Palmitoylated Cysteine 341 Modulates Phosphorylation of the β2-Adrenergic Receptor by the cAMP-dependent Protein Kinase*

(Received for publication, April 15, 1996, and in revised form, May 29, 1996)

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We previously showed that substitution of a glycine residue for the palmitoylated cysteine 341 of the human β2-adrenergic receptor (Gly341β2AR), increases the basal level of the receptor phosphorylation and reduces its ability to functionally interact with Gs. In the present study, we show that additional mutation of serines 345 and 346 (Ala345,346Gly341β2AR) restored normal phosphorylation and receptor-Gs coupling, thus suggesting that the increased phosphorylation of this site, rather than the lack of palmitoylation per se, is responsible for the poor coupling of the unpalmitoylated receptor. This is supported by the observation that chemical depalmitoylation of purified β2AR did not affect the ability of the receptor to stimulate adenyl cyclase in reconstitution assays. Furthermore, mutation of Ser345,346 in a wild type receptor background (Ala345,346β2AR) significantly decreased the rate of agonist-promoted desensitization of the receptor-stimulated adenyl cyclase activity, supporting a role for this phosphorylation site in regulating the functional coupling of the receptor. Since serines 345 and 346 are located in a putative cyclic AMP-dependent protein kinase (PKA) phosphorylation site immediately downstream of the palmitoylated cysteine 341, the hypothesis that the accessibility of this site may be regulated by the receptor palmitoylation state was further assessed in vitro. In membrane phosphorylation assays, Gly341β2AR was found to be a better substrate for PKA than the wild type receptor, thus supporting the notion that palmitoylation restrains access of the phosphorylation site to the enzyme. Taken together, the data demonstrate that palmitoylation of cysteine 341 controls the phosphorylation state of the PKA site located in the carboxyl tail of the β2AR and by doing so modulates the responsiveness of the receptor.

Post-translational modification of transmembrane receptors has been shown to play important roles in the proper regulation of hormonal signaling. For the human β2-adrenergic receptor (β2AR),1 a large body of evidence implicates phosphorylation in the rapid desensitization of the β-adrenergic receptor-stimulated adenyl cyclase activity that follows sustained stimulation (1–3). In particular, phosphorylation of the receptor by both cAMP-dependent protein kinase (PKA) and the β-adrenergic receptor kinase (βARK) has been shown to functionally uncouple the receptor from Gs (4).

Another post-translational modification of the β2AR, the palmitoylation of its cysteine 341, has also been shown to influence the ability of the receptor to functionally interact with Gs and to stimulate adenyl cyclase (5). Indeed, substitution of a glycine residue for the cysteine 341 (Gly341β2AR) was found to prevent receptor palmitoylation and greatly reduced its ability to stimulate adenyl cyclase activity. This mutation also led to a loss of the guanine nucleotide-sensitive high affinity binding state of the receptor for agonists, thus suggesting that the unpalmitoylated receptor was largely uncoupled from Gs. More recently, we showed (6) that the decreased responsiveness of Gly341β2AR was accompanied by a constitutive elevation of the basal level of this receptor phosphorylation. These data suggested that concerted interactions between palmitoylation and phosphorylation could play an important role in the regulation of the β2AR function. Such a hypothesis is further supported by the observation that, as for phosphorylation, the palmitoylation state of the β2AR is dynamically regulated by agonist stimulation (7).

One of the two potential PKA phosphorylation sites present in the β2AR is located one amino acid downstream of the palmitoylated cysteine (341GlyR2SS). This observation and the fact that the unpalmitoylated receptor is already phosphorylated and uncoupled from Gs raises the possibility that the phosphorylation of this site may be influenced by the palmitoylation state of the receptor and may contribute to its desensitization. The present study was designed to test this hypothesis.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, streptomycin, glutamine, fungizone, trypsin, and Dulbecco’s phosphate-buffered saline (PBS) were obtained from Life Technologies, Inc. ([125I]CYP, [α-32P]ATP, [-32P]ATP, [3H]AMP, and [32P]H2PO4) were purchased from Du Pont NEN. (−)-isoproterenol, (−)-alcohol, ATP, GTP, cAMP, forskolin, isobutylmethylxanthine, phosphonomypuvate, cAMP-dependent protein kinase, agarose-bound al-

1 The abbreviations used are: β2AR, human β2-adrenergic receptor; Gs, stimulatory GTP-binding protein; PKA, cyclic AMP-dependent protein kinase; Gpp(NH)p, guanosine 5′-[(3′,5′)-monophosphate]; βARK, β-adrenergic receptor kinase; [125I]CYP, [125I]iodocyanopindolol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid.

