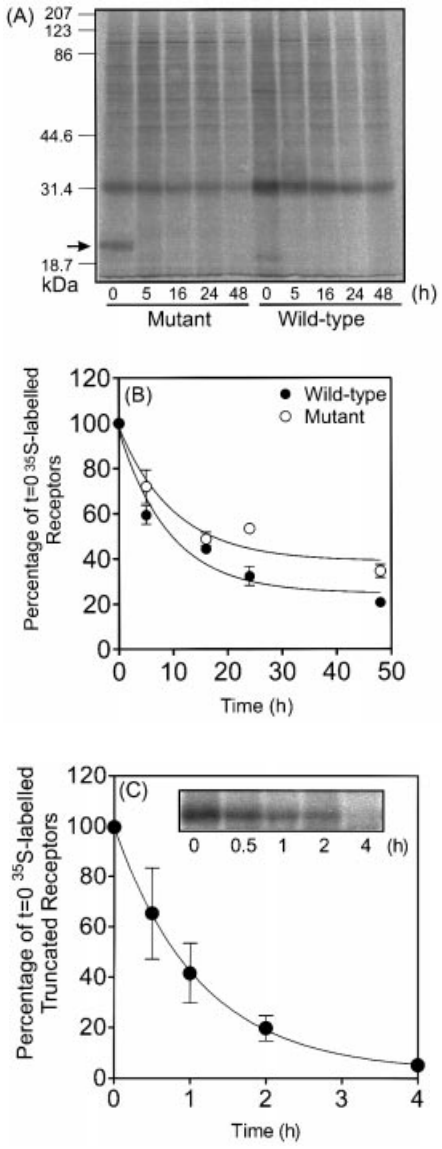

Figure 5 Determination of the half-life of full-length wild-type and $C^{309} \rightarrow A$ mutant H/F-A_1ARs (A and B) and the truncated receptor (C)

HEK-293 cells stably transfected with wild-type or mutant H/F-A_1ARs were pulse-labelled with [^{35}S]methionine. After removal of radioactive medium and a rapid wash, cells were incubated in complete medium supplemented with 1 mM unlabelled methionine for various times as indicated. Purified ^{35}S-labelled wild-type or mutant receptors were treated with N-glycosidase F and subjected to SDS/PAGE followed by expose to Phosphor Screen (Molecular Dynamics) for 2 days (**A**). The arrow in (**A**) indicates the position of a ^{35}S-labelled receptor proteolytic fragment. The relative amount of radiolabelled A_1ARs present at each time point of the chase period was quantified by volume integration using Image Quant™ software. Normalized data, expressed as a percentage of the signal at $t = 0$, were pooled from three independent experiments and were fitted into a one-rate exponential-decay equation (**B** and **C**). The $t_{1/2}$ values for full-length wild-type and mutant A_1AR are 6.4 and 7.1 h respectively, and the $t_{1/2}$ for truncated mutant receptor is 0.8 h.

type H/F-A_1AR expression in CHO-K1 and HEK-293 cells were 25 and 35 pmol/mg of protein respectively.

One wild-type and one $C^{309} \rightarrow A$ mutant line of stably transfected HEK-293 cells with similar levels of A_1AR expression ($B_{max} \cong 4$ pmol/mg of protein) were chosen for comparative analyses. The $C^{309} \rightarrow A$ mutated receptor was not labelled by [^{3}H]palmitate, confirming that Cys^{309} is the unique palmitoylation site on the H/F-A_1AR (Figure 4A). In addition to the predominant 40 kDa mutant receptor, a 25 kDa apparent receptor fragment was observed by Western blotting. The 25 kDa fragment was detected with antibodies directed against the N-terminally engineered FLAG epitope. Deglycosylation of receptors causes a further reduction in the apparent molecular mass of the fragment (results not shown). These results indicate that the 25 kDa protein is probably a C-terminally truncated fragment of the H/F-A_1AR. In initial experiments the 25 kDa protein was observed in Western blots of the $C^{309} \rightarrow A$ mutant, but not the wild type H/F-A_1AR (Figure 4B). By overloading gels it was possible to detect a small amount of receptor fragment in wild-type as well as palmitoylation-deficient mutant receptors (Figure 4C). Densitometric analysis indicates that the amount of this receptor fragment is 11.3 ± 2.2-fold higher in palmitoylation-deficient mutant than in wild-type receptors (mean $\pm$ S.E.M., $n = 3$). At steady state, the ratio of (truncated receptor)/(full-length receptors) is about 1:6 for mutant receptors and 1:66 for wild-type receptors. These findings suggest that the palmitoylation-deficient mutant H/F-A_1ARs are more prone to proteolytic degradation than are wild-type receptors.

