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# Interaction réciproque entre la palmitylation et la phosphorylation du récepteur $\beta_2$-adrénergique.

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## INTERACTION RÉCIPROQUE ENTRE LA PALMITYLATION ET LA PHOSPHORYLATION DU RÉCEPTEUR $\beta_2$-ADRÉNERGIQUE

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# Sommaire

Le récepteur $\beta_2$-adrénergique est, parmi les récepteurs couplés aux protéines G, le mieux connu et, par conséquent, sert de prototype pour cette grande famille de récepteurs. Les différents mécanismes responsables de régulariser l'activité fonctionnelle du $\beta_2$AR sont relativement bien établis. Par exemple, à la suite d'une stimulation soutenue par un agoniste, la phosphorylation et la palmitylation du $\beta_2$AR sont modifiées. Cependant, contrairement à la phosphorylation, peu d'études se sont attardées à caractériser la palmitylation du récepteur. Cette modification consiste à l'ajout d'un acide gras saturé de seize atomes de carbone sur le résidu cystéine 341 du récepteur *via* une liaison thioester. Selon les résultats d'études antérieures, la palmitylation pourrait correspondre à un niveau supplémentaire de régulation de l'activité fonctionnelle du $\beta_2$AR.

Le premier objectif vise donc à mieux comprendre la dynamique de palmitylation du récepteur. À la suite d'expériences de radiomarquages et de "pulse-chase", nous avons démontré que la palmitylation est une modification post-traductionnelle réversible durant la vie du récepteur indépendamment du système d'expression utilisé (cellules de mammifères et cellules d'insectes). De plus, l'état d'activation du récepteur influence grandement sa palmitylation. L'activation du récepteur par un agoniste aboutit à une augmentation nette de la vitesse de renouvellement du palmitate associé au récepteur. Dans ce contexte d'activation, nous avons mis en évidence par mutagenèse dirigée que le site de phosphorylation de la PKA

($^{343}$RRSS$^{346}$) dans la portion C-terminale du récepteur y joue un rôle déterminant. La phosphorylation de ce site de la PKA serait responsable, par une répulsion entre les charges négatives des phospholipides de la membrane plasmique, et celles des sérines phosphorylées 345 et 346, de la diminution de la stabilité de l'ancrage du palmitate dans la membrane. Ultimement, la phosphorylation du récepteur favoriserait une forme non palmitylée du $\beta_2$AR. La déphosphorylation du $\beta_2$AR serait nécessaire pour permettre au récepteur d'être de nouveau palmitylé. Dans l'ensemble, ces résultats suggèrent que la palmitylation est régulée non seulement à la suite de l'activation du récepteur, mais également par l'état de phosphorylation du $\beta_2$AR.

Cette interaction entre la phosphorylation et la palmitylation du récepteur a été observée en utilisant des cellules de mammifère et des cellules d'insectes (Sf9). Cependant, une différence existe entre ces deux systèmes en ce qui à trait à la vitesse de renouvellement du palmitate associé au récepteur. Dans les cellules de mammifères, la vitesse de renouvellement du palmitate associé au récepteur est beaucoup plus lente que celle observée dans les cellules d'insectes (Sf9). Pour cette raison, nous croyons que la palmitylation pourrait avoir d'autres fonctions. Cependant, le ou les rôles pouvant être rattachés à cette modification demeurent à être déterminés.

Nous avons également démontré que la palmitylation du $\beta_2$AR pouvait être régulé indépendamment de l'activation du récepteur. Nous avons mis en évidence le fait que

le monoxyde d'azote exerce une modulation sur l'état de palmitylation du $\beta_2$AR. Les résultats de cette étude ont démontré que la présence de monoxyde d'azote diminue l'incorporation du palmitate tritié au niveau du récepteur activé ou pas par un agoniste tel que l'isoprotérénol. Cet effet du monoxyde d'azote est accompagné du découplage fonctionnel du récepteur avec la protéine G$\alpha$s. Selon nos résultats, le monoxyde d'azote module directement la voie $\beta$-adrénergique en régulant l'état de palmitylation du $\beta_2$AR.

Mots clés du sommaire: récepteur $\beta_2$-adrénergique, palmitylation, phosphorylation, monoxyde d'azote, régulation.

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# Liste des sigles et abréviations

| | |
|---|---|
| A | alanine |
| AKAP | protéine d'ancrage, en autre, de la PKA |
| AMPc | adénosine monophosphate 3'-5'- cyclique |
| $\beta_2AR$ | récepteur $\beta_2$-adrénergique |
| C | cystéine |
| CoA | coenzyme A |
| G | glycine |
| GABA | acide aminobutyrique gamma ($\gamma$) |
| G$\alpha$s | sous unité alpha stimulatrice de la protéine G trimérique |
| G$\alpha$i | sou unité alpha inhibitrice de la protéine G trimérique |
| GDP | guanosine diphosphate |
| GMPc | guanosine monophosphate 3'-5'- cyclique |
| GTP | guanosine triphosphate |
| GRK | kinases couplées aux RCPG |
| FT | farnésyltransférase |
| GGT | géranylgéranyltransférase |
| HEK293 | cellules rénales embryonnaires humaines |
| $[^{125}I]ICYP$ | $[^{125}I]$iodocyanopindolol |
| K | lysine |
| kDa | kilo dalton |
| L | leucine |
| M | méthionine |
| NO | monoxyde d'azote (nitric oxide) |
| NOSe | NO synthase endothéliale |

NOSi            NO synthase inductible

PAT             palmityl acyltransférase

Pi              phosphate inorganique

PKA             protéine kinase dépendante de l'AMPc

PKC             protéine kinase dépendante du calcium

PPT             protéine palmityl thioestérase

RCPG            récepteurs couplés aux protéines G

S               sérine

SDS             dodécylsulfate de sodium

SDS-PAGE        électrophorèse en gel de polyacrylamide en présence de SDS

Sf9             *spodoptera frugiperda 9*

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À Robert…

*Ah ! comme la neige a neigé !*

*Ma vitre est un jardin de givre.*

*Émile Nilligan*
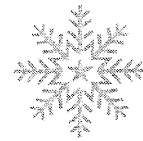
# 1. Introduction

## 1.1 Introduction aux récepteurs couplés aux protéines G

Les Récepteurs Couplés aux Protéines G (RCPG) constituent de loin la plus grande famille de récepteurs membranaires dont la fonction est de transformer sélectivement un signal biologique extracellulaire en une cascade d'événements biologiques intracellulaires.

Ces récepteurs répondent à des ligands aussi variés que des amines, des peptides, des hormones, des dérivés lipidiques, des dérivés d'acides aminés, des nucléosides ou des nucléotides, des ions, une variété pratiquement infinie de substances odorantes ainsi que des agents physiques (photons pour la rhodopsine et les pigments visuels). Cette grande variété de signaux extracellulaires provoque l'activation de cascades intracellulaires, dans la plupart des cas par l'intermédiaire de protéines trimériques (protéine G) liant le GTP. Parmi les protéines effectrices intracellulaires dont l'activité est modulée par les protéines G mentionnons l'adénylyl cyclase (stimulation ou inhibition), les phospholipases C et $A_2$ (activation), la GMPc phosphodiestérase, ainsi que les canaux calciques (ouverture ou fermeture) et potassique (ouverture) (Parmentier et al., 1995).

La transmission des signaux par des RCPG est constamment régularisée par des modifications de leur capacité fonctionnelle et par leur densité à la surface cellulaire. Ces mécanismes de régulation permettent de moduler la réponse cellulaire d'un récepteur en modifiant l'interaction fonctionnelle de celui-ci avec une protéine G.

L'intérêt premier de cette thèse, est de mieux comprendre d'obtenir une meilleure compréhension des modifications posttraductionnelles impliquées dans la régulation de l'activité fonctionnelle du récepteur $\beta_2$-adrénergique ($\beta_2AR$). De nombreuses études portant sur les caractéristiques pharmacologiques, cellulaires, génétiques, biochimiques et structurales du $\beta_2AR$ en font un des RCPG les mieux connus. Donc, le $\beta_2AR$ est considéré comme un modèle par rapport à cette grande famille de récepteurs. Dans les prochaines sections, tout d'abord, les différents membres des récepteurs adrénergiques ainsi que leur pharmacologie seront décrits. Ensuite, la voie de signalisation $\beta$-adrénergique sera explicitée.

## 1.2 Les récepteurs adrénergiques

### 1.2.1 L'historique et la classification des récepteurs adrénergiques

En 1948, et suite à des études de liaison effectuées à l'aide d'amines sympathomimétiques sur des tissus isolés, Ahlquist (Ahlquist, 1948) propose l'existence de deux types de récepteurs des catécholamines qu'il nomme $\alpha$ et $\beta$ adrénergiques. Vingt ans plus tard, une subdivision des récepteurs $\beta$-adrénergiques en deux sous types ($\beta_1$ et $\beta_2$) basée sur l'affinité de différents agonistes $\beta$-adrénergiques est suggérée (Lands et al., 1967). Dans les années 1970, une différence entre les caractéristiques pharmacologiques des récepteurs $\alpha$-adrénergiques ($\alpha$-AR) est observée. En 1974, Langer (Langer, 1974) utilise les termes $\alpha_1$ et $\alpha_2$ pour désigner les différences d'affinités $\alpha$-adrénergiques. Le développement de ligands spécifiques a ensuite permis de mettre en évidence l'existence d'une hétérogénéité des sites de liaison $\alpha_1$- et $\alpha_2$-adrénergiques (Bylund, 1985). Au cours des années 1980, il devenait évident qu'un troisième sous-type de récepteurs $\beta$-adrénergiques avec ses propres caractéristiques pharmacologiques

existait. Ce récepteur fut nommé le récepteur β-adrénergique atypique (Arch et al., 1984).

Depuis environ une quinzaine d'années, le développement rapide et l'accessibilité à des techniques de biologie moléculaire ont permis de cloner et de séquencer les gènes codants pour un grand nombre de ces récepteurs adrénergiques. En 1986, le premier gène cloné codant pour un RCPG fut celui du $\beta_2$AR de hamster (Dixon et al., 1986). Par la suite, les techniques de clonages moléculaires ont permis de mettre en évidence trois sous-types $\alpha_1$AR ($\alpha_{1A}$, $\alpha_{1B}$ et $\alpha_{1D}$) (Cotecchia et al., 1988; Schwinn et al., 1990; Lomasney et al., 1991), trois sous-types $\alpha_2$AR ($\alpha_{2A}$, $\alpha_{2B}$ et $\alpha_{2C}$) (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990) et deux sous-types $\beta$AR ($\beta_1$ et $\beta_3$) (Frielle et al., 1987; Emorine et al., 1989), le $\beta_3$AR correspondant au point de vue pharmacologique au récepteur atypique mentionné plus haut. Considérant l'ensemble des récepteurs adrénergiques clonés, il a été possible d'établir une corrélation entre les sous-types de récepteurs adrénergiques et les différents profils pharmacologiques déjà connus.

## 1.2.2    La pharmacologie des récepteurs adrénergiques

Les récepteurs adrénergiques sont stimulés *in vivo* par les catécholamines endogènes qui sont l'adrénaline et la noradrénaline. Les sous-types β-adrénergiques possèdent différentes affinités pour les catécholamines (Tableau 1). Par exemple, l'affinité de l'adrénaline pour le $\beta_2$AR comparativement au $\beta_1$AR est significativement supérieure (facteur de 100) (Bylund et al., 1994). En ce qui à trait au $\beta_3$AR, la noradrénaline possède une meilleure affinité pour ce récepteur que l'adrénaline (Emorine et al., 1989).

Plusieurs agonistes ou antagonistes sélectifs ont été développés pour la majorité des sous-types de récepteurs adrénergiques (Tableau 1). Il existe également des ligands non-sélectifs très utilisés tels que l'isoprotérénol, un puissant agoniste β-adrénergique, le propanolol, un antagoniste possédant une très bonne affinité pour le $\beta_1$AR et, le $\beta_2$AR. Les radioligands les plus utilisés pour les $\beta_1$AR et $\beta_2$AR sont le [$^3$H]-dihydroalprenolol, le [$^{125}$I]-iodopindolol et le [$^{125}$I]-iodocyanopindolol. Ce dernier radioligand est également utilisé pour détecter la présence du $\beta_3$AR mais avec une affinité moindre que les deux autres récepteurs adrénergiques (Bylund et al., 1994).

**Tableau I**      **Pharmacologie des récepteurs adrénergiques**

| Sous-types | Agonistes | Antagonistes | Ordre de Puissance |
|---|---|---|---|
| $\alpha_1 AR_{(A, B, D)}$ | Methoxamine, Phényléphrine | Prasozin | A > NA > ISO |
| $\alpha_2 AR_{(A, B, C)}$ | Clonidine, Tramazoline | Rauwolscine, Yohimbine | A > NA > ISO |
| $\beta_1 AR$ | Xamoterol, | Metoprolol, CGP 20712 | ISO > NA $\cong$ A |
| $\beta_2 AR$ | Salmeterol, Salbutamol | Cyanopindolol, propanolol | ISO > A >> NA |
| $\beta_3 AR$ | BRL37344 | --------- | ISO $\cong$ NA > A |

A ; adrénaline, NA ; noradrénaline, ISO ; isoprotérénol

Les récepteurs adrénergiques sont exprimés dans la majorité des tissus de mammifères et diverses fonctions leur sont attribuées. Le développement de ligands spécifiques a permis de mieux caractériser leurs rôles respectifs. Cependant, la coexistence de plusieurs sous-types de récepteurs adrénergiques dans un même tissu ou dans une même cellule peut rendre l'interprétation des événements physiologiques complexe. Nous allons résumer quelques effets physiologiques observés suite à l'activation de ces récepteurs. Au niveau des effets cardio-vasculaires, la phényléphrine, agoniste $\alpha$-sélectif, augmente la pression artérielle, diminue le rythme cardiaque et conduit à une vasoconstriction. L'isoprotérénol, agoniste $\beta$-sélectif, augmente le rythme et le débit cardiaque et produit une vasodilatation des vaisseaux des muscles squelettiques. Le $\beta_2 AR$ régularise la relaxation des muscles lisses suivant: muscles bronchiques, utérins et intestinaux. En revanche, l'$\alpha_1 AR$ entraîne une contraction des sphincters intestinaux et viscéraux, de l'urètre et du myomètre. De plus, des effets métaboliques ont été mesurés dans différents tissus. Notons par exemple; une hyperglycémie médiée par les $\beta_2 AR$ et/ou $\alpha_1 AR$ selon l'espèce et l'étape de développement, une hyperlactacidémie impliquant le $\beta_2 AR$ au niveau des muscles lisses, une lipolyse dans les tissus adipeux impliquant le $\beta_3 AR$, et finalement la sécrétion de l'insuline régulée par le $\beta_2 AR$ et, le $\alpha_2 AR$ dans le pancréas. La multiplicité de ces actions physiologiques démontre l'importance du système adrénergique.

Nous venons de décrire très brièvement la classification, la distribution ainsi qu'un certain nombre de rôles associés à ces récepteurs adrénergiques. L'essentiel de cette thèse portera sur la voie de signalisation $\beta$-adrénergique. Dans un premier temps, nous détaillerons les protéines impliquées dans cette voie de signalisation. Dans un deuxième temps, nous traiterons de l'aspect dynamique de la régulation de cette voie de signalisation.

### 1.2.3 Les composantes de la voie de signalisation β-adrénergique

D'une manière classique, la voie de signalisation du $\beta_2AR$ est composée de trois protéines essentielles: le récepteur, la protéine G et l'adénylyl cyclase. Dans les prochaines sections, nous allons décrire chacune de ces protéines ainsi que leurs interactions afin de produire un signal intracellulaire.

### 1.2.3.1 Le $\beta_2AR$

Comme nous l'avons déjà mentionné, le $\beta_2AR$ est un récepteur à sept domaines transmembranaires. La structure primaire de la protéine contient sept domaines d'une vingtaine d'acides aminés chacun pouvant former des hélices α hydrophobes dans la membrane cytoplasmique. Le domaine N-terminal ainsi que les boucles situées entre les hélices α II-III, IV-V et VI-VII sont orientées du côté extérieur de la cellule. La figure 1 représente un modèle schématique proposé pour le $\beta_2AR$ basé sur des résultats d'études d'hydropathicité de la séquence en acides aminés ainsi que les résultats d'études de protéolyse du récepteur (Dohlman et al., 1987). Nos connaissances concernant la structure tridimensionnelle du $\beta_2AR$ sont très limitées puisque jusqu'à maintenant aucun RCPG n'a été cristallisé. La principale difficulté expérimentale rencontrée est la forte hydrophobicité de ces RCPG. Les seules références actuelles, obtenues par des techniques de cryomicroscopie électronique, sont la bactériorhodopsine (Henderson et al., 1990) et la rhodopsine bovine (Schertler et al., 1993).

La portion extracellulaire du $\beta_2AR$ subit des N-glycosylations sur les asparagines 6 et 15. Le rôle proposé pour cette modification est de favoriser le transport efficace du récepteur à la membrane plasmique. Dans la portion extracellulaire du récepteur, on

retrouve un premier pont disulfure entre la cystéine 106 et la cystéine 184 et un deuxième entre la cystéine 190 et la cystéine 191. La formation de ces liens confère au récepteur une telle structure qui favorise la reconnaissance et la liaison du ligand (Dohlman et al., 1990). Dans la portion intracellulaire du récepteur, il y a formation de trois boucles cytoplasmiques, connectant les hélices I-II, III-IV et V-VI, et la présence de la queue C-terminale. La troisième boucle intracellulaire est nécessaire à l'activation de la protéine G. Des déterminants moléculaires participant à la modulation de l'activité du récepteur ont également été identifiés dans la région cytoplasmique ainsi que dans la portion C-terminale.

**Figure 1**        **Modèle schématique du récepteur $\beta_2$-adrénergique.**

sites de phosphorylation PKA

sites de phosphorylation βARK

Domaine extracellulaire

Domaine intracellulaire

NH₂

COOH

### 1.2.3.1.1  Les sites d'interaction ligand-$\beta_2$AR

L'équipe de Dixon (Dixon et al., 1987) a été le premier groupe à tenter d'identifier les sites de liaison du $\beta_2$AR. Ils ont démontré par des mutations aléatoires que les boucles extracellulaires ne participent pas à la liaison des agonistes ou des antagonistes. Ces résultats ont permis de suggérer que le domaine de liaison des ligands se retrouve dans la portion hydrophobe du récepteur composée des sept segments transmembranaires formant un cylindre. Ce modèle était plutôt surprenant en raison de la nature hydrophile des catécholamines. Des études de mutagenèse dirigée ont permis de raffiner ce modèle en démontrant l'importance de certains résidus hydrophiles chargés localisés au niveau des hélices $\alpha$ du récepteur. Ainsi, le ligand doit plonger à environ 11 A° de la surface cellulaire afin d'interagir avec les résidus aspartate 113, sérine 204, sérine 207 et phénylalanine 290 {Collins 1993 ID: 1706}. Le résidu aspartate 113 de l'hélice III est conservé parmi la famille des récepteurs liant les catécholamines. Il est d'ailleurs essentiel à la liaison de celles-ci {Tota, Candelore, et al. 1991 ID: 2149}. Ce résidu favorise par sa charge négative un contre-ion à la fonction ammonium des catécholamines. En ce qui concerne les deux résidus sérine 204 et 207 de l'hélice V du $\beta_2$AR, ceux-ci permettent de stabiliser le ligand par la formation de ponts hydrogènes avec les groupements hydroxyls en position *méta* et *para* du noyau catéchol du ligand. Au niveau de l'hélice VI, la phénylalanine 290 semble induire une interaction hydrophobe avec la partie aryle du catéchol. Finalement, une cavité est formée par des acides aminés aromatiques conservés dans les hélices III et VI favorisant une meilleure solvatation autour de l'interaction ammonium-aspartate 113. Une représentation des interactions entre l'adrénaline et le $\beta_2$AR est illustré à la figure 2.

**Figure 2**    **Représentation des interactions entre l'adrénaline et le récepteur $\beta_2$-adrénergique**
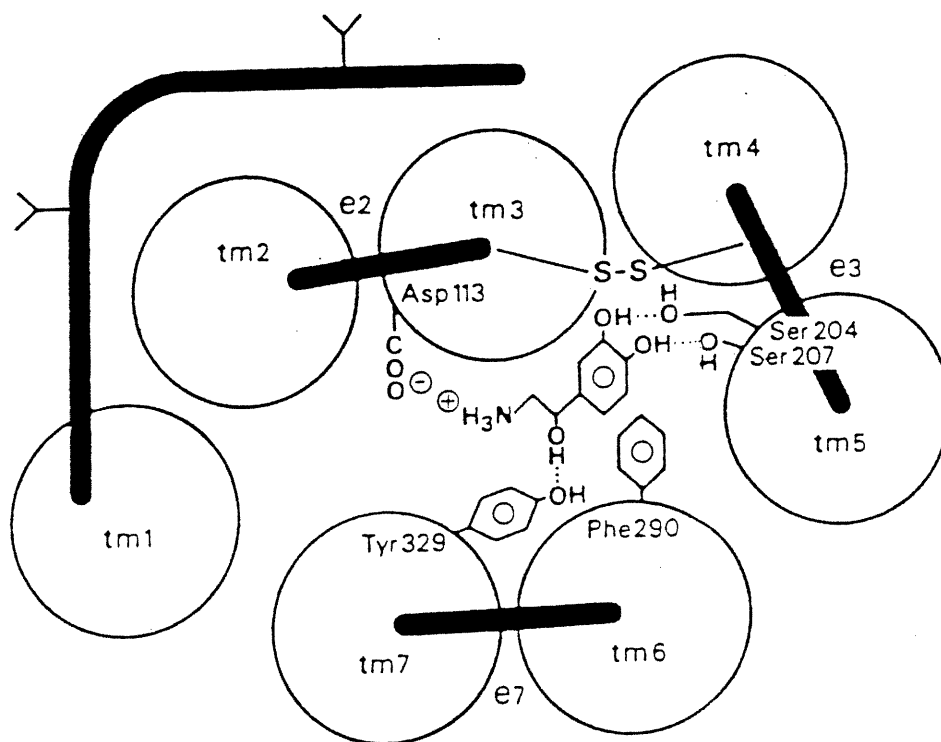
### 1.2.3.1.2 Les sites d'interaction $\beta_2$AR-protéine G

Parallèlement à la mise en évidence des déterminants moléculaires de l'interaction ligand-récepteur, des expériences de mutagenèse ont permis de localiser et d'identifier les déterminants moléculaires de l'interaction récepteur-protéine G.

Les régions du récepteur interagissant avec la sous-unité $\alpha$ des protéines G, devraient en principe se situer sur la face intracytoplasmique. La construction de récepteurs chimériques a permis de confirmer ce postulat. En effet, des constructions de récepteurs chimériques entre le $\beta_2$AR et le $\alpha_2$AR (Kobilka et al., 1988; Liggett et al., 1991) ou le $\alpha_1$AR (Cotecchia et al., 1990) ont permis de démontrer que la troisième boucle cytoplasmique participe significativement au couplage avec la protéine G. Des mutations ponctuelles réalisées au niveau de la troisième boucle cytoplasmique du $\beta_2$AR provoquent une diminution de l'efficacité de stimulation de l'adénylyl cyclase confirmant ainsi les résultats découlant des études réalisées avec les récepteurs chimériques (Hausdorff et al., 1990).

La troisième boucle cytoplasmique n'est pas la seule région impliquée dans le couplage du $\beta_2$AR avec la sous-unité $\alpha$. De fait, la substitution du résidu proline 138 (deuxième boucle cytoplasmique) par une sérine diminue de 60 % la stimulation de l'adénylyl cyclase (O'Dowd et al., 1988). Les domaines transmembranaires V et VI influencent également le couplage fonctionnel du $\beta_2$AR (Dohlman et al., 1991). En réalisant des chimères entre $\beta_2$AR et $\alpha_2$AR, Kobilka *et coll.* (Kobilka et al., 1988)

ont démontré l'importance de tous les domaines cytoplasmiques du $\beta_2AR$ pour stimuler l'adénylyl cyclase à un niveau maximal. Dans cet ordre d'idées, l'utilisation de peptides synthétiques entrant en compétition avec le récepteur pour la liaison à Gs s'est révélée une autre approche fructueuse pour élucider les sites d'interaction du $\beta_2AR$ avec la protéine Gs. Les résultats de ces études ont permis de corroborer l'importance de la deuxième boucle cytoplasmique, les portions C-terminale et N-terminale de la troisième boucle cytoplasmique, la région juxta-membranaire de la queue C-terminale (Munch et al., 1991) et la région transmembranaire VI (Hebert et al., 1996).

### 1.2.3.2   Les protéines G

En 1971, Rodbell et coll. (Rodbell et al., 1971) ont observé que l'activation de l'adénylyl cyclase suite à la stimulation du récepteur du glucagon (RCPG) nécessitait la présence de GTP. Cette observation a permis à l'équipe de Gilman (Ross and Gilman, 1977; Gilman, 1987; Hepler and Gilman, 1992) de mettre en évidence l'importance des protéines liant les nucléotides guanyliques (protéines G) dans la transduction du signal des RCPG.

Ces protéines G associées à la membrane plasmique sont composées de trois sous-unités polypeptidiques; la sous-unité $\alpha$ lie le GTP et possède une activité GTPasique permettant l'hydrolyse du GTP en GDP, et un dimère stable formé par les sous-unités $\beta$ et $\gamma$ (G$\beta\gamma$). Les protéines hétérotrimériqes forment une très vaste famille. On dénombre dix-sept sous-unités $\alpha$, cinq sous-unités $\beta$ et douze sous-unités $\gamma$. Par

homologie de séquence, les sous-unités $\alpha$ sont regroupées en quatre classes: Gs, Gi, Gq et G12. La plupart des protéines G$\alpha$ sont exprimées de façon ubiquitaire sauf les G$\alpha_t$, G$\alpha_g$ et G$\alpha_{15-16}$. Les protéines G$\alpha_t$ et G$\alpha_g$ sont respectivement exprimées au niveau des cellules de la rétine et de l'épithélium gustatif. En ce qui concerne les protéines G$\alpha_{15-16}$ exprimées dans des cellules hématopoïétiques, leurs rôles associés à leur expression demeurent inconnus (Dhanasekaran et al., 1998). Parmi les sous-unités $\beta$ (1 à 5), seul le profil d'expression de la sous-unité $\beta$5 semble spécifique. Cette dernière se retrouve au niveau de la rétine, des cellules neuronales et des plaquettes.

Plusieurs études ont démontré que la sous unité G$\beta$ en s'associant à la sous- unité G$\gamma$ peut activer différents effecteurs indépendamment de la sous unité G$\alpha$. En raison d'un profil d'expression cellulaire spécifique, il a été proposé que les nombreuses sous-unités $\gamma$ pourraient procurer aux dimères G$\beta\gamma$ une spécificité permettant l'activation d'effecteurs particuliers. Les dimères G$\beta\gamma$ peuvent activer une variété d'effecteurs comme la phospholipase A$_2$ (Jelsema and Axelrod, 1987), des canaux calciques (Lotersztajn et al., 1992) et potassiques (Yamada et al., 1993), l'adénylyl cyclase de type II et IV (Federman et al., 1992) et la phospholipase C (PLC) (Blank et al., 1992). Récemment, le groupe de Lefkowitz (Della Rocca et al., 1997) a démontré l'importance de l'activation de la PLC par G$\beta\gamma$ afin d'activer la voie de signalisation de la MAP kinase (*Mitogen-Activated Protein kinase*). Ces résultats viennent confirmer qu'il existe bien une interaction entre différentes voies de signalisation. Cette régulation croisée ne sera pas détaillée ici puisque ces travaux

demeurent extérieurs aux questions soulevées dans cette thèse. Si, pendant plusieurs années, l'activation d'effecteurs fût associée aux protéines Gα, il est certain maintenant que les dimères Gβγ jouent un rôle important dans la transduction du signal qui suit l'activation de RCPG.

Différents mécanismes permettent de réguler l'activité des protéines G. L'hydrolyse du GTP en GDP augmente l'affinité de la protéine Gα avec le dimère Gβγ favorisant un complexe hétérotrimérique (Gαβγ) inactif. Certaines protéines ayant une activité GAP (*GTPase activating protein*) peuvent moduler la vitesse d'hydrolyse des protéines Gα. Ces protéines appartiennent à la famille des RGS (*regulators of G-protein signaling*). Jusqu'à maintenant, seize RGS ont été identifiés dans des cellules de mammifères. Certains RGS vont augmenter d'un facteur de dix l'hydrolyse du GTP en GDP favorisant ainsi la formation du complexe inactif Gαβγ.

Récemment, des études de cristallographie ont révélé la structure et l'arrangement moléculaire d'un des membres de la famille des protéines G, $G\alpha_t\beta\gamma$ (Lambright et al., 1996). Cette étude a permis de mettre en évidence deux surfaces d'interaction entre les sous-unités $G\alpha_t$ et $G_t\beta$. Aucune interaction n'est observée entre $G\alpha_t$ et $G_t\gamma$. L'interface avec le récepteur entraîne l'interaction des trois sous-unités $G\alpha_t\beta\gamma$ favorisant ainsi une stabilité récepteur-protéine G. Enfin, il faut mentionner que la liaison du GTP modifie seulement la structure de la sous-unité $G\alpha_t$. Ce changement de conformation attribuable à la liaison du GTP sur $G_t\alpha$ permettrait un relargage passif des sous-unités $G_t\beta\gamma$ sans modifier leurs conformations très rigides.

### 1.2.3.3    L'adénylyl cyclase

C'est en travaillant sur les mécanismes d'action de l'adrénaline que Sutherland et

coll. (Sutherland, 1970) ont découvert l'adénosine monophosphate cyclique (AMPc)

et élaboré le concept de second messager hormonal. Il a fallu attendre environ quinze

ans avant d'avoir les premières preuves biochimiques et moléculaires concernant

l'existence d'une activité catalytique responsable de la formation d'AMPc (Pfeuffer

et al., 1985; Kruprinski et al., 1989).


L'adénylyl cyclase catalyse la transformation de l'ATP cytoplasmique en AMPc.

Cette enzyme  permet de produire une grande quantité d'AMPc suite à un seul cycle

d'activation de la protéine. L'adénylyl cyclase joue donc un rôle dans le phénomène

d'amplification du signal. Parmi les nombreux événements intracellulaires associés à

la production d'AMPc, un effet prédominant est l'activation de la protéine kinase

dépendante de l'AMPc (PKA). Cette enzyme peut moduler l'activité de nombreuses

protéines qui participent à la signalisation cellulaire et/ou à la régulation de

l'expression des gènes (Taussig and Gilman, 1995).


Chez les mammifères, neuf isoformes de l'adénylyl cyclase ont été identifiées

jusqu'à maintenant. Ces protéines contiennent deux domaines hydrophobes reliés par

une large portion cytoplasmique. Chaque domaine hydrophobe est composé de six

segments transmenbranaires. Afin d'identifier les régions de l'enzyme responsable de

l'activité catalytique, différentes portions de l'adénylyl cyclase ont été exprimées

dans *Escherichia coli* (Tang and Gilman, 1995). Les régions cytosoliques seules peuvent catalyser la formation de l'AMPc. Des travaux récents de cristallographie des portions cytoplasmiques de l'adénylyl cyclase ont permis de confirmer que ces régions forment effectivement un site de liaison pour l'ATP (Zhang et al., 1997; Tesmer et al., 1997). Ce même environnement protéique est également responsable de lier la forskoline, une molécule de diterpène activant directement l'adénylyl cyclase. Les connaissances de la structure moléculaire de cette famille d'enzymes permettent une meilleure compréhension des mécanismes de la catalyse. Cependant plusieurs questions demeurent sans réponses comme, par exemple, quelles sont les fonctions associées aux domaines transmembranaires de la protéine.

Plusieurs mécanismes ont été proposés pour expliquer la régulation de l'activité de l'adénylyl cyclase. D'une part, la phosphorylation par la PKA ou par la PKC influence l'activité de ces enzymes. D'autre part, les sous-unités $\beta\gamma$ peuvent, *via* une interaction directe, moduler l'activité de certaines adénylyl cyclases. Finalement, des variations de concentrations intracellulaires de $Ca^{2+}$ de l'ordre du nanomolaire peuvent influencer la production d'AMPc. Il faut mentionner que l'activité de chaque adénylate cyclase semble être modulée différemment par ces mécanismes (Tang and Hurley, 1998).

### 1.2.4    Aspect dynamique de la voie de signalisation du $\beta_2AR$

Le modèle d'activation le mieux accepté, nommé le modèle du complexe ternaire (agoniste-récepteur-G$\alpha$s) (De Lean et al., 1980) propose que la liaison de l'agoniste

sur le récepteur induit un changement conformationnel propagé jusqu'aux boucles intracytoplasmiques, capable alors d'interagir et de former un complexe avec les sous-unités $\alpha s\beta\gamma$ des protéines G. Suite à la formation d'un tel complexe, les sous-unités $\alpha s$ et $\beta\gamma$ seront libérées afin d'activer respectivement leurs effecteurs. En ce qui concerne la sous-unité $\alpha s$, la propagation du signal conformationnel agit sur $G\alpha s$ en modifiant l'affinité du site de liaison des nucléotides guanyliques favorisant l'échange du GDP par du GTP. Le complexe actif $G\alpha s$-GTP interagit directement avec l'adénylyl cyclase pour favoriser la formation d'AMPc. L'activité GTPasique de $G\alpha s$ permet l'hydrolyse du GTP en GDP favorisant ainsi la dissociation de $\alpha s$ avec l'adénylyl cyclase. La sous-unité $\alpha s$ liant le GDP peut de nouveau se lier aux sous-unités $\beta\gamma$. Les protéines G trimériques sont ainsi disponibles pour un autre cycle d'activation (Figure 3). Ce modèle propose l'existence chez le récepteur de deux états d'affinités différentes pour un agoniste dépendant de la liaison du récepteur avec $G\alpha s$. Il a été démontré qu'une activation de $G\alpha s$ par un analogue non hydrolysable (Gpp(NH)p) produit la conversion des sites de haute affinité en basse affinité suggérant ainsi que la haute affinité correspond au complexe agoniste-récepteur-$G\alpha s$. Lors de la formation du complexe Gs-GTP, la diminution de couplage entre le récepteur et $G\alpha s$ amènerait cette baisse d'affinité du récepteur pour l'agoniste. Une méthode très utilisée pour déterminer l'état de couplage du récepteur avec $G\alpha s$ consiste en l'analyse d'une courbe représentant l'inhibition de la liaison d'un antagoniste radioactif par des concentrations croissantes d'un agoniste (Figure 4). Ces courbes de compétition biphasiques représentent deux populations de récepteurs ayant des affinités distinctes pour l'agoniste. Il est généralement accepté que la proportion de récepteurs à haute affinité pour son agoniste est un index représentant la capacité du récepteur à se coupler à la protéine G. Lors du découplage récepteur-$G\alpha s$, la courbe ne présente qu'un seul site, celui de basse affinité pour l'agoniste comme il est illustré à la figure 4.