2 T. P. Loisél, T. E. Hébert, and M. Bouvier, submitted for publication.
Palmitoylation Modulates β2AR Phosphorylation

Kaline phosphatase, and myokinase were obtained from Sigma. Pyruvate kinase was obtained from Calbiochem.

Construction of Mutated β2AR cDNAs and Cell Transfection—A human cDNA encoding a glycine substitution at site 341 (Gly341b) was obtained from O'Dowd et al. (5) and subcloned into the eukaryotic expression vector pcB128I (9). To generate the other β2AR mutants, the cDNAs encoding wild type β2AR (10), Gly341bβ2AR (5), and Ala261,262,345,346β2AR (11) were subcloned into pSP65 (Promega) by introduction in the Ncol-Sall sites. Ala345b,346β2AR was constructed by digestion of pSP65 plasmids containing wild type β2AR and Ala345b,346β2AR cDNAs respectively, with Avil. The appropriate restriction fragments were isolated and ligated, and a resulting pSP65 plasmid containing a cDNA for Ala345b,346β2AR was obtained. All of the constructs were subcloned in the expression vector pcB128Iβ2AR (10) by introduction in the Ncol-Sall sites. The identities of all mutants were confirmed by direct sequenceto nucleotide sequencing. The wild type β2AR and all of the mutants were stably expressed in cultured fibroblasts (mouse LTK- cells or Chinese hamster ovary, CHO) cells. The fibroblasts were prepared for SDS-PAGE, and were labeled using 10% Slab gels (16). Following electrophoresis, proteins were transferred to nitrocellulose in order to reduce the free phosphate background, and the membranes were exposed to Kodak XAR-5 films at -70°C for several days. Laser densitometric scanning of the autoradiographs (UltraScan, Pharmacia Biotech Inc.) was used to quantitate the incorporation of 32P into the receptor.

Whole Cell Phosphorylation and Purification of the β2AR—Phosphorylation experiments were performed essentially as described (10). Briefly, 2 h before the labelling, cells were cultured in phosphate-free DMEM. Following this period, cells were incubated at 37°C with ~8 mCi of [32P]H3PO4 in DMEM containing 50 μM NaH2PO4 for 1 h. The cells were then rinsed with ice-cold PBS, mechanically detached, and resuspended in 10 ml of buffer C (20 mM Tris-HCl, 7.4), 5 mM EDTA, 100 mM NaCl, 1 mM benzylmalonyl-CoA, 1 mM L-tryptophan, 1 mM chymostatin, and 5 μg/ml leupeptin. The cells were sonicated by 4,000 x g for 20 min at 4°C. The pellets were rinsed twice, and the resulting membranes were solubilized in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA and 2% digitonin for 2 h at 4°C. Solubilized receptors were recovered in the supernatant and resuspended in 20 mM Tris-HCl (pH 7.4) for 20 min at 4°C. The receptors were then purified by alperno-Sepharose affinity chromatography as described previously (15) and, purified receptors were concentrated by membrane filtration over Centriprep and Centri concentrators (Amicon). The amount of purified receptors was assessed by soluble [32P]CTP binding assays. Aliquots were then weighed for SDS-PAGE, and electrophoresed using 10% Slab gels (16). Following electrophoresis, proteins were electro transfeted into nitrocellulose in order to reduce the free phosphate background, and the membranes were exposed to Kodak XAR-5 films at -70°C for several days. Laser densitometric scanning of the autoradiographs (UltraScan, Pharmacia Biotech Inc.) was used to quantitate the incorporation of 32P into the receptor. In some experiments the specificity of the cellular [32P]ATP pool was assessed by high pressure liquid chromatography using a reverse phase nucleotide/nucleoside 7U (Alltech Inc.) column. The stoichiometry of phosphorylation could then be calculated by liquid scintillation of the excised receptor band for a known quantity of β2AR loaded on SDS-PAGE.