Determination of the half-life of wild-type and $C^{309} \rightarrow A$ palmitoylation-deficient mutant receptors

We next set out to determine whether palmitoylation affects the half-life of receptors. HEK-293 cells stably expressing wild-type or $C^{309} \rightarrow A$ mutant A_1ARs were pulse-labelled with [^{35}S]-methionine. After a chase period, H/F-A_1ARs were purified by anti-FLAG affinity chromatography, deglycosylated and analysed by SDS/PAGE. As shown in Figure 5(A), two forms of H/F-A_1ARs were metabolically labelled with [^{35}S]methionine, i.e. full-length receptors (both wild type and $C^{390} \rightarrow A$ mutant receptors) and corresponding C-terminally truncated mutant receptors. This proteolytic receptor fragment is not readily detected with ^{35}S-labelled wild-type receptors. There is no significant difference in half-life between full-length wild-type ($t_{1/2} = 6.4$ h) and full-length mutant ($t_{1/2} = 7.1$ h) receptors (Figure 5B). Interestingly, the truncated form of the mutant receptor turned over much faster (~ 8 times, $t_{1/2} = 0.8$ h) than either full-length wild-type or full-length mutant receptors (Figure 5C). One possible interpretation of these results is that palmitoylation has little effect on the turn-over rate of a stable subset of full-length receptors (presumably those that are correctly folded and inserted into membrane), but palmitoylation prevents a subset of newly synthesized receptors from entering into a rapidly turning over pool.

Effect of H/F-A_1AR palmitoylation on G-protein coupling

After establishing that Cys^{309} is the palmitoylation site on the H/F-A_1AR, we next set out to determine whether palmitoylation influences receptor coupling to G-proteins. Such an effect of palmitoylation has been suggested for β_2-adrenergic receptors [23]. ^{125}I-ABA is an agonist radioligand that selectively binds to G-protein-coupled A_1ARs [24]. Antagonist radioligands, such as [^{3}H]CPX, bind with similar affinity to all A_1ARs, those that are uncoupled from G-proteins and those that are coupled [25]. We calculated the fractional coupling of A_1ARs to G-proteins from the ratio of high-affinity ^{125}I-ABA to [^{3}H]CPX binding sites. The coupling ratio is similar for wild-type ($41.5 \pm 7.0\%$) and $C^{309} \rightarrow A$ palmitoylation-deficient mutant receptors ($41.8 \pm 4.7\%$). The sensitivity of H/F-A_1ARs to uncoupling by guanine nucleotides was determined by measuring the dependence on concentation of the ability of 5′-[γ-thio]triphosphate ([S]GTP) to inhibit the

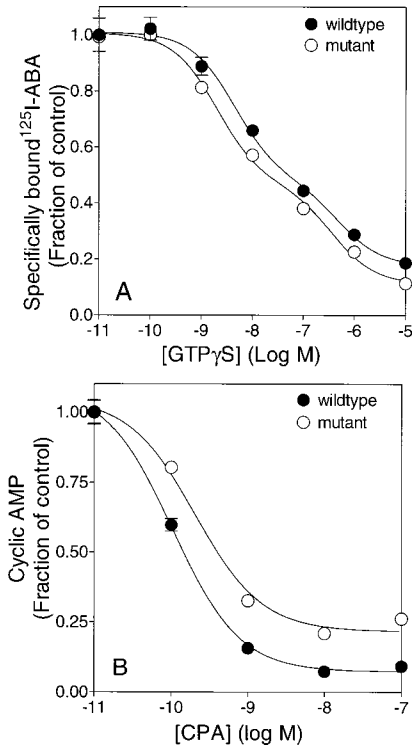

Figure 6 Effect of A_1AR palmitoylation on guanine nucleotide sensitive agonist binding and coupling to inhibition of cAMP accumulation