**Figure 3**     **Voie classique de signalisation du récepteur β$_2$-adrénergique.**
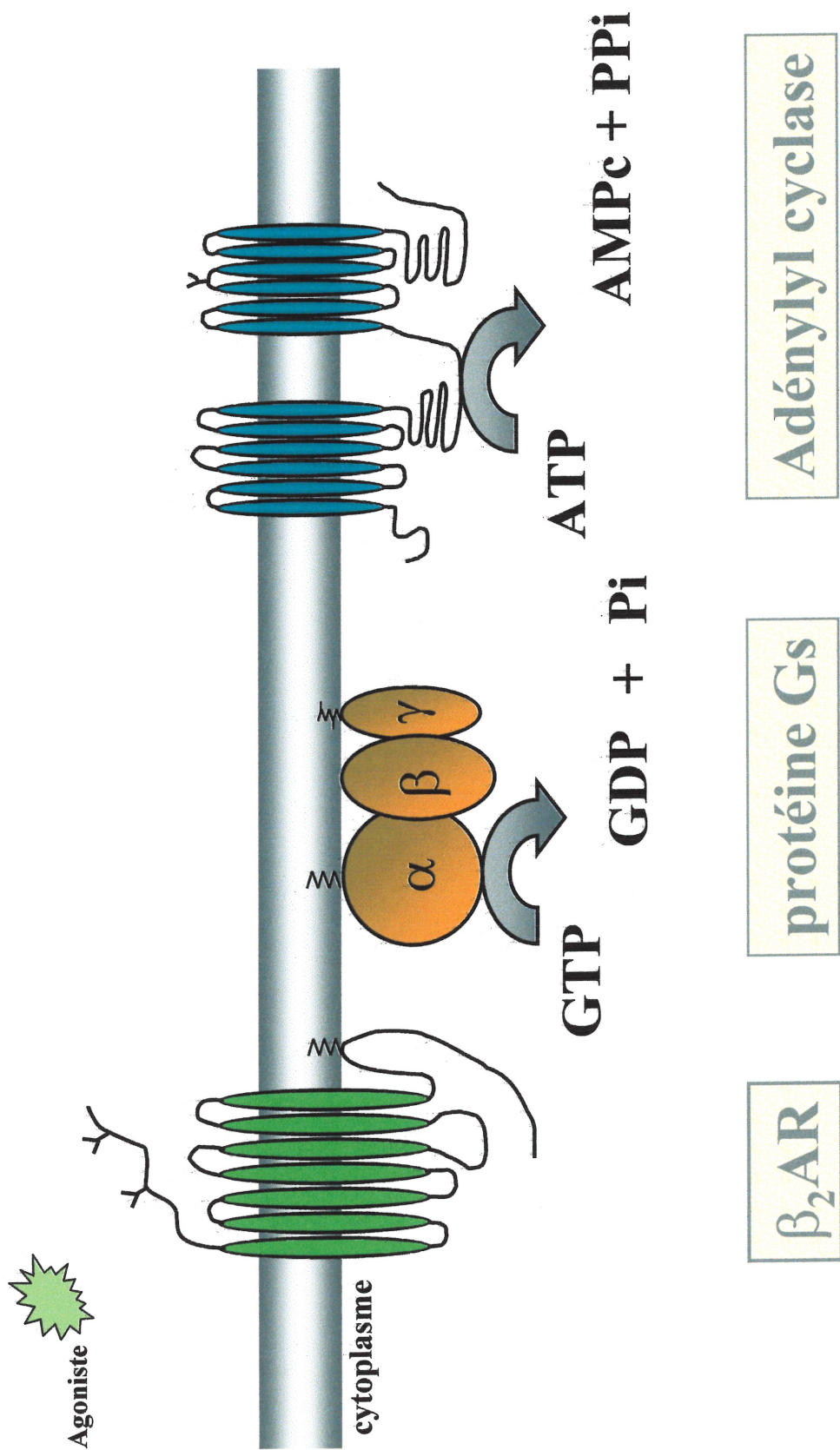
Figure 4       Représentation de la haute et de la basse affinité du récepteur $\beta_2$-adrénergique pour un agoniste.

Haute affinité

βAR   G<sub>S</sub>

Basse affinité

Gpp(NH)p

Basse affinité
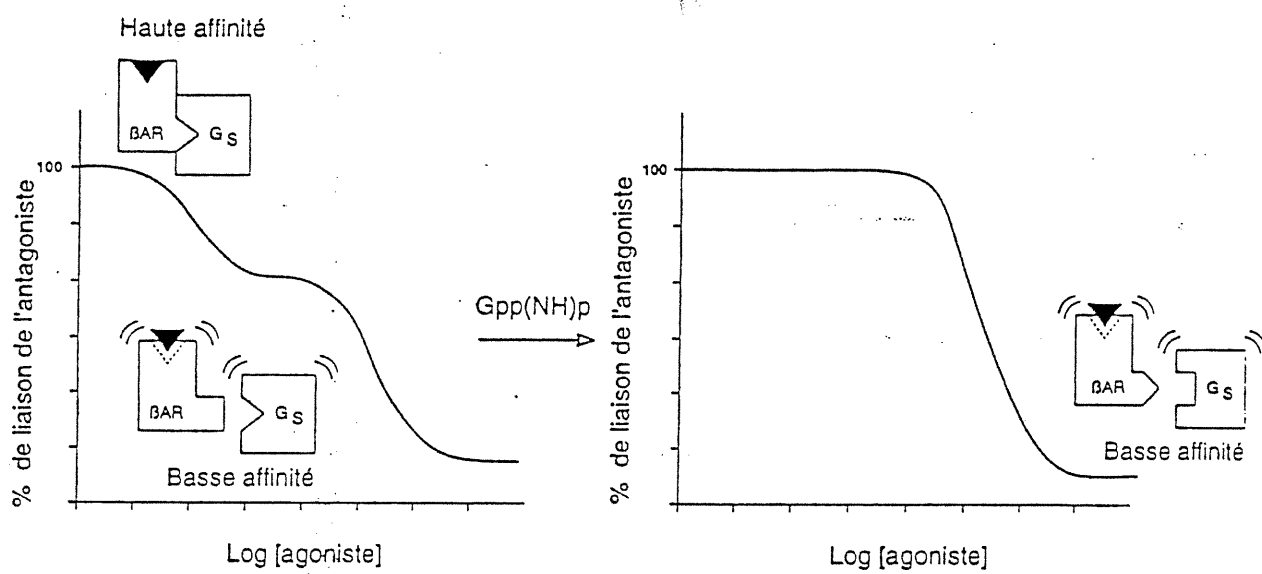
Il faut mentionner que le modèle ternaire ne permet pas d'inclure un certain nombre de résultats récemment publiés. D'une part, Chidiac et coll. (Chidiac et al., 1994) ont mis en évidence, en surexprimant le $\beta_2AR$ dans un système hétérologue, une activité basale élevée de l'adénylyl cyclase. D'autre part, une substitution dans la portion C-terminale de la troisième boucle cytoplasmique du $\beta_2AR$ produit un récepteur constitutivement actif en absence d'agoniste (Samama et al., 1993). Dans ces circonstances, le récepteur, même en absence d'agoniste, entraîne l'activation d'une protéine G et de son effecteur. Afin d'expliquer ces résultats, deux nouveaux modèles ont été proposés (modèle ternaire étendu et modèle cubique) (Samama et al., 1993; Weiss et al., 1996). Il ne s'agit pas ici de développer chacun des modèles mais plutôt de souligner le concept commun aux deux modèles permettant d'expliquer les résultats précédents. Ces modèles propose l'existence du récepteur sous deux états distincts au niveau basal: un état inactif (R) et un état actif (R*). Un équilibre dynamique existerait entre ces deux états. Ainsi, dans le contexte d'une densité accrue du récepteur $\beta_2AR$, l'isomérisation spontanée favorise une quantité suffisante de récepteur à l'état actif (R*) pour permettre la stimulation de $Gs\alpha$ et de l'adénylyl cyclase. En ce qui concerne le récepteur constitutivement actif, la mutation des résidus impliqués dans le maintien de la forme inactive favoriserait un état actif du récepteur. L'hypothèse voulant que le récepteur existe sous deux formes à l'état basal permet également d'expliquer un autre phénomène relativement récent, l'agoniste inverse.

Les antagonistes étaient considérés comme des molécules pouvant se lier aux récepteurs mais ne produisant aucune réponse cellulaire. Plusieurs travaux récents dont ceux de Chidiac et coll. (Chidiac et al., 1994) montrent clairement que des antagonistes peuvent diminuer la capacité du récepteur à stimuler l'adénylyl cyclase. Ces ligands ont été nommés agonistes inverses. Ces agonistes inverses auraient une

affinité supérieure avec la forme inactive du récepteur (R) plutôt qu'avec la forme active (R*). Il faut mentionner que ces modèles (étendu ou cubique) considèrent toujours le ratio : 1 ligand : 1 récepteur : 1 protéine G. Cependant, ces modèles excluent des résultats très récents concernant la dimérisation des RCPG.

L'existence d'homodimères a été démontrée pour plusieurs RCPG dont le $\beta_2AR$ (Hebert et al., 1996). Les études biochimiques et fonctionnelles sur la dimérisation ont montré qu'un peptide dérivé de la VI hélice transmembranaire du $\beta_2AR$ inhibe à la fois la formation de dimères ainsi que la capacité du récepteur à activer l'adénylyl cyclase (Hebert et al., 1996). Des études récemment publiées ont mis en évidence l'existence non pas d'homodimères mais d'hétérodimères des récepteurs GABA. La co-expression de différents sous-types de ces récepteurs ont permis: 1) d'exprimer correctement à la surface cellulaire certains sous-types, 2) d'augmenter l'affinité des récepteurs pour l'agoniste GABA (White et al., 1998), et 3) d'activer des canaux potassiques insensibles aux homodimèr (Jones al., 1998). Ces travaux sont les premiers à proposer des rôles fonctionnels pour des hétérodimères. Maintenant, il reste à savoir si ces observations peuvent également s'appliquer à d'autres RCPG.

## 1.2.5    La régulation du $\beta_2AR$

La voie de signalisation du $\beta_2AR$ est soumise à une régulation dynamique de son activité. Cette modulation peut être d'origine hétérologue si elle est la conséquence de l'activation d'une autre voie de signalisation ou homologue si elle résulte de l'activation de la voie adrénergique elle-même. La régulation homologue englobe plusieurs mécanismes de régulation de l'activité du récepteur, nous allons les décrire en détails dans cette section.

Suite à une stimulation prolongée du récepteur par un agoniste, la diminution de la réponse cellulaire induite est nommée désensibilisation. Dépendant de la durée de la stimulation, trois mécanismes peuvent être impliqués dans une telle désensibilisation: 1) un découplage fonctionnel très rapide entre le récepteur et la protéine Gα, 2) une séquestration du récepteur et 3) une régulation négative du nombre de récepteurs au niveau de la cellule entière.

### 1.2.5.1   Le découplage fonctionnel

Le découplage fonctionnel est caractérisé par une diminution de l'activité de l'adénylyl cyclase dès les premières minutes d'exposition à un agoniste. Au cours de cette désensibilisation à court terme, plusieurs études ont démontré qu'une augmentation de la phosphorylation du $\beta_2$AR est déterminante dans le découplage fonctionnel entre le récepteur et la protéine G$\alpha$s (Stadel et al., 1983; Lohse et al., 1990a; Kobilka, 1992). Parmi les protéines kinases responsables de phosphoryler le récepteur, la protéine kinase dépendante de l'AMPc (PKA) (Benovic et al., 1985) et la kinase du récepteur $\beta$-adrénergique ($\beta$ARK-1 ou GRK2 : *G protein-coupled Receptor Kinases*) (Benovic et al., 1987) sont les deux kinases importantes lors de la désensibilisation à court terme du $\beta_2$AR.

La PKA est un tétramère formé de deux sous-unités régulatrices et de deux sous-unités catalytiques. Ces deux dernières sous-unités seront activées lors de la liaison de quatre molécules d'AMPc favorisant leur séparation des sous-unités régulatrices (Alberts et al., 1989). Il existe respectivement trois et quatre isoformes des sous-unités régulatrices et catalytiques. La localisation dans la cellule de certains isoformes semble être régulée en partie par une protéine d'ancrage appelée AKAP (*A*

*Kinase Anchoring Protein*) (Dell'Acqua and Scott, 1997). Cette dernière favoriserait une compartimentalisation de la PKA permettant ainsi la phosphorylation de certains substrats spécifiques (Dell'Acqua and Scott, 1997).

Suite à l'analyse de la séquence primaire du $\beta_2$AR, il existe deux sites consensus de phosphorylation pour la PKA, l'un situé dans la portion C-terminale de la troisième boucle cytoplasmique ($^{261}$SSRR$^{264}$, premier site de PKA) et l'autre situé dans la région proximale de la queue cytoplasmique ($^{343}$RRSS$^{346}$, deuxième site de PKA) (Dixon et al., 1986). La mutation de ces deux sites de phosphorylation (Hausdorff et al., 1989) ou l'utilisation d'inhibiteur de la PKA (Lohse et al., 1990a) va produire une absence presque complète de la désensibilisation du récepteur dans les conditions expérimentales utilisées. A la lumière de ces résultats, les sites de phosphorylation par la PKA sont donc importants dans le mécanisme de désensibilisation du récepteur. Cependant, ces études ne permettent pas de distinguer le(s) rôle(s) de chaque site de phosphorylation pour la PKA. Plusieurs évidences suggèrent que le premier site de phosphorylation pour la PKA serait responsable du découplage fonctionnel entre le récepteur et la protéine G$\alpha$s. En effet, l'absence de ce site consensus pour la PKA diminue significativement la capacité du récepteur à être désensibilisé (Clark et al., 1989). De plus, une étude utilisant un peptide dont la séquence correspond à la portion C-terminale de la troisième boucle intracytoplasmique a confirmé l'importance du premier site de PKA dans le processus de découplage fonctionnel entre le récepteur et G$\alpha$s. Brièvement, le peptide seul a la capacité de stimuler *in vitro* et sa phosphorylation par la PKA inhibe

fortement son couplage avec la protéine G (Okamoto et al., 1991). Cela démontre le rôle de la phosphorylation des sérines 261 et 262 dans la suppression de l'interaction de cette région du récepteur avec Gαs. Comme nous l'avons précédemment mentionné, cette portion du récepteur est importante pour l'interaction du $\beta_2$AR avec Gαs. Ainsi, une perturbation de la conformation de cette région du récepteur attribuable au changement de polarité des résidus impliqués empêcherait le couplage du récepteur avec Gαs provoquant la désensibilisation du $\beta_2$AR (Clark et al., 1989).

Comme nous l'avons dit ci-dessus, l'activité de la PKA est régulée par la présence d'AMPc cellulaire. Dans ce contexte, la phosphorylation du récepteur par cette kinase peut avoir lieu sans toutefois activer la voie $\beta$-adrénergique. En effet, l'activation de l'adénylyl cyclase par d'autres récepteurs peut moduler l'activité fonctionnelle du $\beta_2$AR. Cette modulation est donc d'origine hétérologue puisqu'elle est la conséquence de l'activation d'une autre voie de signalisation (Kobilka, 1992).

En ce qui concerne le deuxième site de phosphorylation pour la PKA situé dans la portion C-terminale du récepteur, ce dernier peut être phosphorylé seulement dans des conditions de désensibilisation homologue. En effet, des études de phosphorylation sur des cellules entières ont démontré qu'en utilisant un analogue de l'AMPc, un activateur spécifique de la PKA, il n'y a pas d'augmentation de la phosphorylation chez le récepteur dont le premier site de PKA est muté comparativement au récepteur de type sauvage (Bouvier et al., 1989). Ces résultats suggèrent que le premier site de PKA est responsable de l'augmentation de la phosphorylation observée chez le récepteur de type sauvage. Des conclusions

semblables sont rapportées suite à des expériences de phosphorylation *in vitro* à l'aide de la PKA purifiée (Clark et al., 1989). À la lumière de ces résultats, il a été proposé que la phosphorylation de ce deuxième site de PKA situé dans la région proximale de la queue cytoplasmique dans la portion C-terminale du récepteur soit accessible pour être phosphorylé seulement au cours d'une désensibilisation homologue. Les mécanismes moléculaires sous-jacents à une telle spécificité de la phosphorylation du $\beta_2AR$ nous échappent toujours.

La famille des GRK, ces protéines kinases (sérine/thréonine) associées aux RCPG, comprend six isoformes dont un des prototypes est la kinase du récepteur $\beta$-adrénergique (GRK2) (Pitcher et al., 1998). La GRK2 est une enzyme cytosolique, dont l'ancrage membranaire se fait par l'interaction avec les sous-unités $\beta\gamma$ des protéines G, la sous-unité $\gamma$ étant elle-même associée à la membrane par isoprénylation (Pitcher et al., 1992). C'est seulement lorsque le $\beta_2AR$ est activé par son agoniste qu'il devient substrat pour la GRK2. Celle-ci peut alors le phosphoryler sur des sérines et thréonines spécifiques dans la portion C-terminale du récepteur (Fredericks et al., 1996). À la suite de cette phosphorylation, la $\beta$-arrestine, une protéine cytosolique est à son tour recrutée par le récepteur (Lohse et al., 1990b). La liaison de la $\beta$-arrestine sur le récepteur phosphorylé empêche ce dernier de se lier à la sous-unité $G\alpha s$. Comme nous l'avons mentionné antérieurement, une dissociation du récepteur avec la $G\alpha s$ provoque une diminution importante de l'affinité des ligands pour le $\beta_2AR$ (Pitcher et al., 1998).

Parmi les membres de la famille des GRK, la GRK3 et la GRK4 peuvent également phosphoryler le $\beta_2AR$. La spécificité de liaison de ces différents isoformes pour le

$\beta_2$AR semble être coordonnée par les différents isoformes $\beta\gamma$. L'activité GRK2 varie en effet en fonction des différents isomères G$\beta$ et G$\gamma$ composant le complexe G$\beta\gamma$ (Pitcher et al., 1998). Ainsi, les différentes isoformes $\beta\gamma$ dans une cellule permettraient de cibler les GRK appropriées aux RCPG .

Comme nous venons de le décrire, la désensibilisation homologue implique la participation de deux protéines kinases responsables de phosphoryler le $\beta_2$AR à la suite d'une stimulation par un agoniste. Une représentation schématique est illustrée à la figure 5. Ce mécanisme de désensibilisation permet de réguler rapidement l'activité non seulement du $\beta_2$AR mais également d'autres RCPG (Jaber and Giros, 1998). Cependant, il faut mentionner que la présence d'inhibiteurs de la PKA et de la GRK2 ensemble ne permet pas d'abolir complètement le découplage fonctionnel du $\beta_2$AR (Lohse et al., 1989; Lohse et al., 1990a). Dans ces conditions, il est possible que la spécificité des inhibiteurs soit douteuse. Cependant, en absence de tous les sites de phosphorylation pour la PKA et les GRK une désensibilisation est toujours apparente (Hausdorff et al., 1989). Ces résultats peuvent être expliqués par un phénomène de séquestration du récepteur.

Figure 5     Représentation schématique du découplage fonctionnel du récepteur $\beta_2$-adrénergique de la protéine G$\alpha$s par la phosphorylation.
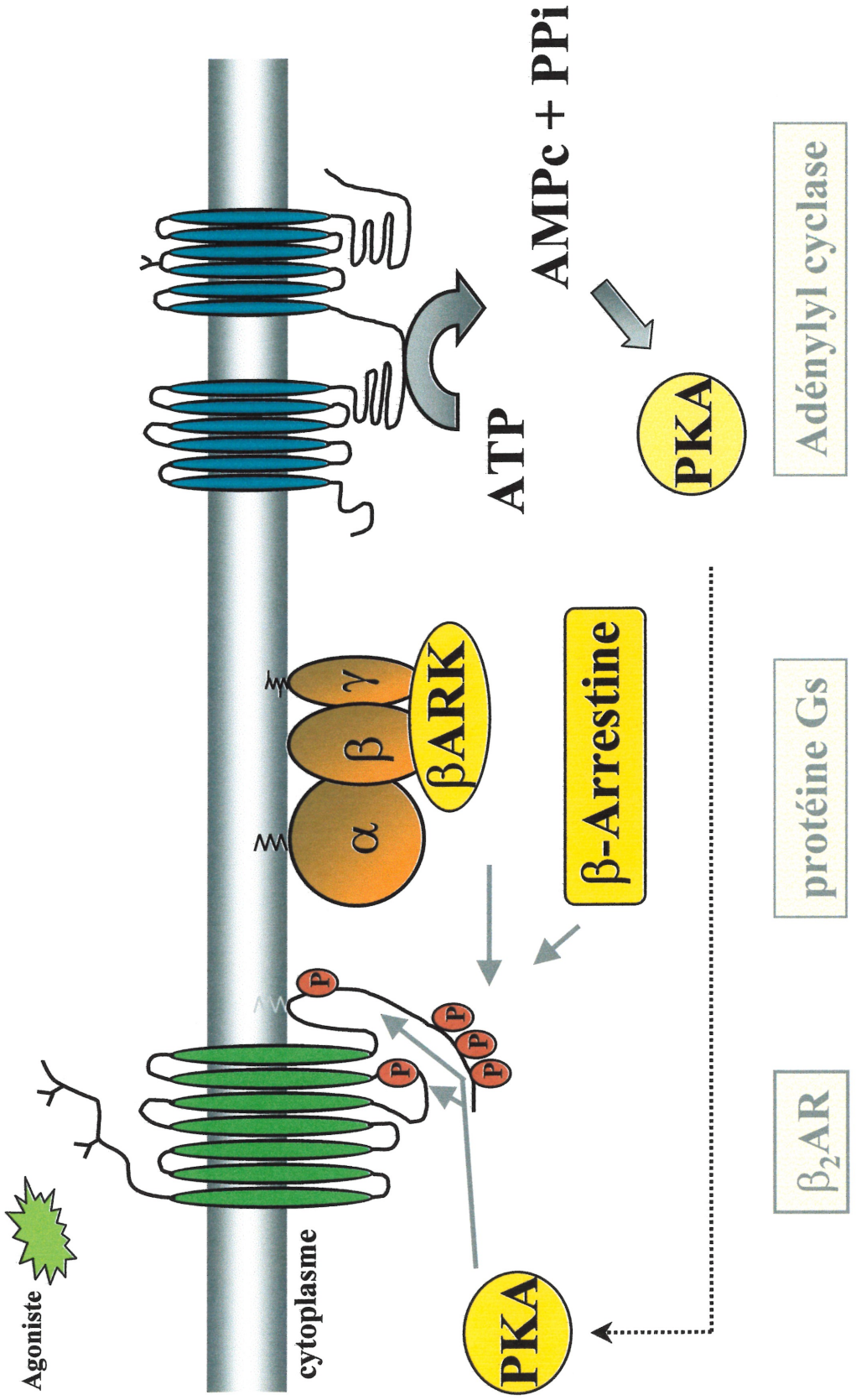
### 1.2.5.2   La séquestration

La séquestration survient rapidement après le découplage fonctionnel et se caractérise par une redistribution des récepteurs dans la cellule. Cette relocalisation des récepteurs provoque une diminution de l'accessibilité des récepteurs aux ligands hydrophiles tandis que la liaison reste toujours possible pour les ligands lipophiles. L'interprétation généralement acceptée veut que le récepteur suite à son activation se retrouve dans des vésicules légères à l'intérieur de la cellule. Ainsi, le récepteur ne peut plus stimuler l'adénylyl cyclase puisque la protéine Gαs est exclue de ces vésicules. Des études d'immunocytochimie et de fractionnements subcellulaires ont permis de localiser une proportion des récepteurs à l'intérieur de la cellule (Strosberg, 1992). Cette internalisation est-elle une conséquence de l'augmentation de la phosphorylation du $\beta_2$AR à la suite de son activation par un agoniste ou par un événement indépendant ?

Malgré de nombreuses publications concernant la séquestration, ce phénomène fait encore l'objet de nombreuses controverses. Des études rapportent que l'internalisation du $\beta_2$AR est indépendante de sa phosphorylation. En effet, l'addition d'inhibiteurs de sérine/thréonine kinases (Lohse et al., 1990a) ou la mutation des sites de phosphorylation pour la PKA et les sites potentiels pour la GRK ne préviennent pas l'internalisation induite par un agoniste (Hausdorff et al., 1989; Bouvier et al., 1988). De plus, le couplage du récepteur à Gαs ne semble pas nécessaire à la séquestration puisque dans la lignée cellulaire mutante de lymphome de souris S49 où Gαs est absente, le $\beta_2$AR a un profil de séquestration normal

(Mahan et al., 1985). Ainsi, l'internalisation pourrait être un phénomène indépendant de la phosphorylation permettant un découplage physique de sa voie de signalisation.

Cependant, Ferguson et coll. (Ferguson et al., 1995) ont mis en évidence qu'une substitution de la tyrosine 326 ($\beta_2$AR-Y$^{326}$A) dans le septième domaine membranaire du $\beta_2$AR provoque une diminution importante de l'internalisation accompagnée par une baisse de la phosphorylation du récepteur par la GRK2. La surexpression de cette kinase ramène à un niveau normal l'internalisation du mutant $\beta_2$AR-Y$^{326}$A. Des résultats similaires ont été observés en surexprimant GRK3, GRK4, GRK5 et GRK6 (Menard et al., 1996). Ainsi, l'ensemble de ces travaux permettent de supposer qu'une modification de la conformation engendrée par la phosphorylation permettrait à ce complexe protéique de pouvoir interagir avec d'autres protéines pour faciliter l'internalisation du $\beta_2$AR.

Récemment, la participation d'une telle protéine, la $\beta$-arrestine, a été mise en évidence (Ferguson et al., 1996). Un dominant négatif de la $\beta$-arrestine bloque l'internalisation du $\beta_2$AR même si la GRK2 est surexprimée. La $\beta$-arrestine, en plus de pouvoir se lier au récepteur phosphorylé par la GRK2, a la capacité de se lier directement à la clathrine (Goodman et al., 1996). Cette dernière protéine est une composante importante des vésicules tapissées de clathrines, une voie d'endocytose utilisée par plusieurs types de récepteurs comme par exemple les récepteurs des LDL (*Low Density Lipoprotein*) (Anderson et al., 1975). L'implication de la $\beta$-arrestine dans l'internalisation du $\beta_2$AR est directement reliée à sa capacité à lier la clathrine

puisque la substitution de différentes séquences de cette protéine par celles de l'arrestine-1, son homologue du système visuel, inhibe sa liaison à la clathrine et l'internalisation du récepteur. Ces études ont permis d'identifier les régions de liaison entre ces deux protéines. Ainsi la portion C-terminale de la $\beta$-arrestine se lie à la région N-terminale de la clathrine *via* des interactions ioniques et hydrophobes (Goodman et al., 1997). De plus, des essais d'immunofluorescence ont permis de colocaliser le $\beta_2AR$ et la $\beta$-arrestine avec la clathrine. Ainsi, la $\beta$-arrestine en se liant au $\beta_2AR$ phosphorylé favoriserait l'internalisation du récepteur *via* les vésicules tapissées de clathrines. À la suite de ces travaux, de nombreuses questions demeurent sans réponses: comment se forme le complexe récepteur-$\beta$arrestine-clathrine et quels sont les mécanismes de régulation ? Des travaux récents de Ménard et coll. (Ménard et al., 1997) ont corrélé la proportion d'internalisation du $\beta_2AR$ avec les différentes concentrations de GRK2 et de $\beta$-arrestines disponibles dans la cellule. Ainsi, la cinétique d'internalisation semble être influencée par la concentration des différentes protéines impliquées dans ce processus mais également par leurs affinités respectives entre elles. Ce nouveau modèle pourrait permettre de concevoir l'étiologie de certaines pathologies associées aux RCPG. Par exemple, dans certains cas de défaillances cardiaques, on détecte un niveau élevé de GRK2 responsable de l'augmentation de la phosphorylation du récepteur provoquant sa désensibilisation et donc une diminution de l'activité adrénergique (Jaber and Giros, 1998).

Plusieurs auteurs proposent que l'internalisation pourrait être un mécanisme favorisant la déphosphorylation du récepteur permettant ainsi au récepteur d'être

recyclé à la membrane plasmique. L'internalisation induirait donc une resensibilisation du récepteur. Cette hypothèse est soutenue par un ensemble de résultats, comme par exemple l'utilisation d'agents bloquant l'internalisation, tels que la concanavaline A ou le sucrose qui prévient la déphosphorylation et la resensibilisation des récepteurs (Yu et al., 1993). Des résultats similaires sont obtenus en inhibant l'activité phosphatase avec la caliculyne A (Pippig et al., 1995). Récemment des travaux de Krueger et coll. (Krueger et al., 1997) ont permis de colocaliser une phosphatase de type 2A (P2A) avec le $\beta_2$AR à la suite d'une stimulation par l'isoprotérénol. Un milieu acide serait nécessaire pour déphosphoryler le récepteur par la P2A. On retrouve ce pH au niveau des vésicules impliquées dans l'internalisation des RCPG. Ces auteurs (Krueger et al., 1997) indiquent qu'un environnement acide induirait un tel changement de conformation du récepteur pouvant ainsi être reconnu par la P2A.

En dépit des efforts déployés par de nombreux groupes de recherches, les déterminants moléculaires du $\beta_2$AR responsables de l'internalisation ne sont pas encore complètement définis. Des travaux récents de Jockers et coll. (Jockers et al., 1996) suggèrent que plusieurs régions cytoplasmiques sont impliquées dans le phénomène d'internalisation du $\beta_2$AR. Ils ont construit une série de récepteurs chimériques entre le $\beta_2$AR et le $\beta_3$AR mettant à profit le fait que le $\beta_3$AR n'est pas internalisé malgré sa séquence primaire similaire au $\beta_2$AR. La présence individuelle des différentes boucles intracellulaires ou de la partie C-terminale du $\beta_2$AR ne confère qu'un faible niveau d'internalisation au $\beta_3$AR. Toutefois, la présence de la

première et de la deuxième boucle cytoplasmique ainsi que la queue C-terminale du $\beta_2$AR permet au $\beta_3$AR d'avoir un profil d'internalisation similaire au $\beta_2$AR. Ainsi, plusieurs domaines cytoplasmiques sont requis suggérant qu'une conformation appropriée soit nécessaire pour l'internalisation.

Jusqu'à maintenant plusieurs évidences suggèrent que le $\beta_2$AR soit internalisé *via* les vésicules tapissées de clathrines. Cependant, certaines d'études proposent une deuxième voie d'internalisation pour le $\beta_2$AR *via* des vésicules non tapissées de clathrines (Raposo et al., 1989; Dupree et al., 1993). Ces études ont montré qu'en perturbant la voie d'internalisation des vésicules tapissées de clathrines, l'activation par un agoniste induirait toujours un certain pourcentage d'internalisation du $\beta_2$AR. De plus, des études de microscopie électronique ont permis de localiser le $\beta_2$AR associé à des vésicules lisses, non tapissées de clathrines. Il a été proposé que ces vésicules pouvaient être des caveolaes (Lisanti et al., 1994b). Ces vésicules (ouvertes) que l'on retrouve au niveau de la membrane cytoplasmique suscitent beaucoup d'intérêt depuis quelques années puisque plusieurs protéines impliquées dans la signalisation cellulaire y ont été localisées. Le $\beta_2$AR a été identifié dans une telle fraction membranaire riche en cavéoline, une protéine marqueur des caveolaes (Dupree et al., 1993). D'autres RCPG y ont été également localisés: le récepteur de l'endothéline A (Chun et al., 1994), le récepteur de la bradykinine (De Weerd and Leeb-Lundberg, 1997), le récepteur muscarinique M2 (Pals-Rylaarsdam et al., 1997) et le récepteur de la cholécystokinine (Roettger et al., 1995). En ce qui à trait à ces deux derniers récepteurs, ils peuvent être internalisés par la voie classique de

vésicules tapissées de clathrines mais également par une autre voie apparentée aux caveolaes. Ces résultats préliminaires permettent de suggérer que les RCPG pourraient emprunter une voie d'internalisation autre que celle des vésicules tapissées de clathrines, ces autres voies demeurant pour l'instant peu caractérisées.

### 1.2.5.3    La régulation négative

Un autre mécanisme responsable de la désensibilisation du $\beta_2AR$ correspond à une diminution du nombre total de récepteurs exprimés au niveau de la cellule. Ce phénomène appelé régulation négative peut être observé suite à une stimulation prolongée par un agoniste (Benovic et al., 1988). Au moins deux phénomènes contribueraient à cette régulation négative. Une dégradation du $\beta_2AR$ (Doss et al., 1981; Frederich et al., 1983) ainsi qu'une diminution de l'ARNm correspondant au récepteur (Hadcock and Malbon, 1988; Danner and Lohse, 1997) sont les deux mécanismes sous-jacents à la régulation négative. Ces derniers seront successivement discutés.

### 1.2.5.3.1    Dégradation du $\beta_2AR$

Le niveau d'expression du $\beta_2AR$ résulte d'un équilibre dynamique entre la synthèse et la dégradation du récepteur (Mahan et al., 1985). Plusieurs études ont dévoilé qu'à l'état basal la demi-vie du $\beta_2AR$ est généralement supérieure à 20 heures (Kobilka, 1992). La demi-vie du $\beta_2AR$ est grandement affectée par la présence d'agonistes. En effet, une période de stimulation de quelques heures du récepteur par un agoniste provoque une diminution significative de sa demi-vie (Kobilka, 1992). Une dégradation des récepteurs constituerait une conséquence de l'occupation du $\beta_2AR$

par un agoniste. Néanmoins, une diminution du nombre total de récepteurs peut également être observée indépendamment de la présence d'un agoniste. En effet, une augmentation d'AMPc dans la cellule soit par des analogues perméables d'AMPc (comme par exemple le dibutyryl AMPc ou le 8-bromo AMPc), soit par des agents favorisant l'augmentation d'AMPc comme par exemple la forskoline ou, soit par des inhibiteurs des phosphodiestérases peuvent diminuer jusqu'à 50% le nombre de récepteurs (Hadcock and Malbon, 1988; Collins et al., 1989). Ces résultats suggèrent fortement que la PKA soit responsable de cette diminution. Afin d'évaluer l'importance de la phosphorylation du $\beta_2AR$ par la PKA dans ce phénomène de régulation négative, Bouvier et coll. (Bouvier et al., 1989) ont produit un récepteur muté pour lequel les sites consensus de la PKA sont absents. Ce mutant possède un profil de dégradation inférieur à celui du récepteur de type sauvage. Néanmoins, la régulation négative résiduelle de ce mutant suggère l'implication d'un deuxième mécanisme. De fait, les auteurs observent une diminution importante du niveau d'ARNm du $\beta_2AR$ associée à la perte de récepteurs à la suite de l'augmentation de la concentration d'AMPc intracellulaire. Ainsi, ces résultats proposent que l'AMPc favorise la régulation négative par deux mécanismes différents : la phosphorylation du récepteur par la PKA et la réduction de son niveau d'ARNm. Il faut toutefois souligner qu'une stimulation du récepteur par un agoniste favorise une régulation négative supérieure (80%) à celle obtenue suite à une augmentation d'AMPc cellulaire (50%) (Bouvier et al., 1989; Danner and Lohse, 1997). Ainsi, l'AMPc contribuerait à la diminution du niveau d'ARNm du $\beta_2AR$ alors que la stimulation du récepteur par l'agoniste favoriserait en plus la dégradation de ce dernier.

Sachant que l'activation du récepteur par un agoniste favorise sa phosphorylation par la GRK, des études ont été réalisées afin de vérifier l'implication de cette

augmentation de la phosphorylation du récepteur sur la régulation négative (Strader et al., 1987; Campbell et al., 1991). Cette dernière n'est aucunement affectée chez le récepteur pour lequel les sites potentiels de la GRK ont été mutés. Toutefois, des études récentes ont démontré que la GRK et la β-arrestine sont des facteurs déterminants pour l'internalisation du récepteur (Ferguson et al., 1995; Ferguson et al., 1996; Goodman et al., 1996; Goodman et al., 1997) (voir section Le découplage fonctionnel). Considérant le fait que l'internalisation du récepteur soit requise pour la dégradation du récepteur (Von Zastrow and Kobilka, 1992), il serait plausible dans ces conditions que la phosphorylation du récepteur par la GRK puisse être impliquée dans la régulation négative. Des études plus approfondies sont requises afin d'évaluer l'implication réelle de la GRK dans la régulation négative.

### 1.2.5.3.2   Régulation du niveau d'ARNm du $\beta_2$AR

Une stimulation du récepteur par un agoniste ou une augmentation de l'AMPc cellulaire favorise une diminution du nombre de récepteurs accompagnée par une baisse du niveau de l'ARNm du $\beta_2$AR. Cette régulation au niveau de l'ARNm a été observée dans une variété de cellules de mammifères telles les cellules CHW et LTK$^-$ (Nantel et al., 1994; Bouvier et al., 1989), les DDT1 MF-2 (Hadcock and Malbon, 1988) et les S49 (Hadcock et al., 1989a). Cela souligne l'importance de ce phénomène régulateur. Parmi les mécanismes suggérés pour expliquer cette baisse d'ARNm, une régulation au niveau de la stabilité de l'ARNm du $\beta_2$AR a été proposée. Ce type de régulation négative de l'ARNm a été mis en évidence la première fois par le groupe de Lefkowitz (Bouvier et al., 1989). Dans cette étude, les auteurs ont exprimé le $\beta_2$AR sous le contrôle du promoteur SV40 et non de son

promoteur original de façon à éviter tout effet de l'AMPc sur des événements transcriptionnels. Ainsi, une régulation négative de la transcription du gène ne peut donc être considérée comme un facteur influençant la diminution de l'ARNm du β2AR. Une baisse importante de l'ARNm observée suite à une augmentation de l'AMPc cellulaire a permis aux auteurs de conclure à une diminution de la stabilité de l'ARNm. Cet effet sur la demi-vie de l'ARNm a également été observé par Hadcock et coll. (Hadcock et al., 1989b). L'utilisation de l'actinomycine D a été prônée par ce groupe afin d'inhiber la transcription. Dans ces conditions expérimentales, un traitement avec l'isoprotérénol a provoqué une diminution de l'ordre de 50% de la demi-vie de l'ARNm du β2AR. En somme, ce processus de dégradation de l'ARNm joue donc un rôle important dans la régulation négative.