Phosphorylation of β2AR in Membrane Preparations Derived from Sf9 Cells—Cells were cultured at 27°C in Grace supplemented insect medium up to a density of 1.5–2.0 x 10^7 cells/ml. The cells were then infected with recombinant baculoviruses encoding wild type β2AR, Gly341bβ2AR, or Gly341b,346β2AR at a multiplicity of infection of 2–5. The virus encoding the wild type β2AR was generated as described before (7), while the two other viruses were constructed by subcloning the mutant receptor coding regions into the pVJ5AR recombinant vector. Recombinant baculovirus was purified by successive plaque assays using the β-galactosidase assay. Cells were harvested 48 h after the infection, and membranes were prepared as described above. The final membrane pellets were resuspended in a phosphorylation buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 5 mM phosphoenolpyruvate, 1 unit pyruvate kinase, 0.2 mM ATP, and 0.025 mM [γ-32P]ATP (200 cpm/nmol). Phosphorylation assay was initiated by adding 3 x 10^-3 units of protein kinase A catalytic subunit (Sigma). Maximum phosphorylation was attained following a 30-min incubation at 30°C. Thus, this incubation time was used for routine assays. At the end of the incubation, membranes were centrifuged at 43,000 x g for 20 min at 4°C, and the pellets were resuspended in 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 20 mM NaH2PO4. Receptors were then solubilized in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.3% deoxycholate for 90 min at 4°C. Affinity purification was then carried out as described above. The level of phosphorylation was assessed following SDS-PAGE of the purified receptor. For this purpose, the bands corresponding to the β2AR were excised from the gel and counted by liquid scintillation. Simultaneously, the phosphorylation could be calculated based on the specific activity of the [γ-32P]ATP used in the phosphorylation assays. In some experiments, membranes were treated with agarose-bound alkaline phosphatase (200 units/ml) for 45 min at 37°C in 20 mM Tris-HCl (pH 8.0), 1 mM MgCl2, and 0.1 mM ZnCl2 prior to the phosphorylation assay. Alkaline phosphatase was removed from the samples by centrifugation. The samples were then washed extensively and desalted by filtration dialysis using Centricon 30 (Amicon). For receptor reconstitution, membranes derived from untransformed LLCMK2 cells were prepared as described above and reconstituted at a concentration of 2–3 μg/ml in a buffer containing 20 mM Hepes (pH 7.4), 2 mM MgCl2, 1 mM EDTA, 0.4% CHAPS. Purified β2ARs

Isolated by sequential chromatography on a Dowex cation exchange resin and aluminum oxide. Data calculated as pmol of cAMP produced/mg protein were analyzed using nonlinear least squares regression.
treated as above were then incubated with this membrane preparation (8 fmol/μl) under mild agitation at 15°C. After 1 h, an equal volume of a freshly prepared buffer containing 10 mM Hepes (pH 7.4), 10 mM MgCl₂, 2 mM ATP, 0.2 mM GTP was added to the mixture and incubated for an additional 20 min at 30°C. The preparation was then centrifuged at 180,000 × g for 40 min. The pellet membranes were then washed twice in a buffer containing 75 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM EDTA. The reconstituted membranes were finally diluted to 2–5 mM MgCl₂, 2 mM ATP, 0.2 mM GTP was added to the mixture and incubated.

Enol-stimulated adenylyl cyclase activity as described above. Twenty-four hours after this treatment, [9,10-3H]palmitic acid dissolved in dimethyl sulfoxide was added to the culture to a final concentration of 0.2 mM/ml. After 1.5 h the labeling period was terminated by centrifugation of the cells at 500 × g for 5 min at 4°C followed by two washes with ice-cold PBS. Cell membranes were then prepared, and the receptor was solubilized and purified by Sepharose-alprenolol affinity chromatography. The concentrated receptor preparation was then treated or not treated with hydroxyamine as above.

**RESULTS AND DISCUSSION**

Mutant forms of the human β₂-AR and Gly³⁴¹β₂-AR in which serine 345 and 346 were replaced by alanine residues were constructed (see Fig. 1) and stably expressed in mouse LTK⁻ cells. Membranes from cellular clones, selected by virtue of a co-transfected neomycin resistance marker, were prepared and assayed for [¹²⁵I]CYP binding activity. As seen in Table I, no significant difference in the affinity for the radioiodinated antagonist was observed among the receptors tested. The [¹²⁵I]CYP bound to the wild type and mutant β₂-ARs with K_d values in good agreement with those reported for human β₂-AR expressed in other cell systems (7, 17). No specific binding of [¹²⁵I]CYP to membranes from untransfected LTK⁻ cells was detected (data not shown). Since apparent coupling properties can vary with receptor expression levels (10, 18), cellular clones expressing similar numbers of receptors for wild type and mutant forms of human β₂-AR were selected for further characterization.