Top: membranes prepared from HEK-293 cells expressing wild-type or $C^{309} \rightarrow A$ mutant receptors were incubated with ^{125}I-ABA ($\sim$ 0.5 nM final concentration) and various concentrations of [S]GTP for 3 h. The assays were performed in triplicate and results are expressed as a fraction of specific ^{125}I-ABA binding in the absence of added [S]GTP. The total binding was 20 000–30 000 c.p.m. and the non-specific binding was 600–900 c.p.m. Competition curves were fitted to a two-site model. The calculated IC_{501} and IC_{502} values of [S]GTP for wild-type or mutant receptors are as follows: wild-type, $IC_{501} = 3.0 \pm 0.73$ nM, $IC_{502} = 450 \pm 181$ nM; mutant, $IC_{501} = 2.3 \pm 0.64$ nM, $IC_{502} = 516 \pm 275$ nM. Bottom: inhibition of cAMP accumulation in HEK-293 cells expressing wild-type and $C^{309} \rightarrow A$ mutant H/F-A_1AR. Cells (40 000/tube) were incubated without and with 10 μM forskolin and various concentrations of CPA for 15 min at 37 °C as described in the Experimental procedures section. The results are normalized to the forskolin-stimulated component of the responses. The basal levels of cAMP were: 0.59 ± 0.08 pmol/tube ($n = 3$, wild type) and 0.80 ± 0.04 pmol/tube ($n = 3$, mutant). Forskolin elevated cAMP levels to 10.75 ± 0.52 ($n = 3$, wild type) and 10.0 ± 0.29 ($n = 3$, mutant). The IC_{50} of CPA to reduce forskolin-stimulated cAMP accumulation is: wild type, 0.11 ± 0.02 nM ($n = 3$); mutant, 0.19 ± 0.04 nM ($n = 3$).

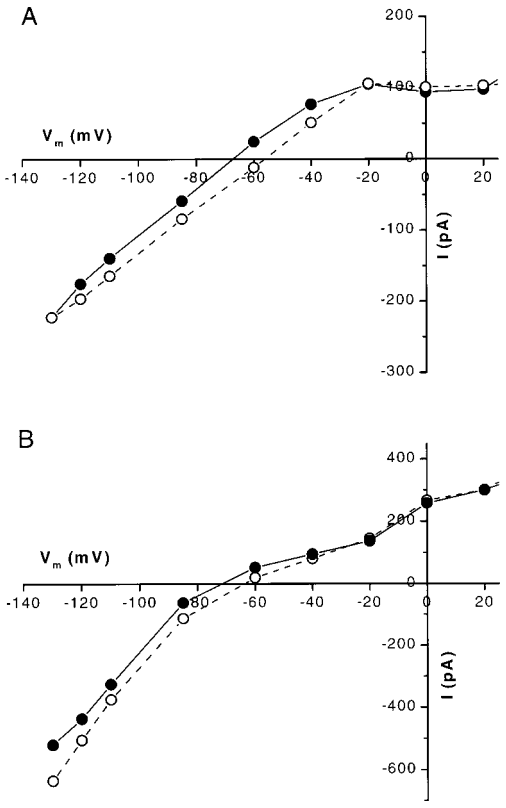

Figure 7 Lack of effect of palmitoylation on CPA-induced K^+ currents

CPA- (●) and ACh (○)-induced whole cell currents were recorded from HEK-293 cells stably expressing CIR and transiently transfected with GIRK1, m2 muscarinic receptor, green fluorescent protein and either wild-type (**A**) or palmitoylation-deficient mutant (**B**) H/F-A_1ARs. *I–V* curves were constructed from measurements taken at the end of 250-ms steps from a holding potential of -85 mV to the indicated test potentials in the absence and presence of 1 μM CPA or 1 μM ACh. The agonist-induced currents shown are the difference between the currents measured in the presence and absence of agonists.

binding of ^{125}I-ABA to wild-type and mutant receptors. The effect of [S]GTP on binding was biphasic, as noted previously in porcine cardiac membranes [26]. The existence of two apparent affinity states for [S]GTP may be indicative of two populations of G-proteins that couple to A_1ARs. IC_{501} and IC_{502} were similar for wild-type and palmitoylation-deficient mutant receptors (Figure 6, top), suggesting that palmitoylation does not alter the ability of [S]GTP to uncouple receptor–G protein complexes.

