Agonist Stimulation Increases the Turnover Rate of β_2AR-Bound Palmitate and Promotes Receptor Depalmitoylation†

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ABSTRACT: We have characterized the dynamic nature of β_2-adrenergic receptor palmitoylation in Sf9 cells. Under basal conditions, the turnover of receptor-bound palmitate is rapid (half-life = 9.8 ± 1.8 min) compared to the turnover rate of the receptor protein itself (half-life = 109 ± 10 min). This suggests that an equilibrium between the palmitoylated and nonpalmitoylated forms of the receptor exists at resting state. Stimulation of the receptor by the agonist isoproterenol reduces the half-life of the β_2-adrenergic receptor-bound palmitate by 1.8 fold without affecting the turnover rate of the receptor itself. Upon sustained stimulation, this increased palmitate turnover rate shifted the equilibrium toward the nonpalmitoylated form of the receptor, suggesting that prolonged activation either increases the rate of depalmitoylation or prevents receptor palmitoylation. Consistent with the latter possibility, pretreatment of cells with agonist, prior to metabolic labeling, reduced the incorporation of [^{3}H]palmitate into the β_2-adrenergic receptor by more than 80%. This suggests a link between receptor desensitization occurring upon sustained agonist stimulation and the decrease in receptor palmitoylation. Supporting this hypothesis, mutation of PKA phosphorylation sites known to be involved in receptor desensitization abolished the agonist-promoted reduction in palmitate incorporation. We have previously reported that palmitoylation of the β_2-adrenergic receptor is important in controlling receptor phosphorylation by PKA [Moffett, S., et al. (1993) *EMBO J. 12,* 349−356; Moffett, S., et al. (1996) *J. Biol. Chem. 271,* 21490−21497]. The present study now demonstrates that the receptor palmitoylation state is regulated by agonist stimulation and suggests the existence of concerted reciprocal regulatory interactions between palmitoylation and phosphorylation upon sustained receptor stimulation.

In recent years, a number of proteins involved in signal transduction have been shown to be modified by covalent fatty acylation [for a review, see Casey (1995)]. In addition to promoting membrane association for a number of these molecules, prenylation, myristylation, and palmitoylation have also been proposed to regulate their function. Palmitoylation of a number of G protein-coupled receptors has been reported. These include: rhodopsin (O'Brien & Zatz, 1984), the β_2-adrenergic receptor (β_2AR)[1] (O'Dowd et al., 1989), the α_2-adrenergic receptor (α_2AR) (Kennedy & Limbird, 1993), the D1 dopamine receptor (Ng et al., 1994), the serotonin 5HT$_{1B}$ receptor (Ng et al., 1993), the LH/hCG receptor (Kawate & Menon, 1994), and the glutamate mGluR4 (Alaluf et al., 1995). A regulatory function in receptor−G protein coupling has been attributed to this modification for some of these receptors (O'Dowd et al., 1989; Moffett et al., 1993; Morrison et al., 1991; Jensen et al., 1995) while a role in receptor trafficking, targeting, and internalization has been demonstrated for others (Eason et al., 1994; Nussenzveig et al., 1993). For some nonreceptor protein tyrosine kinases, palmitoylation has been shown to promote association with caveolae (Robbins et al., 1995; Shenoy-Scarcia et al., 1994). Enrichment of G protein-coupled receptors in these structures has also recently been reported (Chun et al., 1994). However, whether or not palmitoylation of G protein-coupled receptors is involved in promoting this subcellular localization remains to be determined.

Unlike myristylation and prenylation which are usually irreversible modifications, palmitoylation is a reversible posttranslational modification which may facilitate potentially important acylation−deacylation cycles (Casey, 1995). This type of dynamic regulation has been suggested for G protein-coupled receptors (Mouillac et al., 1992; Kennedy & Limbird, 1994). Given the proposed functional roles for receptor palmitoylation, a detailed understanding of the dynamics of palmitoylation is of primary importance.

In a first attempt to determine if receptor stimulation could modulate the palmitoylation state of the β_2AR, Mouillac et al. (1992) studied the effect of agonist stimulation on the

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[1] Abbreviations: PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; [^{125}I]CYP, [^{125}I]iodocyanopindolol; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; β_2AR, β_2-adrenergic receptor; α_2AR, α_2-adrenergic receptor; C16-CoA, palmitoyl-coenzyme A; PKA, cAMP-dependent protein kinase; Sf9, *Spodoptera frugiperda*; MDCK, Madin−Darby canine kidney; CHW, Chinese hamster fibroblasts.

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incorporation of [^{3}H]palmitate into the β_2AR during pulse-labeling experiments. A short (15 min) agonist stimulation of the receptor led to an increased incorporation of labeled fatty acid. These observations were made in both Sf9 (insect) and CHW (mammalian) cells, suggesting that this is a general property of the β_2AR, independent of the expression system. More recently, Kennedy et al. (1994) showed, in MDCK cells, that stimulation of the α_{2A}-adrenergic receptor shortens the half-life of the receptor-bound radiolabeled palmitate. Unfortunately, they reported a half-life for the palmitate moiety that exceeded the half-life of the receptor molecule itself. This casts doubt on the interpretation of the results and did not permit firm conclusions about the existence of dynamic turnover of receptor-bound palmitate to be drawn. Similarly, the experimental conditions originally used by Mouillac et al. (1992) could not distinguish whether the increased palmitate incorporation into agonist-stimulated β_2-AR resulted from an enhanced stoichiometry of palmitoylation or from an accelerated turnover rate.

In the present study, a detailed kinetic analysis of β_2AR palmitoylation was performed using both pulse-labeling and pulse−chase experiments. Taking into account the half-life of both palmitate and receptor, two specific questions were directly addressed: (i) is β_2AR palmitoylation a reversible process in whole cells, and if so (ii) does agonist stimulation modulate the palmitoylation/depalmitoylation cycle of the receptor? Our results demonstrate that palmitoylation of the β_2AR is indeed a reversible process and that the receptor exists in equilibrium between palmitoylated and depalmitoylated states. We also found that agonist stimulation of the receptor increases the receptor-bound palmitate turnover rate and that long-term stimulation which promotes receptor phosphorylation and desensitization shifts the equilibrium toward the depalmitoylated state.

EXPERIMENTAL PROCEDURES

Construction of β_2AR Recombinant Baculoviruses. The recombinant baculovirus encoding the c-*myc*-tagged human β_2AR was constructed, screened, and amplified as previously described (Mouillac et al., 1992). To generate the baculovirus encoding a receptor lacking the consensus PKA sites, cassette subcloning of a mutant β_2AR cDNA, pTZ18R-β_2-AR-Ala$^{261-262}$, Ala$^{345-346}$ (Hausdorff et al., 1989), into the Sf9 recombination plasmid pJV-c-myc-β_2AR (Mouillac et al., 1992) was carried out. Briefly, the *Nco*I-PpUM1 fragment from the pTZ18R-β_2AR-Ala$^{261-262}$, Ala$^{345-346}$ plasmid was subcloned into the *Nco*I-PpUM1 site of the pJV-c-myc-β_2AR. Positive clones were confirmed by dideoxy sequencing. The recombinant virus was then generated by homologous recombination with the linear AcMNPV genome by cotransfection in Sf9 cells using the Cationic Liposome Mediated Transfection kit (Invitrogen). Recombinant baculoviruses were purified by plaque assay using the β-galactosidase detection system (Vialard et al., 1990). Confirmation of β_2AR protein expression was assessed by whole cell binding assays.