Nous avons décrit jusqu'à maintenant plusieurs mécanismes régulant l'activité du β2AR. Il a été question entre autres de la phosphorylation, cette modification post-traductionnelle responsable du découplage fonctionel entre le récepteur et la protéine Gαs. Récemment, une autre modification post-traductionnelle a été identifiée sur le β2AR, la palmitylation. Dans la prochaine section, la palmitylation sera traitée afin de mieux cerner sa nature, sa fonction et sa régulation dynamique. Sachant que la palmitylation n'est pas la seule modification des protéines par les lipides, nous allons décrire brièvement ces autres modifications lipidiques.

## 1.3    L'acylation des protéines

En 1951, la solubilisation de protéines dans des solvants organiques a permis de mettre en évidence des protéines modifiées par des lipides (Folch and Lees, 1951). Par leur nature lipidique, ces modifications ont longtemps été associées à un rôle

passif d'ancrage à la membrane. Aujourd'hui, quatre types principaux de modifications covalentes des protéines par les lipides ont été identifiés et caractérisés. Ces modifications sont la glypiation, l'isoprénylation, la myristylation, la palmitylation (Casey, 1995) et la cholestérylation (Porter et al., 1996b). Le tableau II résume les différentes caractéristiques de ces modifications lipidiques.

**Tableau II**      **Les caractéristiques des modifications covalentes des protéines par les lipides.**

| Modification | Structure | Résidu modifié / Type de liaison | Stade | Réversibilité de la liaison | Localisation |
|---|---|---|---|---|---|
| glypiation | GPI | cystéine / amide | Co-traductionnelle | non | membrane extracellulaire |
| isoprénylation | isoprène | | | | |
| •farnésylation | 15 carbones | cystéine / thioéther | post-traductionnelle | non | cytosol et membrane |
| •géranylgéranylation | 20 carbones | cystéine / thioéther | post-traductionnelle | non | cytosol et membrane |
| myristylation | acide gras saturé 14 carbones | glycine / amide | co-traductionnelle | non | cytosol et membrane |
| Palmitylation | acide gras saturé 16 carbones | cystéine / thioester | post-traductionnelle | oui | membrane |
| cholestérylation | cholestérol | ester | post-traductionnelle | non | membrane extracellulaire |

## 1.3.1    La glypiation

La glypiation consiste en l'ajout d'un groupement glycosylphosphatidylinositol (GPI) à des protéines qui seront sécrétées et ancrées via cette modification à la surface extracytoplasmique de la membrane. Le groupement GPI se compose d'un phosphatidylinositol inséré dans la membrane plasmique et séparé de la protéine par des sucres et des groupements éthanolamines (Udenfriend, 1995).

Les protéines soumises à une telle modification co-traductionnelle peuvent être identifiées selon leur profil d'hydropathicité. En général, deux domaines hydrophobes situés aux extrémités N- et C-terminales caractérisent ces protéines. La séquence en N-terminale permet le ciblage et la translocation de la protéine dans le réticulum endoplasmique (RE). Par la suite, cette séquence sera clivée par une peptidase située dans la lumière du RE. La séquence en C-terminale est reconnue par une enzyme de la classe des transamidases dont la séquence primaire n'est pas encore connue. Le GPI ancré dans la membrane du RE est transféré en entier sur la protéine par l'intermédiaire de cette enzyme. Finalement, la protéine sera sécrétée et ancrée par l'intermédiaire du GPI à la membrane plasmique extracellulaire où elle pourra exercer ses fonctions (Udenfriend, 1995; Nosjean et al., 1997).

La glypiation n'est pas limitée à une seule classe de protéines. On retrouve des récepteurs (récepteur du folate, récepteur d'immunoglobuline GFcγ) et des enzymes (phosphatase alcaline, lipase lipoprotéine) dont les fonctions sont très variées (Nosjean et al., 1997). Même si les rôles exacts de la glypiation ne sont pas clairement définis, une des fonctions potentielles de cet ancrage est d'augmenter la mobilité membranaire de ces protéines modifiées avec le GPI comparativement aux protéines associées à la membrane par une séquence peptidique (Udenfriend, 1995).

## 1.3.2    La prénylation

La prénylation (ou l'isoprénylation) des protéines consiste en l'ajout d'un groupement farnésyl ou géranylgéranyl *via* une liaison thioéther sur une cystéine en position C-terminale. Le farnésyl et le géranylgéranyl, deux lipides non saturés, sont des enchaînements linéaires de molécules d'isoprènes. Le farnésyl se compose de quinze carbones et représente l'assemblage de trois unités d'isoprène, alors que le géranylgéranyl comporte vingt carbones et correspond à l'addition de quatre unités d'isoprène (Clarke, 1992).

Les enzymes responsables de ces modifications post-traductionnelles sont connues sous le nom d'isoprényltransférase. Deux classes d'enzymes cytoplasmiques ont été caractérisées: la farnésyltransférase (FT) et la géranylgéranyltransférase (GGT). La première catégorie correspond à une enzyme catalysant la farnésylation sur des protéines possédant un motif CAAX à l'extrémité C-terminale (C: cystéine, A: résidu à caractère aliphatique) où X représente une méthionine, une sérine, une glutamine ou une alanine (Casey, 1992). Contrairement à la farnésyltransférase, deux classes de géranylgéranyltransférase  (classe I et II) ont été identifiées. La GGT de classe I reconnaît le motif CAAL situé à l'extrémité C-terminale de la protéine (C: cystéine, A: résidu à caractère aliphatique et L: leucine). Les séquences CC ou CXC se retrouvant soit à l'extrémité C-terminale où soit à l'intérieure de la séquence protéique seront reconnues par la GGT de classe II. Il faut souligner que les protéines dont la séquence en C-terminale est AAX subiront à la suite de la prénylation, une méthylation. Cette dernière réaction implique une protéolyse des trois derniers acides aminés (AAX) avant la carboxyméthylation de la cystéine à l'extrémité C-terminale (Casey, 1992). Jusqu'à très récemment, aucune évidence de l'hydrolyse du

groupement prényl n'avait été rapportée. Cependant, le groupe de recherche du Dr P. Casey vient de cloner une protéase humaine ayant la capacité de cliver l'extrémité C-terminale des protéines prénylées (Otto et al., 1999). Une caractérisation approfondie de cette protéase permettra de déterminer son rôle exact dans le processus de la prénylation.

Plusieurs protéines impliquées dans la signalisation cellulaire sont prénylées. Parmi celles-ci, nous retrouvons la kinase de la rhodopsine (Inglese et al., 1992), la sous-unité γ de certaines protéines G trimériques (Ohguro et al., 1991), les sous-unités α et β de la GMPc phosphodiestérase de la rétine (Qin et al., 1992) et les protooncogènes de la famille *ras* (Newman and Magee, 1993). Pour plusieurs protéines prénylées, dont celles mentionnées précédemment, le rôle de la prénylation est de favoriser l'ancrage des protéines acylées à la surface cytosolique des membranes lipidiques où elles peuvent exercer leurs fonctions. Par exemple, la mutation de la cystéine prénylée chez le protooncogène *ras* provoque son relargage dans le cytosol et son inactivation (Hancock et al., 1989). Toutefois, les protéines prénylées ne sont pas nécessairement associées à la membrane. Dans certain cas, l'acylation permettrait à des protéines cytosoliques d'interagir entre elles. À cet égard, les protéines solubles Rab3A et p21$^{rho}$ forment un complexe stœchiométrique 1:1 seulement si ces dernières sont prénylées (Araki et al., 1991). Ce second rôle de la prénylation permet d'envisager qu'une protéine pourrait osciller entre la membrane et le cytosol en interagissant avec des récepteurs cytosoliques ou même membranaire par l'intermédiaire de son groupement prényl. Un tel mécanisme a d'ailleurs été proposé pour les petites protéines G (Marshall, 1993).

### 1.3.3    La myristylation

Le myristate est un acide gras saturé de 14 carbones transféré sur les glycines N-terminales de certaines protéines *via* la formation d'une liaison amide (Schmidt, 1989). Cette modification est catalysée par la N-myristyl transférase (NMT) cytosolique. Celle-ci reconnaît la séquence MGXXXS/T (M: méthionine, G: glycine, X n'importe quel acide aminé, S: sérine ou T: thréonine) à l'extrémité N-terminale de la protéine. Suite au clivage de la méthionine initiatrice, l'enzyme transférera le myristate à partir du donneur, myristyl-CoA, sur la glycine en N-terminale de la protéine. Cette modification co-traductionnelle est irréversible durant la vie de la protéine. Cependant, une activité enzymatique responsable de cliver le myristate a été identifiée récemment dans une fraction cytosolique de synaptosomes de cerveau bovin (Manenti et al., 1994).

À l'instar des protéines prénylées, une proportion importante de protéines myristylées font parties de la signalisation cellulaire. Parmi celles-ci, on retrouve des protéines de la famille des tyrosine kinases (src, lyn, yes, fyn, yrk, hck, fgr, lck et blk ) (Resh, 1994), la sous-unité $\alpha$ de nombreuses protéines G ($G_i$, $G_o$, $G_z$ et $G_t$) (Casey, 1994) et la sous-unité catalytique de la protéine kinase A (PKA) (Resh, 1996). Un grand nombre de protéines myristylées se retrouvent ancrées à des membranes de divers compartiments cellulaires où elles peuvent ainsi exercer leurs fonctions (Bhatnagar and Gordon, 1997). Toutefois, il faut mentionner que l'énergie de liaison d'un myristate seul est insuffisante pour assurer l'ancrage d'une protéine à la membrane. Ainsi, la présence d'une deuxième modification lipidique (prénylation ou palmitoylation) ou encore une séquence d'acides aminés hydrophobes ou basiques permettra d'augmenter l'énergie de liaison requise pour lier une protéine myristylée

à la membrane (Silvius and L'Heureux, 1994; Resh, 1996). Parmi les protéines myristylées, MARCKS (M*yristoylated Alanine-rich C Kinase Substrate),* est une des premières protéines qui a permis aux chercheurs d'observer ce phénomène de coopération entre la myristylation et des résidus basiques (Thelen et al., 1991; Buser, 1994; Swierczynski and Blackshear, 1995).

Il est certain que la myristylation d'une protéine facilite son interaction avec la membrane. Mais toutes les protéines myristylées ne sont pas nécessairement ancrées à des membranes (Resh, 1996). Donc, il doit exister d'autres fonctions associées à la myristylation des protéines cytosoliques. À cet égard, il a été proposé que la myristylation puisse favoriser une plus grande stabilité de la recoverine cytosolique. Celle-ci fait partie de la grande famille des protéines liant le calcium (Bhatnagar and Gordon, 1997). En absence de calcium, le myristate de la recoverine s'insère dans une cavité hydrophobique formée par la protéine favorisant ainsi la stabilité de la forme cytosolique. La liaison du calcium à la recoverine provoque une translocation de cette dernière à la membrane. Ainsi, le calcium semble responsable de cette relocalisation en induisant un changement conformationnel de la protéine et par le fait même de l'orientation du myristate. Ce dernier n'étant plus enfoui dans la cavité hydrophobique de la recoverine, il contribuerait à la liaison de la protéine à la membrane (Ames et al., 1995). À la suite de la résolution de la structure cristallographique de la PKA (protéine kinase A), un phénomène de stabilité structurale engendré par sa myristylation a également été proposé pour cette protéine (Sowadski et al., 1996).

### 1.3.4 La cholestérylation

### 1.3.5 La palmitylation

La palmitylation est l'ajout d'un acide gras saturé de 16 carbones sur une cystéine d'une protéine *via* une liaison thioester. La distinction majeure entre la palmitylation et les autres modifications lipidiques se retrouve au niveau de sa liaison thioesther labile. Cette liaison est sensible à des pH basiques et peut être clivée par des agents réducteurs. Afin de vérifier la présence du lien thioester, l'hydroxylamine à pH neutre est couramment utilisé pour hydrolyser ce lien et libérer ainsi le palmitate de la protéine (Schmidt, 1989). Son utilisation permet de distinguer la présence de liaisons thioesthers responsables de la palmitylation des liaisons amides (myristylation ou glypiation) ou des liaisons thioéthers (isoprénylation) insensibles à une telle hydrolyse.

La palmitylation est particulièrement fréquente pour des protéines impliquées dans le processus d'adhérence cellulaire, de régulation de la croissance et de la signalisation cellulaire. Parmi ces protéines on retrouve H-RAS (Hancock et al., 1989), GAP-43 (Skene and Virag, 1989), des tyrosines kinases de la famille p60$^{src}$ tels que p56$^{lck}$ (Paige et al., 1993) et p56$^{hck}$ (Shenoy-Scarcia et al., 1994) ainsi que plusieurs sous-unités $\alpha$ des protéines G hétérotrimériques (Mumby, 1997). Pour la majorité de ces protéines, la réversibilité de cette modification post-traductionnelle module leurs interactions avec la face interne de la membrane plasmique où elles peuvent exercer leurs fonctions biologiques (Dunphy and Linder, 1998). Cependant, plusieurs

## La cholestérylation

La cholestérylation correspond à l'ajout d'une molécule de cholestérol sur une cystéine d'une protéine via une liaison ester. Cette modification permet l'ancrage de la protéine à la membrane plasmique du côté extracellulaire. Pour l'instant, la cholestérylation a été rapportée pour une seule protéine, la protéine Hedgehog (Porter et al. 1996a). Cette protéine impliquée dans la signalisation lors du développement embryonnaire s'ancrerait dans la membrane plasmique pour exercer son rôle grâce à l'ajout d'une molécule de cholestérol. Les mécanismes moléculaires responsables de la cholestérylation sont très peu caractérisés. Il a été cependant proposé qu'un mécanisme autocatalytique soit responsable de la cholestérylation de la protéine Hedgehog (Porter et al. 1996b).

protéines intrinsèques de la membrane sont aussi palmitylées, ce qui suggère que des fonctions autres que le ciblage des protéines vers la membrane pourraient être attribuées à cette modification. Les mécanismes de régulation de la palmitylation ne sont pas encore très bien identifiés. Même si aucune séquence primaire spécifique ne semble être requise pour la palmitylation des protéines, plusieurs groupes de chercheurs ont proposé qu'une activité enzymatique soit responsable du cycle de la palmitylation-dépalmitylation.

Au cours des prochaines sections, nous discuterons de la régulation de la palmitylation, de ses différents rôles proposés pour un ensemble de protéines impliquées dans la signalisation cellulaire dont les RCPG. Finalement, les objectifs de cette thèse seront élaborés en relation avec nos connaissances actuelles concernant la palmitylation du $\beta_2$AR.

## 1.3.5.1 Enzymologie de la palmitylation

Depuis l'identification des premières protéines palmitylées au début des années 1980 (Schmidt and Schlesinger, 1979; Schlesinger et al., 1980; Omary and Trowbridge, 1981; Magee and Schlesinger, 1982; Magee and Courtneidge, 1985) deux caractéristiques de cette modification semblent être communes à plusieurs protéines. D'une part, la demi-vie de l'acide palmitique sur les protéines est plus courte que celle des protéines elles-mêmes (Magee et al., 1987; Paige et al., 1993; Wedegaertner and Bourne, 1994). D'autre part, la vitesse du cycle de la palmitylation-dépalmitylation est grandement influencée par l'état d'activation de ces

protéines (Wedegaertner and Bourne, 1994; Robinson et al., 1995; Hayashi and Haga, 1997). À la suite de ces constatations, une des questions qui se pose est de savoir quelle est la nature de la régulation de cette modification. Plusieurs groupes de recherche ont proposé que des activités enzymatiques pouvant catalyser la palmitylation (palmityl-transférase (PAT)) et la dépalmitylation (palmityl-thioestérase (PTT)) des protéines pourraient réguler la réversibilité de cette modification.

Depuis plusieurs années de nombreuses équipes ont essayé d'isoler et de caractériser la protéine responsable de l'activité PAT (Berger and Schmidt, 1984; Berger and Schmidt, 1985; Bizzozero and Lees, 1986; Gutierrez and Magee, 1991; Schmidt et al., 1995; Berthiaume and Resh, 1995; Dunphy et al., 1996; Veit et al., 1998). En utilisant des extraits cellulaires en présence d'un donneur de palmitate, le palmityl-CoA, ces études *in vitro* ont permis d'identifier des activités PAT membranaires. Récemment, deux protéines membranaires avec une telle activité PAT ont été identifiées (Liu et al., 1996; Das et al., 1997). L'équipe de Liu et coll. (Liu et al., 1996) a purifié une protéine de foies de rats ayant la capacité *in vitro* de palmityler la protéine H-RAS. En SDS-PAGE, cette protéine correspond à un doublet de 30 et 33 kDa. Cependant, il faut mentionner que cette dernière a récemment été identifiée comme étant la 3-oxoacyl-CoA thiolase A (Liu et al., 1999). La deuxième protéine purifiée ayant une activité PAT a été isolée à partir d'érythrocytes humains (Das et al., 1997) et son poids moléculaire est de 70 kDa. Dans cette dernière étude, la spectrine a servi de substrat pour réaliser les expériences de palmitylation. Celle-ci

est une protéine palmitylée abondante du cytosquelette. Même si la caractérisation de ces deux protéines PAT demeurent préliminaires, il faut mentionner que le palmityl-CoA est le donneur acylé avec lequel les deux protéines ont démontré la plus grande affinité (comparativement au myristyl-CoA et au stearyl-CoA) (Liu et al., 1996; Das et al., 1997). La spécificité des ces protéines pour leur substrat renforce l'hypothèse voulant que des protéines soient responsables de la dépalmitylation. De plus, l'isolement de ces protéines avec une telle activité PAT à la membrane correspond aux résultats d'un grand nombre d'études suggérant que le ciblage à la membrane plasmique de plusieurs protéines est un pré-requis à leur palmitylation (Dunphy and Linder, 1998).

Il faut toutefois mentionner qu'une controverse existe à l'effet que la palmitylation soit une réaction nécessitant la présence d'une protéine avec une activité PAT. En effet, certaines protéines peuvent être palmitylées *in vitro* en absence d'une telle activité enzymatique (O'Brien et al., 1987; Bizzozero.O.A. et al., 1987; Quesnel and Silvius, 1994; Duncan and Gilman, 1996; Banó, 1998). Parmi ces études, il faut mentionner la caractérisation détaillée de la palmitylation *in vitro* non enzymatique de la protéine $G_{i\alpha 1}$ (Duncan and Gilman, 1996). À un pH et à une température physiologique, la présence d'un donneur de palmitate ($^3$H-palmityl-CoA) favorise la palmitylation spécifique de la cystéine en position 3 de la protéine purifiée $G_{i\alpha 1}$, ce même résidu étant palmitylé *in vivo*. Dans les conditions expérimentales *in vitro*, l'incorporation du palmitate au niveau de la protéine $G_{i\alpha 1}$ est dépendante de sa myristylation et peut être augmentée d'environ cinq fois en présence des sous-unités

βγ. Selon ces auteurs, cela favoriserait la liaison de la protéine $G_{i\alpha 1}$ aux micelles formées par le détergent et pourrait ainsi créer un environnement favorable à la palmitylation en forçant la rencontre entre la cystéine de la protéine à palmityler et le palmityl-CoA inséré dans ces micelles. Les résultats de cette étude démontrent clairement qu'une autoacylation de la protéine $G_{i\alpha 1}$ peut être observé *in vitro*.

La question qui demeure est de savoir si ce mécanisme de palmitylation non enzymatique existe au niveau de la cellule. Si tel est le cas, quel serait le rôle d'une protéine ayant une activité PAT ? Des travaux récents de Veit et coll. (Veit et al., 1998) ont permis de répondre en partie à cette question. Ces auteurs ont vérifié l'état de palmitylation de la rhodopsine en absence et en présence d'une activité PAT partiellement purifiée provenant de membranes de placentas bovins. Une palmitylation non-enzymatique a pu être observée pour ce récepteur mais la présence d'une activité PAT a provoqué une augmentation de l'incorporation maximale du palmitate de l'ordre de 10 fois. Ainsi, une régulation fine de la palmitylation *in vivo* pourrait nécessiter la présence d'une telle activité PAT. Néanmoins, il n'est pas exclu qu'une réaction de palmitylation *in vivo* en absence d'activité PAT puisse avoir lieu. Dans ces conditions, un mécanisme de régulation pourrait être attribué à la présence d'une palmityl-thioestérase.

L'existence d'un mécanisme de palmitylation dynamique suggère aussi l'existence d'une réaction inverse pouvant catalyser l'hydrolyse du palmitate. Très peu d'équipes se sont intéressées à l'isolement et à la caractérisation d'une telle enzyme.

En 1984, Berger et coll. (Berger and Schmidt, 1984) ont été les premiers à déceler une activité palmityl-thioestérase sur des protéines virales palmitylées. Presque 10 ans se sont écoulés avant que l'équipe de Hofmann (Camp and Hofmann, 1993) réussisse à purifier une protéine pouvant catalyser *in vitro* la dépalmitylation de H-RAS et de la sous-unité Gαo. Cependant, la sécrétion extracellulaire de cette protéine, suite à son expression, indique que H-RAS et la sous-unité Gαo ne peuvent pas être des substrats endogènes pour cette enzyme (Camp et al., 1994). Récemment, Duncan et coll. (Duncan and Gilman, 1998) ont purifié à partir de foies de rats, une protéine de 27 kDa pouvant *in vitro* dépalmityler H-RAS et la sous-unité Gαs. De plus, des études de marquage métabolique avec du palmitate tritié ont permis de confirmer que cette protéine transfectée dans des cellules de mammifères (HEK293) peut dépalmityler la protéine Gαs. Par ailleurs, cette protéine de 27 kDa avait été préalablement clonée et une activité lysophospholipase lui avait été associée (Sugimoto et al., 1996). Les substrats connus de cette enzyme correspondaient à une variété de lysophospholipides (lyso-PC, lyso-PE, lyso-PI et lyso-PS) mais aucun substrat palmitylé n'avait alors été caractérisé. Une meilleure caractérisation de l'activité palmityl-thioestérase de cette protéine permettra de vérifier les fonctions exactes de cette enzyme.

En somme, l'étude de la régulation de la palmitylation se révèle ardue. Pour l'instant, aucune enzyme responsable de la palmitylation ou de la dépalmitylation n'a été analysée en profondeur. Il est certain qu'une amélioration des connaissances à propos de la régulation de cette modification permettra de mieux connaître les fonctions qui

y sont associées. Le développement d'inhibiteurs potentiels du cycle de palmitylation/dépalmitylation pourrait servir à cette fin. Malgré l'absence presque totale d'outils disponibles permettant de contrôler l'état de palmitylation des protéines, d'autres stratégies telles que l'utilisation de peptides palmitylés, de la mutagenèse dirigée et de la dynamique de palmitylation *in vivo* ont été des approches utilisées afin de déterminer les fonctions potentielles de cette modification. Nous traiterons de ces différentes stratégies dans la prochaine section.

### 1.3.5.2    Rôles de la palmitylation

La fonction associée le plus souvent aux protéines modifiées par des lipides est la capacité de ces protéines à s'associer à une membrane cellulaire suite à l'ancrage de l'acide gras ou du groupement prénylé dans la bi-couche lipidique (Casey, 1995). Toutefois, comme nous l'avons mentionné antérieurement, les protéines myristylées et prénylées peuvent être cytosoliques ou liées à une membrane cellulaire. En ce qui à trait aux protéines palmitylées, cette modification lipidique semble confiner ces protéines à l'association à une membrane cellulaire. Le rôle d'ancrage à la membrane proposé pour la palmitylation a été confirmé par plusieurs groupes utilisant des approches expérimentales différentes (Dunphy and Linder, 1998).

Un des modèles d'étude a été l'utilisation de peptides modifiés par des lipides afin de vérifier leur capacité de liaison avec des membranes lipidiques reconstituées. Cette approche a permis de démontrer que la myristylation ou la farnésylation conférait aux peptides une liaison transitoire avec les liposomes ($t_{1/2}$ < 1 min) (Silvius and

L'Heureux, 1994; Peitzsh, 1993) bien inférieure à celle mesurée pour ces mêmes peptides sur lesquels un groupement palmitate était ajouté. En effet, une liaison presque irréversible avec les membranes est observée pour ces peptides doublement modifiés par des lipides ($t_{1/2} < 70$ heures) (Shahinian and Silvius, 1995). Ces résultats peuvent s'expliquer par le fait que l'énergie de liaison estimée d'un peptide myristylé (Shahinian and Silvius, 1995) ou farnésylé (Silvius and L'Heureux, 1994) (8kcal/mol) est inférieure d'environ 10 fois à un peptide palmitylé. Il semble assez évident que la palmitylation pourrait jouer un rôle déterminant dans l'ancrage des protéines à la membrane.

Afin de vérifier le ou les rôles de la palmitylation *in vivo*, l'approche la plus utilisée a été la mutagenèse dirigée. Pour plusieurs protéines palmitylées dont les protéines H-RAS (Cadwallader et al., 1994), des tyrosines kinases de la famille de p60[scr] (p59[fyn] et p56[lck]) (Alland et al., 1994; Shenoy-Scarcia et al., 1994) ainsi que plusieurs sous-unités $\alpha$ des protéines G hétérotrimériques (Mumby, 1997), la mutation de la cystéine portant cette modification affecte leurs localisations cellulaires. Ces protéines non palmitylées sont retrouvées majoritairement dans le cytosol plutôt qu'associées à la surface interne de la membrane plasmique, là même où elles exercent leurs fonctions. Un des exemples récemment publié concerne un membre de la famille des protéines AKAP (A-Kinase Anchoring Proteins), AKAP18 (Fraser et al., 1998). Cette protéine myristylée (glycine 1) et doublement palmitylées (cystéines 4 et 5 ) est liée à la membrane plasmique par l'intermédiaire de ces modifications lipidiques. Il a été proposé que certaines protéines telle que la PKA puisse se lier à la

protéine AKAP afin de se localiser près de leur substrat (Gray et al., 1998). Suite à la mutation des résidus responsables des modifications lipidiques de la protéine AKAP, cette dernière devient cytosolique. Dans ces conditions, une perte de l'activité de la PKA a été observée. Selon les auteurs, une relocalisation de la protéine AKAP18 mutante serait responsable de la diminution de l'activité kinase de la PKA. Les résultats de cette étude ne permettent pas de distinguer les rôles respectifs de la myristylation et de la palmitylation mais il est clair que ces modifications lipidiques jouent un rôle déterminant pour les fonctions de la protéine AKAP18.

Il faut souligner que la palmitylation d'un grand nombre de protéines est dépendante d'une deuxième modification lipidique. Une des hypothèses proposée par la communauté scientifique est qu'une première modification lipidique favoriserait le rapprochement de la protéine à la membrane là où elle pourrait être palmitylée grâce à la présence potentielle d'une activité PAT (Dunphy and Linder, 1998). À cet égard, les premières évidences qui ont été rapportées concerne la protéine H-Ras. La mutation de la séquence CAAX de H-Ras a clairement démontré la nécessité de l'isoprénylation pour favoriser la palmitylation subséquente (Hancock et al., 1989). Il faut mentionner que la palmitylation de H-Ras est dépendante de son ancrage à la membrane mais indépendante de la nature de l'acide gras qui favorise cet ancrage. Ainsi, l'isoprénylation peut être remplacée par une myristylation sans influencer la palmitylation (Cadwallader et al., 1994). Dans une étude récente, l'isoprénylation a été remplacée par une séquence de résidus basiques (six lysines) (Booden et al., 1999). Il a été proposé que ces résidus peuvent, en interagissant avec les

phospholipides chargés négativement de la bi-couche lipidique, favoriser le rapprochement de la protéine à la membrane (Bouvier et al., 1995a). Ainsi, ce mutant de la protéine H-Ras est localisé à la membrane, palmitylé et pleinement actif. Pour les protéines de la famille Src (Alland et al., 1994) et quelques sous-unités $\alpha$ des protéines G ($G_z$, $G_o$ et $G_i$) (Galbiati et al., 1994), la myristylation doit survenir en premier en favorisant l'ancrage à la membrane et permettre ainsi leur palmitylation. Cependant, des études plus récentes ont démontré que la myristylation n'était pas nécessairement requise pour la palmitylation de la sous-unité $G_{i1}$ (Galbiati et al., 1996). D'ailleurs, certaines sous-unité $\alpha$ des protéines G sont seulement palmitylées ($G_s$, $G_q$ et $G_{12}$). Ces dernières sont ancrées à la membrane et correctement palmitylées (Mumby, 1997). Il a été proposé que la présence des sous-unités $\beta\gamma$, la sous unité $\gamma$ étant prénylée, pourrait favoriser le rapprochement des sous-unités $G\alpha$ à la membrane et permettre ainsi la palmitylation des $G\alpha$ {Ponimaskin, Harteneck, et al. 1998 ID: 2144}.

Jusqu'à maintenant la palmitylation a toujours été associée à un rôle d'ancrage à la membrane permettant aux protéines d'interagir avec d'autres pour exercer leurs activités biologiques. Pour certaines protéines palmitylées, une régulation négative de leur fonction a été proposée. À cet égard, la protéine GAP-43 a été la première protéine pour laquelle un telle observation a été faite (Sudo et al., 1992). Cette protéine abondante des cônes de croissance neuronale peut moduler positivement l'activité GTPasique de la protéine G$\alpha$o. La mutation des deux cystéines responsables de la palmitylation de GAP-43 induit un gain d'activité de cette dernière

(Sudo et al., 1992). Des fonctions similaires ont récemment été rapportées concernant la palmitylation des protéines Gz et Gi (Tu et al., 1997). En effet, la palmitylation de ces dernières provoque une diminution importante de leur affinité pour certains membres (GAP, GAIP, RGS4 et RGS10) de la famille des RGS (Regulators of G-protein signalling) importantes pour leur fonctionnement. Les résultats de ces deux études découlent d'expériences *in vitro*. Il faudra confirmer ces résultats dans un modèle *in vivo* afin de valider ce rôle potentiel de séquestration pour la palmitylation.

Depuis le début de cette section, il a été question des différentes stratégies utilisées pour identifier la ou les fonctions importantes de la palmitylation. D'après l'ensemble des expériences, la fonction première est sans aucun doute un rôle d'ancrage à la membrane. Pour de nombreuses protéines, la perte de cette association avec la membrane aboutit à une modification de leurs activités biologiques. Dans ce contexte, une des dernières stratégies utilisées a été d'étudier le caractère réversible de la palmitylation afin de mieux cerner ses fonctions. Parmi toutes les protéines palmitylées identifiées jusqu'à ce jour, le $\beta_2$AR (Bouvier et al., 1995b), la sous-unité G$\alpha$s (Mumby, 1997) et la NO ("Nitric Oxide") synthase endothéliale (NOSe) (Michel and Feron, 1997) sont les protéines pour lesquelles de nombreuses études sur la dynamique de palmitylation/dépalmitylation ont été réalisées. Nous commencerons par détailler les études concernant les deux dernières protéines afin de terminer avec le $\beta_2$AR.

Plusieurs groupes (Degtyarev et al., 1993b; Wedegaertner and Bourne, 1994; Mumby et al., 1994) ont observé que l'activation du β₂AR permet de moduler la vitesse de renouvellement du palmitate sur la cystéine 3 de la protéine Gαs. À l'état basal, la demi-vie du palmitate sur la protéine Gαs varie entre 20 et 90 minutes. L'activation de Gαs, par l'intermédiaire du β₂AR, produit une augmentation de la vitesse du taux de renouvellement du palmitate sur la protéine G d'un facteur de dix (Wedegaertner and Bourne, 1994; Mumby et al., 1994). Des résultats similaires sont obtenus lorsque la protéine Gαs est stimulée directement par la toxine cholérique ou suite à une mutation activatrice (Mumby, 1997). Un traitement avec un activateur de l'adénylyl cyclase, la forskoline, ni une augmentation de la concentration d'AMPc cellulaire n'affecte la palmitylation de Gαs (Degtyarev et al., 1993b).

L'activation de la voie β-adrénergique aboutirait à la dépalmitylation de la protéine Gαs. Cette dernière n'étant plus palmitylée, elle se retrouverait dans le cytosol incapable de se coupler au récepteur et à l'adénylyl cyclase (Wedegaertner et al., 1993). Cependant, ces résultats ne font pas l'unanimité puisque certains scientifiques n'observent pas ce changement de répartition de Gαs suite à un traitement avec un agoniste β-adrénergique (Degtyarev et al., 1993a; Mumby et al., 1994). De plus, l'état de dépalmitylation de Gαs semble être transitoire puisque son activation ne produit pas de changements significatifs au niveau de sa stœchiométrie de palmitylation (Jones et al., 1997). Ces observations rejoignent les travaux récemment publiés par le groupe du Dr Mumby (Huang et al., 1999) suggérant que la protéine Gαs demeure toujours associée à la membrane même lorsqu'elle est dépalmitylée et/ou constitutivement active. Dans cette étude, la palmityl-thioestérase clonée récemment par Duncan et col. (Duncan and Gilman, 1998) a été utilisée pour dépalmityler à 85% la protéine Gαs. L'activation et l'association à la membrane de

cette dernière semblent indépendantes de sa palmitylation. De plus, ce groupe a démontré, à la suite d'études d'immunofluorescences, que la protéine Gαs constitutivement active (Q213L ou R187C) demeure toujours associée à la membrane. Les auteurs ont soulevé la possibilité que la protéine Gαs en demeurant toujours liée à la membrane plasmique suite à son activation, pourrait subir une relocalisation dans une sous région membranaire. Ce phénomène serait analogue aux observations de l'équipe du Dr Lisanti suggérant que certaines sous-unités Gα, dont Gαs, pourraient, suite à leur activation, subir une relocalisation dans un sous-domaine de la membrane plasmique appelée caveolae (Lisanti, 1994a). Ce même groupe a démontré avec des expériences *in vitro* que la forme inactive de la protéine Gαs (liant le GDP) pourrait se lier avec la cavéoline (Li et al., 1995; Kurzchalia et al., 1994). Dans ce contexte, un rôle potentiel des caveolaes seraient de séquestrer spécifiquement des protéines inactives.

Les rôles potentiels des caveolaes dans la signalisation cellulaire ne sont pas très bien définis mais un certain nombre de protéines palmitylées impliquées dans la signalisation y ont été localisées. Parmi ces protéines, on retrouve des membres de la famille des protéines kinases (LCK, HCK, YES, FYN) (Shenoy-Scarcia et al., 1993; Robbins et al., 1995; Shenoy-Scarcia et al., 1994; Alland et al., 1994), la sous-unité Gαi (Song et al., 1997) et la NOSe ( (Michel and Feron, 1997)). Il a été proposé que la palmitylation de ces protéines permettrait leur ciblage dans les caveolaes. À cet égard, la mutation des cystéines responsables de cette modification diminue significativement leur présence dans les caveolaes (Shenoy-Scarcia et al., 1993; Michel and Feron, 1997). La NOSe est la protéine la plus étudiée à propos de sa

localisation dans les caveolaes. Nous allons décrire comment la dynamique de palmitylation de l'enzyme influence sa localisation et son activité.

Jusqu'à maintenant, la NOSe est la seule des trois isoformes de la NOS clonée à s'être révélée acylée. Cette enzyme est myristylée et doublement palmitylée (cystéines 15 et 26). À l'état basal, l'enzyme palmitylée inactive se retrouve au niveau des caveolaes, plus spécifiquement liée avec la cavéoline. Une augmentation de la concentration intracellulaire en calcium, causée par l'activation du récepteur de la bradykinine ou par la présence d'un ionophore de calcium, favorise l'activation de la protéine NOSe. Celle-ci catalyse la formation du monoxyde d'azote (NO, nitric oxide) à partir de la L-arginine et de l'oxygène (Lander et al., 1995). Dans ces conditions d'activation, l'équipe du Dr Michel (Robinson et al., 1995) a observé une diminution de la demi-vie de liaison du palmitate associé à la NOSe alors que la demi-vie de la protéine n'est pas influencée par cette augmentation de calcium intracellulaire. Cette activation favorise un relargage de la NOSe au niveau du cytosol. Il a été proposé que la dépalmitylation induite par l'activation de l'enzyme provoque sa relocalisation au niveau du cytosol. Une augmentation de la phosphorylation est également observée suite à sa relocalisation dans le cytosol. Cependant, ni le rôle ni les sites de cette phosphorylation sont connus. Une diminution de la concentration du calcium intracellulaire provoque la réassociation de l'enzyme déphosphorylée avec la membrane cytoplasmique. À la membrane, la protéine NOSe est de nouveau palmitylée et se retrouve majoritairement dans les caveolaes (Michel and Feron, 1997). Toutefois, il faut mentionner que la

palmitylation de NOSe n'est pas essentielle à sa localisation dans les caveolaes mais cette modification semble la stabiliser dans cette sous-région membranaire (Feron et al., 1998). Ainsi, un des rôles de la palmitylation serait de diriger des protéines dans certaines régions de la cellule. Récemment, il a été démontré qu'en effet la palmitylation permettrait de cibler les protéines, en autre, au niveau du Golgi et de la membrane plasmique (Choy et al., 1999; McCabe and Berthiaume, 1999).