Receptor–effector coupling to adenylate cyclase was evaluated by comparing the potency of CPA to inhibit cAMP production in cells expressing similar numbers of wild-type or palmitoylation-deficient H/F-A_1ARs. There was a modest difference in the potency and efficacy of CPA to inhibit forskolin-stimulated cAMP accumulation in the two cell lines. This could reflect a subtle effect of palmitoylation on receptor function (Figure 6, bottom), but the difference is small enough that it might be attributed in part to the 22% higher receptor expression in the wild-type compared with the palmitoylation-deficient mutant cells (Table 1), i.e. to the difference in the number of spare receptors. Both the basal and forskolin-stimulated levels of cAMP were similar in the cell lines expressing wild-type or mutant receptors. In triplicate experiments, the calculated IC_{50} values for cells expressing wild-type and mutant receptors differed by a factor of less than 2 (0.11 ± 0.02 nM compared with 0.19 ± 0.04 nM). As a second measure of receptor–effector coupling we examined the ability of CPA to activate K_{ACh} channels (GIRK1/CIR) co-expressed with both H/F-A_1ARs and m2 muscarinic receptor. CPA (1 μM) stimulated K^+ channel conductance in cells expressing either wild-type or palmitoylation-deficient H/F-A_1ARs (Figure 7). To our knowledge this is the first instance in which recombinant ARs have been demonstrated to couple to recombinant K^+ channels. Representative results are shown in Figure 7 in which the agonist-induced current is plotted as a function of voltage. The *I–V* curves in Figures 7(A) and 7(B) exhibit inward rectification typical of the GIRK1/CIR channel. Stimulation by CPA of K^+ channel conductance is absent in non-transfected cells and is blocked by the selective A_1AR antagonist CPX in transfected cells (results not shown). Considering that various factors may affect protein expression levels, and therefore affect the CPA-induced K^+ current, CPA responses were normalized to ACh responses in each cell. Normalized CPA responses were compared between wild-type and palmitoylation-

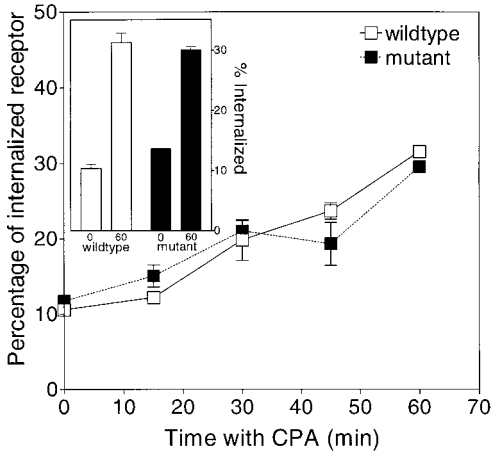

Figure 8 Effect of palmitoylation on CPA-induced H/F-A_1AR internalization

HEK-293 cells expressing wild-type or $C^{309} \rightarrow A$ mutant A_1ARs were treated with either vehicle (0.1% DMSO) or 10 μM CPA for various times at 37 °C in the presence of 0.5 units/ml adenosine deaminase. After washing, whole cell [^{3}H]CPX binding was performed with or without 1 mM 8-SPT or 1 μM CPX. Receptor internalization is defined as the percentage of specific [^{3}H]CPX binding sites not displaced by 8-SPT. Data points are the means $\pm$ S.E.M. of triplicate determinations. Inset: percentage of internalized receptors measured after treatment of cells with vehicle or CPA for 1 h. The results given in the inset were pooled from five independent experiments, each assayed in triplicate.

deficient mutant H/F-A_1AR groups using an unpaired *t*-test. The results showed that there is no significant difference between these two groups [CPA/ACh, 0.82 ± 0.13 ($n = 5$) compared with 1.23 ± 0.21 ($n = 4$); wild type compared with mutant, $P > 0.05$]. We conclude from these results that palmitoylation has little effect on the coupling of A_1ARs to two effectors, adenylate cyclase and K^+-channels.

Palmitoylation has no effect on agonist-induced A_1AR internalization

To determine whether palmitoylation has any influence on H/F-A_1AR internalization, binding of the hydrophobic antagonist [^{3}H]CPX to intact cells was measured in the absence and presence of the cell-impermeant ligand, 8-SPT. We anticipated that [^{3}H]CPX would bind to both cell surface and internalized receptors, whereas 8-SPT would be restricted to a subset of receptors, presumably on the cell surface. Figure 8 shows that the percentage of internalized receptors increases approx. 3-fold, from 10% to 30%, during a 1 h treatment of cells with CPA, and that the palmitoylation state of the H/F-A_1AR has no significant effect on the baseline level of internalized receptors, the magnitude or the kinetics of CPA-induced receptor internalization.