To assess the functional properties of the various receptor mutants, their ability to confer an isoproterenol-sensitive adenylyl cyclase activity to LTK⁻ cells was tested. In membranes derived from cells transfected with wild type β₂-AR cDNA, isoproterenol stimulated adenylyl cyclase activity in a dose-dependent manner (Fig. 1). The EC₅₀ of isoproterenol was 67 ± 10 nM (n = 9), consistent with its potency in other cellular systems (5, 7). No isoproterenol-stimulated adenylyl cyclase activity could be detected in untransfected LTK⁻ cells (data not shown). As previously reported in CHW cells (5, 6), the ability of Gly³⁴¹β₂-AR to stimulate the adenylyl cyclase was found to be markedly reduced relative to wild type β₂-AR in LTK⁻ cells. This is illustrated by the blunted isoproterenol dose-response curve observed in membranes derived from Gly³⁴¹β₂-AR expressing cells (Fig. 1). The marked difference between β₂-AR- and Gly³⁴¹β₂-AR-stimulated adenylyl cyclase activity did not result from the small (statistically nonsignificant) difference in receptor number expressed in the two cell lines studied (Table I), since in several experiments the number of Gly³⁴¹β₂-ARs was higher than that of wild type β₂-AR; yet, the level of stimulation

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**Table I**

| Receptor | [¹²⁵I]CYP binding parameters | Adenylyl cyclase stimulation |
|----------|-----------------------------|-------------------------------|
|          | (nM)                        |                                |
|          | K_d[^a]                     | B_max[^b]                     | E_C₅₀[^c]  | R_max[^d]   |
|          | (fmol/mg)                   | (pmol CAMP/min/mg)            | nM        | pmol CAMP/min/mg |
| Wild type β₂-AR | 3 | 51.3 ± 0.7 | 168 ± 17 | 67 ± 10 | 24.8 ± 1.8 |
| Gly³⁴¹β₂-AR   | 4 | 70.0 ± 6.5 | 125 ± 20 | 61 ± 15 | 4.9 ± 0.8  |
| Ala³⁴⁵β₂-AR   | 3 | 62.2 ± 22  | 210 ± 12 | 35 ± 10 | 27.7 ± 3.6 |
| Gly³⁴¹Ala³⁴⁵β₂-AR | 3 | 84.0 ± 20  | 290 ± 30 | 33 ± 8  | 22.2 ± 1.9 |

[^a] Equilibrium dissociation constant for [¹²⁵I]CYP.
[^b] Maximal specific [¹²⁵I]CYP binding.
[^c] Agonist potency defined as the concentration yielding 50% of the maximal response.
[^d] Maximal isoproterenol-stimulated adenylyl cyclase activity.
[^e] p < 0.05, compared to wild type β₂-AR.
Palmitoylation Modulates β₂-AR Phosphorylation

The isoproterenol binding parameters were determined by competition of [¹²⁵I]CYP binding using increasing concentrations of isoproterenol (0–100 μM) in the presence of 100 μM Gpp(NH)p. The estimates were obtained from analysis of three to six competition experiments using the program Ligand (13). n, number of independent experiments.

|  | Kᵢ(H) (nM)ᵃ,b | Kᵢ(L) (nM)ᵃ,b | % Bmax₀(H)ᵃ,c |
|---|---|---|---|
| Wild type β₂-AR | 6.0 ± 0.3 | 33 ± 7 | 35 ± 7 |
| Control | 3 | — | 84 ± 10 |
| Gly341β₂-AR | 3 | — | 73 ± 8 |
| Gpp(NH)p | 3 | — | 95 ± 15 |
| Ala345,346β₂-AR | 6.1 ± 0.2 | 55 ± 7 | 48 ± 6 |
| Control | 3 | — | 81 ± 12 |
| Gpp(NH)p | 3 | — | 81 ± 12 |
| Gly341Ala345,346 β₂-AR | 5.1 ± 0.4 | 105 ± 17 | 42 ± 2 |
| Control | 3 | — | 138 ± 10 |

a H and L refer to high and low affinity binding parameters of isoproterenol, respectively.
b Equilibrium inhibition constant for isoproterenol.
c Percentage of site in the high affinity state for isoproterenol.
d Binding was performed in the presence of 300 μM Gpp(NH)p.
ea, not detectable.