Cell Culture, Infection with Recombinant β_2AR Baculoviruses, and Metabolic Labeling. Sf9 cells were cultured in Grace's supplemented media (Gibco) containing 10% fetal serum (FBS) and 0.001% pluronic acid in 100 mL spinner flasks (Bellco Glass) at 27 °C. Cells (2×10^6/mL) were infected with the recombinant baculoviruses at a multiplicity of infection varying between 2 and 5. Thirty hours after infection, cells were harvested and placed in 100 mL of serum-free media for 17 h. One milliliter of FBS was added 1 h prior to the start of metabolic labeling. Since receptor density reached its maximum at 48 h and remained stable for up to 72 h post-infection, metabolic labelings were started 48 h post-infection. Selecting this infection time allowed experiments to be carried out at steady state. Cell viability was found to be greater than 85% at 48 h post-infection. For palmitate labeling experiments, 25 mCi of [9,10-^{3}H]-palmitate dissolved in 200 μL of dimethyl sulfoxide (DMSO) was added. For [^{35}S]protein labeling experiments, 5 mCi of Tran^{35}S-Label (ICN) was added. Cells were then split into 8 × 50 mL tubes (Falcon) (12 mL/tube). Isoproterenol (1 μM final concentration) (Sigma), dissolved in 10 μM ascorbate or ascorbate only (vehicle), was immediately added, and the cells were incubated for the indicated times. Following the labeling period, cells were centrifuged at 500*g* for 5 min at 4 °C, rinsed once with ice-cold phosphate-buffered saline (PBS), and resuspended in 15 mL of ice-cold lysis buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.4, containing 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor and 10 μg/mL benzamidine). Cells were then disrupted by sonication, the lysates were centrifuged 5 min at 500*g* at 4 °C and the supernatants centrifuged at 45,000 X g for 20 min at 4 °C. Pellets were resuspended in 10 mM Tris-HCl, 100 mM NaCl, and 2 mM EDTA, pH 7.4, containing either 0.3% digitonin (Gallard-Schleisinger) or 0.3% n-dodecyl maltoside (Boehringer Mannheim), with protease inhibitors. Solubilization was carried out for 90 min at 4 °C. Solubilized receptors were then purified as described below.

For pulse−chase experiments with palmitate, labeling was conducted as described above. The cells were then harvested, and rinsed, and the chase was started at room temperature in 100 mL of complete Grace's media with 1% FBS containing 0.2 mM unlabeled palmitate. Cells were then stimulated or not with isoproterenol as described above. The chase was stopped by placing the cells on ice, and membranes were immediately prepared and receptors solubilized as above. For ^{35}S pulse−chase experiments, 5 mCi of Tran^{35}S-Label was added to methionine- and cysteine-free Grace's supplemented media following a 30 min preincubation in this media. The chase was initiated by adding complete Grace's media containing 1 mM methionine and 1 mM cysteine.

To determine the β_2AR-bound palmitate and the receptor half-lives, data from [^{3}H]palmitate and Tran^{35}S-Label pulse−chase experiments were analyzed by nonlinear least-squares regression analysis (SIGMA PLOT 4.17). The equation used for the fit is a modification of the one-compartment metabolic turnover equation (Simon, 1972) as follows: $q(t) = q(t\rightarrow\infty) + q(t=0)e^{(-R)t}$ where t is the time of incubation (in minutes), R is the rate of decay, and q represents the level of labeling (in percent of control). The half-lives were estimated as t where $q(t) = 50\%$.

Purification of β_2AR. Alprenolol−Sepharose affinity purification matrix was synthesized according to the method of Benovic et al. (1987). This matrix was used to purify the β_2AR as described (Mouillac et al., 1992) with a slight modification. Buffers contained either digitonin (0.05%) or *n*-dodecyl maltoside (0.05%) depending on the detergent used

for solubilization. The affinity-purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon), and the amount of β_2AR in each sample was determined by radioligand binding assay using [^{125}I]iodocyanopindolol ([^{125}I]CYP) as described (Mouillac et al., 1992). SDS−PAGE was conducted under nonreducing conditions according to the method of (Laemmli, 1970) using 10% slab gels. Fixed gels were incubated in Enlightning (DuPont) for 30 min, dried, and exposed to DuPont REFLECTION film at room temperature. Fluorographs were scanned with an Ultroscan XL laser densitometer (LKB).

Whole Cell Lipid Labeling. Thirty milliliters of Sf9 cells expressing β_2AR was incubated with 7 mCi of [^{3}H]palmitate for different times. Total lipids were extracted from 1 mL cell suspensions. Prior to extraction, cells were rinsed once with ice-cold PBS and resuspended in 250 μL of PBS. Next, 200 μL of organic phase $CHCl_3$/MeOH/H_2O (5:5:1 by volume) was added, and the mixture was vortexed thoroughly. The two phases were separated by centrifugation, and the organic phase was quantitatively recovered and dried under vacuum. The desiccant was resuspended in 100 μL of $CHCl_3$/MeOH/50 mM Tris, pH 7.5 (6:6:1 by volume). A 20 μL aliquot was then loaded on a thin layer silica gel (Aldrich) for ascending chromatography using *n*-butanol/CH_3COOH/H_2O (5:2:3 by volume) as the mobile phase (Juguelin & Cassagne, 1995). The air-dried thin-layer was then dipped into LSC cocktail (Dupont) and sealed into a hybridization bag prior to fluorography. Total radioactivity was evaluated from a 10 μL aliquot of the extraction.

Incorporation of [^{3}H]Palmitate into Total Protein. Thirty milliliters of Sf9 cells expressing β_2AR was incubated with 7 mCi of [^{3}H]palmitate for different times with or without isoproterenol as described above. Aliquots of 3 mL of cell suspensions were taken and membranes prepared as described above. Membrane pellets were resuspended in sample buffer, and 20 μg of protein was loaded on a 10% SDS−PAGE slab gel. Protein concentration was assessed by the method of Bradford (1976) using bovine serum albumin as a standard. Five hundred μL of aliquoted cell suspensions was also taken for each condition, and total lipids were extracted, chromatographed, and detected as described above.