Effect of palmitoylation on agonist-induced receptor down-regulation

CPA-induced down-regulation [18] was examined in wild-type and palmitoylation-deficient H/F-A_1ARs. As shown in Figure 9, 24 h of exposure to CPA (10 μM) had similar effects on wild-type and mutated receptors. The CPA-induced decrease in receptor number, as determined by binding of the antagonist [^{3}H]CPX, was 48.0 ± 8.0% for wild-type receptors and 37.3 ± 3.9% for palmitoylation-deficient mutant receptors (Table 1). We used ^{125}I-ABA to evaluate the effect of CPA pretreatment on the number of G-protein-coupled receptors. CPA-induced down-regulation of ^{125}I-ABA binding was similar for wild-type and palmitoylation-deficient H/F-A_1ARs: wildtype, 64.3 ± 5.4%; mutant, 60.0 ± 4.4% (Table 1).

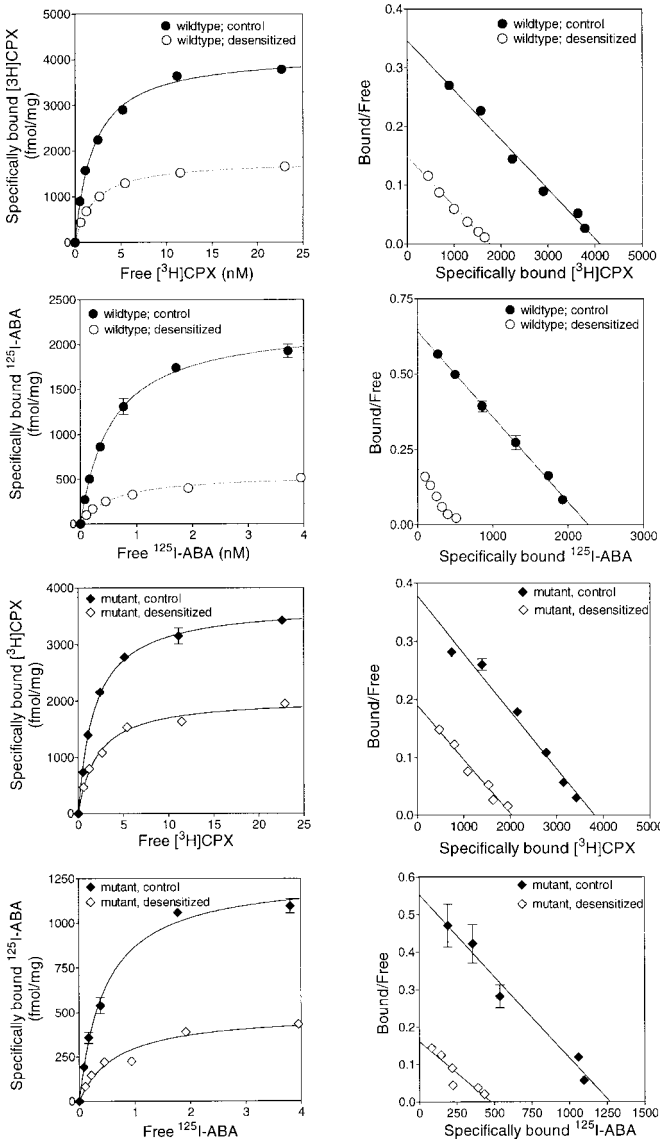

Figure 9 Effect of palmitoylation on CPA-induced down-regulation of H/F-A_1ARs

Stably transfected HEK-293 cells were pretreated for 24 h with vehicle (0.1% DMSO) or 10 μM CPA in the presence of 0.5 units/ml adenosine deaminase. Membranes were prepared and used for radioligand binding assays. Left: specific binding isotherms; right: Scatchard-transformed specific binding. Each point is the mean $\pm$ S.E.M. of triplicate determinations. The results are representative of three independent experiments. Binding parameters are summarized in Table 1.

DISCUSSION

Palmitoylation influences receptor trafficking and effector coupling in ways that appear to vary among individual G-protein-coupled receptors. In this study we set out to establish that the A_1AR is palmitoylated and to investigate thoroughly the influence of this modification on receptor expression, trafficking and function. We found that depalmitoylation appears to cause

a subset of receptors to undergo rapid degradation, although the remaining receptors turn over at a normal rate.

The A_1AR is particularly well suited for this type of study. The evaluation of radioligand binding and G-protein coupling is facilitated by the existence of high-affinity agonist and antagonist radioligands and photoaffinity labels that bind to A_1ARs with high specificity [27,28]. The H/F-receptor can be purified to near homogeneity in high yield [17], so its labelling with [^{3}H]palmitic acid can be assessed. A polar antagonist, 8-sulphophenyltheophylline, can be used to clearly distinguish between A_1ARs on the cell surface and those in the cell interior [29]. In addition, A_1ARs are known to couple to multiple effectors. This provides an opportunity to determine whether palmitoylation has a differential effect on receptor coupling to different effectors. The results of this study indicate that palmitoylation of the A_1AR has little effect on the regulation of effector coupling of recombinant receptors.