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Fig. 2. Agonist binding characteristics of wild type β₂-AR, Gly³⁴ⁱβ₂-AR, Gly³⁴¹Ala³⁴⁵,³⁴⁶β₂-AR, and Ala³⁴⁵,³⁴⁶β₂-AR. Isoproterenol competition of [¹²⁵I]CYP binding was assessed in the absence (□) or in the presence (●) of 100 μM Gpp(NH)p in membrane preparations derived from wild type or mutant β₂-AR-expressing cells. The curves shown are computer-generated fits of data obtained in representative experiments, using the program Ligand (13).

Additional replacement of serines 345 and 346 by alanine residues into Gly³⁴¹β₂-AR almost completely restored the ability of the receptor to stimulate adenyl cyclase activity. This is illustrated by the normal isoproterenol dose-response curve observed in membranes derived from Gly³⁴¹Ala³⁴⁵,³⁴⁶β₂-AR-expressing cells (Fig. 1). In fact, the maximal isoproterenol-stimulated adenyl cyclase activities were virtually identical for wild type β₂-AR and Gly³⁴¹Ala³⁴⁵,³⁴⁶β₂-AR (Table I). This contrasts with the much reduced isoproterenol-stimulated activity observed for Gly³⁴¹β₂-AR. These results suggest that the absence of the palmitoylated cysteine 341 decreases the ability of the receptor to stimulate the adenyl cyclase only when the PKA phosphorylation site located downstream of the palmitoylation site is intact. Mutation of the two serines alone in the wild type β₂-AR did not affect the binding parameters of isoproterenol. Kᵢ values and the proportion of sites in high and low affinity states are summarized in Table I. These data suggest that the absence of serines 345 and 346 restores the ability of the unpalmitoylated receptor to interact with Gₛ, consistent with the effects of this mutation on the capacity of the β₂-AR to stimulate the adenyl cyclase activity.

Since serines 345 and 346 represent a potential phosphorylation site for PKA, it could be hypothesized that phosphorylation of this site contributes to the uncoupled phenotype of Gly³⁴¹β₂-AR. To test this hypothesis, the basal level of phosphorylation of the wild type β₂-AR, Gly³⁴¹β₂-AR and Gly³⁴¹Ala³⁴⁵,³⁴⁶β₂-AR was assessed in LTK⁻ cells. Following metabolic labeling with inorganic [³²P]phosphoric acid (³²Pᵢ), the cells were broken, membranes were prepared, and the receptors were purified by alprenolol-Sepharose affinity chromatography. An identical number of receptors, as determined by [¹²⁵I]CYP binding, was prepared for SDS-PAGE. Purified receptors migrated with an apparent molecular mass of ~70 kDa as previously reported (20). Fig. 3 illustrates the level of phosphorylation of the three receptors. The labeling intensity of purified Gly³⁴¹β₂-AR was found to be much higher than that of the wild type receptor, thus confirming that, as found in CHW cells (6), Gly³⁴¹β₂-AR has a significantly elevated basal level of phosphorylation. In contrast, the level of phosphorylation of Gly³⁴¹Ala³⁴⁵,³⁴⁶β₂-AR was not different from that of the wild type receptor. Thus, the mutation of serines 345 and 346 completely prevented the elevation of the basal phosphorylation state induced by the mutation of cysteine 341. Taken together, the data support the hypothesis that this PKA phosphorylation site contributes to the increased phosphorylation and uncoupling of the unpalmitoylated Gly³⁴¹β₂-AR. The low

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*Table I*

|  | Kᵢ(H) (nM)ᵃ,b | Kᵢ(L) (nM)ᵃ,b | % Bmax₀(H)ᵃ,c |
|---|---|---|---|
| Wild type β₂-AR | 6.0 ± 0.3 | 33 ± 7 | 35 ± 7 |
| Control | 3 | — | 84 ± 10 |
| Gly³⁴¹β₂-AR | 3 | — | 73 ± 8 |
| Gpp(NH)p | 3 | — | 95 ± 15 |
| Ala³⁴⁵,³⁴⁶β₂-AR | 6.1 ± 0.2 | 55 ± 7 | 48 ± 6 |
| Control | 3 | — | 81 ± 12 |
| Gpp(NH)p | 3 | — | 81 ± 12 |
| Gly³⁴¹Ala³⁴⁵,³⁴⁶ β₂-AR | 5.1 ± 0.4 | 105 ± 17 | 42 ± 2 |
| Control | 3 | — | 138 ± 10 |