RESULTS

Incorporation of [^{3}H]palmitate into the β_2AR was studied as a function of time in whole cells. As shown in Figure 1A, [^{3}H]palmitate was rapidly incorporated into the receptor and the labeling intensity increased steadily to reach a maximum between 30 and 60 min. Despite the continuous presence of labeled palmitate in the medium, the intensity of labeling started to slowly decrease after 60 min and continued to decline for as long as labeling was pursued. Similarly, the amount of labeled free palmitate taken up by cells also increased as a function of time for up to 1 h of labeling before decreasing steadily between 1 and 3 h (Figure 1B). The rapid incorporation of tritiated fatty acids into cellular phospholipids, which is also illustrated in Figure 1B, indicates that the [^{3}H]palmitate is distributed quickly into cellular pools and is rapidly metabolized. As a consequence, the specific activity of the free palmitate never reached true equilibrium under the conditions studied. Interestingly, the changes in receptor labeling intensity paralleled those observed for the cellular free-palmitate pool. The almost superimposable kinetics of incorporation indicate a rapid exchange between the palmitate donor pool and receptor-bound palmitate, thus suggesting that rapid palmitate turnover occurs at the receptor level under basal conditions. Alternatively, the fact that the level of palmitate incorporation into the β_2AR closely parallels the cellular content of free [^{3}H]palmitate could suggest that palmitate transfer occurs only on newly synthesized β_2AR and that a rapid degradation of the receptor is responsible for the good correlation existing between the specific activity of the free and receptor-bound palmitate.

To distinguish between the two possibilities evoked above, pulse−chase experiments were undertaken to determine the turnover rate both of the receptor-bound palmitate and of the β_2AR itself. Following a labeling period of 1 h with [^{3}H]palmitate, the cells were washed and incubated in the presence of excess unlabeled palmitate. Figure 2 shows that the labeling intensity of the β_2AR rapidly declined during the chase period. The half-life for the receptor-bound palmitate was found to be 9.8 $\pm$ 1.8 min. Under these experimental conditions, the receptor half-life determined by [^{35}S]methionine/cysteine pulse−chase experiments was found to be 109 $\pm$ 10 min (data not shown). This indicates that receptor-bound palmitate turns over 11 times more rapidly than the receptor itself and is consistent with the idea that dynamic palmitoylation/depalmitoylation cycles occur on the receptor under basal conditions. Also consistent with this idea is the observation that the initial incorporation rate of [^{3}H]palmitate into the receptor, calculated from pulse-labeling experiments (Figure 1), was 6.3-fold faster than the incorporation of [^{35}S]methionine/cysteine (Figure 5), demonstrating that most of the palmitoylation occurs on receptor already synthesized. Thus, it is reasonable to assume that the decrease in incorporated palmitate, after 60 min of labeling, is not due to receptor degradation but rather reflects a rapid equilibrium between the receptor-bound and free palmitate resulting from a rapid palmitate turnover on the receptor itself.

To determine if receptor activation influences the palmitoylation state of the β_2AR, the kinetics of [^{3}H]palmitate incorporation were studied in the presence or absence of the β-adrenergic agonist isoproterenol. As seen in Figure 3A, agonist activation significantly increased the rate of [^{3}H]palmitate incorporation into the β_2AR. This is particularly evident at 5 and 10 min where the labeling was increased by 2.1- and 2.0-fold, respectively, by agonist treatment. Similar agonist-promoted increases in palmitate incorporation were also seen for the short labeling periods presented in Figure 3B. However, no such effects were observed when labeling was carried out for longer periods (e.g., 30 and 60 min; Figure 3B). In contrast, as seen in Figure 3B, the maintained presence of agonist markedly reduced the [^{3}H]palmitate incorporation observed after 60 min of labeling.

Figure 3C summarizes the effect of agonist treatment on the kinetics of [^{3}H]palmitate incorporation into the receptor. As seen in the inset, isoproterenol treatment clearly increases the initial rate of [^{3}H]palmitate incorporation into the β_2-AR. Indeed, during the initial 10 min of labeling, the rate of incorporation was increased 2.9 fold by the agonist. In the presence of isoproterenol, the maximal incorporation was attained between 10 and 30 min while in its absence [^{3}H]-palmitate incorporation was still increasing at these times to