We found for the first time that non-palmitoylated A_1ARs are substantially more prone ($>$ 11-fold) to form a 25 kDa presumed receptor proteolytic fragment, suggesting that palmitoylation may affect receptor stability. Consistent with the finding were pulse–chase experiments in which we found that some palmitoylation-deficient receptors are more prone to rapid degradation than are wild-type receptors. The half-life of truncated receptors is eight times shorter than that of full-length receptors. This difference in the turn-over rate indicates that the proteolytic cleavage of the mutant A_1ARs occurs intracellularly, and is not an artifact of *in vitro* receptor purification. It is striking that there is little difference in the half-lives between full-length wild-type and full-length palmitoylation-deficient mutant A_1ARs. Why might this be so? If palmitoylation prevents receptors from rapid degradation, one might expect that non-palmitoylated receptors would all become more labile. One possible interpretation is that newly synthesized palmitoylation-deficient receptors enter into two different subcellular compartments. A proportion of receptors are stabilized, presumably by correctly folding and anchoring into a membrane. These stable full-length mutant receptors turn over at a slow rate, comparable with that for full-length wild-type receptors. Another fraction of newly synthesized palmitoylation-deficient receptors are incorrectly folded and/or enter into a cellular compartment marked for rapid degradation. The palmitoylation-deficient full-length receptors appear to degrade with a single exponential time course with $t_{1/2} = 7.1$ h, i.e. we could not detect a rapidly turning over pool. This suggests that the fraction of receptors destined for rapid turn-over are very rapidly truncated to the 25 kDa fragment size. The steady-state ratio of full-length to truncated receptors is 6:1; the $t_{1/2}$ of the full-length receptor pool is 7.1 h; and the $t_{1/2}$ of the truncated receptor 0.8 h. If k_1 and k_2 depict the rates of degradation of the full-length and truncated receptor respectively, then $k_1/k_2 = (1/0.8)/(6/7.1) \approx 1.5$. By this calculation, 60% of the total palmitoylation-deficient receptors appear to be rapidly degraded.

If this interpretation is correct, then palmitoylation apparently protects nascent receptor peptides from proteolysis and thus shifts the distribution of receptors so that more are delivered to a stable pool. Protection from degradation by palmitoylation might occur if palmitoylation reduces the delivery of receptors into cellular compartments, where they are rapidly degraded, or if it reduces the affinity of the receptor for proteases or regulators of proteases that degrade receptors. Palmitoylation may facilitate the association of receptors to docking sites that escort receptors to the cell surface. It is notable in this regard that removal of palmitate from rhodopsin with hydroxylamine dramatically increases its sensitivity to proteases [3], and palmitoylation of the α-subunit of the G-protein, Gz, inhibits by more than 90% its affinity for Gz GTPase-activating protein, a member of the regulators of G-protein signalling family of proteins [30].

The notion that palmitoylation of target proteins may regulate their affinity for regulatory factors can be extended to the β_2-adrenergic receptor, where palmitoylation of the receptor is thought to decrease its affinity for protein kinase A [31,32]. However, the effect of palmitoylation to reduce β_2-receptor phosphorylation cannot be generalized to other G-protein-coupled receptors. For example, palmitoylation has no effect on the phosphorylation of the α_{2A} adrenergic receptor [33] or the V2 vasopressin receptor [34]. The general absence of an effect of palmitoylation on receptor phosphorylation is consistent with the general lack of an effect of palmitoylation on receptor desensitization.

We observed a large ($>$ 6-fold) discrepancy in the highest B_{max} values detected in multiple ($>$ 20) stably transfected clones of palmitoylated wild-type receptors compared with palmitoylation-deficient $C^{309} \rightarrow A$ mutant receptors. This was noted both in HEK-293 cells and in CHO-K1 cells and is consistent with our suggestion that depalmitoylation enhances receptor proteolysis and average turn-over rate, and decreases receptor stability. However this effect of palmitoylation on receptor expression is not observed in transiently transfected cells. Why might this be so? Newly synthesized receptors enter an endosomal compartment and are either delivered to the cell surface or degraded. We have suggested that palmitoylation could have the effect of shifting the distribution of receptors so that more are delivered to the cell surface and stabilized. Since transiently transfected cells express far more receptors than do stably transfected cells, the results suggest that palmitoylation influences a cell-surface targeting process that may be saturated when the rate of new receptor synthesis is high, as in transient transfection. Of note in this regard are previous studies of luteinizing hormone receptors. In stably transfected cells, palmitoylation-deficient mutants were reported to be trapped intracellularly [35]. In a later study of transiently transfected cells, no evidence for intracellular trapping of palmitoylation-deficient mutants was detected [10]. This is consistent with the possibility that the effect of palmitoylation varies markedly between stably and transiently transfected cells.