Un rôle de ciblage réversible à la membrane plasmique ne peut pas être proposé pour la palmitylation de protéines intrinsèques de la membrane plasmique. Par exemple, la palmitylation des RCPG ne peut pas justifier l'association à la membrane de ces récepteurs puisque celle-ci est déjà assurée par leurs sept segments transmembranaires. Jusqu'à maintenant, pour les dix études portant sur la palmitylation des RCPG, le site de palmitylation a toujours été localisé dans la portion proximale de la queue C-terminale des récepteurs. Donc, il a été proposé que cette modification puisse favoriser la formation d'une quatrième boucle intracellulaire. Cette hypothèse est supportée par une étude utilisant un analogue fluorescent du palmitate démontrant que les deux palmitates de la rhodopsine sont insérés dans la membrane plasmique (Moench et al., 1994). Même si la palmitylation d'un nombre restreint de RCPG a été confirmée, une cystéine est conservée dans la portion C-terminale de la plupart des RCPG (Tableau III). Pour cette grande famille de récepteurs, la palmitylation dans la portion C-terminale des récepteurs pourrait être ubiquitaire.

**Tableau III**     **Séquence de la portion C-terminale de plusieurs RCPG.**

Les résidus cystéines conservés dans la région proximale de la queue C-terminale

sont soulignés.

| RCPG | cystéines | Séquences de la portion C-terminale |
|---|---|---|
| HUM Rho | 310 | NKWFRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVA |
| HUM β₁AR | 388 | DFRKAFQGLLCCARRAARRRHTATHGDRPPRASGCLA...+ |
| HUM β₂AR | 329 | PDFRIAFQELLCLRRSSLKAYGNGYSSNGNGNGEQSG...+ |
| HUM β₃AR | 350 | PDFRSAFRRLLCRCGRRRLPPEPCAAARPALFPSGVPAQP...+ |
| HUM α₁ₐAR | 406 | SREFKRAFLRLLRCQCRRRRRRRPLWRVYGHHWRASTSS...+ |
| HUM α₁ᵦAR | 351 | SSKEFRAFVRILGCQCRGRRRRRRRRRLGCAYTYRPWT...+ |
| HUM α₁cAR | 330 | SQEFKKAFQNVLRIQCLRRKQSSKHALGYTLHPPSQA...+ |
| HUM α₂ₐAR | 430 | NHDFRRAFKKILCRGDRKRIV |
| HUM α₂ᵦAR | 431 | NQDFRRAFRRILCRPWTQTAW |
| HUM D₁ | 334 | NADFRKAFSTLLGCYRLCPATNNAIETVSINNNGAAM...+ |
| HUM D₂ | 430 | IEFRKAFLKILHC |
| HUM 5HT₁ₐ | 404 | NKDFQNAFKKIIKCNFCRQ |
| HUM 5HT₁ᵦ | 374 | EDFKQAFHKLIRFKCTS |
| HUM 5HT₂ₐ | 385 | NKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSS...+ |
| HUM 5HT₂ᵦ | 384 | NKTFRDAFGRYITCNYRATKSVKTLRKRSSKIYFRNP... |
| HUM 5HT₂ | 373 | NKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGR... |
| HUM M₁ₐAch | 415 | NKAFRDTFRLLLLCRWDKRRWRKIPKRPGSVHRTPSKRC |
| HUM M₂ₐAch | 443 | NATFKKTFKHLLMCHYKNIGATR |
| HUM M3AACh | 456 | NATFKKTFRHLLLCQYRNIGTAR |
| HUM M₄ₐAch | 547 | NTFRTTFKTLLLCQCDKRKRRKQQYQQRQSVIFHKRVPEQAL |
| HUM M₅ₐAch | 499 | NRTFRKTFKMLLLCRWKKKKVEEKLYWQGNSKLP |
| RAT mGluR₄ | 848 | HPEQNVPKRKRSLKAVVTAATMSNKFTQKGNFRPNGEAKSELCENLETPAL... |
| HUM mGluR₄ | 848 | HPEQNVPKRKRSLKAVVTAATMSNKFTQKGNFRPNGEAKSELCENLEAPAL... |
| HUM PGE_EP2 | 334 | RKTVLSKAIEKIKCLFCRIGGSRRESGQHCSDSQRTSSAMSGHSRSFISRE...+ |
| HUM PGE_EP3 | 350 | RKILLRKFCQIRYHTNNYASSSTSLPCQCSSTLMWSDHLER |
| HUM PAF | 297 | TKKFRKHLTEKFYSMRSSRKCSRATTDVTEVVVPFNQIPGNSLKN |
| HUM ETA | 383- | SKKFKNCFYSCLCCCCTQSKSLMTSVPMNGTSIQWKNHDYNNHNTD... |
| HUM ETB | 402- | RFKNCFKSCLCCWCYSFEEKQSLEEKQSCLKFKANDHGYDNFRSSNKYSSS |
| HUM NK1 | 310 | DRFRLGFKAAFRCCPFISAGDYEGLEMKSTRYLQTQGSVYKVSRLETTISTV...+ |
| HUM NK2 | 311 | NHRFRSGFRLAFRCCPWVTPTKEDKLELTPTTSLSTRVNRCHTKETLFMAG... |
| HUM NK3 | 360 | NKRFRAGFKRAFRWCPFIKVSSYVELELKTTRFHPNRQSSMYTVTRMESMT... |

| | | |
|---|---|---|
| HUM AT1a | 304 | FLGKKFKRYFLQLLKYIPPKAKSHSNLSTKMSTLSYRPSDNVSSSTK...+ |
| RAT AT1a | 304 | FLGKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSSAK...+ |
| HUM AT2 | 314 | NPFLYCFVGNRFQQKLRSVFRVPITWLQGKRETMSCRK... |
| HUM V1a | 355 | SGHLLQDCVQSFPCCQNMKEKFNKEDTDSMSRRQTFY... |
| HUM V1b | 342 | NSHLLPRPLRHLACCGGPQRMRRRLSDGSLSSRHTTLL... |
| HUM V2 | 328 | SSSVSSELRSLLCCARGRTPPASLGPQDECTTASSSLA... |
| HUM OXYTOCIN | 333 | TGHLFHELVQRFLCCSASYLKGRRLGETSASKKSNSSSFVL...+ |
| HUM NPY1 | 323 | KNFQRDLQFFFNFCDFRSRDDDYETIAMSTMHTDVSKLSKKA... |
| HUM NPY2 | 328 | MNSNYRKAFLSAFRCEQRLDAIHSEVSVTFKAKKNLEVNKNSG... |
| HUM □opioid | 340 | DENFKRCFREFCIPTSSTIEQQNSTRVRQNTREHPSTANTVDRTN... |
| HUM □opioid | 324 | ENFKRCFRGLCRKPCGRPDPSSFSRPREATARERVTACTPSDGPGGGRAA |
| HUM □opioid | 324 | DENFKRCFRDFCFPLKMRNERQSTSRVRNTVQDPAYLRDIDGMNKPV |
| RAT LH/hCG | 606 | KAFQRDFLLLLSRFGCCKRRAELYYRRKEFSAYTSNCKNGFPGASKP...+ |
| HUM LH/hCG | 628 | KTFQRDFFLLLLSKFGCCKRRAELYRRKDFSAYTSNCKNGFTGSNKP...+ |
| HUM Thrombin | 375 | SECQRYVSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT |
| HUM TRH-R | 319 | YNLMSQKFRAAFRKLCNCKQKPTEKPANYSVALNYSVIK...+ |
| HUM TSH-R | 682 | KAFQRDVFILLSKFGICKRQAQAYRGQRVPPKNSTD... |

C : cystéines conservés dans les premiers vingt acides aminés de la queue C-terminale.
C : cystéine retrouvée après les vingt premiers acides aminés.
+ : présence d'au moins une autre cystéine

Un nombre limité d'études a porté sur les rôles potentiels de la palmitylation pour les RCPG. L'impact fonctionnel de cette modification a été évalué pour onze récepteurs. Parmi ceux-ci, il y a, celui de la rhodopsine (O'Brien and Zatz, 1984), du $\beta_2$-adrénergique (O'Dowd et al., 1989), du $\alpha_{2A}$-adrénergique ($\alpha_{2A}$A) (Kennedy and Limbird, 1993), de la LH/hCG (Luteinizing Hormone/humain Chorionic Gonadotropin) (Kennedy and Limbird, 1993), de la dopamine D1 (Ng et al., 1994), de l'endothéline A (Horstmeyer et al., 1996) et B (Okamoto et al., 1997), du muscarinique $M_2$ (Hayashi and Haga, 1997), le vasopressine $V_2$ (Sadeghi et al., 1997), le TSH (Thyrotropin) (Tanaka et al., 1998) et l'adénosine A1 (Gao et al., 1999). Pour le récepteur $\alpha_{2A}$-adrénergique, la mutation de la cystéine 442 responsable de la palmitylation inhibe sa régulation négative (Eason et al., 1994). Ce mécanisme est également modifié pour le récepteur LH/hCG lorsque les cystéines 621 et 622 sont remplacées par des sérines. Cependant, pour ce récepteur non palmitylé, une augmentation de la régulation négative et de l'internalisation a été observée (Kawate and Menon, 1994). En ce qui à trait au récepteur de la vasopressine $V_2$, le remplacement par mutagénèse dirigée des cystéines 341 et 342 par des sérines diminue l'internalisation ainsi que le niveau d'expression à la surface cellulaire (Schulein et al., 1996; Sadeghi et al., 1997) . Pour les récepteurs $\beta_2$AR (O'Dowd et al., 1988), dopamine D1 (Jensen et al., 1995), endothéline A et B (Horstmeyer et al., 1996; Okamoto et al., 1997) et muscarinique $M_2$ (Hayashi and Haga, 1997), la mutation des sites de palmitylation conduit à une diminution du couplage à leurs protéines G respectives. Finalement, deux publications récentes ont révélé que l'absence des sites de palmitylation du récepteur TSH (Tanaka et al., 1998) et du récepteur de l'adénosine A1 (Gao et al., 1999) provoque une diminution de la forme mature des récepteurs. Les études entreprises pour évaluer les rôles fonctionnels de la palmitylation ont presque toutes été réalisées à la suite de

l'abolition des résidus responsables de cette modification, sauf pour la rhodopsine. Pour ce récepteur, deux approches ont été utilisées: 1) un traitement avec l'hydroxylamine pour abolir la palmitylation et 2) le remplacement par mutagénèse dirigée des cystéines 322 et 323 responsables de cette modification. La première étude a démontré qu'un traitement avec l'hydroxylamine provoque une augmentation de la capacité du récepteur à stimuler la protéine Gαt (Morrison et al., 1991). En revanche, la deuxième étude a mis en évidence le fait que la mutation des résidus responsables de la pamiltylation (cystéines 322-323) n'affecte pas la capacité de ce récepteur mutant de se coupler à la protéine Gαt (Karnik et al., 1993). Avec ces résultats contradictoires, il faut demeurer critique face aux différentes approches utilisées et à leurs limites. Malgré tout, l'ensemble de ces travaux suggère que plusieurs fonctions des RCPG sont affectées par leur palmitylation.

**Tableau IV**     Énumération des RCPG pour lesquels la palmitylation a été rapportée.

| RCPG | Palmitylation (cystéine #) | Types cellulaires | Fonction(s) associée(s) à la palmitylation |
|---|---|---|---|
| 1) **Rhodopsine** | C322 et C333 | Bâtonnets | Diminution de la phosphorylation et augmentation de la stimulation Gt |
| 2) **$\beta_2$-adrénergique** | C341 | CHW, Sf9 HEK293 | Augmentation de la phosphorylation, diminution du couplage avec Gs et de l'activité adénylyl cyclase |
| 3) **$\alpha_{2A}$-adrénergique** | C442 | COSM6, CHO | Absence de régulation négative |
| 4) **LH / hCG** | C621 et C622 | HEK293 | Augmentation de l'internalisation et de la régulation négative |
| 5) **Muscarinique M$_2$** | C457 | Sf9 | Diminution du couplage avec Gi |
| 6) **Vasopressine V$_2$** | C341 et C342 | COS | Diminution du nombre de récepteurs membranaires |
| 7) **Endotheline B** | C402, C403 et C405 | COS | Diminution du couplage avec Gq et Gi |
| 8) **hTSH** | C699 | HEK293 | Ralentissement de l'expression de la forme mature du récepteur |
| 9) **Adénosine A$_1$** | C309 | HEK293 | Diminution de la forme mature |
| 10) **Endotheline A** | C383*, C285*, C386*, C387* et C388* | Sf9 | Diminution du couplage avec Gq et couplage avec Gs non-modifiée |
| 11) **Dopamine D1** | C347* | Sf9, COS | Diminution de l'activité adénylyl cyclase |
| 12) **Opioïde $\mu$** | C346*, C351* et autres | CHO | N.D. |
| 13) **5-HT$_{1A}$** | N.D. | Sf9 | N.D. |
| 14) **5HT$_{1B}$** | N.D. | Sf9 | N.D. |
| 15) **Dopamine D2$_L$** | N.D. | Sf9 | N.D. |
| 16) **mGLUR$_4$** | N.D. | BHK | N.D. |

(N.D. : non-déterminé, * site potentiel pour la palmitylation )

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En plus des rôles fonctionnels proposés pour la palmitylation, cette modification pourrait affecter la phosphorylation de certains RCPG. Une telle observation a été rapportée pour le $\beta_2AR$ et la rhodopsine. Pour ce dernier, l'abolition de sa palmitylation à la suite d'un traitement avec l'hydroxylamine provoque une augmentation de sa phosphorylation par la caséine kinase I (Ohguro et al., 1996). Selon les auteurs de cette étude, ce résultat de phosphorylation serait la conséquence d'un changement de conformation attribuable à l'absence de la palmitylation. En ce qui concerne le $\beta_2AR$, la mutation de la cystéine 341 responsable de la palmitylation ($\beta_2AR$-C341G) confère aux récepteurs un profil de phosphorylation beaucoup plus élevé que celui du récepteur de type sauvage (Moffett et al., 1993). Une désensibilisation causée par le traitement à l'isoprotérénol du $\beta_2AR$ produit un niveau de phosphorylation similaire à celui du $\beta_2AR$-C341G à l'état basal. Comme il a été mentionné, une diminution importante de la capacité de ce récepteur mutant à se coupler à la protéine G a été observée. Ce résultat découle du fait que le profil de phosphorylation du $\beta_2AR$-C341G à l'état basal est similaire au récepteur de type sauvage désensibilisé. Les auteurs de ces études ont proposé que l'ancrage du palmitate à la membrane plasmique pourrait former une quatrième boucle cytoplasmique. Comme pour la rhodopsine, l'absence du palmitate viendrait altérer la structure de la queue cytoplasmique du $\beta_2AR$ et modifier l'exposition de sites de phosphorylation pour la PKA ou la GRK2. Ce modèle suggérant une interaction entre la palmitylation et la phosphorylation devient potentiellement valide si la palmitylation du récepteur peut être modulée. À ce sujet, le groupe du Dr Bouvier a démontré que l'état de palmitylation du $\beta_2AR$ pouvait être modifié suite à son

activation (Mouillac et al., 1992). En effet, la présence d'un agoniste pendant les quinze dernières minutes d'un marquage de soixante-quinze minutes avec du palmitate tritié provoque une augmentation de l'incorporation du radiotraceur par rapport au récepteur non stimulé. Toutefois, les mécanismes responsables d'une telle modulation de la palmitylation du $\beta_2AR$ ne sont pas connus.

## 1.4   Les objectifs de la thèse

Au cours des premières études portant sur la palmitylation du $\beta_2AR$, Bernard Mouillac (Mouillac et al., 1992) du laboratoire du Dr Michel Bouvier a mis en évidence l'existence d'une régulation de la palmitylation du récepteur dépendante de son état d'activation. Il était évident que nos connaissances sur la palmitylation du $\beta_2AR$ étaient très limitées. Dans ce contexte, une étude approfondie de la palmitylation du récepteur constituait une suite logique à ce projet.

**1)** Mon premier objectif a été de caractériser le processus de la palmitylation du $\beta_2AR$. Pour ce faire, deux systèmes d'expression ont été utilisés (cellules d'insectes (Sf9) et cellules de mammifères (HEK293)). En parallèle, j'ai étudié la dynamique de palmitylation à la suite de l'activation du récepteur par l'isoprotérénol, un agoniste $\beta$-adrénergique (articles 2 et 4).

Il avait été démontré que le $\beta_2AR$ non palmitylé ($\beta_2AR$-C341G) possédait un profil de couplage avec la protéine G inférieur à celui du récepteur de type sauvage (O'Dowd et al., 1989). Cette diminution importante de la capacité du $\beta_2AR$-C341G à stimuler l'effecteur, l'adénylyl cyclase, a été en partie expliquée par les travaux réalisés dans le laboratoire par Serge Moffett (Moffett et al., 1993). Les résultats de cette étude ont démontré que le récepteur non palmitylé possédait un profil de phosphorylation à l'état basal bien supérieur à celui d'un récepteur de type sauvage. Ainsi, le récepteur non-palmitylé présentait donc les caractéristiques d'un récepteur désensibilisé. Dans ces conditions, la palmitylation du récepteur semblait pouvoir influencer sa phosphorylation.

**2)** Mon deuxième objectif a été de vérifier si la palmitylation du $\beta_2AR$ pouvait influencer la phosphorylation en modifiant l'accessibilité à des kinases pour leurs sites de phosphorylation dans la portion C-terminale du récepteur (article 1). Dans ce contexte, il était également pertinent de vérifier si une régulation inverse pouvait exister. En d'autres termes, les sites de phosphorylation à proximité de la palmitylation pouvaient-ils influencer la dynamique de palmitylation (article 2 et 4) ?

Les notions de régulation de la palmitylation des protéines étaient très restreintes. En ce qui concerne le $\beta_2AR$, une seule étude avait rapporté une modulation de la palmitylation du récepteur. Comme nous l'avons déjà mentionné, l'activation du récepteur était responsable de modifier l'état de palmitylation du $\beta_2AR$. Par ailleurs, certaines évidences suggéraient que le monoxyde d'azote (nitric oxide : NO) pouvait influencer l'état de palmitylation de nombreuses protéines (Hess et al., 1993). Ainsi, le NO pouvait devenir un agent potentiel pour réguler la palmitylation du récepteur.

**3)** Mon troisième objectif a été d'évaluer si le NO pouvait influencer l'état de palmitylation du $\beta_2AR$. Dans ce contexte, il était justifié de vérifier si le NO pouvait influencer l'activité fonctionnelle du récepteur.

L'ensemble des études présenté dans le cadre de cette thèse ont permis la réalisation de quatre articles dont deux en tant que premier auteur.

1)      Palmitoylated cystein 341 modulates phosphorylation of the $\beta_2$-adrenergic receptor by the cAMP-dependent protein kinase.

Serge Moffett, **Lynda Adam**, Hélène Bonin, Thomas P. Loisel, Michel Bouvier et Bernard Mouillac.

(1996) *J. Biol. Chem.* **271** : 21490-21497

2) Agonist stimulation increases the turn-over rate of $\beta_2$AR-bound palmitate and promotes receptor depalmitylation.

Thomas P. Loisel, **Lynda Adam**, Terence E. Hebert et Michel Bouvier

(1996) *Biochemistry* **35** : 15923-15932

3) Dynamic palmitoylation of the human $\beta$2-adrenergic receptor in mammalian cells; Effects of agonist stimulation and receptor phosphorylation.

**Lynda Adam** and Michel Bouvier

Soumettre à Biochemistry

4) Nitric oxide modulates $\beta_2$-adrenergic receptor palmityolation and signaling.

**Lynda Adam**, Michel Bouvier and Teresa L. Z. Jones

(1999) *J. Biol. Chem.* 274 : 26337

# 2. Résultats

## 2.1 Article 1: Palmitoylated cysteine 341 modulates phosphorylation of the $\beta_2$-adrenergic receptor by the cAMP-dependent protein kinase.

Dans le cadre de ce projet, j'ai développé un protocole de phosphorylation *in-vitro* pour le récepteur $\beta_2$-adrénergique. Les résultats des ces expériences (figures 5 et 6) ont contribué grandement à l'élaboration du mécanisme par lequel la palmitylation du récepteur influencerait son état de phosphorylation et, par conséquent, ont facilité la publication de cet article dans le *Journal of Biological Chemistry*.

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

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We previously showed that substitution of a glycine residue for the palmitoylated cysteine 341 of the human β2-adrenergic receptor (Gly$^{341}$β2AR), 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 (Ala$^{345,346}$Gly$^{341}$β2AR) 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 adenylyl cyclase in reconstitution assays. Furthermore, mutation of Ser$^{345,346}$ in a wild type receptor background (Ala$^{345,346}$β2AR) significantly decreased the rate of agonist-promoted desensitization of the receptor-stimulated adenylyl 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, Gly$^{341}$β2AR 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 adenylyl 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 adenylyl cyclase (5). Indeed, substitution of a glycine residue for the cysteine 341 (Gly$^{341}$β2AR) was found to prevent receptor palmitoylation and greatly reduced its ability to stimulate adenylyl 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 Gly$^{341}$β2AR 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).[2]

One of the two potential PKA phosphorylation sites present in the β2AR is located one amino acid downstream of the palmitoylated cysteine ($^{341}$C$^{palm}$LRRSS). 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. [$^{125}$I]CYP, [α-$^{32}$P]ATP, [γ-$^{32}$P]ATP, [$^3$H]cAMP, and [$^{32}$P]H$_3$PO$_4$ were purchased from DuPont NEN. (−)-isoproterenol, (−)-alprenolol, ATP, GTP, cAMP, forskolin, isobutylmethylxanthine, phosphoenolpyruvate, 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'-(β,γ-imido)triphosphate; βARK, β-adrenergic receptor kinase; [$^{125}$I]CYP, [$^{125}$I]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. Loisel, T. E. Hébert, and M. Bouvier, submitted for publication.

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

*Construction of Mutated $\beta_2AR$ cDNAs and Cell Transfection* — A human $\beta_2AR$ cDNA clone encoding a glycine substitution of cysteine 341 (Gly[341]$\beta_2AR$) was constructed as in O'Dowd *et al.* (5) and subcloned into the eukaryotic expression vector pBC12BI (9). To generate the other $\beta_2AR$ mutants, the cDNAs encoding wild type $\beta_2AR$ (10), Gly[341]$\beta_2AR$ (5), and Ala[261,262,345,346]$\beta_2AR$ (11) were subcloned into pSP65 (Promega) by introduction in the *NcoI-SalI* sites. Ala[345,346]$\beta_2AR$ was constructed by digestion of pSP65 plasmids containing wild type $\beta_2AR$ and Ala[261,262,345,346]$\beta_2AR$ cDNAs respectively, with *AviII*. The appropriate restriction fragments were isolated and ligated, and a resulting pSP65 plasmid containing a cDNA for Ala[345,346]$\beta_2AR$ was obtained. Gly[341]Ala[345,346]$\beta_2AR$ was constructed by digestion of pSP65 plasmids containing Gly[341]$\beta_2AR$ and Ala[261,262,345,346]$\beta_2AR$ respectively, with *AviII*. The appropriate restriction fragments were isolated and ligated and a resulting pSP65 plasmid containing a Gly[341]Ala[345,346]$\beta_2AR$ cDNA was obtained. All of the constructs were subcloned in the expression vector pBC12BI$\beta_2AR$ (10) by introduction in the *NcoI-SalI* sites. The identities of all mutants were confirmed by direct dideoxynucleotide sequencing. The wild type $\beta_2AR$ and all of the mutants were stably expressed in cultured fibroblasts (mouse LTK⁻ cells or Chinese hamster CHW cells) by co-transfection with the neomycin resistance plasmid pSV2-Neo (Pharmacia) using the calcium phosphate precipitation procedure (12). Neomycin-resistant cells were selected by culturing in DMEM supplemented with 10% fetal bovine serum containing G418 (450 $\mu$g/ml for LTK⁻ cells or 150 $\mu$g/ml for CHW cells). Clones were then screened for $\beta_2AR$ expression by radioligand assays using [125I]CYP as the ligand (10). Cell lines expressing a similar number of receptors were selected for the study.

*Cell Culture and Membrane Preparation* — LTK⁻ and CHW cells were grown as a monolayer in 75-cm² Corning or Nunc plastic flasks containing DMEM supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 500 units/ml penicillin, 500 units/ml streptomycin, and 0.25 $\mu$g/ml amphotericin B in an atmosphere of 95% air and 5% $CO_2$ at 37 °C. Attached cells were washed 3 times with ice-cold PBS, mechanically detached into 10 ml of buffer A (5 mM Tris-HCl (pH 7.4), 2 mM EDTA, 10 $\mu$g/ml benzamidine, 5 $\mu$g/ml soybean trypsin inhibitor, and 5 $\mu$g/ml leupeptin) on ice. Cell suspensions were homogenized with a Polytron homogenizer (Ultra-Turrax T-25; Janke and Kunkel) using a single burst of 7 s at maximum setting. Homogenates were centrifuged at 43,000 × $g$ for 20 min at 4 °C, and the pellets were rinsed twice in buffer A. Membranes were finally resuspended in buffer B (75 mM Tris-HCl (pH 7.4)), 5 mM $MgCl_2$, 2 mM EDTA, 10 $\mu$g/ml benzamidine, 5 $\mu$g/ml soybean trypsin inhibitor, and 5 $\mu$g/ml leupeptin) at a concentration of ~1 mg/ml and used immediately for binding or adenylyl cyclase assays. Protein concentrations were determined by the method of Bradford (Bio-Rad), using bovine serum albumin as a standard.

For agonist-induced desensitization experiments, cell medium was changed for fresh media containing isoproterenol and ascorbic acid to a final concentration of 1 $\mu$M and 100 $\mu$M, respectively, for the indicated time. Control cells were treated with media containing ascorbic acid only.

*Radioligand Binding Assays* — Radioligand binding assays were conducted essentially as described in Bouvier *et al.* (10) using 10 $\mu$l of membrane suspension (~10 $\mu$g of protein) in a total volume of 0.5 ml of buffer B. For saturation experiments, concentrations of [125I]CYP ranging from 5 to 250 pM were used. Competition binding assays with (−)-isoproterenol were conducted using ~50 pM [125I]CYP as the radioligand. The concentration of the $\beta$-adrenergic agonist was varied from 0 to 100 $\mu$M in the presence or absence of 100 $\mu$M Gpp(NH)p. Binding data were analyzed by nonlinear least squares regression using the computer program Ligand (13). For routine determination of receptor number, triplicate assay tubes contained a nearly saturating concentration of [125I]CYP (200 pM) in the presence or absence of 10 $\mu$M (−)-alprenolol to define specific binding.

*Adenylyl Cyclase Assays* — Adenylyl cyclase activity was determined in crude membrane preparations according to the method of Salomon *et al.* (14). Membranes (~4 $\mu$g of protein) were incubated in a reaction mixture containing 45 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 1.2 mM EDTA, 53 $\mu$M GTP, 100 $\mu$M cAMP, 120 $\mu$M ATP, 100 $\mu$M isobutylmethylxanthine, 2.7 mM phosphoenolpyruvate, 1 unit myokinase, 0.2 unit of pyruvate kinase and 0.13 $\mu$Ci of [$\alpha$-32P]ATP in a final volume of 50 $\mu$l. The enzyme activity was determined in the absence of activator (basal activity) or in the presence of increasing concentrations (0–100 $\mu$M) of (−)-isoproterenol for 30 min at 37 °C. The reaction was stopped by the addition of 1 ml of stop solution (360 $\mu$M ATP, 280 $\mu$M cAMP, and [³H]cAMP (25,000 cpm) to monitor cAMP recovery). cAMP was then

isolated by sequential chromatography on a Dowex cation exchange resin and aluminum oxide. Data calculated as pmol of cAMP produced/min/mg were analyzed using nonlinear least squares regression.

*Whole Cell Phosphorylation and Purification of the $\beta_2ARs$* — Phosphorylation experiments were performed essentially as described (10). Briefly, 2 h before the labeling, 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 $\mu$M $NaH_2PO_4$ 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 (pH 7.4), 5 mM EDTA, 10 mM $Na_4P_2O_7$·10 $H_2O$, 10 $\mu$g/ml benzamidine, 5 $\mu$g/ml soybean trypsin inhibitor, and 5 $\mu$g/ml leupeptin). The cells were disrupted by sonication at 4 °C, and membranes were isolated by centrifugation at 43,000 × $g$ 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 of a 43,000 × $g$ centrifugation for 20 min at 4 °C. The receptors were then purified by alprenolol-Sepharose affinity chromatography as described previously (15) and, purified receptors were concentrated by membrane filtration over Centriprep and Centricon concentrators (Amicon). The amount of purified receptors was assessed by soluble [125I]CYP binding assays. Aliquots were then prepared for SDS-PAGE, and electrophoresis was conducted using 10% slab gels (16). Following electrophoresis, proteins were electrophoretically 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 (UltroScan, Pharmacia Biotech Inc.) was used to quantitate the incorporation of ³²P into the receptor. In some experiments, the specific activity of the cellular [³²P]ATP pool was determined 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 scintigraphy of the excised receptor band for a known quantity of $\beta_2AR$ loaded on SDS-PAGE.

*Phosphorylation of $\beta_2AR$ 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 × 10⁶ cells/ml. The cells were then infected with recombinant baculoviruses encoding wild type $\beta_2AR$, Gly[341]$\beta_2AR$, or Gly[341]Ala[345,346]$\beta_2AR$ at a multiplicity of infection of 2–5. The virus encoding the wild type $\beta_2AR$ was generated as described before (7), while the two other viruses were constructed by subcloning the mutant receptor coding regions into the pJV$\beta_2AR$ recombinant vector. Recombinant baculovirus was purified by successive plaque assays using the $\beta$-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 $MgCl_2$, 5 mM phosphoenolpyruvate, 0.2 unit/50 $\mu$l pyruvate kinase, 0.2 mM ATP, and 0.025 mM [$\gamma$-32P]ATP (2000 cpm/pmol). Phosphorylation assay was initiated by adding 3 × 10⁻⁵ 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 × $g$ for 20 min at 4 °C, and the pellets were rinsed 3 times in a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 20 mM $NaH_2PO_4$. Receptors were then solubilized in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.3% dodecylmaltoside 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 $\beta_2AR$ were excised from the gel and counted by liquid scintigraphy. The stoichiometry of phosphorylation could be calculated based on the specific activity of the [$\gamma$-32P]ATP used in the phosphorylation assays. In some experiments, membranes were treated with agarose-bound alkaline phosphatase (600 units/ml) for 45 min at 37 °C in 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ prior to the phosphorylation assay. Alkaline phosphatase was removed from the samples by centrifugation at 500 × $g$ for 5 min at 4 °C by extensive washing and repeated centrifugation.

*Hydroxylamine Treatment and Reconstitution of the $\beta_2AR$* — Human recombinant $\beta_2AR$ was purified from Sf9 cells as described above. The concentrated purified receptor was incubated for 1 h at room temperature in a 10 mM Tris-HCl solution (pH 7.0) containing or not containing hydroxylamine at a final concentration of 1 M. Samples were then washed extensively and desalted by filtration dialysis using Centricon 30 (Amicon). For receptor reconstitution, membranes derived from untransfected LTK⁻ cells were prepared as described above and resuspended at a concentration of 2–3 $\mu$g/$\mu$l in a buffer containing 20 mM Hepes (pH 7.4), 2 mM $MgCl_2$, 1 mM EDTA, 0.4% CHAPS. Purified $\beta_2ARs$

treated as above were then incubated with this membrane preparation (8 fmol/$\mu$l) under mild agitation at 15 °C. After 1 h, an equal volume of a freshly prepared buffer containing 100 mM Hepes (pH 7.4), 10 mM MgCl$_2$, 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 pelleted membranes were then washed twice in a buffer containing 75 mM Tris-HCl (pH 7.4), 5 mM MgCl$_2$, 2 mM EDTA. The reconstituted membranes were finally diluted to 2–5 $\mu$g/20 $\mu$l in the same buffer and assayed for [$^{125}$I]CYP binding and isoproterenol-stimulated adenylyl cyclase activity as described above.
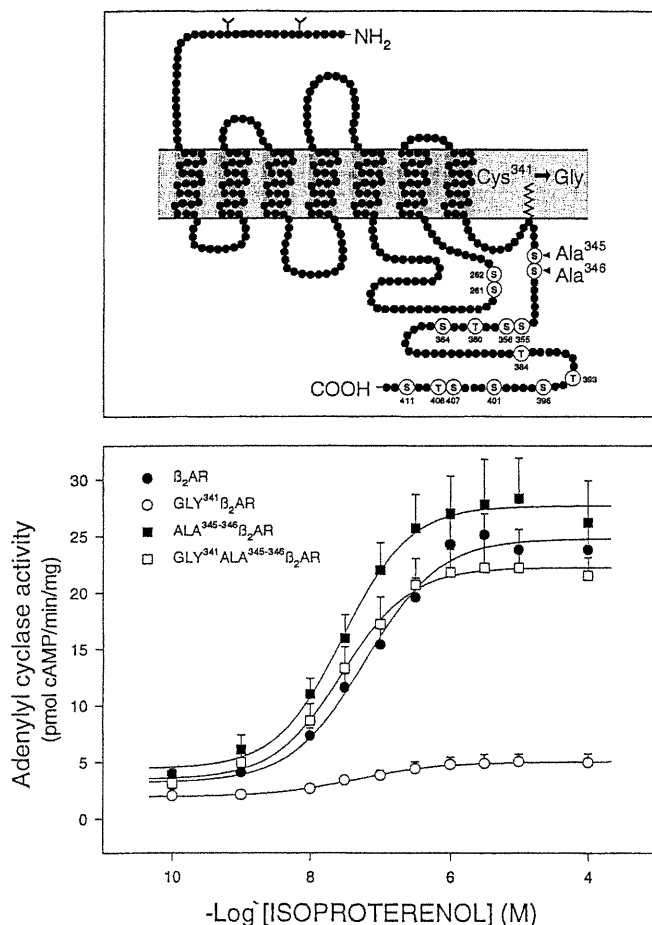


FIG. 1. *Upper panel*, schematic representation of the $\beta_2AR$. The position of the regulatory phosphorylation sites and of the palmitoylated cysteine are illustrated. *Lower panel*, isoproterenol-stimulated adenylyl cyclase activity in cells expressing wild type $\beta_2AR$, Gly$^{341}\beta_2AR$, Ala$^{345,346}\beta_2AR$ and Gly$^{341}$Ala$^{345,346}\beta_2AR$. Activities were measured in membranes derived from cells expressing a comparable number of receptors and are expressed as pmol of cAMP produced per min per mg of protein. Data shown represent the mean ± S.E. of five to nine independent experiments conducted in duplicate.

*Whole Cell Palmitoylation of the $\beta_2AR$*—[$^3$H]palmitate labeling experiments were performed essentially as described in Ref. 7. Briefly, Sf9 cells (2 × 10$^6$/ml) grown in suspension were infected with wild type $\beta_2AR$ recombinant baculovirus at a multiplicity of infection of 2–5. Twenty-four hours after this treatment, [9,10-$^3$H]palmitic acid dissolved in dimethyl sulfoxide was added to the culture to a final concentration of 0.2 mCi/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 hydroxylamine as above.