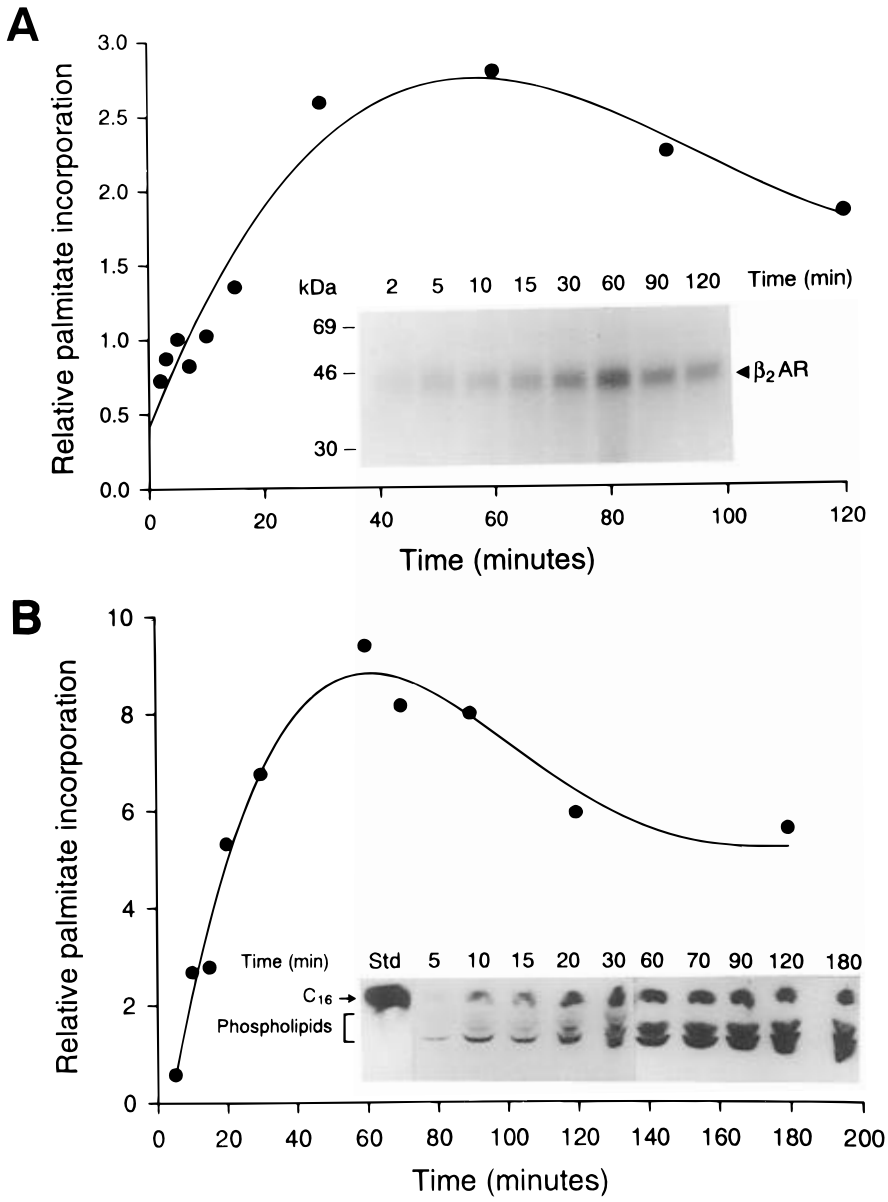

FIGURE 1: Time course of palmitate incorporation into β_2AR. Sf9 cells expressing the human β_2AR were metabolically labeled with [^{3}H]-palmitate prior to receptor purification. (A) Purified receptors were resolved by SDS−PAGE, and incorporated palmitate was detected by fluorography. The inset shows a representative fluorograph for labeling periods from 2 to 120 min. Equal amounts of purified receptor (1.6 pmol) were loaded into each lane (fluorogram shown was exposed for 3 weeks). Relative incorporation of [^{3}H]palmitate into the receptor was estimated by densitometric analysis of fluorographs by arbitrarily setting to 1 the labeling intensity observed at 5 min. The data shown are representative of 8 separate experiments. (B) Cellular lipid incorporation of [^{3}H]palmitate was determined by thin-layer chromatography following organic extraction of total lipids. The inset shows a representative fluorograph for labeling periods from 5 to 180 min. The position of palmitic acid, as determined by using commercial [^{3}H]palmitate as a standard (Std), is indicated by an arrow. The labeled species running slower than palmitate represent cellular phospholipids (Juguelin & Cassagne, 1995). Relative cellular incorporation of [^{3}H]palmitate was assessed by densitometric analysis of fluorographs. The data shown are representative of 2 independent experiments.

reach a maximum following 60 min of labeling. However, as can be seen in Figure 3B, the maximal level of labeling reached in the absence of isoproterenol is significantly higher than the maximal labeling observed in its presence. This is well illustrated in Figure 3C where it can be seen that the labeling intensity of the β_2AR in the absence of isoproterenol reached and exceeded that obtained for the agonist-stimulated receptor after 15 min of labeling. In the presence of agonist, the labeling had already reached its maximum by 30 min, and the labeling slowly declined thereafter. In contrast, the intensity of labeling continued to increase between 30 and 60 min in the absence of isoproterenol, reaching 3 times the intensity observed for the agonist-stimulated receptor. Thus, agonist stimulation transiently increased the rate of [^{3}H]-palmitate incorporation but sustained stimulation appears to favor the unpalmitoylated form of the receptor.

The changes in receptor labeling upon agonist stimulation did not result from a general metabolic effect of isoproterenol on the [^{3}H]palmitate cellular pool. Indeed, neither the overall cellular incorporation of [^{3}H]palmitate (Figure 4A) nor the incorporation of [^{3}H]palmitate into total membrane proteins (Figure 4B) was affected by the agonist treatment for times varying between 5 and 120 min. It should also be noted that isoproterenol treatment did not change the rate of receptor synthesis. Indeed, as shown in Figure 5, [^{35}S]methionine/cysteine incorporation into the β_2AR was not affected by agonist over the time period studied.

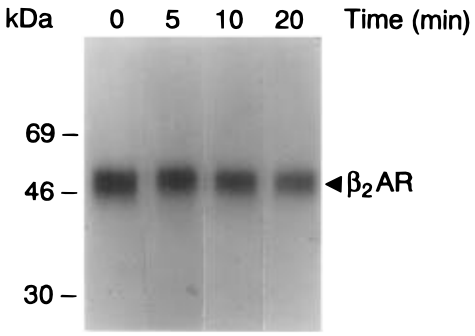

FIGURE 2: β_2AR-bound palmitate turnover. Pulse−chase experiments were carried out as described under Experimental Procedures. Receptors were purified and resolved by SDS−PAGE. The intensity of labeling was assessed by fluorography and a typical labeling decay pattern is shown. Identical amounts of purified β_2AR (5 pmol) were loaded into each lane (fluorogram shown was exposed for 3 weeks). labeling levels were determined by densitometric analysis of the fluorographs. The data shown are representative of three independent experiments. The half-life of the β_2AR-bound palmitate was determined as described under Experimental Procedures. The average half-life calculated from 3 independent experiments was 9.8 ± 1.8 min (mean ± SD).

Due to the rapid turnover rate of the free palmitate in cells, the agonist-promoted increase in receptor labeling, observed upon short time stimulation, may reflect either an elevation in the stoichiometry of palmitoylation or a faster exchange between labeled and unlabeled palmitate. To distinguish between these possibilities, the effect of β_2AR stimulation on the half-life of the receptor-bound palmitate was investigated in pulse−chase labeling experiments. As seen in Figure 6, isoproterenol stimulation reduced the receptor-bound palmitate half-life by 40% from 9.0 ± 1.6 to 5.4 ± 0.4 min. Under these conditions, no receptor down-regulation occurred (data not shown) and no change in the receptor half-life accompanied the treatment (109 ± 10 vs 101 ± 14 min), thus indicating that receptor stimulation increased the rate of palmitate turnover on the receptor. Although the Sf9 expression system provided sufficient material to carry out the biochemical experiments described above, the quantity of β_2AR obtained is still insufficient to directly measure the stoichiometry of receptor-bound palmitate. However, taken together with the results obtained in pulse-labeling experiments, the above data strongly suggest that biological activation of the β_2AR by an agonist increases the turnover rate of the receptor-bound palmitate and ultimately favors the unpalmitoylated form of the receptor.

Figure 3C clearly shows that the effects of agonist stimulation on the receptor palmitate incorporation is biphasic. In the early phase, the stimulation increases [^{3}H]-palmitate incorporation while it reduces it in the latter phase. Although the increase in receptor-bound palmitate turnover rate can explain the initial increased label incorporation (because of the accelerated exchange between nonradioactive and [^{3}H]palmitate), this alone cannot explain the reduction in labeling observed following longer stimulation.

The effects of maintained stimulation on receptor phosphorylation and desensitization have been well characterized (Benovic et al., 1988). It may therefore be hypothesized that desensitization taking place during sustained stimulation influences the dynamics of palmitoylation. To directly test this hypothesis, cells were pretreated or not with isoproterenol for 15 min prior to the metabolic labeling. As seen in Figure 7, this desensitizing treatment inhibited [^{3}H]palmitate incorporation by more than 80% in labeling experiments carried out for either 5 or 15 min. This effect of desensitization was reversible as removal of isoproterenol prior to the labeling restored [^{3}H]palmitate incorporation in a time-dependent fashion (Figure 7). These data indicate that desensitization of the receptor may be responsible for the reduction in [^{3}H]palmitate incorporation observed in Figure 3C. Since a link between the palmitoylation of cysteine-341 and the phosphorylation of the β_2AR by PKA has already been proposed (Moffett et al., 1993, 1996), the palmitoylation state of a mutant form of the β_2AR lacking the PKA sites (β_2AR-Ala$^{261-262}$, Ala$^{345-346}$) was assessed. As shown in Figure 8A, the extent of [^{3}H]palmitate incorporation following a 60 min labeling was found to be 37% higher in the mutant than in the wild-type receptor suggesting that the basal level of phosphorylation of wild type receptor is sufficient to partially inhibit palmitoylation. More importantly, the [^{3}H]palmitate incorporation into the mutant receptor was increased following sustained stimulation with isoproterenol (for 30, 60, and 90 min; Figure 8B) in contrast with the agonist-promoted decrease observed for the wild-type receptor (Figure 3C), thus further supporting the notion that agonist-promoted phosphorylation inhibits receptor palmitoylation.