a H and L refer to high and low affinity binding parameters of isoproterenol, respectively.
b Equilibrium inhibition constant for isoproterenol.
c Percentage of site in the high affinity state for isoproterenol.
d Binding was performed in the presence of 300 μM Gpp(NH)p.
ea, not detectable.
number of receptors expressed in LTK− cells and the very small basal level of phosphate incorporation into the wild type receptor makes it impossible to accurately determine the stoichiometry of phosphorylation in this system. In order to quantify the increase in the basal phosphorylation level observed in Gly341β2AR, metabolic labeling experiments with 32P, were conducted in CHW cells expressing higher levels of wild type β2AR and Gly341β2AR (−2.0 pmol/mg of protein in each case). Assessment of the specific activity of the cellular [32P]ATP pool allowed us to determine the basal stoichiometry of phosphorylation. A stochiometry of 0.34 mol of phosphate/mol of receptor was found for the wild type β2AR, while 1.1 mol of phosphate/mol of receptor were incorporated into Gly341β2AR.

The above results strongly suggest that mutation of glycine 341 does not affect receptor coupling by itself but rather as a consequence of the increased phosphorylation level resulting from the mutation. To further test the hypothesis that the absence of the palmitate moiety does not directly affect the coupling of the receptor, the effect of chemical removal of the palmitate was assessed. Recombinant human β2AR expressed in SF9 cells was purified, treated or not treated with hydroxylamine, and reconstituted in membranes derived from native LTK− cells devoid of endogenous β2AR. The efficacy of the hydroxylamine treatment to remove the receptor-bound palmitate was tested using [3H]palmitate-labeled β2AR. As shown in the inset to Fig. 4, the treatment led to a complete depalmitoylation of the receptor. However, such depalmitoylation was without effect on the ability of the receptor to stimulate adenylyl cyclase activity. Indeed, reconstitution of an equal number of native or hydroxylamine-treated β2AR conferred identical isoproterenol-sensitive adenylyl cyclase activity to the LTK−-derived membranes (Fig. 4). No isoproterenol-stimulated adenylyl cyclase activity could be detected using nonreconstituted LTK− membranes (data not shown). These results confirm that the presence of receptor-bound palmitate is not required per se to allow productive interaction between the β2AR and Gs.

It has been proposed that post-translational modification of the β2AR by palmitoylation of cysteine 341 promotes the association of the amino-terminal portion of the carboxyl tail with the plasma membrane, thus forming a fourth intracellular loop (5). Such a model was originally proposed for rhodopsin (21), and the insertion of the rhodopsin-bound palmitate moieties into the membrane lipid bilayer was experimentally confirmed using fluorescent analogues of the fatty acid (22). The results presented above clearly demonstrate that the formation of this fourth loop is not required for proper coupling of the receptor to Gs. Indeed, Gly341Ala345,346β2AR, which lacks the palmitoylated cysteine and the chemically depalmitoylated receptor, are perfectly able to stimulate the Gs/adenylyl cyclase system. Nevertheless, the formation of such a loop undoubtedly modifies the topology of the carboxyl tail, a region that is known to act as substrate for regulatory kinases. In particular, serine 345 and 346 would be located very near the plasma membrane, where they might not be easily accessible to PKA. This model is consistent with the observation that only the PKA site located in the third cytoplasmic loop of the β2AR (359RRSS) and not the one in the carboxyl tail (346CLRSS) has been shown to be phosphorylated upon direct activation of PKA by dibutyryl-cAMP (23), or following incubation of broken cell preparations with the PKA catalytic subunit (24). Following this model, mutation of Cys341 may provide an easier access of the carboxyl tail site to PKA by preventing the formation of the putative fourth intracellular loop.