## RESULTS AND DISCUSSION

Mutant forms of the human $\beta_2AR$ and Gly$^{341}\beta_2AR$ 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 [$^{125}$I]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 [$^{125}$I]CYP bound to the wild type and mutant $\beta_2$ARs with $K_d$ values in good agreement with those reported for human $\beta_2AR$ expressed in other cell systems (7, 17). No specific binding of [$^{125}$I]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 $\beta_2AR$ 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 $\beta_2AR$ cDNA, isoproterenol stimulated adenylyl cyclase activity in a dose-dependent manner (Fig. 1). The EC$_{50}$ 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 Gly$^{341}\beta_2AR$ to stimulate the adenylyl cyclase was found to be markedly reduced relative to wild type $\beta_2AR$ in LTK$^-$ cells. This is illustrated by the blunted isoproterenol dose-response curve observed in membranes derived from Gly$^{341}\beta_2AR$ expressing cells (Fig. 1). The marked difference between $\beta_2AR$- and Gly$^{341}\beta_2AR$-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 Gly$^{341}\beta_2$ARs was higher than that of wild type $\beta_2AR$; yet, the level of stimulation

TABLE I

*[$^{125}$I]CYP binding parameters and adenylyl cyclase stimulation for wild type and mutant $\beta_2AR$ expressed in LTK$^-$ cells*

Affinities for [$^{125}$I]CYP were determined by saturation experiments using membrane reperations derived from cells expressing wild type or mutant $\beta_2AR$. Adenylyl cyclase activities were determined as in Fig. 1 and analyzed using nonlinear least squares regression. The data represent the mean ± S.E. n, number of independent experiments.

| Receptor | [$^{125}$I]CYP binding parameters | | | Adenylyl cyclase stimulation | | |
| --- | --- | --- | --- | --- | --- | --- |
| | n | $K_d$ [a] | $B_{max}$ [b] | n | EC$_{50}$ [c] | $R_{max}$ [d] |
| | | pM | fmol/mg | | nM | pmol cAMP/min/mg |
| Wild type $\beta_2AR$ | 3 | 51.3 ± 0.7 | 168 ± 17 | 9 | 67 ± 10 | 24.8 ± 1.8 |
| Gly$^{341}\beta_2AR$ | 4 | 70.0 ± 6.5 | 125 ± 20 | 5 | 61 ± 15 | 4.9 ± 0.8 |
| Ala$^{345,346}\beta_2AR$ | 3 | 62.2 ± 22 | 210 ± 12 | 5 | 35 ± 10 | 27.7 ± 3.6 |
| Gly$^{341}$Ala$^{345,346}\beta_2AR$ | 3 | 84.0 ± 20 | 290 ± 30[e] | 5 | 33 ± 8 | 22.2 ± 1.9 |

[a] Equilibrium dissociation constant for [$^{125}$I]CYP.
[b] Maximal specific [$^{125}$I]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 $\beta_2AR$.
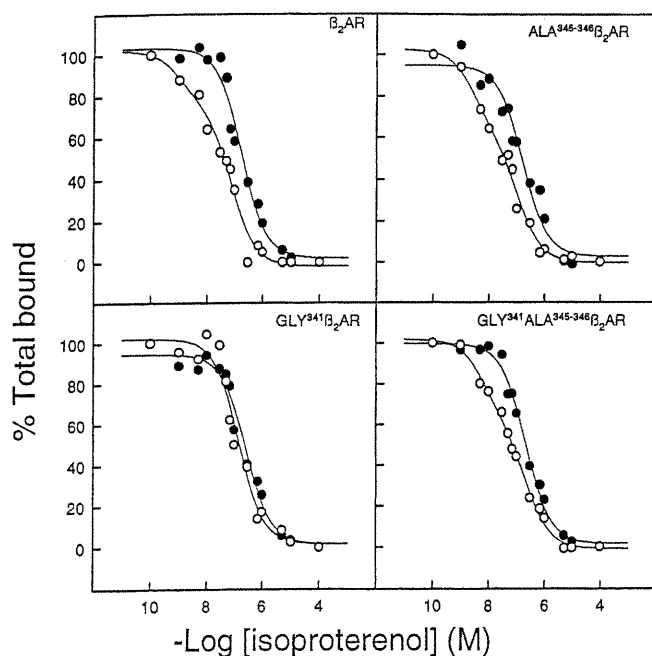
FIG. 2. **Agonist binding characteristics of wild type $\beta_2AR$, Gly$^{341}\beta_2AR$, Gly$^{341}$Ala$^{345,346}\beta_2AR$, and Ala$^{345,346}\beta_2AR$.** Isoproterenol competition of [$^{125}$I]CYP binding was assessed in the absence (O) or in the presence (●) of 100 $\mu$M Gpp(NH)p in membrane preparations derived from wild type or mutant $\beta_2AR$-expressing cells. The curves shown are computer-generated fits of data obtained in representative experiments, using the program Ligand (13).

TABLE II

*Isoproterenol binding parameters for wild type and mutant $\beta_2AR$*

The isoproterenol binding parameters were determined by competition of [$^{125}$I]CYP binding using increasing concentrations of isoproterenol (0–100 $\mu$M) in the presence or the absence of 100 $\mu$M 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.

| | $n$ | $K_{i(H)}$ (nM)$^{a,b}$ | $K_{i(L)}$ (nM)$^{a,b}$ | % Bmax$_{(H)}$$^{a,c}$ |
|---|---|---|---|---|
| Wild type $\beta_2AR$ | | | | |
| Control | 6 | 0.8 ± 0.3 | 33 ± 7 | 35 ± 7 |
| Gpp(NH)p$^d$ | 3 | —$^e$ | 84 ± 10 | — |
| Gly$^{341}\beta_2AR$ | | | | |
| Control | 3 | — | 73 ± 8 | — |
| Gpp(NH)p$^d$ | 3 | — | 95 ± 15 | — |
| Ala$^{345,346}\beta_2AR$ | | | | |
| Control | 6 | 1.3 ± 0.2 | 55 ± 7 | 48 ± 6 |
| Gpp(NH)p$^d$ | 3 | — | 81 ± 12 | — |
| Gly$^{341}$Ala$^{345,346}$ $\beta_2AR$ | | | | |
| Control | 5 | 2.1 ± 0.4 | 105 ± 17 | 42 ± 2 |
| Gpp(NH)p$^d$ | 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 100 $\mu$M Gpp(NH)p.
$^e$ —, not detectable.

obtained for Gly$^{341}\beta_2AR$ never reached that of the wild type receptor.

Additional replacement of serines 345 and 346 by alanine residues into Gly$^{341}\beta_2AR$ almost completely restored the ability of the receptor to stimulate adenylyl cyclase activity. This is illustrated by the normal isoproterenol dose-response curve observed in membranes derived from Gly$^{341}$Ala$^{345,346}\beta_2AR$-expressing cells (Fig. 1). In fact, the maximal isoproterenol-stimulated adenylyl cyclase activities were virtually identical for wild type $\beta_2AR$ and Gly$^{341}$Ala$^{345,346}\beta_2AR$ (Table I). This contrasts with the much reduced isoproterenol-stimulated activity observed for Gly$^{341}\beta_2AR$. These results suggest that the absence of the palmitoylated cysteine 341 decreases the ability of the receptor to stimulate the adenylyl 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 receptor modestly increased the ability of the receptor to stimulate the adenylyl cyclase activity (Fig. 1 and Table I).

Agonist binding properties of the receptors were then assessed by competition of [$^{125}$I]CYP binding with isoproterenol. As shown in Fig. 2 and Table II, competition of [$^{125}$I]CYP binding by isoproterenol was biphasic for the wild type $\beta_2AR$, and the curve could be best resolved by a two-affinity state model using iterative nonlinear least squares fitting of the nontransformed data (19). The high affinity state was guanyl nucleotide-sensitive. Indeed, when the binding was conducted in the presence of 100 $\mu$M Gpp(NH)p, the curve was best fitted to a single low affinity state model. The existence of guanyl nucleotide-sensitive high affinity sites for agonist is generally believed to reflect functional coupling of the receptor to G proteins. In membranes derived from L cells expressing Gly$^{341}\beta_2AR$, the displacement curves were best fitted to a single low affinity site whether the binding was conducted in the presence or the absence of Gpp(NH)p, thus suggesting that Gly$^{341}\beta_2AR$ is largely uncoupled from G$_s$. This is consistent with the modest adenylyl cyclase stimulation mediated by this

mutant receptor. The additional mutation of serines 345 and 346 completely restored the ability of the unpalmitoylated receptor to bind agonist with high affinity. Indeed, for Gly$^{341}$Ala$^{345,346}\beta_2AR$ the competition of [$^{125}$I]CYP binding by isoproterenol was best resolved by a two-affinity state model (Fig. 2, Table II). As was the case for the wild type receptor, the high affinity state was sensitive to Gpp(NH)p, and a single population of low affinity sites was found when the binding was carried in the presence of the GTP analog. Mutation of the two serines alone in the wild type $\beta_2AR$ did not affect the binding parameters of isoproterenol. $K_i$ values and the proportion of sites in high and low affinity states are summarized in Table II. These data suggest that the absence of serines 345 and 346 restores the ability of the unpalmitoylated receptor to interact with G$_s$, consistent with the effects of this mutation on the capacity of the $\beta_2AR$ to stimulate the adenylyl 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 Gly$^{341}\beta_2AR$. To test this hypothesis, the basal level of phosphorylation of the wild type $\beta_2AR$, Gly$^{341}\beta_2AR$ and Gly$^{341}$Ala$^{345,346}\beta_2AR$ was assessed in LTK$^-$ cells. Following metabolic labeling with inorganic [$^{32}$P]phosphoric acid ($^{32}$P$_i$), 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 [$^{125}$I]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 Gly$^{341}\beta_2AR$ was found to be much higher than that of the wild type receptor, thus confirming that, as found in CHW cells (6), Gly$^{341}\beta_2AR$ has a significantly elevated basal level of phosphorylation. In contrast, the level of phosphorylation of Gly$^{341}$Ala$^{345,346}\beta_2AR$ 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 Gly$^{341}\beta_2AR$. The low
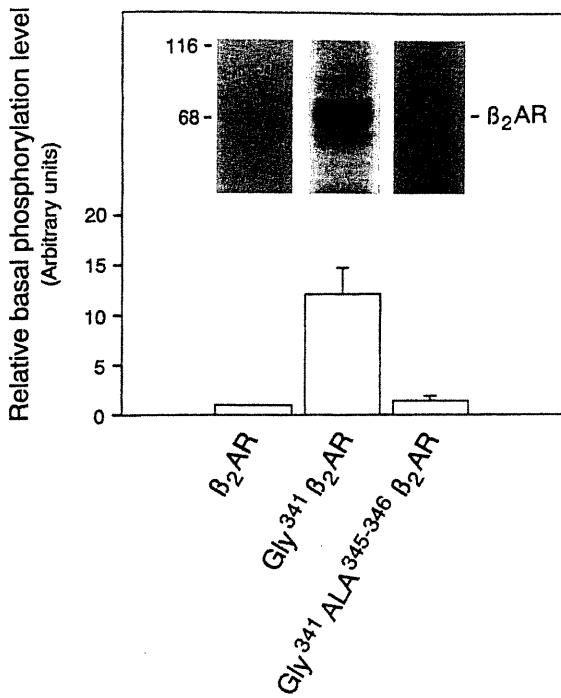
FIG. 3. **Basal phosphorylation level of wild type $\beta_2AR$, $Gly^{341}\beta_2AR$, and $Gly^{341}Ala^{345,346}\beta_2AR$.** LTK$^-$ cells stably expressing either the wild type or mutant $\beta_2AR$ were metabolically labeled with $^{32}P_i$. Solubilized $\beta_2AR$ were then purified by alprenolol-Sepharose affinity chromatography and subjected to electrophoresis on 10% SDS-PAGE. Shown is a representative autoradiograph. 295 fmol of $\beta_2AR$ as determined by [$^{125}$I]CYP binding were loaded in each lane. The *bar graph* compares the relative phosphorylation level of the receptors as determined by laser densitometric analysis of the autoradiograms. In each experiment, an arbitrary value of 1 was assigned to the phosphorylation level of wild type $\beta_2AR$. Values represent means ± S.E. of three independent experiments.

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 quantitate the increase in the basal phosphorylation level observed in $Gly^{341}\beta_2AR$, metabolic labeling experiments with $^{32}P_i$ were conducted in CHW cells expressing higher levels of wild type $\beta_2AR$ and $Gly^{341}\beta_2AR$ (~2.0 pmol/mg of protein in each case). Assessment of the specific activity of the cellular [$^{32}$P]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 $\beta_2AR$, while 1.1 mol of phosphate/mol of receptor were incorporated into $Gly^{341}\beta_2AR$.

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 $\beta_2AR$ expressed in Sf9 cells was purified, treated or not treated with hydroxylamine, and reconstituted in membranes derived from native LTK$^-$ cells devoid of endogenous $\beta_2AR$. The efficacy of the hydroxylamine treatment to remove the receptor-bound palmitate was tested using [$^3$H]palmitate-labeled $\beta_2AR$. 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 $\beta_2AR$ conferred identical
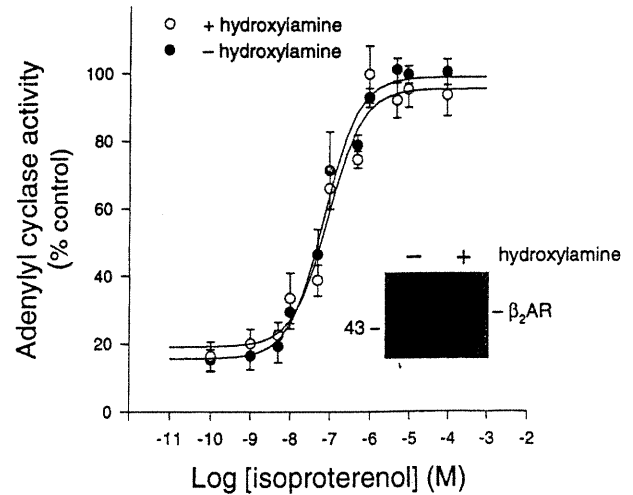


FIG. 4. **Effect of hydroxylamine treatment on $\beta_2AR$-stimulated adenylyl cyclase activity.** Affinity-purified receptor was treated with 1 M hydroxylamine (*open circles*) or the vehicle (*closed circles*) for 1 h. Desalted receptor preparations were then reconstituted in membrane preparations derived from untransfected LTK$^-$ cells. The preparations were then assayed for isoproterenol-stimulated adenylyl cyclase activity. The dose response curves shown represent the mean ± S.E. of eight independent experiments. *Inset*, [$^3$H]palmitate-labeled $\beta_2AR$ was purified from Sf9 cells by alprenolol-Sepharose affinity chromatography. 1 pmol of receptor was treated or not treated with 1 M hydroxylamine and then subjected to electrophoresis on 10% SDS-PAGE. The autoradiogram of a representative experiment is shown.

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 $\beta_2AR$ and G$_s$.

It has been proposed that post-translational modification of the $\beta_2AR$ 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 G$_s$. Indeed, $Gly^{341}Ala^{345,346}\beta_2AR$, which lacks the palmitoylated cysteine and the chemically depalmitoylated receptor, are perfectly able to stimulate the G$_s$/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, serines 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 $\beta_2AR$ ($^{259}$RRSS) and not the one in the carboxyl tail ($^{341}$CLRRSS) 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 Cys$^{341}$ 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 $\beta_2AR$, $Gly^{341}\beta_2AR$, and $Gly^{341}Ala^{345,346}\beta_2AR$. Membranes were incubated with [$\gamma$-$^{32}$P]ATP in the presence of PKA catalytic subunit. As shown
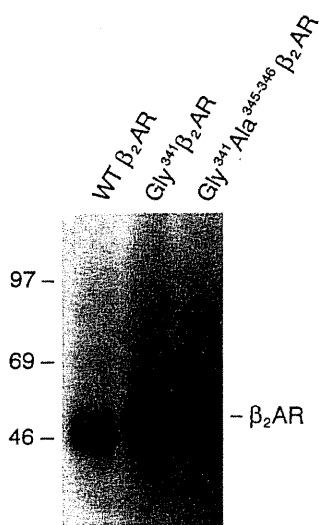
FIG. 5. **In vitro phosphorylation of wild type $\beta_2$AR, Gly$^{341}\beta_2$AR, and Gly$^{341}$Ala$^{345,346}\beta_2$AR by PKA.** Sf9 membrane expressing wild type $\beta_2$AR, Gly$^{341}\beta_2$AR, and Gly$^{341}$Ala$^{345,346}\beta_2$AR was phosphorylated with purified PKA catalytic subunit and [γ-$^{32}$P]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.

in Fig. 5, PKA catalyzed the phosphorylation of wild type $\beta_2$AR, Gly$^{341}\beta_2$AR, and Gly$^{341}$Ala$^{345,346}\beta_2$AR. The phosphorylated receptors migrated with an apparent molecular mass of 45–55 kDa, consistent with the reported electrophoretic mobility of the human $\beta_2$AR 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 p21$^{N\text{-}ras}$ (8), the unpalmitoylated Gly$^{341}\beta_2$AR and Gly$^{341}$Ala$^{345,346}\beta_2$AR have a slightly lower electrophoretic mobility then the wild type receptor. When comparing the maximum level of phosphorylation, Fig. 5 clearly shows that Gly$^{341}\beta_2$AR 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 $\beta_2$AR, thus suggesting that phosphorylation of this site contributed to the elevated phosphorylation observed in membranes expressing Gly$^{341}\beta_2$AR.

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 $^{343}$RRSS to the kinase. However, the stoichiometry of phosphorylation reached under the conditions studied were relatively modest (0.11 mol/mol for wild type *versus* 0.17 mol/mol for Gly$^{341}\beta_2$AR). We reasoned that the preexisting phosphorylation state of the receptors may interfere with the *in vitro* assay. To make sure that this did not bias the interpretation of the data, the receptors were dephosphorylated prior to the *in vitro* phosphorylation assay. For that purpose, membranes were treated with alkaline phosphatase for 45 min. This treatment was found to remove more than 80% of the phosphate incorporated into the receptor following whole cell metabolic labeling with inorganic [$^{32}$P]phosphoric acid (data not shown). As can be seen in Fig. 6, the phosphatase treatment dramatically increased the amount of phosphate that could be transferred by PKA for the two receptors, but the higher phosphorylation observed for Gly$^{341}\beta_2$AR was maintained. The stoichiometry of phosphorylation reached following pretreatment with the phosphatase was 0.31 mol/mol for wild type $\beta_2$AR and 0.70 mol/mol
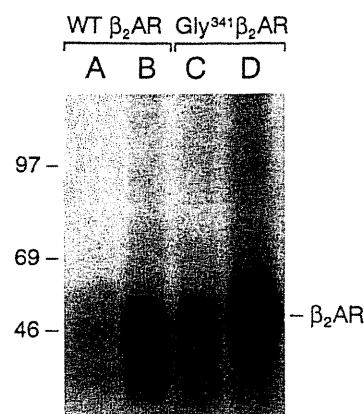


FIG. 6. **In vitro phosphorylation of dephosphorylated wild type $\beta_2$AR and Gly$^{341}\beta_2$AR.** Sf9 membranes expressing either the wild type $\beta_2$AR (*A* and *B*) or Gly$^{341}\beta_2$AR (*C* and *D*) were dephosphorylated (*B* and *D*) or not dephosphorylated (*A* and *C*) with alkaline phosphatase-agarose prior to the *in vitro* phosphorylation assay described in Fig. 5. Identical numbers of receptor (4.0 pmol), as assessed by radioligand binding, were loaded in each lane. The autoradiogram shown is representative of four independent experiments.

for Gly$^{341}\beta_2$AR. The amount of [$^{32}$P]PO$_4$ 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 Gly$^{341}\beta_2$AR than into the wild type $\beta_2$AR, 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 Ala$^{345,346}\beta_2$AR was compared with that of wild type $\beta_2$AR. As shown in Fig. 7, mutation of Ser$^{345,346}$ 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 (EC$_{50}$ of control, $1.6 \times 10^{-7}$ M; 1 min, $8.8 \times 10^{-7}$; 2 min, $1.3 \times 10^{-6}$) were observed. This desensitization was receptor-specific, since no significant change in either basal or forskolin-stimulated activity were observed (data not shown). In Ala$^{345,346}\beta_2$AR-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 Ala$^{345,346}\beta_2$AR and wild type receptor were similar (data not shown), suggesting that Ser$^{345}$ and Ser$^{346}$ 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 Cys$^{341}$ regulates the accessibility of a PKA phosphorylation site in the carboxyl tail of the $\beta_2$AR. 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 G$_o$ (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 $\beta_2$AR.
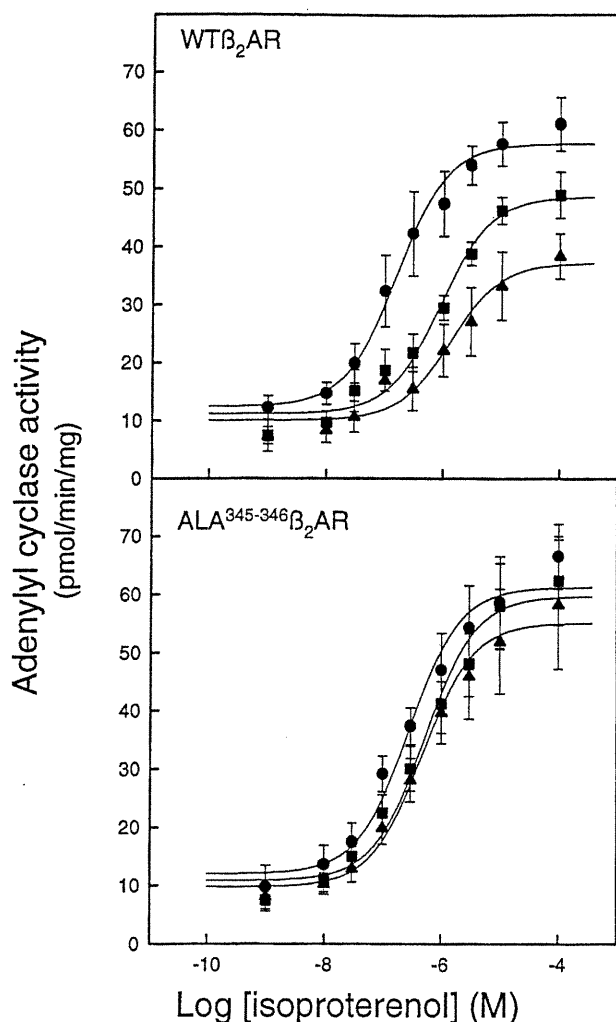
Indeed, Gly$^{341}$Ala$^{345,346}\beta_2AR$, which is lacking cysteine 341, interacts normally with $G_s$ and stimulates the adenylyl cyclase activity to levels comparable with that of wild type receptor.

For many proteins involved in signal transduction, palmitoylation serves to regulate membrane attachment. Good examples are $G\alpha$ subunits whose subcellular distribution has been shown to be modified following mutation of the palmitoylated cysteine (26, 27). Interestingly, stimulation of the $\beta_2AR$ has been shown to increase the turnover rate of $G\alpha_s$-bound palmitate, which may lead to a reduced palmitoylation of the protein (28–30). This regulation of the palmitoylation state of $G\alpha_s$ has been proposed to promote translocation of $G\alpha_s$ from the plasma membrane to the cytosol and thus could act as a process modulating signaling efficacy (28). A similar agonist-promoted regulation of the $\beta_2AR$ palmitoylation state has recently been proposed (7).[2] 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, providing better access for PKA to the phosphorylation site neighboring cysteine 341. The favored phosphorylation of this site may then contribute to the rapid onset of desensitization. Interestingly, 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, studies show that this site could not be phosphorylated upon direct activation of PKA (23). Also, deletion 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 desensitization (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 reconcile these data is to propose that serine 345 and 346 become 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 population) or with cAMP analogues would not promote phosphorylation 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 homologous 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 $\beta$ARK 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 $\beta$ARK like enzyme (33). The authors suggested a sequential interplay between PKA and the receptor-specific kinase. Similar 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 anaphylatoxin receptor (37).

Cysteine residues located in a position similar to that of cysteine 341 in the $\beta_2AR$ are found in most of the G protein-coupled receptors, and the occurrence of palmitoylation has been demonstrated for a number of these. They include rhodopsin (38), the $\alpha_{2A}$-adrenergic receptor (39), the dopamine $D_1$ receptor (40), the serotonin $5HT_{1B}$ (41) and $5HT_{1A}$ receptors (42), the luteinizing hormone receptor (43), and the metabotropic mGluR4 glutamate receptor (44). Although the precise site of palmitoylation has been identified only for a few receptors (5, 39, 44, 45), a number of studies have assessed the functional effects of mutating cysteines that were either known palmitoylation sites or assumed as potential sites. Similarly to what has been observed for the $\beta_2AR$, mutation of cysteine 347 of the $D_1$-dopamine receptor decreases its ability to interact with $G_s$ and to stimulate the adenylyl cyclase activity in COS cells (46). Furthermore, this mutant receptor becomes resistant to agonist-promoted desensitization, suggesting that it is constitutively desensitized. It is noteworthy that a potential PKA phosphorylation site is located four amino acids upstream of cysteine 347. In contrast, mutation of cysteine 442 in the $\alpha_{2A}AR$, which has been shown to abolish its palmitoylation, is without effect on the receptor-G protein coupling (39). Similarly, mutations of conserved cysteines in the lutropin/choriogonadotropin (43), thyrotropin-releasing hormone (47), and $M_2$-muscarinic receptors (48) were found not to affect coupling to their cognate G proteins. However, these mutations markedly affected the rate of agonist-promoted internalization. These observations suggest that the functional consequences of the lack of palmitoylation may vary among different G protein-

coupled receptors. The nature of the molecular motif(s) present in the carboxyl tail of a given receptor might determine these consequences. This is to be expected if the absence of the palmitoylated cysteines exposes the surface of the receptor carboxyl terminus and influences its interaction with various regulatory proteins.

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## 2.2 Article 2: Agonist stimulation increases the turn-over rate of $\beta_2$AR-bound palmitate and promotes receptor depalmitylation.

Dans le cadre de ce projet concernant la caractérisation de la palmitylation du récepteur exprimé dans les cellules d'insectes (Sf9), mon apport a été déterminant. En effet, mes résultats (figures 7 et 8) ont mis en évidence que la phosphorylation du récepteur, par la protéine kinase A, régule son état de palmitylation. Ainsi, l'ajout de ces résultats à cet article a permis de proposer un mécanisme de régulation entre ces deux modifications post-traductionnelles. Cela a été essentiel pour la publication de cet article dans *Biochemistry*.

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# Agonist Stimulation Increases the Turnover Rate of $\beta_2$AR-Bound Palmitate and Promotes Receptor Depalmitoylation[†]

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ABSTRACT: We have characterized the dynamic nature of $\beta_2$-adrenergic receptor palmitoylation in Sf9 cells. Under basal conditions, the turnover of receptor-bound palmitate is rapid (half-life $= 9.8 \pm 1.8$ min) compared to the turnover rate of the receptor protein itself (half-life $= 109 \pm 10$ 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 $\beta_2$-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 $\beta_2$-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 $\beta_2$-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 $\beta_2$-adrenergic receptor ($\beta_2$AR)[1] (O'Dowd et al., 1989), the $\alpha_2$-adrenergic receptor ($\alpha_2$AR) (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 $\beta_2$AR, 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; $\beta_2$AR, $\beta_2$-adrenergic receptor; $\alpha_2$AR, $\alpha_2$-adrenergic receptor; C16-CoA, palmitoyl-coenzyme A; PKA, cAMP-dependent protein kinase; Sf9, *Spodoptera frugiperda*; MDCK, Madin–Darby canine kidney; CHW, Chinese hamster fibroblasts.

incorporation of [³H]palmitate into the $\beta_2$AR 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 $\beta_2$AR, independent of the expression system. More recently, Kennedy et al. (1994) showed, in MDCK cells, that stimulation of the $\alpha_{2A}$-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 $\beta_2$-AR resulted from an enhanced stoichiometry of palmitoylation or from an accelerated turnover rate.

In the present study, a detailed kinetic analysis of $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR Recombinant Baculoviruses.* The recombinant baculovirus encoding the c-*myc*-tagged human $\beta_2$AR 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 $\beta_2$AR cDNA, pTZ18R-$\beta_2$-AR-Ala²⁶¹⁻²⁶², Ala³⁴⁵⁻³⁴⁶ (Hausdorff et al., 1989), into the Sf9 recombination plasmid pJV-c-myc-$\beta_2$AR (Mouillac et al., 1992) was carried out. Briefly, the NcoI-PpUM1 fragment from the pTZ18R-$\beta_2$AR-Ala²⁶¹⁻²⁶², Ala³⁴⁵⁻³⁴⁶ plasmid was subcloned into the NcoI-PpUM1 site of the pJV-c-myc-$\beta_2$AR. 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 $\beta$-galactosidase detection system (Vialard et al., 1990). Confirmation of $\beta_2$AR protein expression was assessed by whole cell binding assays.

*Cell Culture, Infection with Recombinant $\beta_2$AR 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 × 10⁶/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 [9,10-³H]-palmitate dissolved in 200 $\mu$L of dimethyl sulfoxide (DMSO) was added. For [³⁵S]protein labeling experiments, 5 mCi of Tran³⁵S-Label (ICN) was added. Cells were then split into 8 × 50 mL tubes (Falcon) (12 mL/tube). Isoproterenol (1 $\mu$M final concentration) (Sigma), dissolved in 10 $\mu$M 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 $\mu$g/mL leupeptin, 5 $\mu$g/mL soybean trypsin inhibitor and 10 $\mu$g/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 ³⁵S pulse−chase experiments, 5 mCi of Tran³⁵S-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 $\beta_2$AR-bound palmitate and the receptor half-lives, data from [³H]palmitate and Tran³⁵S-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 $\beta_2$AR.* Alprenolol−Sepharose affinity purification matrix was synthesized according to the method of Benovic et al. (1987). This matrix was used to purify the $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR 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$_2$O (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$_3$COOH/H$_2$O (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 $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR closely parallels the cellular content of free [$^3$H]palmitate could suggest that palmitate transfer occurs only on newly synthesized $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR rapidly declined during the chase period. The half-life for the receptor-bound palmitate was found to be 9.8 ± 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 ± 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 $\beta_2$AR, the kinetics of [$^3$H]palmitate incorporation were studied in the presence or absence of the $\beta$-adrenergic agonist isoproterenol. As seen in Figure 3A, agonist activation significantly increased the rate of [$^3$H]palmitate incorporation into the $\beta_2$AR. 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 $\beta_2$-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
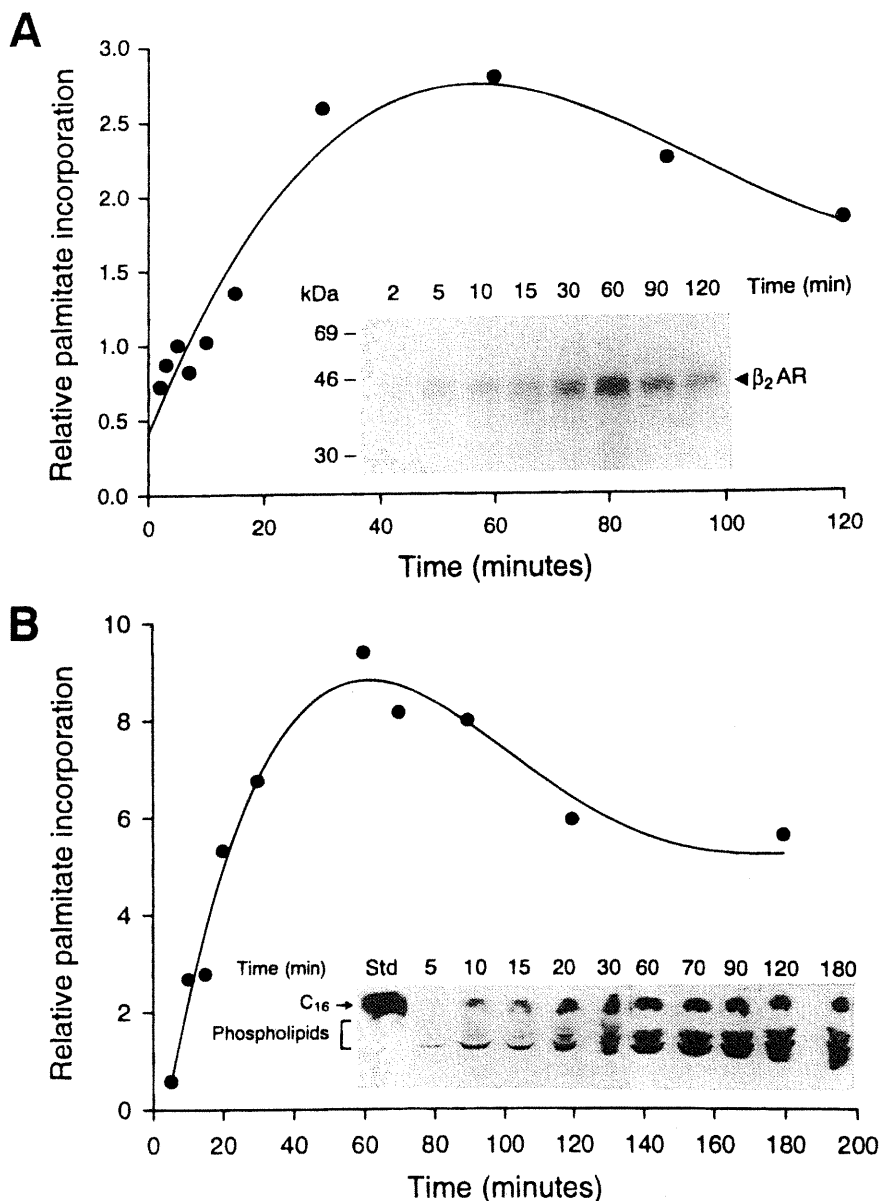
**A**



**B**



FIGURE 1:  Time course of palmitate incorporation into $\beta_2$AR. Sf9 cells expressing the human $\beta_2$AR were metabolically labeled with [³H]-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 [³H]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 [³H]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 [³H]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 [³H]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 $\beta_2$AR 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 [³H]-

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 [³H]palmitate cellular pool.  Indeed, neither the overall cellular incorporation of [³H]palmitate (Figure 4A) nor the incorporation of [³H]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, [³⁵S]me-thionine/cysteine incorporation into the $\beta_2$AR was not affected by agonist over the time period studied.
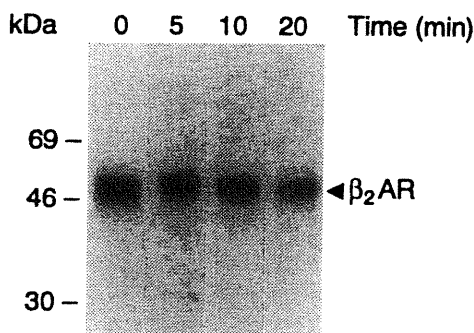
FIGURE 2: $\beta_2$AR-bound palmitate turnover. Pulse—chase experiments were carried out as described under Experimental Procedures. Receptors were purified and resolved by SDS—PAGE. The intensity of labeling was assessed by fluorography and a typical labeling decay pattern is shown. Identical amounts of purified $\beta_2$AR (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 $\beta_2$AR-bound palmitate was determined as described under Experimental Procedures. The average half-life calculated from 3 independent experiments was $9.8 \pm 1.8$ min (mean $\pm$ 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 $\beta_2$AR 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 $9.0 \pm 1.6$ to $5.4 \pm 0.4$ min. Under these conditions, no receptor down-regulation occurred (data not shown) and no change in the receptor half-life accompanied the treatment ($109 \pm 10$ vs $101 \pm 14$ 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 $\beta_2$AR 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 $\beta_2$AR 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 [3H]-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 [3H]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 [3H]palmitate incor-

poration 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 [3H]palmitate incorporation in a time-dependent fashion (Figure 7). These data indicate that desensitization of the receptor may be responsible for the reduction in [3H]palmitate incorporation observed in Figure 3C. Since a link between the palmitoylation of cysteine-341 and the phosphorylation of the $\beta_2$AR by PKA has already been proposed (Moffett et al., 1993, 1996), the palmitoylation state of a mutant form of the $\beta_2$AR lacking the PKA sites ($\beta_2$AR-Ala$^{261-262}$, Ala$^{345-346}$) was assessed. As shown in Figure 8A, the extent of [3H]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 [3H]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 $\beta_2$AR 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 $\beta_2$AR. First, the rapid incorporation of [3H]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 [3H]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 $\alpha$ 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 $\beta_2$AR 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 Gs$\alpha$ (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
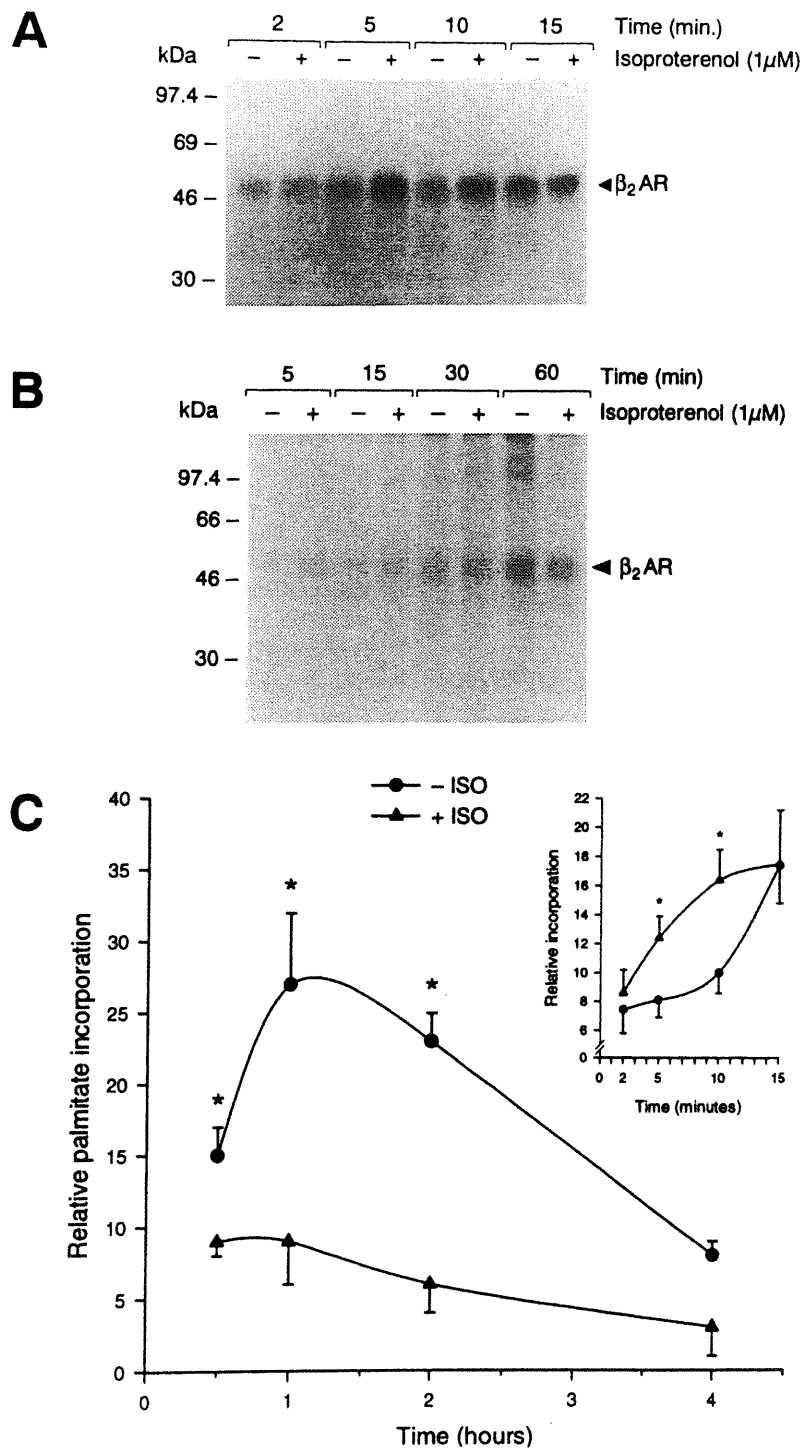
FIGURE 3: Time course of agonist effects on $\beta_2$AR [$^3$H]palmitate incorporation. Sf9 cells expressing $\beta_2$AR were labeled with [$^3$H]palmitate in the presence or absence of isoproterenol for the times indicated. Receptors were purified and resolved by SDS—PAGE. The intensity of labeling 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 $\beta_2$AR 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 $\beta_2$AR 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-

**A**

**B**
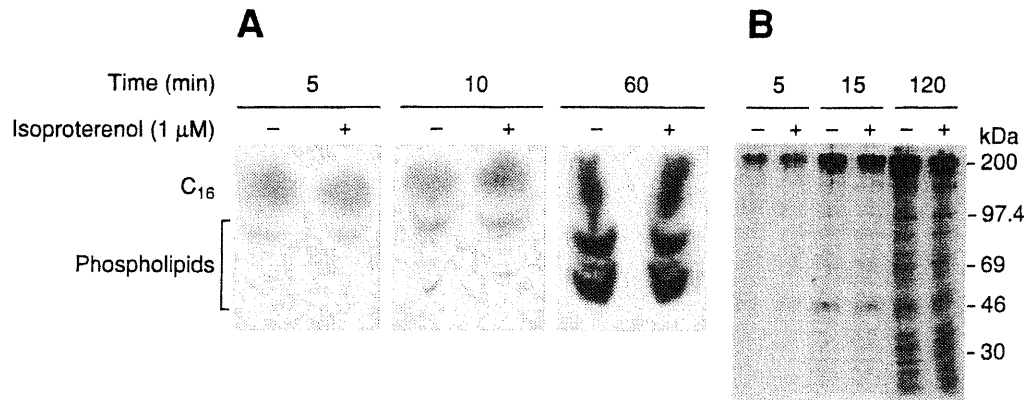


FIGURE 4: Effect of isoproterenol on cellular incorporation of [³H]palmitate. Sf9 cells expressing β2AR were labeled with [³H]palmitate in the presence or absence of isoproterenol for the indicated times. (A) [³H]Palmitate incorporation into cellular lipids was determined by thin-layer chromatography following organic extraction of total lipids as described under Experimental Procedures. (B) [³H]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.