DISCUSSION

The results presented in this study clearly demonstrate that palmitoylation of the β_2AR is a dynamic process which can be modulated by agonist stimulation of the receptor. At least two lines of evidence demonstrate that under basal conditions, a rapid turnover of the palmitate occurs on the β_2AR. First, the rapid incorporation of [^{3}H]palmitate into the receptor closely follows the cellular uptake of free tritiated palmitate. This cannot result solely from the palmitoylation of newly synthesized receptor since the rate of palmitate incorporation was found to be 6 times faster than receptor synthesis. Furthermore, the cellular dilution of [^{3}H]palmitate, which occurs after 1 h of labeling, is paralleled by a decrease in receptor labeling. Second, the half-life of the receptor-bound palmitate, determined in pulse−chase experiments, is 11 times shorter than the half-life of the receptor itself. The existence of palmitate turnover argues that at any given point in time a proportion of the receptor population has a free sulfhydryl group available for palmitate thioesterification at position Cys-341 and that an equilibrium exists between palmitoylated and nonpalmitoylated forms of the receptor.

The dynamic nature of protein palmitoylation was first shown in mammalian cells for p21-ras by Magee et al. (1987). Since then, similar findings have been reported for several proteins involved in signal transduction including the α subunits of heterotrimeric G proteins (Degtyarev et al., 1993; Mumby et al., 1994; Wedeggaertner & Bourne, 1994). Interestingly, the ratio of the receptor-bound palmitate half-life over the half-life of the β_2AR itself under basal conditions was found in the present study to be 0.1, a value very similar to the ratio calculated for the unstimulated palmitoylated Gsα (0.07) (Wedeggaertner & Bourne, 1994). The occurrence of palmitoylation/depalmitoylation cycles suggests the existence of regulatory processes, but the precise mechanism(s) involved has (have) not yet been identified. Membrane-bound palmitoyl acyltransferase activities which catalyze palmitoylation of Fyn (Berthiaume & Resh, 1995), p21-ras (Gutierrez & Magee, 1991), and trimeric G proteins (Dunphy

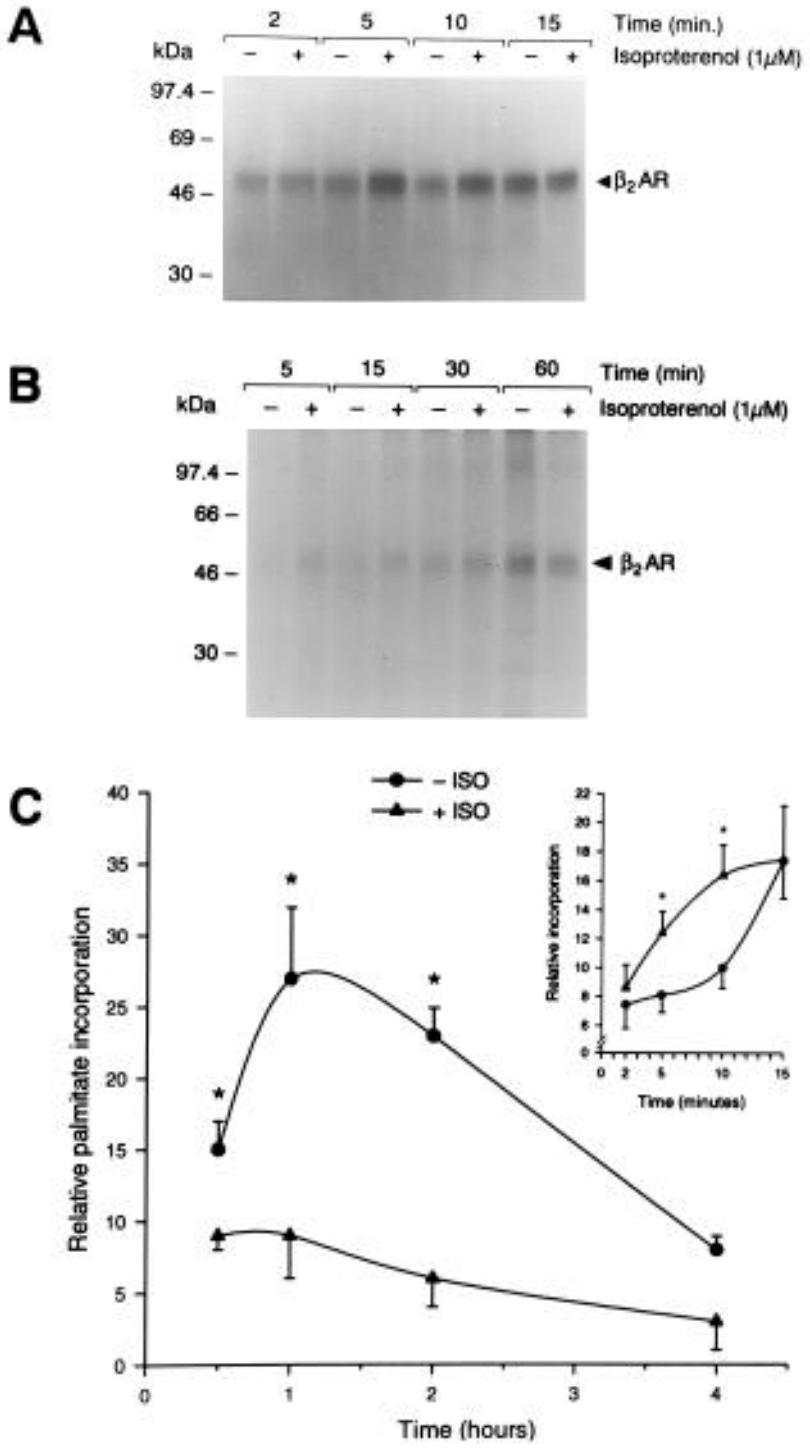

FIGURE 3: Time course of agonist effects on β_2AR [^{3}H]palmitate incorporation. Sf9 cells expressing β_2AR were labeled with [^{3}H]palmitate in the presence or absence of isoproterenol for the times indicated. Receptors were purified and resolved by SDS−PAGE. The intensity of labeling was assessed by fluorography. (A) Fluorograph shown is representative of 4 independent experiments measuring [^{3}H]palmitate incorporation for periods between 2 and 15 min (fluorogram shown was exposed for 6 weeks). (B) Fluorograph shown is representative of 2 independent experiments measuring palmitate incorporation for periods between 5 and 60 min (fluorogram shown was exposed for 4 weeks); 1.4 and 1.3 pmol of receptor was loaded into each well in panels A and B, respectively. (C) Relative [^{3}H]palmitate incorporation into β_2AR for labeling periods from 30 min to 4 h. The data represent the average ± SD of 3 labeling experiments (the asterisk indicates significant differences, $p < 0.05$). The inset shows relative [^{3}H]palmitate incorporation into β_2AR for labeling periods from 2 to 15 min. The data represent the average ± SD of 4 labeling experiments (the asterisk indicates significant differences, $p < 0.05$).