To directly test this hypothesis, the accessibility of the phosphorylation site to PKA was assessed in vitro using SF9 cell membranes expressing wild type β2AR, Gly341β2AR, and Gly341Ala345,346β2AR. Membranes were incubated with γ-32P]ATP in the presence of PKA catalytic subunit.
Gly341
modest (0.11 mol/mol for wild type receptor. Additional mutation of serines 345 and 346 reduced the phosphorylation to levels even lower than those observed in LTK cells expressing Gly341Ala345,346
b
in Fig. 5, PKA catalyzed the phosphorylation of wild type β2AR, Gly341β2AR, and Gly341Ala345,346β2AR. The phosphorylated receptors migrated with an apparent molecular mass of 45–55 kDa, consistent with the reported electrophoretic mobility of the human β2AR expressed in Sf9 cells (7). The difference with the mobility observed in LTK cells is due to different glycosylation patterns. As previously reported for the unpalmitoylated form of p21N-ras (8), the unpalmitoylated Gly341β2AR and Gly341Ala345,346β2AR have a slightly lower electrophoretic mobility than the wild type receptor. When comparing the maximum level of phosphorylation, Fig. 5 clearly shows that Gly341β2AR acted as a better substrate for PKA than the wild type receptor. Additional mutation of serines 345 and 346 reduced the phosphorylation to levels even lower than those reached for the wild type β2AR, thus suggesting that phosphorylation of this site contributed to the elevated phosphorylation observed in membranes expressing Gly341β2AR.

The above results are consistent with the idea that the lack of palmitoylation favors phosphorylation of the receptor by PKA, most likely by increasing the accessibility of the site to PKA catalytic subunit and [γ-32P]ATP during 30 min at 30°C. At the end of the incubation, the receptors were solubilized and purified by alprenolol-Sepharose affinity chromatography. The inset shows a representative autoradiogram of the SDS-PAGE-resolved receptors. An identical number of receptors (6.4 pmol), as assessed by radioligand binding, were loaded in each lane. The autoradiogram shown is representative of three independent experiments.

for Gly341β2AR. The amount of [32P]P4 incorporated in vitro represented the maximal level of phosphate transfer that could be attained for the two receptors under the condition studied, since no further phosphorylation was observed following longer incubation with the kinase (data not shown). It follows that more phosphate molecules can be transferred by PKA into Gly341β2AR than into the wild type β2AR, consistent with the idea that the unpalmitoylated receptor is a better substrate for PKA.

To determine if phosphorylation of this PKA phosphorylation site contributes to the normal development of agonist-promoted desensitization, the pattern of rapid desensitization of Ala345,346β2AR was compared with that of wild type β2AR. As shown in Fig. 7, mutation of Ser345,346 significantly delayed the appearance of agonist-promoted desensitization. In cells expressing wild type receptor, pretreatment with isoproterenol for 1 and 2 min caused a significant decrease in the ability of the agonist to stimulate the adenylyl cyclase in membranes derived from these cells. Reduction in both the maximal stimulation (16 and 37% at 1 and 2 min, respectively) and in the efficacy of isoproterenol (EC50 of control, 1.6 × 10−7 M; 1 min, 8.8 × 10−7; 2 min, 1.3 × 10−6) were observed. This desensitization was receptor-specific, since no significant change in either basal or forskolin-stimulated activity were observed (data not shown). In Ala345,346β2AR-expressing cells, pretreatment for 1 and 2 min with isoproterenol only led to very marginal changes in the maximal adenylyl cyclase stimulation and isoproterenol efficacy. Following a longer desensitizing period (30 min), the desensitization observed for Ala345,346β2AR and wild type receptor were similar (data not shown), suggesting that Ser345 and Ser346 are not essential for the desensitization to occur but that they contribute to its rapid onset.

The data presented in this study demonstrate that palmitoylation of Cys341 regulates the accessibility of a PKA phosphorylation site in the carboxyl tail of the β2AR. Our data also indicate that such a regulatory influence on the phosphorylation state of the receptor may play an important role in the rapid modulation of the receptor responsiveness.

It has been proposed that palmitoylation of GAP-43 modulates its ability to interact with Go (25). In that study, the authors proposed that the sulfhydryl functions of cysteines 3 and 4 from GAP-43 are essential to activate the G protein and that palmitoylation of the cysteines inhibits their stimulatory activity. Our study shows that this is not the case for the β2AR.
Interestingly, the palmitoylation state of another receptor, the
may then contribute to the rapid onset of desensitization. In-
boring cysteine 341. The favored phosphorylation of this site
viding better access for PKA to the phosphorylation site neigh-
true sites or assumed as potential sites. Similarly to
function of mutating cysteines that were either known
s in mammalian cells (46). Furthermore, this mutant receptor becom-
to agonist-promoted desensitization, suggesting that it is con-
it of palmitoylation, is located four amino acids upstream of
site. This regulation of the palmitoylation state of Gαs has
been proposed to promote translocation of Gαs from the plasma
to the cytosol and thus could act as a process mod-
ulating signaling efficacy (28). A similar agonist-promoted reg-
ulation of the β2AR palmitoylation state has recently been
proposed (7). Based on the observations reported here, it could
therefore be proposed that agonist stimulation, by promoting
the depalmitoylation of the receptor, regulates membrane
attachment of the proximal portion of the its carboxyl tail, pro-
viding better access for PKA to the phosphorylation site neigh-
boring cysteine 341. The favored phosphorylation of this site
may then contribute to the rapid onset of desensitization. In-
terestingly, the palmitoylation state of another receptor, the
ionotropic glutamate receptor GluR6, has also been shown to
regulate its phosphorylation by a second messenger-dependent
protein kinase (32).