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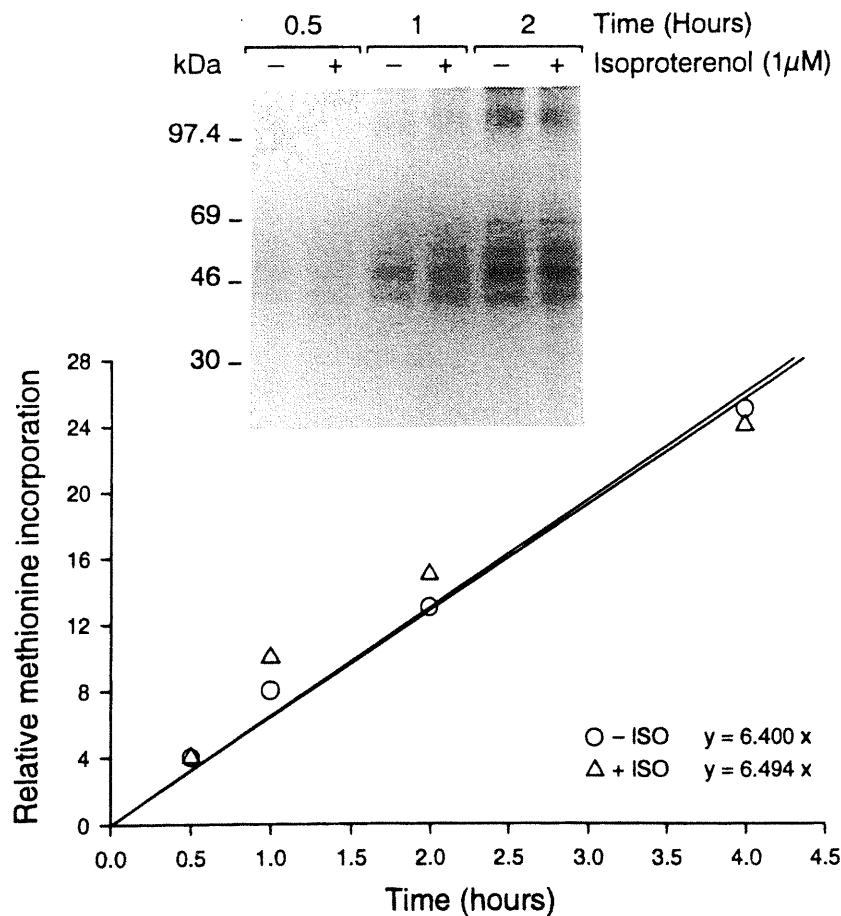
FIGURE 5: Effect of isoproterenol on β2AR synthesis. Sf9 cells expressing β2AR were labeled with [³⁵S]methionine/cysteine in the presence or absence of isoproterenol for the indicated times. Purified receptor was resolved by SDS−PAGE and the relative extent of labeling determined by fluorography. The curves shown represent the average of 2 experiments. The inset shows a representative fluorograph; 3.8 pmol of β2AR 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 β2AR.

tion of this enzymatic activity to the depalmitoylation of the β2AR 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 β2AR 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-
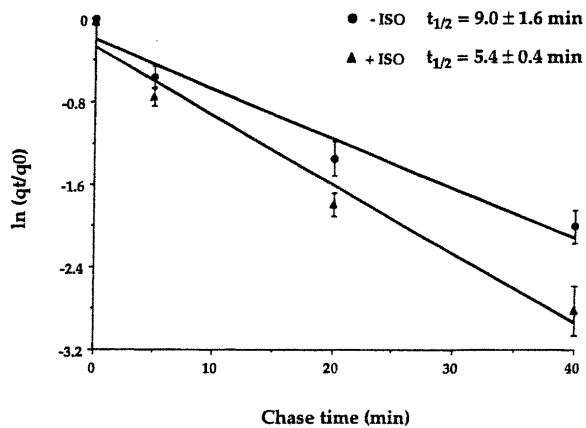
**FIGURE 6:** Effect of isoproterenol on the turnover rate of $\beta_2$AR-bound palmitate. Cells were labeled with [³H]palmitate for 2 h and chased with cold palmitate for the times indicated. Cells were treated (closed triangles) or not (closed circles) with isoproterenol during the chase, and the extent 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).
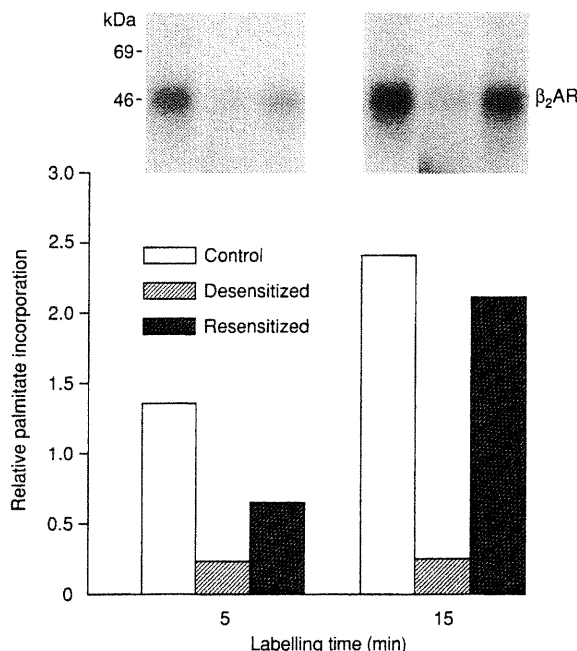


**FIGURE 7:** Effects of agonist pretreatment on $\beta_2$AR [³H]palmitate incorporation. Sf9 cells expressing $\beta_2$AR were treated or not (control) with 1 $\mu$M isoproterenol for 15 min. The cells were then labeled with [³H]palmitate for the indicated times in the continued presence of isoproterenol (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 [³H]-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
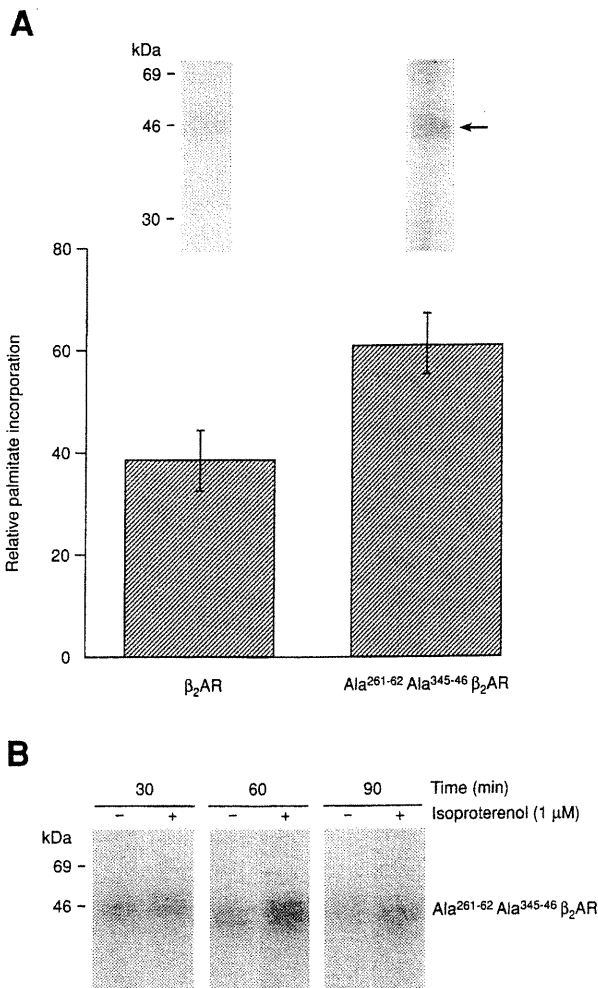




**FIGURE 8:** [³H]palmitate incorporation into $\beta_2$AR-Ala$^{261-262}$, Ala$^{345-346}$. (A) Sf9 cells expressing either wild-type $\beta_2$AR or $\beta_2$-AR-Ala$^{261-262}$, Ala$^{345-346}$ were labeled with [³H]palmitate for 60 min. Receptors were purified and resolved by SDS—PAGE, and the intensity 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 $\beta_2$AR-Ala$^{261-262}$, Ala$^{345-346}$ were labeled with [³H]palmitate in presence or absence of 1 $\mu$M 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 [³H]palmitate into agonist-stimulated $\beta_2$AR 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 [³H]-palmitate incorporation at any of the labeling times studied.

Interestingly, similar to what has been observed here for the $\beta_2$AR, stimulation of Gs has been shown to modulate the turnover rate of the Gs$\alpha$-bound palmitate and has been suggested to favor the nonpalmitoylated form of Gs$\alpha$ (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 $\alpha$ subunit from the plasma membrane to the cytosol. For the $\beta_2$AR, 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 $\beta_2$AR 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 $\beta_2$AR 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 $\beta_2$AR lacking the known PKA phosphorylation sites ($\beta_2$AR-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 $\beta_2$AR 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 $\beta_2$AR 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 ($^{333}$RIAFQELLCLRRSSLK) 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 $\beta_2$AR 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 $\beta_2$AR 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 $\alpha$-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) $\beta_2$AR-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 $\beta_2$AR 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 $\beta_2$AR functions.

## ACKNOWLEDGMENT

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## 2.3 Article 3: Dynamic palmitoylation of the human $\beta_2$-adrenergic receptor in mammalian cells; Effects of agonist stimulation and receptor phosphorylation.

# Dynamic palmitoylation of the human β$_2$-adrenergic receptor in mammalian cells; Effects of agonist stimulation and receptor phosphorylation.

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## SUMMARY

Palmitoylation is a post-translational modification that has been reported for several G protein-coupled receptors. However, very little is known concerning the kinetics and the regulation of this modification. Here, using the $\beta_2$-adrenergic receptor as a model, the turnover rate of the receptor-bound palmitate was investigated both under basal conditions and upon agonist activation. Pulse-chase studies carried out in HEK293 cells show that the basal turnover rate of the receptor-bound palmitate ($t_{1/2}$= 11.5 ± 1.7 hours) is faster than that of the receptor protein itself ($t_{1/2}$= 23 ± 5 hours) indicating that palmitoylation is a reversible process in mammalian cells. The relatively small difference between the two half-life suggest that, under basal conditions, only a few palmitoylation/depalmitoylation cycles occur during the life span of the receptor. Stimulation with the $\beta$-adrenergic agonist isoproterenol, led to a two fold increase in the turnover rate ($t_{1/2}$= 5.3 ± 0.5 hours) of the receptor-bound palmitate indicating that the acylation cycle is affected by the activity of the receptor. This agonist-dependant effect is not mimicked by a treatment with dibutyryl cAMP suggesting that second messenger production is not sufficient to modulate palmitoylation. However, mutation of a PKA phosphorylation site (serines 345-346), located near the acylated cysteine 341, blocked the effect of receptor activation on its palmitoylation state. Taken together, these results demonstrate that palmitoylation of the $\beta_2$AR in mammalian cells is a reversible post-translational modification that can be regulated by agonist stimulation in a phosphorylation-dependant manner.

**INTRODUCTION**

A number of G protein-coupled receptors (1) has been reported to be palmitoylated. In all cases investigated, the site for this postranslational modification was attributed to cysteines located in the carboxyl domain of the receptors. It is currently believed that due to its hydrophobic nature, the palmitate moiety would be inserted in the plasma membrane, providing extra membrane anchorage for GPCRs and creating an additional intracellular loop (2). In many studies, it has been proposed to play regulatory roles for receptor function. For instance, mutations of the palmitoylation site(s) of several receptors have been shown to inhibit receptor-simulated G protein activation (3-7). In others cases, receptor trafficking, targeting, or agonist-promoted internalisation have been found to be affected by such mutations (8-12). Given these facts, the idea that palmitoylation may represent a dynamically regulated postranslational modification that modulates various aspects of receptor function has attracted considerable attention (13).

In several studies, the notion that palmitoylation can be biologically regulated, has been assessed by investigating the effects of agonist stimulation on the incorporation of labelled palmitate in metabolic pulse-labelling experiments. In some cases, the treatment was found to increase the incorporation of the labelled palmitate (4;14;15), whereas in others a decrease (9) or no effect were observed (6;16;17). The interpretation of these results is complicated by the fact that cellular palmitate pools are large and the turnover rapid, making it difficult to reach isotopic equilibrium during pulse-labeling experiments. It follows that variations in the specific affinity of the cellular palmitate donor pool, during the course of the different experiments, may have determined the direction of the changes observed. In one study that took such variations into account and in which pulse-chase labeling experiments were carried out, agonist stimulation of $\beta_2AR$ was shown to promote an increase in the turnover

rate of the receptor-bound palmitate (18), clearly establishing that palmitoylation is a reversible process that can be modulated by receptor activation. However, this study was carried out using the baculovirus/Sf9 insect cell system. Although this system greatly facilitates the study of palmitoylation, due to the very high receptor expression levels that can be attained, it also has its limitations. In particular, the viral infection cycle results in a dramatic shut-off of host gene expression (19) that could influence the palmitoylation process.

Therefore, the present study was undertaken to study the dynamic process of GPCR palmitoylation in mammalian cells. Given that a link between the phosphorylation and the palmitoylation states of the receptor was suggested in Sf9 cells (18), we also investigated if such reciprocal regulation can be seen in mammalian systems. For this purpose, human embryonic kidney (HEK293) cells expressing high levels of the $\beta_2AR$ were used. We found that, as previously reported in Sf9 cells, receptor palmitoylation is a reversible process that can be dynamically regulated upon agonist activation. However, the turnover rate of the receptor-bound palmitate was found to be much slower in mammalian cells indicating a relatively slow process. Despite the apparent difference in the kinetics of palmitoylation and phosphorylation, mutation of a PKA phosphorylation site located near the palmitoylated cysteine was found to prevent the effect of receptor activation on its palmitoylation state confirming the existence of a regulatory link between the two modifications.

## MATERIALS AND METHODS

**Construction of Mutated $\beta_2$AR cDNA and Cell Transfection-** The generation of Ala$^{261\text{-}262}\beta_2$AR and Ala$^{345\text{-}346}\beta_2$AR mutants were constructed using pBacPak-Ala$^{261\text{-}262}\beta_2$AR and pBacPak-Ala$^{345\text{-}346}\beta_2$AR respectively (18). Briefly, pBacPak-Ala$^{261\text{-}262}\beta_2$AR and pBacPak-Ala$^{345\text{-}346}\beta_2$AR were restricted with BamH1/Hpa1 and BamH1/Sal1, respectively. The appropriate restriction fragments were isolated and used to replace the corresponding region in pcDNA3-$\beta_2$AR (wild type). Positive clones were confirmed by sequencing. The wild type and the mutants receptors were stably transfected in Human Embryonic Kidney 293 (HEK293) cells line using the calcium phosphate precipitation method (20). Neomicin resistant cells were selected by culturing in medium containing G418 (450 μg/ml) (Gibco). Resistant clones were then screened for $\beta_2$AR expression by radioligand binding assays using [$^{125}$I]-labeled iodocyanopindolol (CYP) as ligand. Cloned cell lines expressing similar numbers (6 to 7 pmol / mg of membrane protein) of wild type or mutants $\beta_2$AR were selected for the study.

**Mammalian Cell Culture-** HEK293 cells line stably transfected with $\beta_2$AR, Ala$^{261\text{-}262}\beta_2$AR and Ala$^{345\text{-}346}\beta_2$AR were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Metabolic Labelling-** For labelling with [9,10-$^3$H]-palmitate (Mandel), HEK293 cells were incubated in serum-free DMEM for 1 hour followed by serum-free DMEM containing 0.6% DMSO and 250 μCi/ml of [$^3$H]-palmitate with or without 1 μM isoproterenol for different time periods. For the metabolic labelling with [$^{32}$P]P$_i$ (3), cells were cultured in phosphate-free and serum free DMEM for two hours prior

to labelling. Cells were then incubated with 200 μCi/ml of $[^{32}P]P_i$ (Mandel) for 2 hours in the same media and, 1 μM of isoproterenol (Sigma) was added or not for the last 15 minutes. Labelling was stopped by rinsing cells twice in cold phosphate buffered saline (pH 7.4) (PBS), centrifuged at 500 x $g$ for 10 min. Cells were then disrupted by sonication at $4^{o}C$ and membranes prepared (21). Receptors were solubilized in 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, containing 0.3% n-dodecyl maltoside with protease inhibitor cocktail (5 μg/ml leupeptin, 5 μg/ml soybean inhibitor and 10 μg/ml benzamidine) for 90 minutes at $4^{o}C$ and purified as described below.

**Pulse-chase experiments-** For pulse-chase experiments with palmitate, labelling was conducted during a 2 hour period with $[9,10-^{3}H]$-palmitate. Cells were then rinsed 3 times with PBS at room temperature, and the chase was started with DMEM's supplemented with 10% FBS and containing 0.2 mM unlabeled palmitate. Cells were then stimulated or not with final concentration of 1μM isoproterenol dissolved in 10 μM ascorbate or ascorbate alone (vehicle). For the pulse-chase experiments with $[^{35}S]$-methionine/cysteine, cells were incubated in methionine- and cysteine-free DMEM media during 1 hour before to add 150 μCi/ml of $Trans^{35}S$-Label methionine/cysteine (ICN) for labelling of 1 hour. Cells were rinsed 3 times with PBS at room temperature, and the chase was started with DMEM's supplemented with 10% FBS containing 1 mM methionine and 1 mM cysteine. Cells were stimulated or not with 1 μM final concentration of isoproterenol dissolved in 10 μM ascorbate or ascorbate alone (vehicle). At the end of the chase experiments, membranes were prepared and receptors purified.

To determine the $\beta_2AR$-bound palmitate and the receptor half-lives, data from $[^{3}H]$-palmitate and $[^{35}S]$-methionine/cysteine pulse-chase experiments were analysed by a

non linear regression analysis (GraphPad Prism). The equation used for the fit, a modification of the one-compartment metabolic turnover equation (22) is as follow: $q(t) = q(t^{\to 4}) + q\ (t=0)e^{(-R)t}$ where t is the time of incubation, R is the rate of decay, and q represents the level of labelling (in percent of control). The half-lives were estimated where $q(t) = 50\ \%$.

**Purification of $\beta_2AR$-** Alprenolol-Sepharose affinity purification matrix was synthesized according to the method of Benovic *et al.* (23). This matrix was used to purify the $\beta_2AR$ as previously reported (18). The affinity purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon) and the amount of $\beta_2AR$ in each sample was determined by soluble radioligand binding assays using $[^{125}I]$-CYP as described (21). Equal numbers of receptor were then transferred into sample buffer and SDS-PAGE conducted under non-reducing conditions. For metabolic labelling with $[^3H]$-palmitate or $^{35}S$-methionine/cysteine, fixed gels were incubated in 1 M salicylic acid (Sigma) for 45 minutes prior to drying, and exposure to Dupont Reflection$^{TM}$ film and screen at -80°C.

**Whole cell lipid labelling-** $[^3H]$-palmitate was extracted from 1 ml cells suspension after labelling with 250 µCi/ml of $[^3H]$-palmitate for the times indicated. Prior to extraction, cells were rinsed once with ice cold PBS and resuspended in 250 µl PBS. Then, 250 µl of the organic phase of $CHCl_3/MeOH/H_2O$ (5:5:1 by volume) was added and the mixture vortexed. The two phases were separated by centrifugation and the organic phase recovered and dried under vacuum. 100 µl of $CHCl_3/MeOH/50$ mM Tris, pH 7.5 (6:6:1 by volume) was used to resuspend the desiccant. A aliquot (20 µl) was then loaded on a thin layer silica gel (Aldrich) for chromatography using *n*-butanol/ $CH_3OOH/H_2O$ (5:2:3 by volume) as the mobile

phase (24). Then, the air-dried thin-layer dipped into 1 M salicylic acid for 45 minutes prior to fluorography.

## RESULTS

The $\beta_2$AR was stably expressed in human embryonic kidney (HEK293) cells to study the dynamics of receptor palmitoylation. Following metabolic labelling of the cells with [$^3$H]-palmitate the receptor was purified by alprenolol-sepharose chromatography and equal number of receptor were loaded on SDS-PAGE to visualise palmitate incorporation. Under basal conditions, [$^3$H]-palmitate was incorporated into the $\beta_2$AR in a time-dependant manner with a maximal incorporation being observed after two hour of labelling (Fig. 1A). In the presence of the $\beta$-adrenergic agonist, isoproterenol, both the rate and the extent of palmitate incorporation into the receptor were increased. The maximal palmitate incorporation in the presence of the agonist being more than three times that observed in control condition. This effect of the agonist on the labelling intensity did not result from a change in the specific activity of the palmitate donor pool as indicated by the absence of effect of the isoproterenol treatment on the total amount of free [$^3$H]-palmitate detected in cell extracts (Fig 1B).

To determine if the agonist-promoted increase in palmitate incorporation resulted from an increased turnover rate of the receptor-bound palmitate as previously documented in Sf9 cells (18), pulse-chase experiments were carried out. Chases for different period of times were initiated following a two hour metabolic labelling with [$^3$H]-palmitate (Fig. 2A). In the absence of isoproterenol, the half-life of the receptor-bound palmitate was found to be $8.8 \pm 2.0$ hours. This half-life was reduced to $3.7 \pm 0.4$ hours in the presence of the agonist (Fig. 2B).

The half-life of the receptor protein itself was also monitored by pulse-chase metabolic labelling experiments using $^{35}$S-methionine/cysteine as tracer. As shown in figure 2C, the half-life of the receptor was found to be much longer (23 hours $\pm$

5.0 hours) than that of the receptor-bound palmitate indicating that the palmitate associated to the receptor turns over more rapidly than the receptor itself. As expected, the presence of agonist during the chase significantly increases the turnover rate of the receptor protein, decreasing its half-life to $9 \pm 1$ hours. This most likely reflects the well-characterised agonist-promoted down-regulation of the receptor that is usually observed upon sustained agonist treatment (25) (fig. 3). It follows that the turnover rate of the receptor-bound palmitate, deduced in Fig. 2B, includes the turnover of both the palmitate on the receptor and the receptor itself. Figure 2D, illustrates the net turnover of the palmitate on the receptor once the contribution of the protein turnover has been subtracted. It indicates that receptor stimulation with isoproterenol increases the net turnover rate of the palmitate from $11.5 \pm 1.7$ hours to $5.3 \pm 0.5$ hours. Therefore, as it was the case in Sf9 cells, the agonist-promoted increase in [$^3$H]-palmitate incorporation observed in Fig. 1A most likely reflects a faster turnover rate that allows accelerated exchange of the receptor-bound palmitate.

The isoproterenol-induced increase in palmitate turnover requires the continuous presence of the agonist and does not reflect long lasting effect. Indeed, pre-treatment of the cells for 15 minutes with isoproterenol, prior to metabolic labelling, did not influence [$^3$H]-palmitate incorporation into the receptor (Fig. 3). This contrast with the significant increase observed when the agonist is present during the labelling period. A similar increase in the incorporation is also observed when the agonist is added for the last 15 minutes of a two-hour metabolic labelling (Fig. 4). However, the addition of the cell permeable cAMP analogue, dibutyryl cAMP (Bt$_2$cAMP), in the same conditions was without effect on the palmitate incorporation. Thus the activation process itself and not a stable modification of the receptor nor second messenger production mediates the increased turnover rate of the palmitate.

Since phosphorylation represents a rapidly reversible modification that occurs upon receptor activation, we tested the hypothesis that the agonist-dependent phosphorylation of the site located close to the palmitoylated cysteine 341 could be involved in the regulation of receptor palmitoylation. For this purpose, serine 345 and 346, within a consensus phosphorylation site for protein kinase A (PKA), were substituted for alanines using site-directed mutagenesis. The effect of isoproterenol on palmitate incorporation was then tested in HEK293 cells stably expressing the mutant $Ala^{345-346}\beta_2AR$. In contrast with the increased incorporation observed for the wild type receptor (Fig. 4), the agonist treatment was without effect on the $[^3H]$-palmitate labelling of $Ala^{345-346}\beta_2AR$ (Fig. 5). As a control, $[^3H]$-palmitate incorporation was also assessed in cells expressing a mutant $\beta_2AR$ lacking the distal PKA phosphorylation site located in its third intracellular loop $(Ala^{261-262}\beta_2AR)$. In that case, isoproterenol promoted an increase of the $[^3H]$-palmitate incorporation that was identical to that observed for the wild type receptor (Fig. 5). These results are consistent with the notion that phosphorylation of serines 345-346 could play a role in the dynamic regulation of receptor palmitoylation by agonist stimulation.

## DISCUSSION

The results reported in this study demonstrate that 1) palmitoylation of the $\beta_2AR$ expressed in mammalian cells is a reversible process that is dynamically regulated during the life span of the receptor, 2) agonist activation increases the turnover rate of the receptor-bound palmitate and 3) phosphorylation of a PKA consensus site is involved in the agonist-promoted regulation of the receptor palmitoylation.

Although palmitoylation of several G protein-coupled receptors has been documented (26) the dynamic nature of this modification has not been fully characterised. In fact, the present report is among the first directly assessing the turnover rate of GPCR-bound palmitate in mammalian cells. Only one other study measured the half-life of this modification. Kenedy *et al.* (27) determined, using the $\alpha_2$-adrenergic receptor ($\alpha_2$-AR) as a model, that the half-life of receptor-bound palmitate was 13 hours. This value is very similar to that calculated in the present study for the $\beta_2AR$ ($t_{1/2} = 11.5$ hours). However, they reported that the half-life for the palmitate moiety exceeded the half-life of the receptor molecule itself, the calculated half-life of the receptor being 9.5 hours. This obviously casts doubts on the interpretation of the results and did not permit a firm conclusion about the dynamic nature of the palmitoylation process in mammalian cells. In our study, however, the results clearly show that the half-life of the $\beta_2AR$-bound palmitate is shorter than the half-life of the receptor itself. This is consistent with the only other result available for a transmembrane receptor, the transferin receptor, for which a half-life of 60 hours has been calculated whereas the half-life of its bound palmitate was 10 hours (28). Therefore, our results are consistent with the notion that G-protein coupled receptors undergo dynamic cycles of palmitoylation/depalmitoylation in mammalian cells confirming that this modification is truly post-translational.

The relative long half-life of the protein-bound palmitate determined for the $\alpha_2$AR, the $\beta_2$AR and the transferrin receptor may represent a characteristic of trans-membrane proteins since for most cytosolic proteins, GAP-43 (29), N-RAS (30), H-RAS (31), p56$^{lck}$ (32), Gs$\alpha$ (33), NO synthase endotheliale (NOSe) (34) and ankyrine (35), the calculated half-life was significantly shorter. In fact, it was found to be usually less than one hour, even if the half-life of the proteins itself were found to be similar to that of the membrane receptor discussed above (>20 hours). It could be hypothesised that this faster turnover rate of the palmitate bound to cytosilic proteins results from a better accessibility of the reactive thioester bond to a soluble palmitoyl-thioesterase activity. Consistent with this idea, Lu and Hofmann (31) reported an inverse correlation between the palmitate turnover rate and the extent of membrane association for various extrinsic membrane proteins. The recent discovery that a palmitoyl-thioesterase exists in the cytosol of mammalian cells (36) lends additional support to the idea that the subcellular distribution of a given protein may influence the acylation/deacylation cycles.

Although the mechanisms involved in the regulation of the $\beta_2$AR palmitoylation state remain unknown, our results demonstrate that agonist stimulation increases the turnover rate of receptor-bound palmitate by more than 40%. A similar effect of agonist-stimulation has previously been reported for the palmitoylation of the $\beta_2$AR heterologously expressed in Sf9 insect cells. In this latter system, however, the absolute values for the half-life of the receptor-bound palmitate and of the receptor itself were found to be much shorter. This most likely reflects the rapid metabolism associated with the use of a baculovirus-driven expression system.

Modulation of protein-bound palmitate turnover rate upon biological activation has also been observed for two cytosolic proteins: G$\alpha$s and the endothelial nitric oxide

synthase (eNOS). For Gsα, both a constitutively activating mutation (Gsα-R201C) and receptor-mediated stimulation promoted significant increases in the turnover rate of the palmitate linked to the α subunit (33). Similarly, activation of eNOS by increasing intracellular calcium favours the depalmitoylation of the enzyme as a result of an increased palmitate turnover rate (34). Interestingly, for these two proteins, regulation of the palmitoylation state has been shown to provide a mechanism for controlling reversible changes in the sub-cellular localisation and activity of the proteins (33;34). For the $\beta_2AR$, palmitoylation has been proposed as a mechanism regulating the accessibility of its carboxyl tail to regulatory kinases (3;37). Despite these potentially important roles of palmitate turnover regulation for protein function, very little is known concerning the processes involved in such regulation (26).

Considering the mechanisms that underlie the agonist-promoted accelerated turnover of the palmitate linked to the $\beta_2AR$, our results show that production of the second messager, cAMP, is not sufficient. However, they indicate that phosphorylation of the PKA consensus site (serines 345-346) proximal to the palmitoylated cysteine 341 is involved. Indeed, mutation of this site completely blocked the effects of agonist on the palmitoylation kinetics. This may appear difficult to reconcile with the observation that a direct activation of PKA with $Bt_2cAMP$ did not mimic the regulatory action of agonist stimulation. However, it has previously been established that serines 345-346 are not accessible for PKA mediated phosphorylation in the absence of agonist (38-40). Thus one could propose that agonist occupancy of the receptor is required for the phosphorylation of this site.

Although the mechanism by which phosphorylation of serines 345-346 modulates the palmitate turnover remains to be investigated, one could proposed that it

regulates the interaction of this portion of the cytoplasmic tail of the receptor with the plasma membrane. Indeed, the addition of negative charges to this otherwise hydrophobic and positively charged domain would destabilise its interaction with the membrane and thus increase the accessibility of the palmitate moiety to the action of a cytosolic palmitoyl-thioesterase. Such a reciprocal regulation between phosphorylation and palmitoylation has also been observed in a recent study documenting the palmitoylation and phosphorylation of specific sites within the carboxyl tail of the bradykinin receptor (41). Indeed, mass spectroscopy analysis revealed that palmitoylation of cysteine 356 and phosphorylation of tyrosine 352 were mutually exclusive. Reciprocal regulation between phosphorylation and palmitoylation may thus represent a general phenomenon among G protein-coupled receptors.

**Figure 1**: Time course of [$^3$H]-palmitate incorporation by $\beta_2$AR in the presence or absence of isoproterenol.

**A)** HEK293 cells expressing WT $\beta_2$AR were labelled with [$^3$H]-palmitate in the presence or absence of isoproterenol (1 $\mu$M) for the times indicated. Equal amounts of purified receptors (2 pmol) were resolved by SDS-PAGE, and incorporated palmitate was detected by fluorography (representative fluorogram shown was exposed for 3 weeks). Relative [$^3$H]-palmitate incorporation by $\beta_2$AR for labelling periods from one and two hours with or without isoproterenol is shown. The level of [$^3$H]-palmitate incorporation was determined by densitrometric analysis of fluorographs. The data represent the average $\pm$ SEM of three labelling experiments.

**B)** Effect of isoproterenol on cellular incorporation of [$^3$H]-palmitate for the indicated time periods is illustrated. [$^3$H]-palmitate was determined by thin layer chromatography following organic extraction of total lipids as described in Materials and Methods. Result shown is representative of two independent experiments.