et al., 1996) have been reported. In addition, the occurrence of nonenzymatic palmitoylation has been demonstrated *in vitro* using cysteine-containing synthetic lipopeptides as substrates. Indeed, in a recent study, Silvius and collaborators (Quesnel & Silvius, 1994) showed that transfer of palmitate to cysteine residues required only the presence of liposomes and palmitoyl-coenzyme A (C16-CoA). Similarly, nonenzymatic palmitoylation of bovine rhodopsin has also been proposed (O'Brien et al., 1987). Therefore, the mere existence of an enzyme catalyzing the palmitoylation of G protein-coupled receptors remains an open question. A microsomal palmitoyl-protein thioesterase activity that can depalmitoylate several viral capsid proteins has been described (Berger & Schmidt, 1986). However, the contribu-

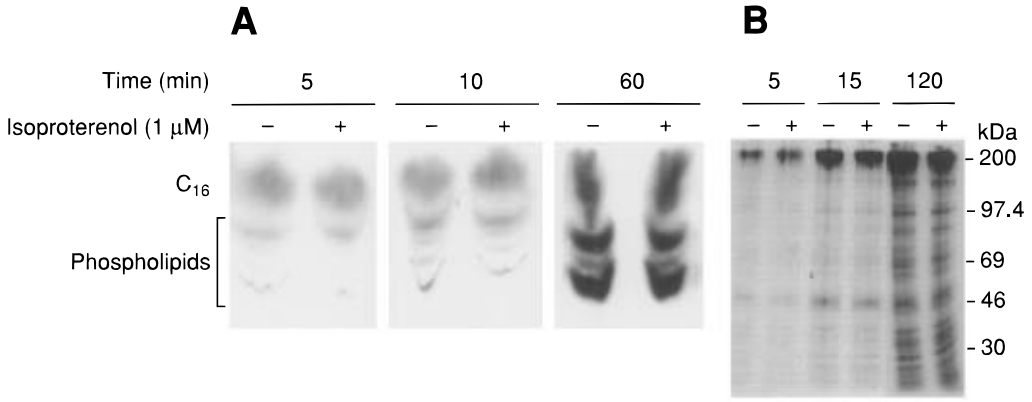

FIGURE 4: Effect of isoproterenol on cellular incorporation of [^{3}H]palmitate. Sf9 cells expressing β_2AR were labeled with [^{3}H]palmitate in the presence or absence of isoproterenol for the indicated times. (A) [^{3}H]Palmitate incorporation into cellular lipids was determined by thin-layer chromatography following organic extraction of total lipids as described under Experimental Procedures. (B) [^{3}H]Palmitate incorporation into total membrane proteins. Membranes were prepared as described under Experimental Procedures, and equal amounts of protein (20 μg) were loaded in each lane of a nondenaturing SDS−polyacrylamide gel (fluorogram shown was exposed for 2 weeks). Data shown are representative of two independent experiments.

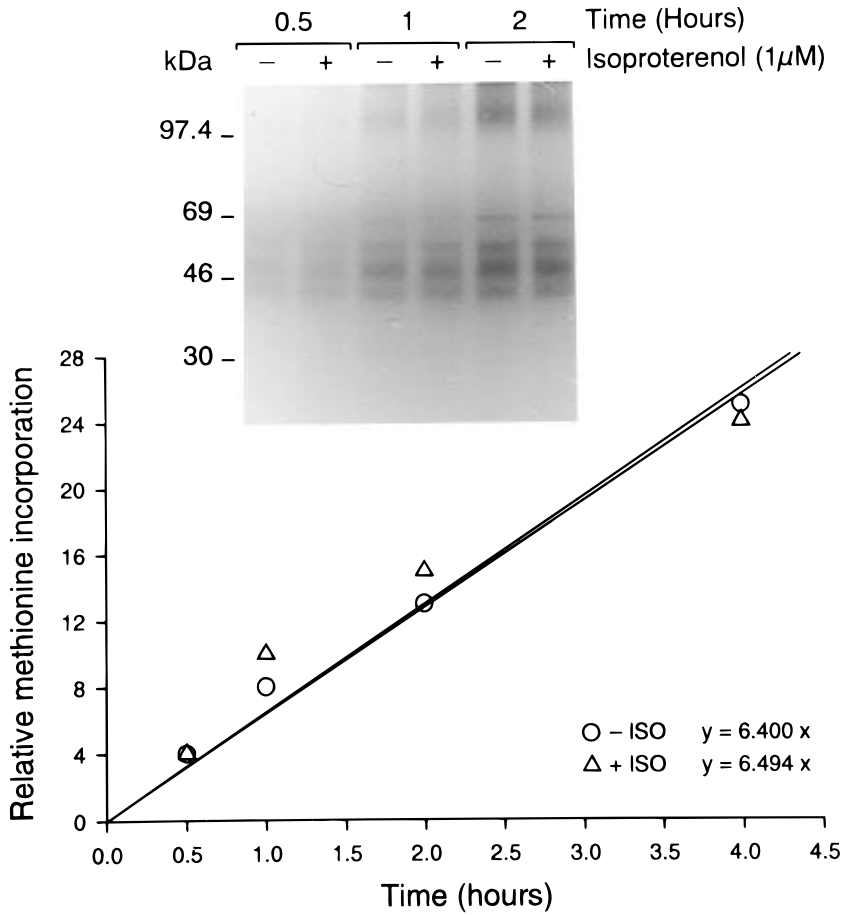

FIGURE 5: Effect of isoproterenol on β_2AR synthesis. Sf9 cells expressing β_2AR were labeled with [^{35}S]methionine/cysteine in the presence or absence of isoproterenol for the indicated times. Purified receptor was resolved by SDS−PAGE and the relative extent of labeling determined by fluorography. The curves shown represent the average of 2 experiments. The inset shows a representative fluorograph; 3.8 pmol of β_2AR was loaded into each well (fluorogram shown was exposed for 2 weeks). The three distinct bands between 43 and 60 kDa represent differentially glycosylated forms of the β_2AR.

tion of this enzymatic activity to the depalmitoylation of the β_2AR remains to be investigated. More recently, a palmitoyl-protein thioesterase that catalyzes the depalmitoylation of H-Ras and Goα *in vitro* has been purified and cloned (Camp & Hofmann, 1993; Camp et al., 1994). However, the fact that this enzyme is secreted suggests that it is not involved in removing protein-bound palmitate imbedded in the inner leaflet of the plasma membrane.