The contribution of the carboxyl tail PKA phosphorylation
site to agonist-promoted desensitization, has previously been
questioned. Indeed, recent studies show that this site may not be
phosphorylated upon direct activation of PKA (23). Also, dele-
tion of this PKA consensus site did not affect desensitization
evoked by the PKA catalytic subunit or low doses (50 nM) of
adrenaline, two conditions known to promote heterologous de-
sensitization (24). However, the present study clearly suggest
that this site becomes phosphorylated and contributes to the
rapid onset of homologous desensitization upon treatment with
micromolar concentrations of agonist. The easiest way to re-
concile these data is to propose that serine 345 and 346 be-
come available for phosphorylation only when receptors are agonist-
bound. Therefore, stimulation with low doses of agonist (which
leads to occupancy of a small proportion of the receptor popu-
lation) or with cAMP analogues would not promote phospho-
rylation of this site. These observations are consistent with a
recent report by Post et al. (31), which showed in S49 cells that
PKA-mediated phosphorylation is important for rapid homolo-
gous desensitization.

The data presented clearly show that phosphorylation of the
PKA site closest to the palmitoylated cysteine is affected by the
palmitoylation state of the receptor. However, further studies
are required to determine whether or not phosphorylation of
more distal sites believed to be phosphorylated by μARK could
also be affected. In olfactory cilia preparations, inhibition of
PKA prevented the phosphorylation and desensitization of
odorant receptors believed to be mediated both by PKA and a
μARK like enzyme (33). The authors suggested a sequential
interplay between PKA and the receptor-specific kinase. Simi-
lar sequential phosphorylation of specific sites involved in
desensitization have also been reported for the rhodopsin (34,
35), the N-formyl peptide receptor (36), and the C5a anapha-
latoxin receptor (37).

Indeed, Gly341Ala345,346β2AR, which is lacking cysteine 341,
interacts normally with Gs and stimulates the adenyl cyclase
activity to levels comparable with that of wild type receptor.

For many proteins involved in signal transduction, palmito-
ylation serves to regulate membrane attachment. Good ex-
amples are Gα subunits whose subcellular distribution has been
shown to be modified following mutation of the palmitoylated
cysteine (26, 27). Interestingly, stimulation of the β2AR has
been shown to increase the turnover rate of Gαs-bound palmi-
tate, which may lead to a reduced palmitoylation of the protein
(28–30). This regulation of the palmitoylation state of Gαs has
been proposed to promote translocation of Gαs from the plasma
membrane to the cytosol and thus could act as a process mod-
ulating signaling efficacy (28). A similar agonist-promoted reg-
ulation of the β2AR palmitoylation state has recently been
proposed (7). Based on the observations reported here, it could
therefore be proposed that agonist stimulation, by promoting
the depalmitoylation of the receptor, regulates membrane
attachment of the proximal portion of the its carboxyl tail, pro-
viding better access for PKA to the phosphorylation site neigh-
boring cysteine 341. The favored phosphorylation of this site
may then contribute to the rapid onset of desensitization. In-
terestingly, the palmitoylation state of another receptor, the

![Time-dependent desensitization of wild type β2AR and Ala345,346β2AR](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**FIG. 7.** Time-dependent desensitization of wild type β2AR and Ala345,346β2AR. LTK− cells stably expressing wild type β2AR (upper panel) or Ala345,346β2AR (lower panel) were incubated for 0 (circle), 1 (square), or 2 (triangle) minutes with 1 μM isoproterenol. Membranes were then prepared, and adenyl cyclase activity was measured as described under “Experimental Procedures.” Data represent the mean ± S.E. of three independent experiments.
Palmitoylation Modulates β2-AR Phosphorylation

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J. Biol. Chem. 1996, 271:21490-21497.
doi: 10.1074/jbc.271.35.21490

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