**A)**
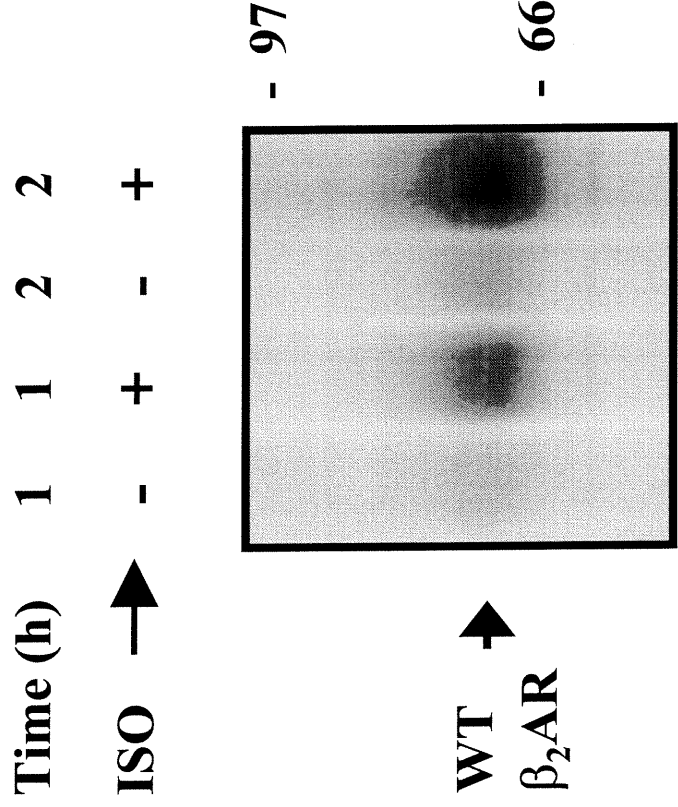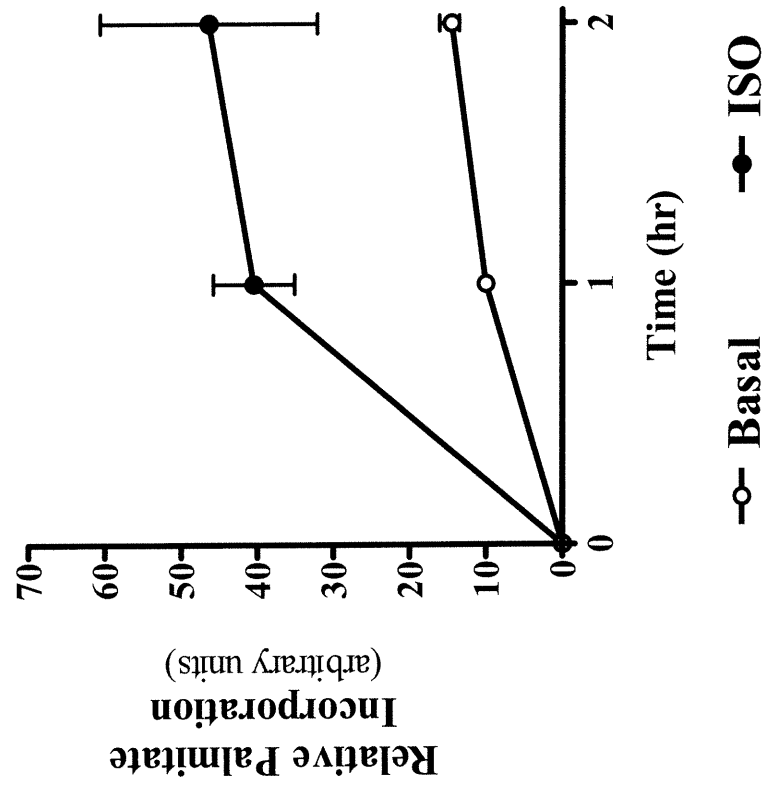
B)
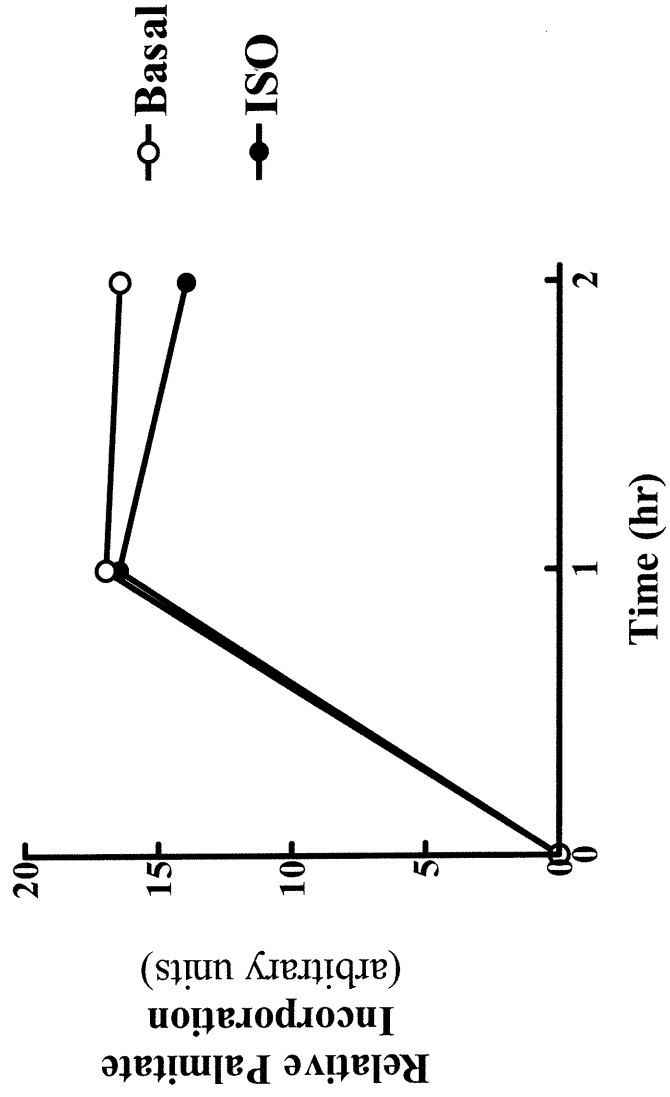
**Figure 2:** Effect of isoproterenol on the turnover rates of $\beta_2$AR-bound palmitate and of receptor itself.

Pulse-chase experiments were carried out as described in Materials and Methods. **A)** Briefly, HEK293 cells expressing WT$\beta_2$AR were labelled with [$^3$H]-palmitate for 2 hours and chase with cold palmitate in the presence or absence of isoproterenol (1 µM) for the indicated times. Equal amounts of purified receptors (4.7 pmol) were resolved by SDS-PAGE, and incorporated palmitate was detected by fluorography (representative fluorogram shown was exposed for 4 weeks). **B)** The graph shows the mean ± SD of two independent experiments. The level of [$^3$H]-palmitate incorporation was determined by densitrometric analysis of fluorographs. The average half-life calculated from three independent experiments is expressed as the mean ± SEM. **C)** HEK293 cells expressing WT $\beta_2$AR were labelled with [$^{35}$S]-methionine/cysteine for 1 hours and chase with cold methionine/cystein in the presence or absence of isoproterenol (1 µM) for the indicated times. Equal amounts of purified receptors (0.35 pmol) were resolved by SDS-PAGE, and labelled receptors were detected by fluorography. The average half-life of the receptor was calculated from three independent experiments expressed as the mean ± SEM. **D)** The graph shows the net turnover of the palmitate on the receptor once the contribution of the protein turnover has been subtracted. The average half-life calculated from three independent experiments is expressed as the mean ± SEM. The slope for each linear regression curve for B), C) and D) is represented by the $y$ value.

**B)** Turnover of receptor-bound palmitate



○ Basal (y = - 0.081x)
$t_{1/2}$ = 8.8 ± 2.0 hours

● ISO (y = - 0.190x)
$t_{1/2}$ = 3.7 ± 0.4 hours
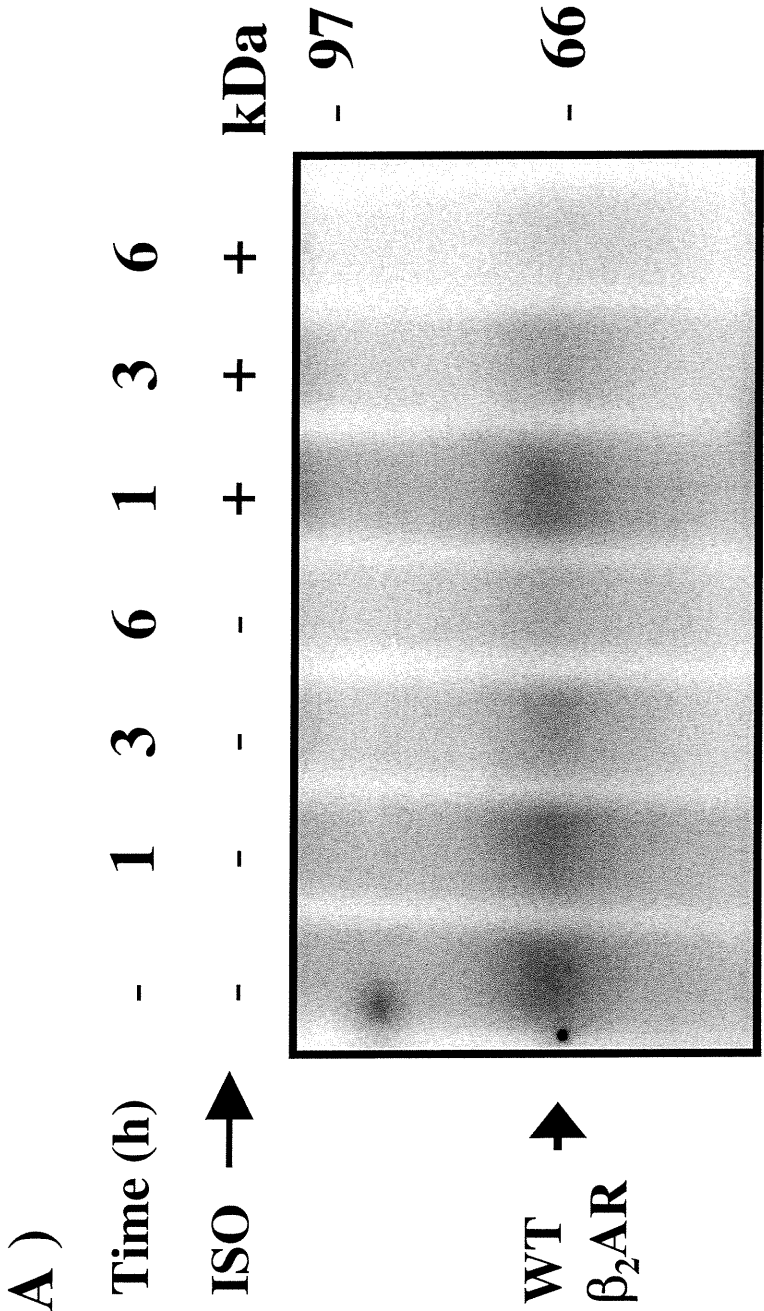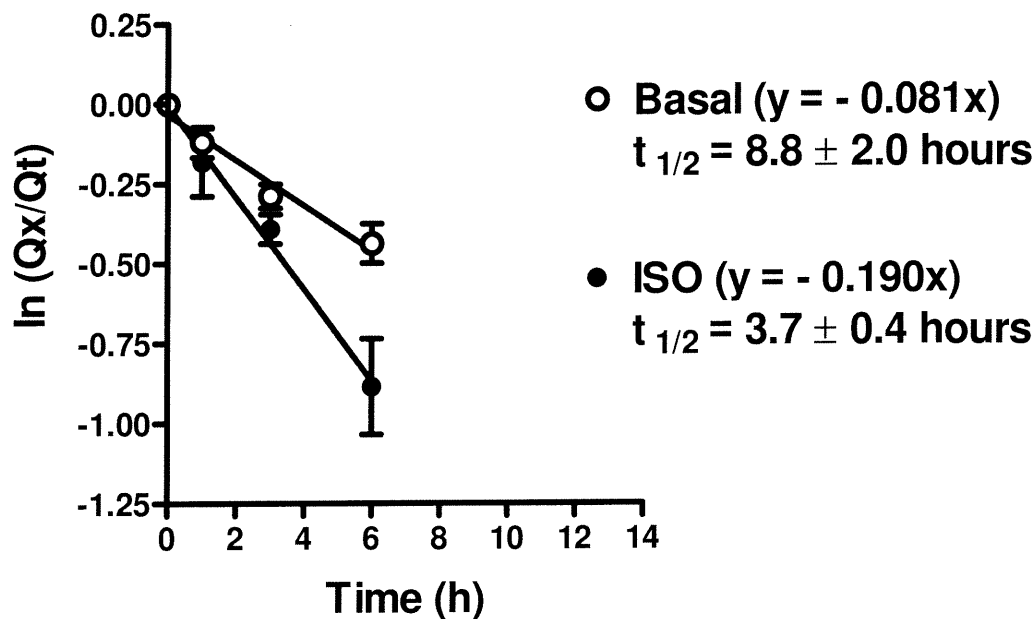
**C)** Turnover of $\beta_2$AR protein



□ Basal (y = - 0.035x)
$t_{1/2}$ = 23 ± 5 hours

■ ISO (y = - 0.077x)
$t_{1/2}$ = 9 ± 1 hours

# D) Net turnover of receptor-bound palmitate



Basal $(y = -0.062)$
$t_{1/2} = 11.5 \pm 1.7$

ISO $(y = -0.130)$
$t_{1/2} = 5.3 \pm 0.5$
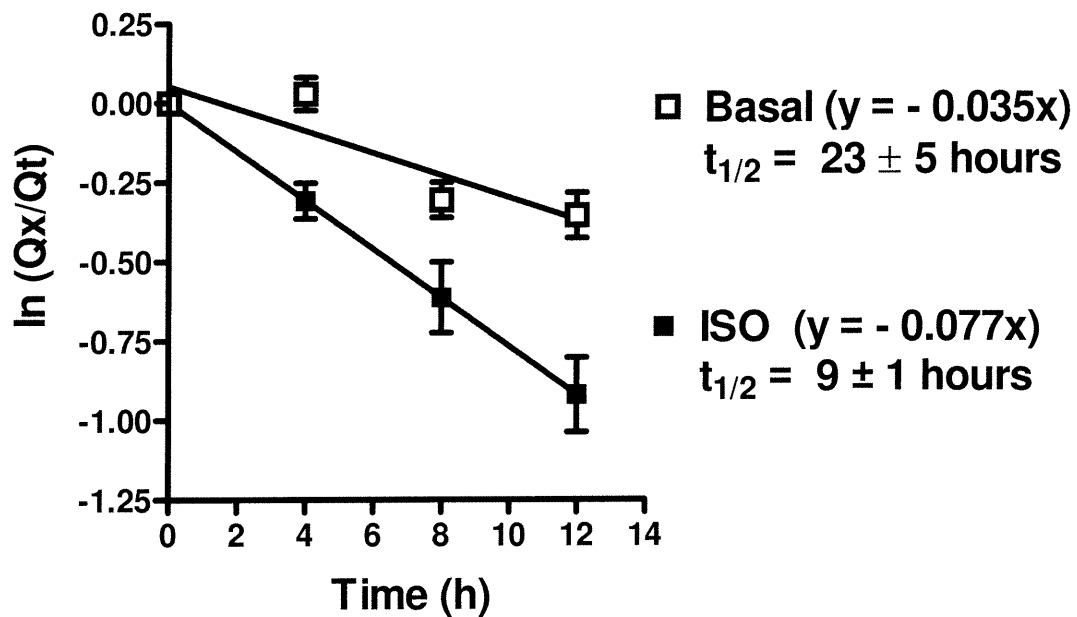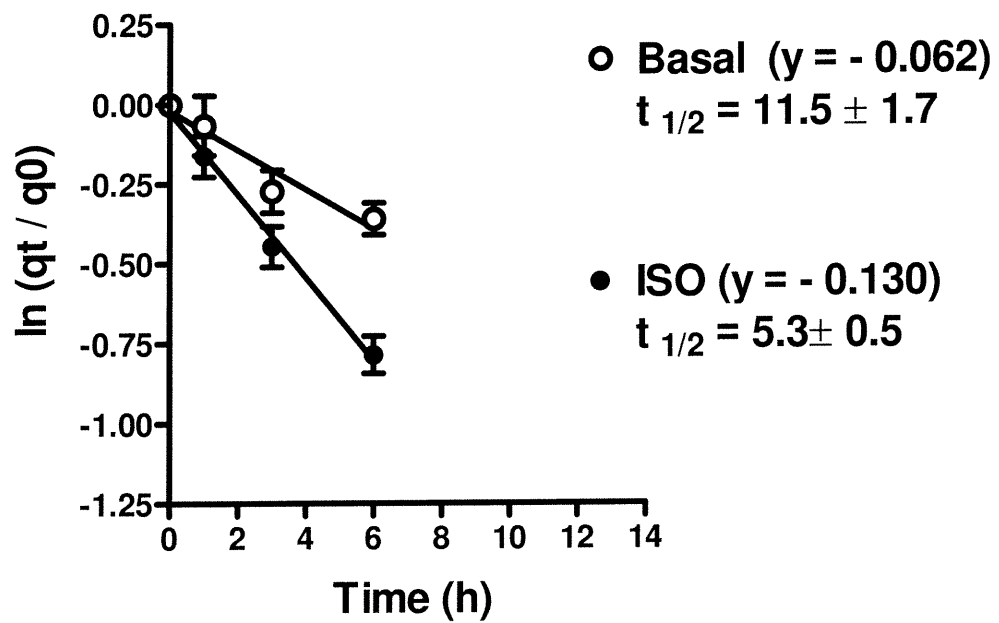
**Figure 3:** Effect of a treatment with isoproterenol on $\beta_2$AR before the labelling with [$^3$H]-palmitate.

HEK293 cells expressing WT$\beta_2$AR were treated or not (control) with isoproterenol (1 µM) for 15 minutes. The cells were then incubated with [$^3$H]-palmitate for 30 minutes in the continued presence of isoproterenol (ISO) (pretreatment (pretrt) and labelling with ISO) of after extensive washes to remove the isoproterenol (pretrt with ISO and labelling without ISO) during the labelling. Receptors were purified and resolved by SDS-PAGE, where, 3.4 pmol of receptor was loaded in each well. The intensity of labelling was assessed by fluorography (representative fluorogram shown was exposed for 4 weeks) and, quantified by densitometric analysis. The *bar graph* shows the mean ± SEM of three independent experiments (the asterisk (*) indicates a significative differences, $p < 0.005$).
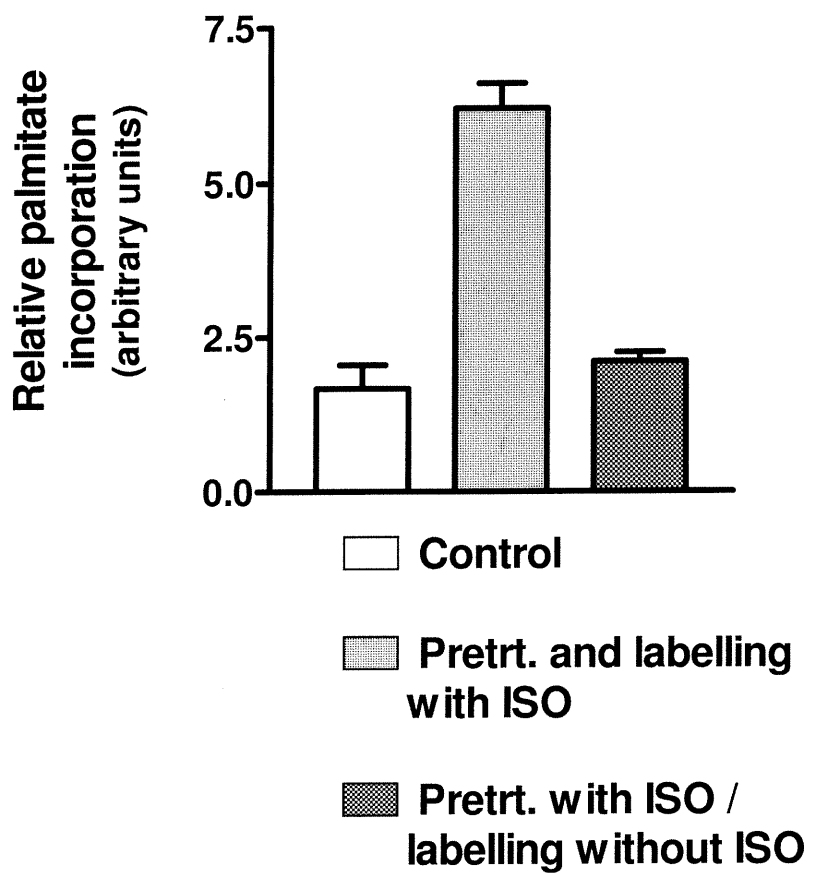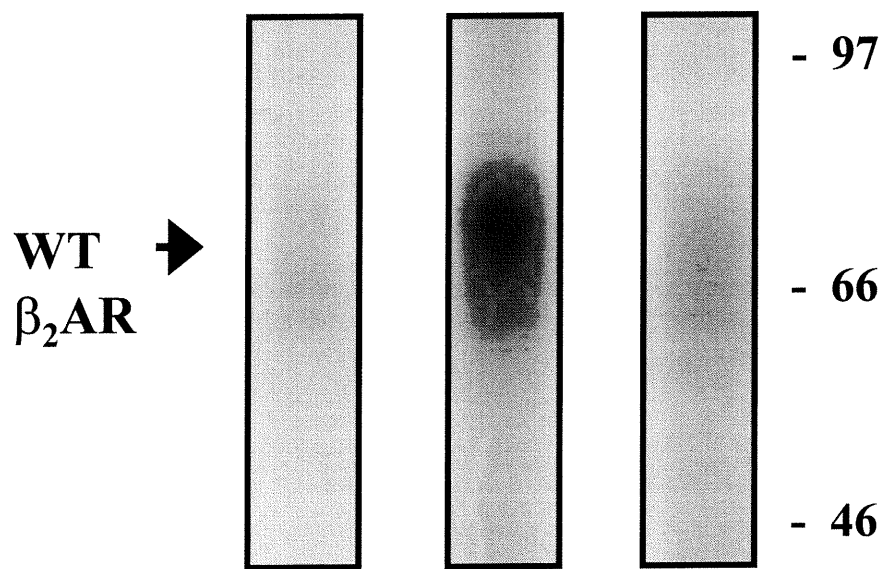
**Figure 4:** Effect of a treatment with isoproterenol or $Bt_2(cAMP)$ on $\beta_2AR$ [$^3H$]-palmitate incorporation .

HEK293 cells expressing $WT\beta_2AR$ pretreated with [$^3H$]-palmitate for 2 hours were exposed to isoproterenol (1 $\mu M$) (ISO), $Bt_2cAMP$ (1 mM) or the vehicle alone (Basal) for 15 minutes. Receptors were purified and resolved by SDS-PAGE, in each well, 0.9 pmol was loaded. The intensity of labelling was assessed by fluorography (representative fluorogram shown was exposed for 5 weeks). The level of [$^3H$]-palmitate incorporation was determined by densitometric analysis of fluorographs. The *bar graph* shows the mean ± SD of two to three independent experiments.

WT
$\beta_2$AR

- 97

- 66

- 46

Relative Palmitate
Incorporation
(arbitrary units)

2.0

1.5

1.0

0.5

0.0

Basal   ISO   Bt$_2$(cAMP)
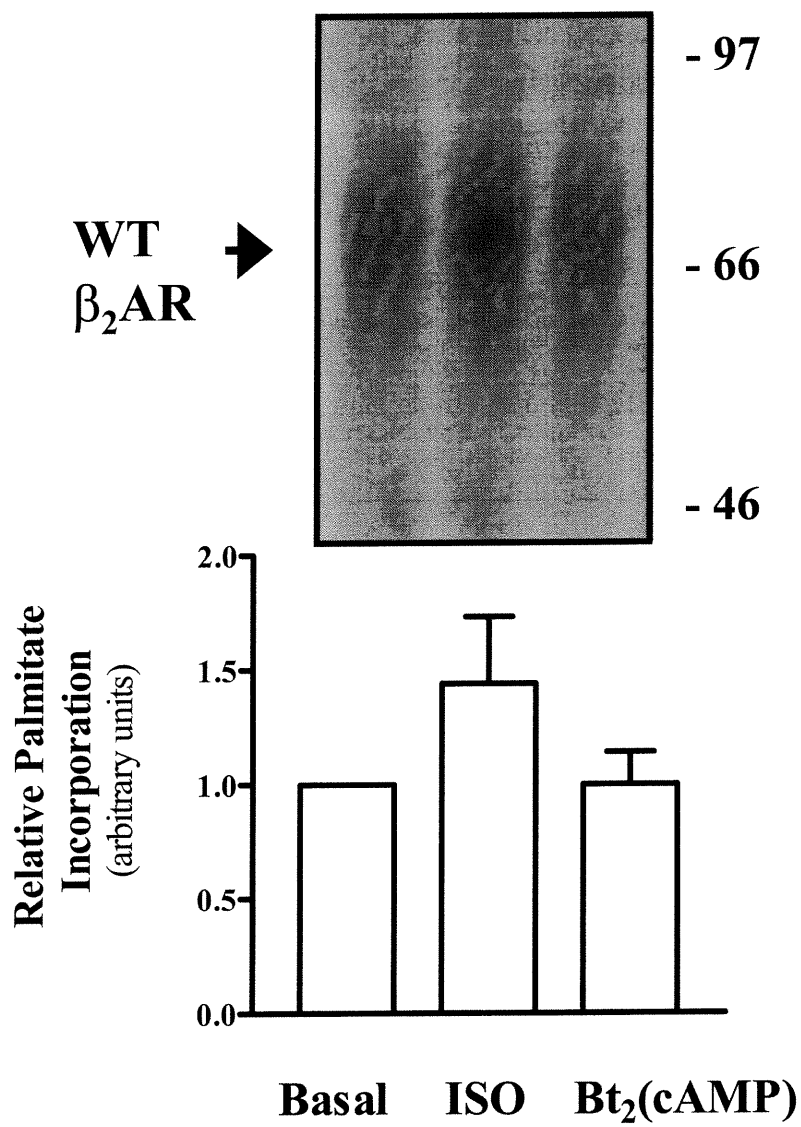
**Figure 5:** Effect of a short treatment with isoproterenol on $Ala^{261-262}\beta_2AR$ and $Ala^{345-346}\beta_2AR$ [$^3H$]-palmitate incorporation.

HEK293 cells expressing mutants $Ala^{261-262}\beta_2AR$ or $Ala^{345-346}\beta_2AR$ pretreated with [$^3H$]-palmitate for 2 hours were exposed to 1 $\mu M$ of isoproterenol (ISO) or the vehicle alone for 15 minutes. Mutant receptors were purified and resolved by SDS-PAGE. In each well, 3 pmol was loaded. The intensity of labelling was assessed by fluorography (representative fluorogram shown was exposed for 4 weeks) and, quantified by densitometric analysis. The *bar graph* shows the mean ± SEM of four independent experiments (the asterisk (*) indicates a significative differences, $p < 0.05$)
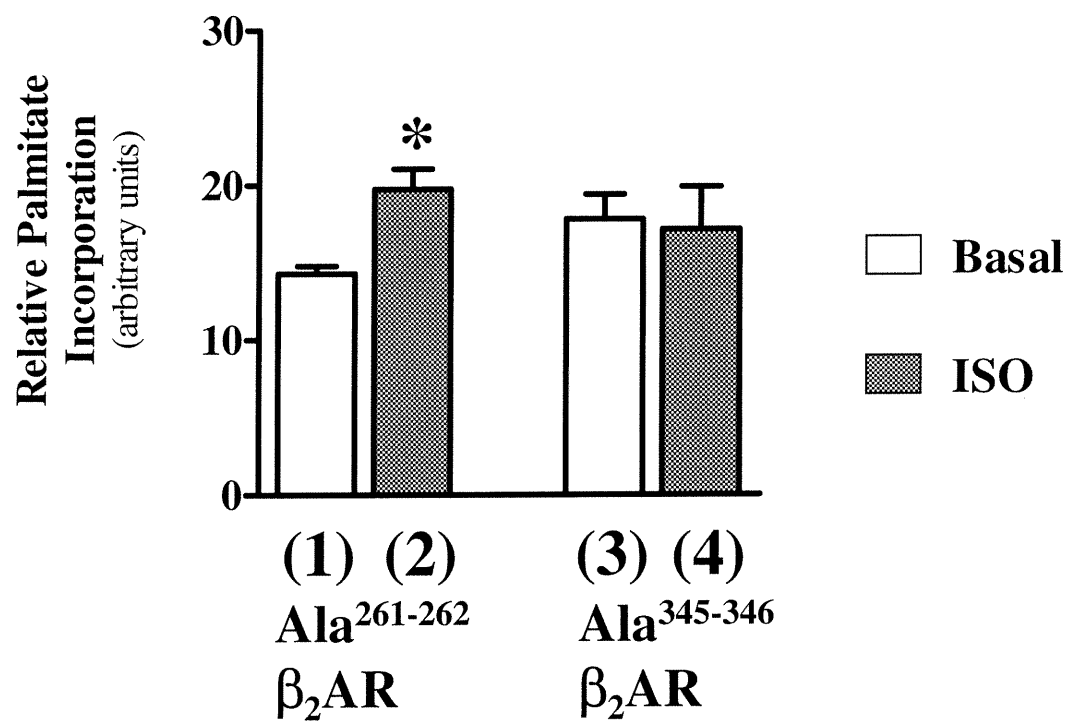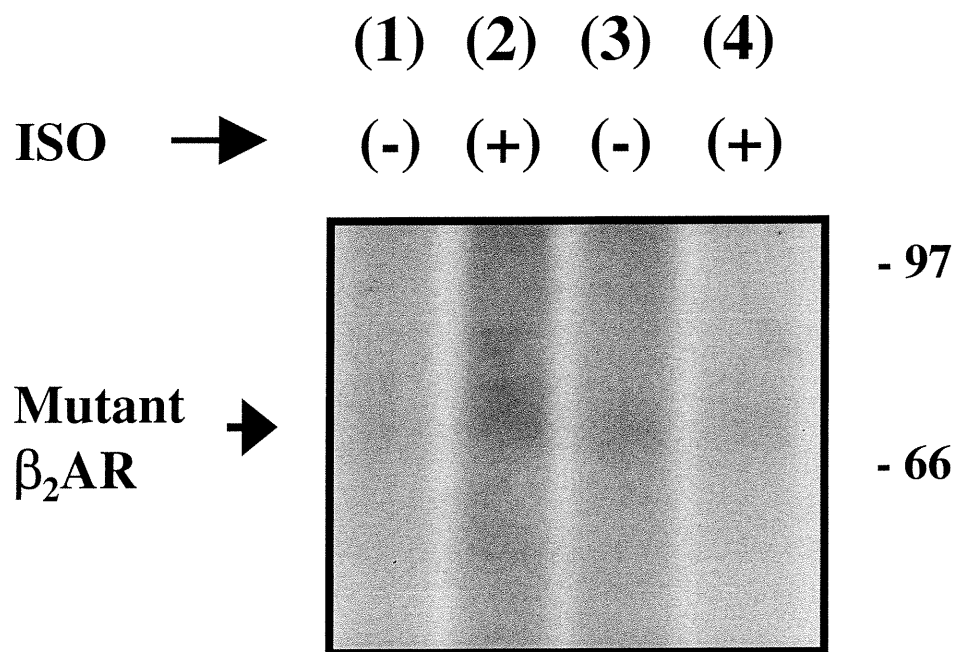
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## 2.4 Article 4: Nitric oxide modulates $\beta_2$-adrenergic receptor palmityolation and signaling.

# Nitric Oxide Modulates $\beta_2$-Adrenergic Receptor Palmitoylation and Signaling*

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To determine whether nitric oxide (NO) modulates the $\beta$-adrenergic signaling pathway, we treated cells expressing $\beta_2$-adrenergic receptors ($\beta_2$AR) with the NO donors, 3-morpholinosydnonimine (SIN-1) and 1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-chloride and determined the intracellular production of cAMP after exposure to $\beta$-adrenergic receptor agonists, cholera toxin and forskolin. NO significantly decreased the potency of the $\beta$-adrenergic agonist, isoproterenol, to stimulate cAMP production without affecting the stimulatory action of forskolin and cholera toxin, which directly activate adenylyl cyclase and $G_s$, respectively. Treatment with the NO donor increased the guanyl nucleotide-sensitive high affinity constant for the agonist, isoproterenol, thus suggesting that it reduced functional coupling between the receptor and $G_s$. Stimulation of endogenous NO production by lipopolysaccharide in RAW 264.7 macrophages also caused a significant increase in the $EC_{50}$ for isoproterenol-stimulated cAMP production. SIN-1 treatment also led to a reduction in both basal and isoproterenol-stimulated incorporation of [³H]palmitate into the $\beta_2$AR. Signaling through the nonpalmitoylated, $Gly^{341}\beta_2$AR mutant was unchanged by SIN-1 treatment. Given the link between $\beta_2$AR palmitoylation and its responsiveness to agonist, these results suggest that the primary action of NO was depalmitoylation of the $\beta_2$AR resulting in decreased signaling through the $\beta_2$AR.

Nitric oxide (NO)[1] is a biologic signal involved in vasodilatation, neurotransmission, and immune defense (1, 2). It is an unstable gas that diffuses across membranes, lacks specific receptors, and is rapidly inactivated by a chemical reaction. Its synthesis by the enzyme NO synthase (NOS) from arginine and oxygen is finely regulated and can be achieved through both calcium-dependent and calcium-independent pathways (3). Both the calcium-dependent endothelial NOS and the calcium-independent inducible NOS (iNOS or NOS2) have been shown to play an important role in the control of vascular tone in normal and inflammatory conditions (4–8), respectively. NO can modulate the action of various vasoactive hormones and transmitters (7). In particular, NO-mediated decreases in $\beta$-adrenergic responsiveness have been described (6, 9). However, the biochemical processes underlying this effect remain largely unexplored.

At the cellular level, the best characterized effect of NO is the activation of guanylate cyclase by the formation of a heme-NO complex that enhances the catalytic activity of the enzyme and increases cGMP production (10). Although several of the effects of NO have been attributed to cGMP formation, NO also leads to nitrosylation of thiol groups on cysteine residues (3, 11) that may contribute to the diverse physiological actions of this gaseous second messenger. Indeed, nitrosylation has been suggested to modulate protein function as a consequence of conformational changes (3, 11), facilitation of ADP-ribosylation (12), and inhibition of protein palmitoylation (13).

The $\beta_2$AR and its cognate G protein ($G_s$) both undergo palmitoylation on cysteine residues. This post-translational modification is dynamically regulated on the receptor and $G_s$ upon $\beta$-adrenergic stimulation (14, 15). Moreover, it has been proposed that regulation of the palmitoylation state of these proteins may have important effects on their biological activity (16–18). The aims of the present study were to determine if NO can directly modulate the signaling efficiency of the $\beta$-adrenergic-$G_s$-adenylyl cyclase signaling pathway and to assess if regulation of the palmitoylation state of the receptor or $G_s$ could play a role in such a regulatory process. We found that NO reduces the potency of a $\beta$-adrenergic agonist to stimulate adenylyl cyclase and promotes depalmitoylation of the $\beta_2$AR.

## EXPERIMENTAL PROCEDURES

*Materials*—Cell culture supplies were from Biofluids, Rockville, MD; COS7 cells were from American Type Culture Collection; [9,10-³H]palmitate (60 Ci/mmol) and [8-³H]adenine (18 Ci/mmol) were from American Radiolabeled Chemicals; pluronic acid was from Life Technologies, Inc.; SIN-1 was from Biomol and Alexis Corp.; GEA and $N^G$-monomethyl-L-arginine (L-NMMA) were from Alexis Corp.; 8-bromo-cGMP, norepinephrine, and epinephrine were from Research Biochemical International; forskolin, isoproterenol, terbutaline, procaterol, hemoglobin-Ao, IBMX, alumina, and lipopolysaccharide (LPS) were from Sigma; $\alpha_2$-macroglobulin was from Roche Molecular Biochemicals; protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech; cholera toxin was from List Biologicals; and AG 50W-X4 (Dowex) resin was from Bio-Rad.

*Construction of Mutated $\beta_2$AR cDNA and Cell Transfection*—The wild type $\beta_2$AR was inserted in the modified pcDNA3, with an RSV promoter, at *Bam*HI and *Eco*RI sites. The generation of

[1] The abbreviations used are: NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; $\beta_2$AR, $\beta_2$-adrenergic receptor; G protein, guanine nucleotide-binding protein; $G_s$, G protein associated with adenylyl cyclase stimulation; DMEM, Dulbecco's modified Eagle's media; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SIN-1, 3-morpholinosydnonimine; GEA, 1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-chloride; IBMX, 1-methyl-3-isobutylxanthine; CYP, cyanopindolol; LPS, lipopolysaccharide; HPLC, high pressure liquid chromatography; WT, wild type; FBS, fetal bovine serum; Gpp(NH)p, guanosine 5'-($\beta,\gamma$-imido)triphosphate; HEK, human embryonic kidney; L-NMMA, $N^G$-monomethyl-L-arginine.

pcDNA3-RSV-Gly[341]$\beta_2$AR, in which cysteine 341 was replaced by a glycine, was constructed by site-directed mutagenesis using wild type pcDNA3-RSV-$\beta_2$AR (19). Positive clones were confirmed by dideoxy sequencing. The wild type $\beta_2$AR and the mutant were stably transfected in human embryonic kidney 293 (HEK293) cells line using the calcium phosphate precipitation method (20). Neomycin-resistant cells were selected by culturing in medium containing G418 (450 $\mu$g/ml) (Life Technologies, Inc.). Resistant clones were then screened for $\beta_2$AR expression by radioligand assays using ^{125}I-labeled iodocyanopindolol (CYP) as the ligand. COS, NIH 3T3, and HEK293 cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Macrophage RAW 264.7 cells were grown in DMEM with 10% FBS without antibiotic. Sf9 cells were grown at 27 °C in Grace's insect medium supplemented with 10% FBS (10%) and pluronic acid to prevent cell tearing due to agitation. They were infected with recombinant baculovirus encoding the wild type human $\beta_2$AR at a density of $1 \times 10^6$ to $2 \times 10^6$ cells per ml for 48 h with multiplicities of infection varying from 2 to 6 (21).