Although the detailed mechanisms involved in regulating the β_2AR acylation/deacylation cycles remain unknown, our results clearly indicate that agonist stimulation modulates the turnover rate of receptor-bound palmitate. An increased turnover rate is evident in pulse−chase experiments in which the agonist treatment reduced by 40% the half-life of receptor-bound palmitate but can also be appreciated in short pulse-labeling experiments. Indeed, agonist treatment en-

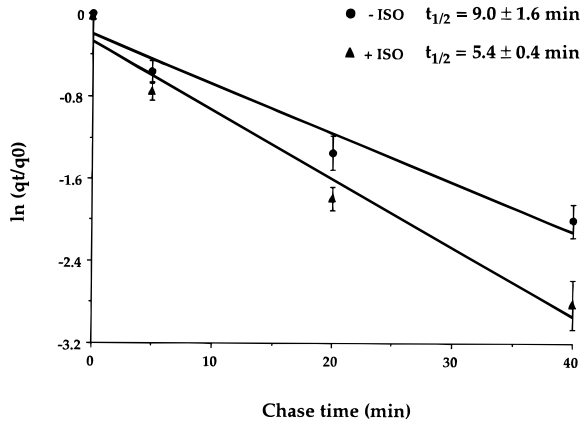

FIGURE 6: Effect of isoproterenol on the turnover rate of β_2AR-bound palmitate. Cells were labeled with [^{3}H]palmitate for 2 h and chased with cold palmitate for the times indicated. Cells were treated (closed triangles) or not (closed circles) with isoproterenol during the chase, and the extent of labeling was determined by fluorography following receptor purification and SDS−PAGE. The graph is derived from densitometric analysis of 2 independent experiments. For each experiment, the same amount of receptor was loaded into each lane. Half-life of incorporated palmitate was determined as described under Experimental Procedures. The average half-life calculated from 2 independent experiments is expressed as the mean ± SD. Correlation coefficients (r^2) of the linear regressions are 0.955 (closed circles) and 0.971 (closed triangles).

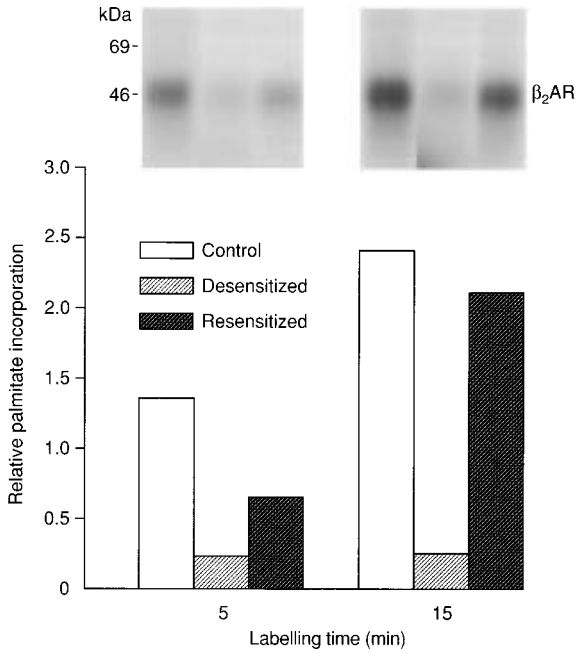

FIGURE 7: Effects of agonist pretreatment on β_2AR [^{3}H]palmitate incorporation. Sf9 cells expressing β_2AR were treated or not (control) with 1 μM isoproterenol for 15 min. The cells were then labeled with [^{3}H]palmitate for the indicated times in the continued presence of isoproterenol (desensitized) or after extensive washes to remove the isoproterenol (resensitized). Receptors were then purified and resolved by SDS−PAGE, and the intensity of labeling was assessed by fluorography; 6.4 pmol of receptor was loaded into each well (fluorogram shown was exposed for 7 weeks). The graph is derived from densitometric analysis of the fluorogram.

hanced the incorporation rate of the radio-labeled palmitate, suggesting that the exchange between nonlabeled and [^{3}H]-palmitate is accelerated upon agonist treatment. These observations do not allow us to determine whether an increase in the rate of palmitoylation, of depalmitoylation, or of both is responsible for the agonist-promoted increased

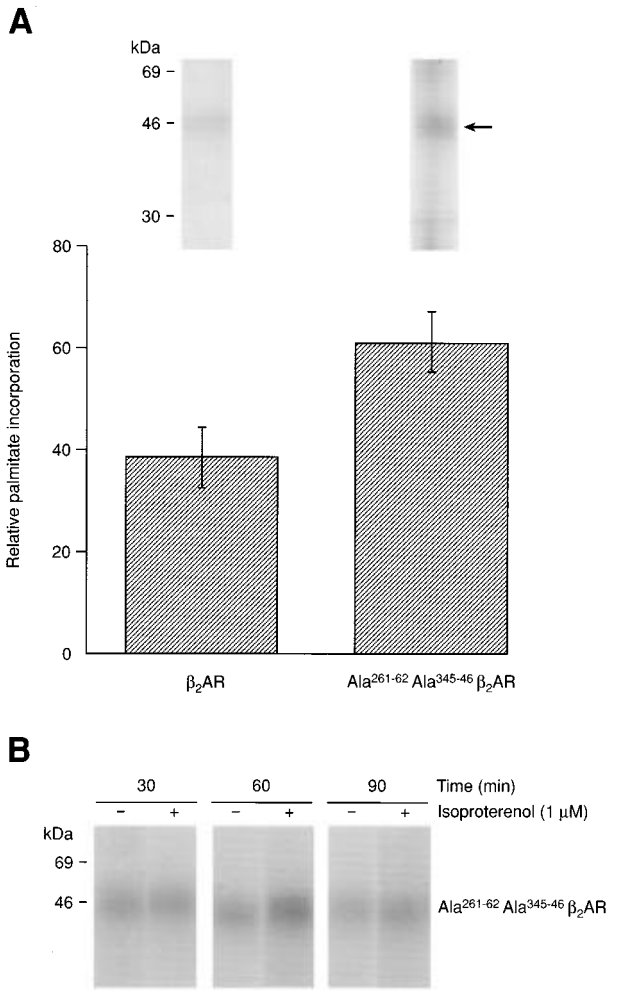

FIGURE 8: [^{3}H]palmitate incorporation into β_2AR-Ala$^{261-262}$, Ala$^{345-346}$. (A) Sf9 cells expressing either wild-type β_2AR or β_2-AR-Ala$^{261-262}$, Ala$^{345-346}$ were labeled with [^{3}H]palmitate for 60 min. Receptors were purified and resolved by SDS−PAGE, and the intensity of labeling was assessed by fluorography; 2.4 pmol of receptor was loaded into each well. The graph is derived from densitometric analysis of the fluorogram and represents the mean ± SEM of 4 independent labeling experiments (the difference between the two conditions was found to be statistically significant, $p < 0.05$). B) Sf9 cells expressing β_2AR-Ala$^{261-262}$, Ala$^{345-346}$ were labeled with [^{3}H]palmitate in presence or absence of 1 μM isoproterenol for the times indicated. Receptors were purified and resolved by SDS−PAGE, and the intensity of labeling was assessed by fluorography; 6.5 pmol of receptor was loaded into each well (the two fluorograms shown were exposed for 3 weeks).

turnover rate. Nevertheless, the observation that the maximal level of incorporation of [^{3}H]palmitate into agonist-stimulated β_2AR never reaches the maximal level attained in unstimulated receptor during prolonged pulse labeling experiments strongly suggests that sustained activation of the receptor shifts the equilibrium toward the nonpalmitoylated form. These effects of isoproterenol are receptor-specific and did not result from general metabolic effects since the agonist treatment did not alter the total protein nor the lipid [^{3}H]-palmitate incorporation at any of the labeling times studied.