The currently published results suggest that palmitoylation has a variable influence on the coupling of various receptors to their effectors. In most instances palmitoylation has been found not to affect G-protein coupling. One possible exception is the palmitoylation-deficient endothelin A receptor, which was reported to selectively lose its ability to couple to Ca^{2+} mobilization, whereas its ability to couple to cAMP accumulation is not affected [11]. The palmitoylation-deficient endothelin B receptor was reported to lose its ability to couple to both Ca^{2+} mobilization and adenylate cyclase inhibition [12]. In the present study we detected minor effects of palmitoylation on A_1AR coupling to inhibition of cAMP accumulation or to coupling to co-transfected GIRK1/CIR K^+ channels. It is possible that palmitoylation may produce subtle effects on receptor trafficking or on the affinity of the interaction between receptors and various regulatory components. Subtle regulatory effects may be masked by the use of overexpressed recombinant receptors.

Based on experiments with α_{2A}-adrenergic receptors, Eason et al. [36] hypothesized that palmitoylation is necessary for agonist-induced down-regulation of G-protein-coupled receptors. The results of the current study represent a counter-example to this suggestion inasmuch as the palmitoylation-resistant $C^{309} \rightarrow A$ mutant A_1ARs undergo down-regulation to nearly the same extent as do the wild-type A_1ARs. We conclude that palmitoylation is not a general requirement for agonist-induced down-regulation of G-protein-coupled receptors.

An agonist-induced increase in receptor palmitoylation has been observed for the β_2-adrenergic receptor [37] and the D_1 dopamine receptor [6], but not for the endothelin A receptor [11] or the mGluR4 receptor [9]. In this study we found that exposure to an A_1AR agonist, CPA, had no effect on the palmitoylation state of the receptor. We conclude that the change in conformational state of G-protein-coupled receptors caused by agonist binding does not generally render G-protein-coupled receptors more susceptible to palmitoylation.

Conclusions about the functional effects of receptor palmitoylation are all based on the assumption that a significant portion of the wild-type receptor is palmitoylated. This assumption has proven to be true in the case of rhodopsin [39]. However, attempts to determine the stoichiometry of palmitoylation of other G-protein-coupled receptors have been unsuccessful due to technical difficulties in accurately determining the specific activity of [^{3}H]palmitate in cells [33]. Although we did not directly determine the stoichiometry of A_1AR palmitoylation in the present study, indirect evidence suggests that, as for rhodopsin, a significant portion ($> 90\%$) of wild-type receptors are palmitoylated. This is based on the fact that at steady state, only a very small fraction ($< 1.4\%$) of wild-type receptors become truncated receptor fragments, compared with 14.2% for palmitoylation-deficient mutant receptors.

In conclusion, the A_1AR has been shown to be palmitoylated on Cys^{309}. A mutant receptor that cannot be palmitoylated appears to be more susceptible to proteolytic degradation. Palmitoylation has little effect on receptor desensitization, internalization or coupling to effectors.

This work was supported by National Institutes of Health Grants RO1-HL37942 (to J.L.) and HL 37127 (to G.S.).