Interestingly, similar to what has been observed here for the β_2AR, stimulation of Gs has been shown to modulate the turnover rate of the Gsα-bound palmitate and has been suggested to favor the nonpalmitoylated form of Gsα (Mumby et al., 1994; Wedeggaertner & Bourne, 1994). Wedegaertner and Bourne (1994) further proposed that such a mechanism may serve a regulatory role in G protein

function by allowing translocation of the α subunit from the plasma membrane to the cytosol. For the β_2AR, modulation of palmitate turnover cannot be involved in directing membrane association as it is an integral membrane protein. However, it has been proposed that the presence of palmitate moieties serves to anchor the proximal portion of the carboxyl tail to the plasma membrane (Ovchinnikov et al., 1988; O'Dowd et al., 1989). In the case of rhodopsin, Moench et al. (1994) confirmed that receptor-bound palmitate is physically imbedded in the lipid bilayer. We have previously shown that the modification of Cys-341 by palmitate modulates the accessibility of this region of the β_2AR to protein kinases most likely by regulating the interaction of the tail with the membrane (Moffett et al., 1993; 1996). It should be noted that a consensus protein kinase A phosphorylation site is located four amino acids downstream of the palmitoylated cysteine which would bring it very close to the membrane surface where it might not be accessible to the kinase. Therefore, agonist-promoted regulation of the palmitoylation state may play an important role in controlling receptor phosphorylation and desensitization. Several lines of evidence are consistent with this notion: (1) replacement of Cys-341 of the β_2AR by a glycine residue, which abolishes receptor palmitoylation, causes the receptor to behave as if it were already desensitized (Moffett et al., 1993); (2) the lack of the palmitoylated cysteine-341 increases the accessibility of the PKA phosphorylation consensus site located four amino acids downstream (^{343}-RRSS) (Moffett et al., 1996); and (3) mutation of this putative phosphorylation site considerably reduces the rate of desensitization promoted by a high level of agonist stimulation (Moffett et al., 1996). It follows that palmitoylation may regulate receptor activity by controlling the accessibility of serine-345,346 to PKA. It should be noted, however, that the contribution of this phosphorylation site in receptor desensitization has been put into question by previous studies. Indeed, mutation of this site did not affect the desensitization induced by direct incubation with cAMP analogues or the PKA catalytic subunit, conditions known to promote heterologous desensitization (Clark et al., 1989; Yuan et al., 1994; Bouvier et al., 1989). To reconcile these apparently contradictory results, it may be suggested that serine-345,346 becomes available for phosphorylation only when the receptor is agonist-bound. This phosphorylation site would thus be involved in homologous but not heterologous desensitization. This is consistent with the recent findings that PKA-mediated phosphorylation plays an important role in rapid homologous desensitization (Post et al., 1996).

Results presented in this study now suggest that PKA phosphorylation may in turn affect the receptor palmitoylation state. Indeed, the extent of [^{3}H]palmitate incorporation into a mutant β_2AR lacking the known PKA phosphorylation sites (β_2AR-Ala$^{261-262}$, Ala$^{345-346}$) was found to be higher than that observed for the wild-type receptor. Furthermore, mutation of the PKA phosphorylation sites abolished the agonist-induced reduction in palmitate incorporation observed upon sustained stimulation . It can therefore be hypothesized that the agonist-promoted phosphorylation of the β_2AR by PKA inhibits palmitoylation. This idea is supported by the observation that desensitizing the receptor prior to the metabolic labeling blocked [^{3}H]palmitate incorporation by more than 80%.

Observations reviewed above suggest that a membrane-bound enzyme (Berthiaume & Resh, 1995; Gutierrez & Magee, 1991; Dunphy et al., 1996) or else the proximity of a phospholipid bilayer interface (Quesnel & Silvius, 1994) is required for protein palmitoylation. It follows that the proximity of the β_2AR Cys-341 to the plasma membrane may be important for covalent attachment of palmitate. The presence of hydrophobic and basic residues flanking the site of palmitoylation (^{333}**RIAF**QE**LL**C**LRR**S**LK**) may stabilize the interaction between this domain of the receptor and the acyl chains and polar head groups of the lipid bilayer, respectively. A polybasic region has been proposed to play a similar role in the attachment of myristoylated MARCKS and farnesylated K-ras to the plasma membrane (Hancock et al., 1990). Phosphorylation of serines-345 and -346 of the β_2AR by PKA may significantly increase the acidic character of this region, thus destabilizing its interaction with the membrane. By doing so, phosphorylation may therefore inhibit the palmitoylation of the proximal cysteine-341. Interestingly, NMR and circular dichroism analyses of a peptide sequence encompassing the turkey β_2AR sequence between arginine-345 and phenylalanine-359 (corresponding to residues 328−342 in the human sequence) have revealed that interaction of this receptor domain with phospholipids could promote the formation of an α-helical structure (Jung et al., 1996). It follows that agonist promoted regulation of the palmitoylation state, by modulating its interaction with the plasma membrane, may have dramatic consequences on the overall structure of the receptor's carboxyl tail.

In summary, the data presented here clearly show for the first time that: (1) β_2AR-bound palmitate turns over more rapidly than the receptor protein itself under basal conditions, (2) agonist stimulation increases this turnover rate, (3) sustained stimulation favors the unpalmitoylated form of the receptor most likely as a result of its desensitization, and (4) these effects are receptor-specific and do not represent general metabolic effects of agonist stimulation. Taken together, the above considerations lead us to propose the following model: upon agonist stimulation, the palmitoylation/depalmitoylation cycle is accelerated, leading initially to a rapid increase in [^{3}H]palmitate incorporation. As stimulation is maintained, the receptor becomes phosphorylated, thus decreasing the ability of the carboxyl tail of the β_2AR to interact with the plasma membrane, therefore inhibiting its repalmitoylation. It follows then that agonist stimulation ultimately favors the phosphorylated unpalmitoylated state of the receptor. Dynamic interactions between palmitoylation and phosphorylation thus appear to play an important role in regulating β_2AR functions.

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