REFERENCES

1 Linden, J., Jacobson, M. A., Hutchins, C. and Williams, M. (1994) in Handbook of Receptors and Channels: G-protein Coupled Receptors (Peroutka, S. J., ed.), pp. 29–44, CRC Press, Boca Raton, FL
2 Papac, D. I., Thornburg, K. R., Bullesbach, E. E., Crouch, R. K. and Knapp, D. R. (1992) J. Biol. Chem. **267**, 16889–16894
3 Ovchinnikov Yu. A., Abdulaev, N. G. and Bogachuk, A. S. (1988) FEBS Lett. **230**, 1–5
4 Kennedy, M. E. and Limbird, L. E. (1993) J. Biol. Chem. **268**, 8003–8011
5 O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. and Bouvier, M. (1989) J. Biol. Chem. **264**, 7564–7569
6 Ng, G. Y., Mouillac, B., George, S. R., Caron, M., Dennis, M., Bouvier, M. and O'Dowd, B. F. (1994) Eur. J. Pharmacol. **267**, 7–19
7 Ng, G. Y., O'Dowd, B. F., Caron, M., Dennis, M., Brann, M. R. and George, S. R. (1994) J. Neurochem. **63**, 1589–1595
8 Ng, G. Y., George, S. R., Zastawny, R. L., Caron, M., Bouvier, M., Dennis, M. and O'Dowd, B. F. (1993) Biochemistry **32**, 11727–11733
9 Alaluf, S., Mulvihill, E. R. and McIlhinney, R. A. (1995) J. Neurochem. **64**, 1548–1555
10 Kawate, N. and Menon, K. M. (1994) J. Biol. Chem. **269**, 30651–30658
11 Horstmeyer, A., Cramer, H., Sauer, T., Muller-Esterl, W. and Schroeder, C. (1996) J. Biol. Chem. **271**, 20811–20819
12 Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S. and Masaki, T. (1997) J. Biol. Chem. **272**, 21589–21596
13 Linden, J. (1994) Trends Pharmacol. Sci. **15**, 298–306
14 Trussel, L. O. and Jackson, M. B. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4857–4861
15 Bünemann, M. and Pott, L. (1995) J. Physiol. (London) **482**, 81–92
16 Linden, J., Patel, A. and Sadek, S. (1985) Circ. Res. **56**, 279–284
17 Robeva, A. S., Woodard, R., Luthin, D. R., Taylor, H. E. and Linden, J. (1996) Biochem. Pharmacol. **51**, 545–555
18 Gao, Z., Robeva, A. S. and Linden, J. (1999) Biochem. J. **338**, 729–736
19 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
20 Brooker, G., Terasaki, W. L. and Price, M. G. (1979) Science **194**, 270–276
21 Pepperberg, D. R., Morrison, D. F. and O'Brien, P. J. (1995) Methods Enzymol. **250**, 348–361
22 Bouvier, M., Chidiac, P., Hebert, T. E., Loisel, T. P., Moffett, S. and Mouillac, B. (1995) Methods Enzymol. **250**, 300–314
23 Moffett, S., Mouillac, B., Bonin, H. and Bouvier, M. (1993) EMBO J. **12**, 349–356
24 Figler, R. A., Graber, S. G., Lindorfer, M. A., Yasuda, H., Linden, J. and Garrison, J. C. (1996) Mol. Pharmacol. **50**, 1587–1595
25 Prater, M. R., Taylor, H., Munshi, R. and Linden, J. (1992) Mol. Pharmacol. **42**, 765–772
26 Leid, M., Schimerlik, M. I. and Murray, T. F. (1989) Mol. Pharmacol. **35**, 450–457
27 Linden, J., Patel, A. and Sadek, S. (1985) Circ. Res. **56**, 279–284
28 Patel, A., Craig, R. H., Daluge, S. H. and Linden, J. M. (1989) Mol. Pharmacol. **33**, 585–591
29 Bhattacharya, S. and Linden, J. (1996) Mol. Pharmacol. **50**, 104–111
30 Wang, J., Tu, Y., Woodson, J., Song, X. and Ross, E. M. (1997) J. Biol. Chem. **272**, 5732–5740
31 Moffett, S., Adam, L., Bonin, H., Loisel, T. P., Bouvier, M. and Mouillac, B. (1996) J. Biol. Chem. **271**, 21490–21497
32 Morello, J. P. and Bouvier, M. (1996) Biochem. Cell. Biol. **74**, 449–457
33 Kennedy, M. E. and Limbird, L. E. (1994) J. Biol. Chem. **269**, 31915–31922
34 Sadeghi, H. M., Innamorati, G., Dagarag, M. and Birnbaumer, M. (1997) Mol. Pharmacol. **52**, 21–29
35 Zhu, H., Wang, H. and Ascoli, M. (1995) Mol. Endocrinol. **9**, 141–150
36 Eason, M. G., Jacinto, M. T., Theiss, C. T. and Liggett, S. B. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 11178–11182
37 Loisel, T. P., Adam, L., Hebert, T. E. and Bouvier, M. (1996) Biochemistry **35**, 15923–15932
38 Mouillac, B., Caron, M., Bonin, H., Dennis, M. and Bouvier, M. (1992) J. Biol. Chem. **267**, 21733–21737
39 Karnik, S. S., Ridge, K. D., Bhattacharya, S. and Khorana, H. G. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 40–44

Received 1 April 1999/21 May 1999; accepted 16 June 1999