*Metabolic Labeling and Cell Fractionation*—For metabolic labeling with [³H]palmitate, COS7 and HEK293 cells were incubated in serum-free medium for 2 and 1 h, respectively. The cells were then labeled with [³H]palmitate (200 to 500 $\mu$Ci/ml) for 1 h. They were rinsed twice in cold phosphate-buffered saline (PBS), pH 7.4, to stop the labeling and centrifuged at $2000 \times g$ for 10 min at 4 °C. Cell pellets were then stored at $-70$ °C. HEK293 cells were thawed in cold lysis buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.4) containing a protease inhibitor mixture (5 $\mu$g/ml leupeptin, 5 $\mu$g/ml trypsin inhibitor, and 10 $\mu$g/ml benzamidine), disrupted by sonication, and the lysates centrifuged 5 min at $500 \times g$ at 4 °C. The supernatants were then centrifuged at $45,000 \times g$ for 20 min at 4 °C. Pelleted membranes were solubilized for 90 min at 4 °C in 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, containing 0.3% $n$-dodecyl maltoside and the above protease inhibitor mixture. COS cells were thawed in buffer composed of 5 mM Hepes, pH 7.4, 50 mM mannitol, 100 $\mu$g/ml soybean trypsin inhibitor, 0.5 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin, 1 mM EDTA, 0.7 $\mu$g/ml pepstatin, and 10 milliunits/ml $\alpha_2$-macroglobulin. They were homogenized by passage through a 25-gauge needle 15 times. The cell lysate was centrifuged at $3000 \times g$ for 3 min. The supernatant was centrifuged at $125,000 \times g$ for 1 h at 4 °C, and the resulting pellet was resuspended in homogenization buffer. Protein concentrations of the cellular fractions were determined by the Bio-Rad Protein Assay Dye kit with IgG as the standard or the method of Bradford (22), using bovine serum albumin as the standard.

*Treatment with NO Donors*—3-Morpholinosydnonimine (SIN-1) and 1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-chloride (GEA) were used to generate NO. Cells were incubated with 1 mM SIN-1 or 1 mM GEA for the indicated time. When in aqueous solution, SIN-1 degrades spontaneously to SIN-1A and subsequently to SIN-1C, with NO being generated in the latter step (23). At 37 °C, the rate constants governing these steps are 0.11 and 0.04 min$^{-1}$, respectively (23), and the half-life of NO is about 10 s (24). NO-depleted SIN-1 was thus generated by keeping a 100 mM solution of SIN-1 at room temperature or at 37 °C for at least 24 h.

*Immunoprecipitation of G$\alpha$s*—The polyclonal, affinity purified RM antibody, which recognizes the carboxyl-terminal decapeptide of G$\alpha$s, was used (25). Immunoprecipitation was performed on equal amounts of the particulate fraction (0.5–1 mg of total protein) in a solubilization buffer consisting of 50 mM Tris-HCl, pH 7.5 (25 °C), 150 mM NaCl, 1% (w/v) Triton X-100, 0.2% (w/v) SDS, and 1 mM EDTA. The immunoprecipitates were recovered by incubation with protein A-Sepharose, washed, and prepared for SDS-PAGE (23). Fixed gels were treated for 45 min with En³Hance (NEN Life Science Products), dried, and exposed to XAR-2 film (Kodak) at $-70$ °C.

*Purification of $\beta_2$AR*—Alprenolol-Sepharose affinity purification matrix was synthesized according to the method of Benovic *et al.* (26). This matrix was used to purify the $\beta_2$AR as previously reported (27) using $n$-dodecyl-maltoside as the detergent instead of digitonin. The affinity purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon), and the amount of $\beta_2$AR in each sample was determined by soluble radioligand binding assays using [^{125}I]iodocyanopindolol ([^{125}I]CYP as described (28). Samples with equal numbers of receptor were then transferred into sample buffer, and SDS-PAGE was conducted under nonreducing conditions. Fixed gels were incubated in 1 M salicylic acid for 45 min, dried, and exposed to Dupont Reflection™ film at $-80$ °C.

*Radioligand Binding Assays*—Radioligand binding assays were conducted essentially as described in Bouvier *et al.* (26) using 10 $\mu$l of membrane suspension (5 $\mu$g) in a total volume of 0.5 ml of 75 mM

Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 2 mM EDTA. Competition binding assays with isoproterenol were conducted using 50 pM [^{125}I]CYP as the radioligand. The concentration of the $\beta$-adrenergic agonist was varied from 0 to 100 $\mu$M in the presence or absence of 100 $\mu$M Gpp(NH)p. For determination of receptor number, the assay was performed in triplicate with a nearly saturating concentration of [^{125}I]CYP (200 pM) in the presence or absence of 10 $\mu$M alprenolol to define specific binding.

*Intracellular cAMP Accumulation Assay*—Approximately $2$–$6 \times 10^5$ cells/well of COS, NIH 3T3, or HEK293 cells were incubated in 24-well plates with 2 $\mu$Ci/ml [³H]adenine in complete DMEM for 24 h. The cells were washed once with PBS and incubated for 15 min in Hepes/DMEM with 0.5 mM IBMX. They were then treated for 45 min with $\beta$-adrenergic agonists, forskolin, cholera toxin, 8-bromo-cGMP or vehicle in Hepes/DMEM with 0.5 mM IBMX. The reaction was stopped following aspiration of the medium by adding 1 ml of 5% trichloroacetic acid or 1 ml of 0.2% (w/v) SDS, 50 mM Tris, pH 7.4. One mM of unlabeled cAMP was added to decrease enzymatic degradation of [³H]cAMP. The cAMP was then separated by sequential chromatography over Dowex and Alumina columns as described previously (29). The cAMP accumulation was expressed as ([³H]cAMP cpm/([³H]cAMP cpm + [³H]ATP cpm)) $\times$ $10^3$).

*Amperometric Detection of Isoproterenol*—To rule out the possibility that treatment with NO donors could promote oxidation of the $\beta$-adrenergic agonist, the concentration of isoproterenol before and after SIN-1 treatment was assessed by amperometry (30, 31). Isoproterenol (10 $\mu$M) was incubated for 45 min at 37 °C in PBS with 1 mM SIN-1. The mixture was then separated by cation-exchange chromatography using a Spherisorb-OD52, 10 cm $\times$ 4.6 mm column, and a Waters HPLC system. Isoproterenol was detected using a Coulochem II (ESA); amperometric system set at +0.6 V. Calibration was carried out using known concentrations of isoproterenol. Since the amperometric method is based on the recording of current produced by voltage-dependent oxidation, only the non-oxidized isoproterenol can be detected (30, 32) thus allowing the assessment of NO-promoted oxidation.

*LPS Treatment and Nitrite Measurement*—Macrophages (RAW 264.7) were treated with LPS (1 $\mu$g/ml) for 24 h to induce nitric oxide synthase and thus stimulate endogenous NO production. Nitrite (NO$_2$) was then used as an indicator of NO synthesis and measured in the culture media as described previously (33). Briefly, 400 $\mu$l of Griess solution (1 part of 0.1% naphthylethylenediamine dihydrochloride and 1 part of 1% sulfanilamide in 5% phosphoric acid) was added to 400 $\mu$l of cell medium. The formation of azochromophore was detected by measuring the absorbance of the reaction mixture at 550 nm ($A_{550}$). Standard curves were generated using known concentrations of sodium nitrite in culture medium.

*Data Analysis*—Palmitoylation levels were estimated by densitometric analysis of the fluorograms using an LKB 2202 UltroScan laser densitometer or an AGFA/ARCUS II scanner and NIH Image 1.61. The Allfit (34) software was used to calculate the EC$_{50}$ from the dose-response curves. Radioligand binding data were analyzed using the computer program GraphPAD Prism.

### RESULTS

*cAMP Accumulation Studies*—To determine if NO can modulate $\beta$-adrenergic signaling, the effect of the NO donor 3-morpholinosydnonimine (SIN-1) on isoproterenol-stimulated adenylyl cyclase activity was assessed in COS cells. As shown in Fig. 1A, treatment with 1 mM SIN-1 for 45 min decreased the potency of the $\beta$-adrenergic agonist from an EC$_{50}$ of $1.7 \pm 0.4$ $\mu$M in control condition to $19.8 \pm 6.0$ $\mu$M in the presence of the NO donor. The isoproterenol potency returned to control values within 30 min following removal of the NO donor indicating that the effect was readily reversible (data not shown). To rule out the possible contribution of SIN-1C, the stable degradation product of SIN-1, a NO-depleted SIN-1C solution obtained by incubation of SIN-1 at 37 °C for 48 h, was used. Such solution was without effect on the $\beta$-adrenergic-stimulated cAMP production (data not shown) thus indicating that the released NO, rather than accumulated SIN-1 C, caused the rightward shift in the isoproterenol dose-response curve. This is also supported by the observation that treatment with another NO donor, 1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-chloride (GEA, 1 mM), for 45 min also inhibited receptor-stimulated cAMP production (Fig. 1B).
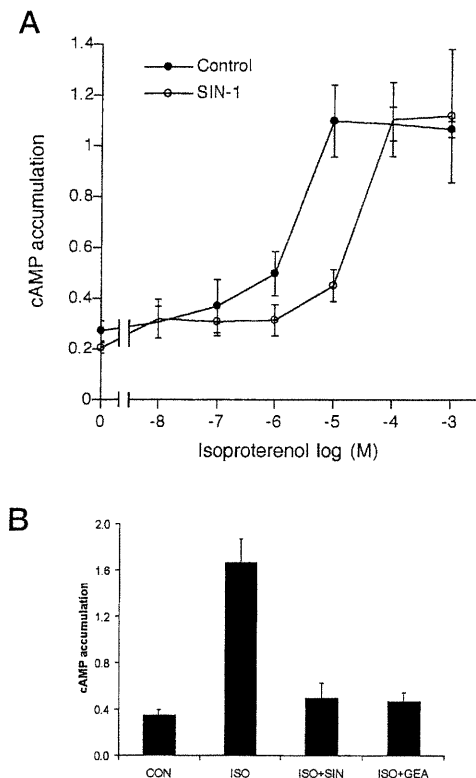
A



B



FIG. 1. **Effects of SIN-1 on isoproterenol-stimulated cAMP production in COS cells.** *A,* COS cells were incubated with 2 μCi/ml [³H]adenine overnight and treated for 45 min with 0.5 mM IBMX and different concentrations of isoproterenol in the presence and absence of 1 mM SIN-1. Following the incubation, cells were harvested, lysed, and cAMP separated using Dowex and alumina columns. cAMP accumulation was calculated as the [³H]cAMP cpm/([³H]ATP cpm + [³H]cAMP cpm) × 10³). Values represent the mean ± S.E. of three independent experiments performed in triplicate. *B,* NIH 3T3 cells were treated with 10 μM isoproterenol *(ISO)* alone or with 1 mM SIN-1 and 1 mM GEA. cAMP accumulation was determined as described above. The values shown are the mean ± S.E. of three independent experiments. *CON,* control.

As shown in Fig. 1*A,* the SIN-1-induced rightward shift in the cAMP accumulation dose-response curve was such that the response evoked by 10 μM isoproterenol in control cells was almost abolished by the SIN-1 treatment. Therefore, further studies aimed at characterizing the effect of the NO donor in other cell types were carried out using this concentration of isoproterenol. SIN-1 blocked the stimulating effect of 10 μM isoproterenol on cAMP production in HEK293, COS, and NIH 3T3 cells (Fig. 2) indicating that the effect was not cell type-specific. This effect was not unique to isoproterenol since SIN-1 treatment substantially decreased the responses to all β-adrenergic agonists tested (epinephrine, norepinephrine, isoproterenol, and procaterol) (Fig. 2*C*).

In contrast to the dramatic effect on the receptor-stimulated cAMP production, SIN-1 was without effect on either forskolin or cholera toxin-stimulated activity (Fig. 2, *B* and *C*) indicating that neither the adenylyl cyclase catalytic activity nor G$_s$-stimulated activity are affected. SIN-1 was also without effect on cAMP accumulation when concentrations of forskolin that gave submaximal cAMP responses were used (data not shown).

NO can increase the hydrolysis of cAMP by activating phosphodiesterases through a cGMP pathway (9). However, the blunting effect of NO on the response to isoproterenol is unlikely to be due to increased degradation of cAMP since a nonspecific phosphodiesterase inhibitor, IBMX, was present in all these experiments. In addition, treatment with 1 mM 8-bromo-cGMP did not alter cAMP production in response to isopro-

A



B



C
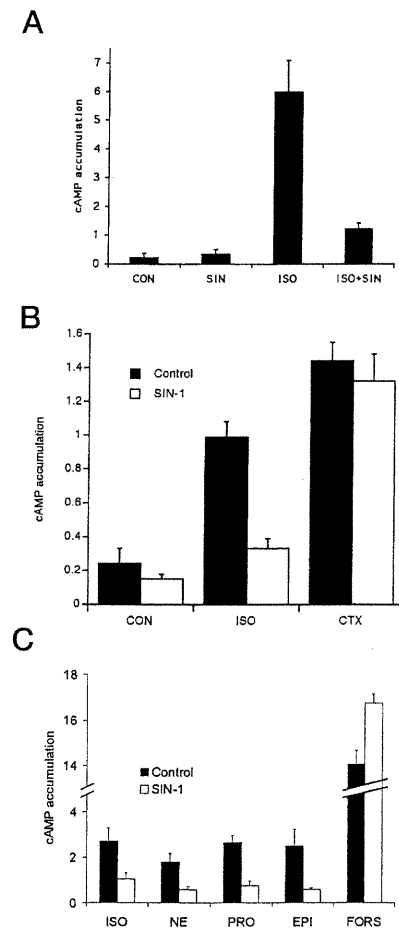


FIG. 2. **Effects of SIN-1 on adenylyl cyclase activation in COS, HEK293, and NIH 3T3 cells.** The intracellular cAMP assay was performed in triplicate on cells exposed to the agents listed below for 45 min as described in the legend to Fig. 1. *A,* HEK293 cells were treated or not with 10 μM isoproterenol *(ISO)* or 1 mM SIN-1. The mean of three independent experiments ± S.E. is shown. *B,* COS cells were treated with 10 μM isoproterenol *(ISO)* or 1 μg/ml cholera toxin *(CTX)* in the presence or absence of 1 mM SIN-1. The mean ± S.E. of four independent experiments is shown. *C,* NIH 3T3 cells were treated with 10 μM isoproterenol *(ISO),* 10 μM norepinephrine *(NE),* 10 μM procaterol *(PRO),* 10 μM epinephrine *(Epi),* and 10 μM forskolin *(FORS)* in the presence or absence of 1 mM SIN-1. *CON,* control. The mean ± S.E. of three independent experiments is shown.

terenol (0.63 for isoproterenol alone and 0.76 with 1 mM 8-bromo-cGMP, the values are the mean of two independent experiments, calculated as [³H]cAMP cpm/([³H]ATP cpm + [³H]cAMP cpm) × 10³). Finally, if the effect of NO resulted from an activator of the phosphodiesterase, reduction in the cholera- and forskolin-stimulated adenylyl cyclase would have also been observed.

*Assay for Oxidized Isoproterenol*—Since it has been previously suggested that NO can promote oxidation of catecholamines (11, 35), we tested whether NO could cause the oxidative degradation of isoproterenol thus reducing its effective concentration. Starting from a concentration of 10 μM isoproterenol, incubation for 1 h at 37 °C with or without 1 mM SIN-1 led to a loss of 12 ± 3 and 8 ± 2%, respectively, of the agonist as assessed by high performance liquid chromatography (HPLC) coupled to electrochemical detection. Since amperometry only detects the non-oxidized form of the agonist, these data indicate that incubation with SIN-1 did not promote any significant oxidation of the β-adrenergic agonist and cannot account for the biological effect observed. In addition, the inhibitory effect on SIN-1 on the isoproterenol-stimulated cAMP accumulation was also observed in the presence of the anti-
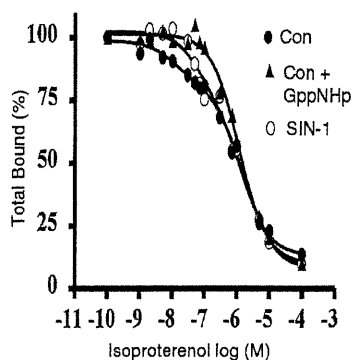
FIG. 3. **Effects of SIN-1 on the agonist binding characteristics of the $\beta_2$AR in HEK293 cells.** Isoproterenol competition of [$^{125}$I]CYP binding was assessed in membrane preparations derived from HEK293-$\beta_2$ AR cells that had been treated for 1 h with or without 1 mM SIN-1. The binding assay was carried out in the presence or absence of 100 $\mu$M Gpp(NH)p. The *curves* shown are computer-generated fits of data obtained in a representative experiment, using the program GraphPad Prism. *Con*, control.

TABLE I
*Effect of SIN-1 on the isoproterenol binding parameters of the $\beta$-AR in HEK293 cells*

The binding assay was performed as described in Fig. 3 legend. The estimates were obtained using the GraphPad Prism software. The values are the mean $\pm$ S.E. $\eta$, number of independent experiments.

|  | $\eta$ | $K_{I(H)}{}^a$ | $K_{i(L)}{}^a$ | $B_{max(H)}{}^b$ |
|---|---|---|---|---|
|  |  | nM | nM | % |
| Control | 4 | $2 \pm 0.6$ | $389 \pm 152$ | $59 \pm 4$ |
| + Gpp(NH)p | 4 | —$^c$ | $315 \pm 20$ | — |
| SIN-1 | 4 | $17 \pm 5^d$ | $375 \pm 206$ | $49 \pm 7$ |
| + Gpp(NH)p | 2 | — | $328$ | — |

$^a$ Equilibrium inhibition constant for isoproterenol. H and L refer to high and low affinity binding parameters.
$^b$ Percentage of sites in the high affinity state for isoproterenol.
$^c$ Not detectable.
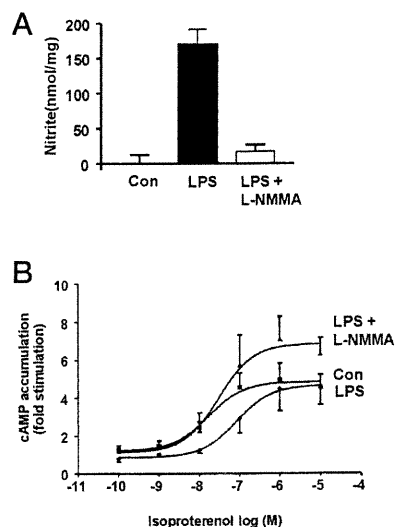$^d$ $p < 0.05$ compared with control.



FIG. 4. **Effects of LPS-stimulated NO production on cAMP accumulation in RAW 264.7 macrophages.** To induce NO synthase, macrophages were incubated with 1 $\mu$g/ml LPS in the presence or absence of 1 mM $N^G$-methyl-L-arginine (*L-NMMA*) for at least 18 h. *A*, nitrite levels in the medium were measured as described under "Experimental Procedures." *B*, intracellular cAMP accumulation was assessed following a 45-min stimulation with isoproterenol ($1 \times 10^{-10}$ -$1 \times 10^{-5}$) as described in the legend to Fig. 1. The cAMP accumulation is expressed as the fold stimulation over the cAMP accumulation without isoproterenol in each of the conditions. Data represents the mean $\pm$ S.E. of 3–8 independent experiments. The *curves* shown are computer-generated fits using the program GraphPad Prism. *Con*, control.

oxidant, ascorbate (10 $\mu$M).

*Agonist Affinity Studies*—The ability of $\beta_2$AR to mediate agonist stimulation of adenylyl cyclase activity has been correlated with the propensity of the receptor to adopt a guanyl nucleotide-sensitive high affinity state that is thought to reflect the formation of a ligand-receptor-$G_s$ ternary complex (36). In order to test if NO could mediate its action by affecting the coupling of the $\beta_2$AR to $G_s$, the agonist binding properties of the receptor were tested in membranes derived from HEK293-$\beta_2$AR cells treated or not with 1 mM SIN-1. As shown in Fig. 3, competition of [$^{125}$I]CYP binding with isoproterenol was biphasic and the curve best resolved by a two-affinity state model. The guanyl nucleotide sensitivity of the high affinity state is illustrated by the observation that upon addition of Gpp(NH)p the curve is best fitted to a single low affinity state model. Both high and low affinity states for agonist were observed in membranes derived from cells treated with SIN-1, but the high affinity constant ($K_{i(H)}$) was found to be significantly increased with SIN-1 treatment compared with control cells (Table I). In contrast, the SIN-1 treatment did not affect the low affinity value ($K_{i(L)}$). Similarly, the total number of binding sites detected by [$^{125}$I]CYP was not changed by the treatment. Neither the high nor the low affinities for isoproterenol were affected by treatment of the cells with SIN-1 depleted of NO (data not shown).

*Effect of Endogenous NO Production in Macrophage Cells*—Experiments described above used chemical NO donors. To determine if endogenously produced NO could also have an effect on receptor-stimulated cAMP production in a more physiologically relevant system, a mouse macrophage cell line (RAW 264.7) naturally expressing the $\beta$-adrenergic receptor was used. As expected, treatment of these cells with lipopolysaccharide (LPS) led to the activation of iNOS as indicated by the marked increase in nitrite accumulation promoted by the treatment (Fig. 4A). In agreement with the results obtained using a NO donor, LPS treatment caused a significant ($p < 0.05$) increase in the EC$_{50}$ for isoproterenol-stimulated cAMP production (156 $\pm$ 64 *versus* 20 $\pm$ 6 nM) (Fig. 4B). Both the nitrite production and the rightward shift of the isoproterenol-stimulated cAMP accumulation were blocked by the NO synthase inhibitor L-NMMA (Fig. 4) suggesting that the endogenous production of NO is responsible for the decrease potency of isoproterenol. Neither receptor number nor forskolin-stimulated cAMP production was affected by LPS (data not shown).

*Palmitoylation of $G_s\alpha$ and $\beta_2$AR in the Presence of SIN-1*—A large body of evidence suggests that NO can modulate the

activity of various proteins by directly interacting with accessible thiol groups within the protein to form nitrosocysteine (13, 37, 38). In one case, it was shown that such a mechanism could promote depalmitoylation (13). Since both $\beta_2$AR and $G\alpha_s$ are palmitoylated and this modification can affect their function, we investigated whether NO could influence the palmitoylation state of these proteins.

The effect of NO on $G\alpha_s$ palmitoylation was assessed by incubating COS cells with [$^3$H]palmitic acid in the presence or absence of SIN-1. Immunoprecipitation of $G\alpha_s$, using the affinity purified RM antibody (25), revealed the incorporation of [$^3$H]palmitate into both the short and long forms of the $\alpha$ subunit (Fig. 5A). As previously reported (15), activation of the G protein via receptor stimulation with isoproterenol or directly with cholera toxin increased palmitate incorporation in both forms of $G\alpha$. The presence of SIN-1 during the metabolic labeling was without effect on the extent of [$^3$H]palmitate incorporation under basal conditions or following cholera toxin stimulation but blocked the stimulatory influence of 10 $\mu$M isoproterenol (Fig. 5, *A* and *B*). The effect of SIN-1 on isopro-

**A**



| SIN-1 | - | + | - | + | - | + |
| ISO | | | + | + | | |
| CTX | | | | | + | + |

42 -

**B**



Optical density (% of control)

- short
- long
- short + SIN
- long + SIN

BASAL    ISO    CTX

**C**



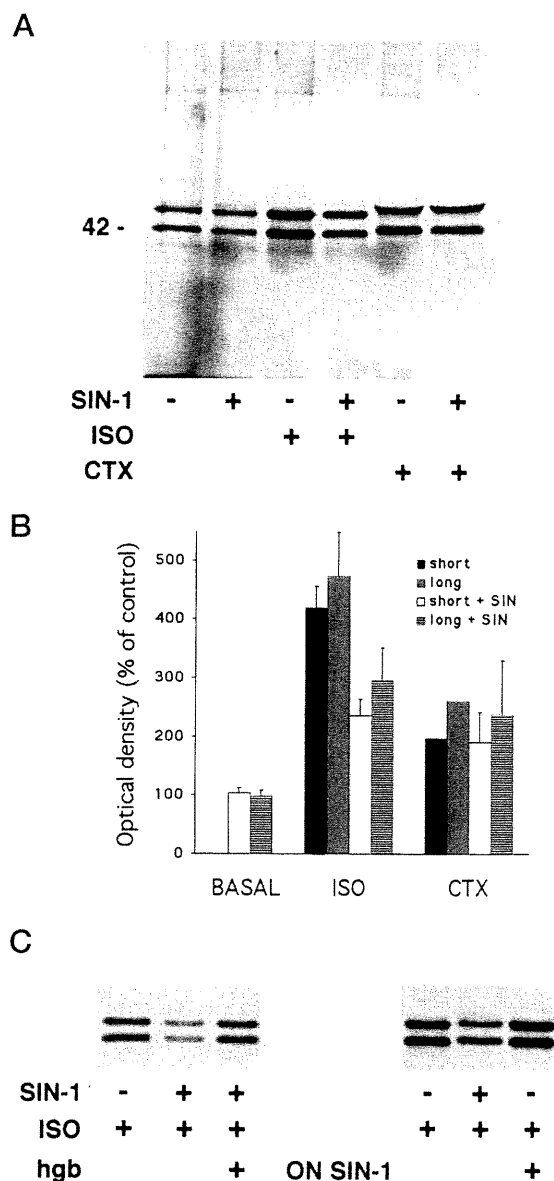| SIN-1 | - | + | + | | - | + | - |
| ISO | + | + | + | | + | + | + |
| hgb | | | + | ON SIN-1 | | | + |

FIG. 5. **Effect of SIN-1 on the basal and isoproterenol-stimulated incorporation of [³H]palmitate into G_sα subunits.** COS cells were incubated with 500 μCi/ml [³H]palmitic acid for 1 h in the presence or absence of 1 mM SIN-1, 10 μM isoproterenol (ISO), 1 μg/ml of cholera toxin (CTX), and 1 mM SIN-1 and 75 μM hemoglobin (hgb) or NO-depleted SIN-1 obtained by storage overnight at room temperature (ON SIN-1). After radiolabeling, the cells were homogenized and separated into particulate and soluble fractions by centrifugation. A and C, G_sα were immunoprecipitated from 1 mg of total protein solubilized from the particulate fractions with an affinity purified antibody. The proteins were separated by SDS-PAGE and prepared for fluorography. The films were exposed for 4 weeks at −70 °C. The long and short forms of G_s, resulting from alternative splicing, migrate at 45 and 42 kDa, respectively. B, densitometric analysis of ³H-labeled G_sα subunit bands on fluorographs are presented. The densitometry measurements are expressed as a percentage of the basal condition without SIN-1 treatment. Results are the mean ± the S.E. for 3–5 independent experiments.

terenol-stimulated palmitate incorporation was blocked by hemoglobin, which binds and inactivates NO (37) (Fig. 5C). Also, SIN-1 depleted of NO was without effect on the isoproterenol-stimulated palmitoylation level (Fig. 5C). These results indicate that NO blunted only the agonist-promoted palmitoylation of Gα_s suggesting that NO did not directly inhibit Gα_s palmitoylation but acted to decrease its activation by the receptor.

Similarly to what was observed for Gα_s, isoproterenol greatly enhanced the incorporation of the [³H]palmitate into the β_2AR

**A**



| | 1 | 2 | 3 | 4 | 5 | 6 | |
| ISO | - | - | + | + | - | + | - 66 |
| SIN-1 | - | + | - | + | - | - | - 46 |
| ON SIN-1 | - | - | - | - | + | + | |

**B**



Densitometry (% of control)

BASAL    ISO    ISO+SIN

**C**



- 66
- 46
- 30

SIN-1    -    +

**D**



Densitometry (% of control)
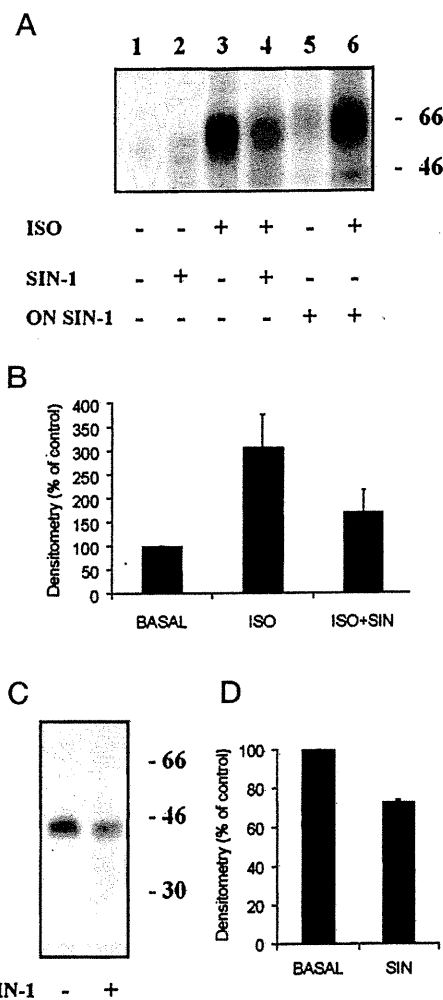
BASAL    SIN

FIG. 6. **Effect of SIN-1 on basal and isoproterenol-stimulated incorporation of [³H]palmitate into the β_2AR.** HEK293 stably transfected with the β_2AR (A) or Sf9 cells infected with recombinant baculovirus encoding the β_2AR (C) were metabolically labeled with [³H]palmitate for 1 h with 1 mM SIN-1, 10 μM isoproterenol, or NO-depleted SIN-1 as indicated. Purified receptors were resolved by SDS-PAGE, and incorporation of [³H]palmitate was detected by fluorography. Equal amounts of purified receptor were loaded into each lane. The fluorographs shown were exposed for 3–4 weeks. B and D, densitometric analyses of ³H-labeled β_2AR bands on fluorographs are presented. The densitometry measurements are expressed as a percentage of the basal condition and represent the mean ± S.E. of 3–4 independent experiments.

(Fig. 6, A and B), and the presence of 1 mM SIN-1 during the metabolic labeling with [³H]palmitate blunted this increase. This action of SIN-1 was not mimicked by a SIN-1 solution depleted of NO (Fig. 6A). The low level of receptor palmitoylation observed under basal conditions in HEK293 cells made it difficult to assess whether SIN-1 could influence the palmitoylation state of the nonstimulated β_2AR. Sf9 cells infected with recombinant baculovirus encoding the human β_2AR were used to verify the influence of SIN-1 on the basal incorporation of [³H]palmitate into the receptor because the basal level of palmitoylation was easier to detect. Sf9 cells were metabolically labeled with [³H]palmitate for 1 h in the presence or absence of 1 mM SIN-1. The presence of SIN-1 promoted a reduction of the basal palmitate incorporation into the receptor (Fig. 6, C and D). This result indicates that, contrary to what is observed for Gα_s, NO can modify the palmitoylation state of the receptor in an agonist-independent manner.

*Effect of SIN-1 Treatment on cAMP Accumulation Stimulated by a Nonpalmitoylated β_2AR Mutant*—To test more for-
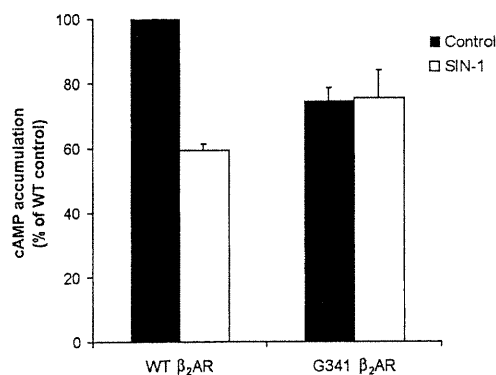
*NO Effects on a GPCR and Signaling*



Fig. 7. **Effect of SIN-1 treatment on cAMP accumulation stimulated by a nonpalmitoylated $\beta_2$AR mutant.** HEK293 cells expressing either the human wild type $\beta_2$AR (*WT*) or the nonpalmitoylated mutant, Gly$^{341}\beta_2$AR, were treated with 1 $\mu$M isoproterenol in the presence or absence of 1 mM SIN-1 for 20 min. cAMP accumulation was determined as described under "Experimental Procedures." The WT $\beta_2$AR and Gly$^{341}\beta_2$AR cells expressed 9 and 12 pmol of receptor per mg of protein, respectively. The values shown are the mean ± S.E. of three independent experiments. The cAMP response to isoproterenol in the cells transfected with the WT $\beta_2$AR was 6-fold greater than isoproterenol stimulation of nontransfected cells and 10-fold greater than basal cAMP accumulation.

mally the hypothesis that the blunting effect of SIN-1 on the $\beta$-adrenergic signaling results, at least in part, from the NO-mediated depalmitoylation of the $\beta_2$AR, the effect of SIN-1 treatment was assessed on a mutant $\beta_2$AR lacking its palmitoylation site (Gly$^{341}\beta_2$AR). As shown in Fig. 7 and as previously reported in other cell types (16, 39, 40), HEK293 cells expressing the nonpalmitoylated Gly$^{341}\beta_2$AR showed a reduced isoproterenol-stimulated adenylyl cyclase activity when compared with cells expressing the wild type receptor despite a modestly higher number of receptors (9 pmol/mg for the wild type *versus* 12 pmol/mg for Gly$^{341}\beta_2$AR). Treatment with SIN-1 that resulted in a 60% decrease in cAMP production in response to isoproterenol in WT $\beta_2$AR-expressing cells did not reduce the cAMP accumulation in response to the same stimuli in Gly$^{341}\beta_2$AR-expressing cells (Fig. 7). This observation is consistent with the hypothesis that palmitoylation of the receptor plays an important role in the modulatory action of NO.

## DISCUSSION

The present study demonstrates that NO produced either using chemical NO donors or following activation of iNOS by LPS significantly inhibits $\beta$-adrenergic-stimulated adenylyl cyclase in cultured cells thus offering a potential mechanism for some of the physiological actions of NO. Indeed, several studies have suggested that NO could act as a functional inhibitor of $\beta$-adrenergic signaling events. For example, acetylcholine-mediated inhibition of the $\beta$-adrenergic-stimulated L-type calcium current in the heart has been shown to involve NO synthesis (41). Also, inhibition of NOS by L-NMMA in rat heart has been shown to potentiate the positive inotropic response to isoproterenol suggesting that NO can dampen the $\beta$-adrenergic stimulation of the contractile response (7). Similarly, interleukin 1 and LPS, which are present during septic shock, can inhibit $\beta$-adrenergic-stimulated cardiac contraction through a NO-dependent mechanism (42, 43). Until now, however, the molecular basis for such antagonistic action of NO on $\beta$-adrenergic responses remained unexplored.

The data reported here suggest that the effect of NO on the $\beta$-adrenergic-stimulated adenylyl cyclase activity occurs upstream of the G protein since neither forskolin- nor cholera toxin-stimulated activities were affected. It follows that NO acts either by changing the effective concentration of agonist or

by modulating coupling between the receptor and G$_s$. NO-induced nitration of catecholamine through an oxidative process has been previously described (32, 35) and thus could be responsible for a reduction in the effective concentration of $\beta$-adrenergic agonist present in the assay. This hypothesis is, however, very unlikely since no significant reduction of the non-oxidized isoproterenol could be observed following NO treatment. Moreover, the addition of the anti-oxidant, ascorbate, which efficiently inhibits catecholamine nitration (32), was without effect on the inhibitory action of NO on the $\beta$-adrenergic-stimulated adenylyl cyclase activity observed in this study. Finally, agonist binding analysis revealed a NO-mediated augmentation of the high but not of the low affinity constant for isoproterenol. Given that the low ($K_L$)/high ($K_H$) affinity constant ratio for agonist is an index of the receptor-G protein coupling state (44), these data suggest that NO promotes uncoupling of the $\beta_2$AR from G$_s$, and this uncoupling is responsible for the blunted $\beta$-adrenergic-stimulated adenylyl cyclase activity. A similar uncoupling between another G protein-coupled receptor, the bradykinin receptor, and its cognate G proteins, G$_q$ and G$_i$, has also been observed following NO donor treatment (45). These effects were mimicked by a cGMP analogue suggesting that activation of the guanylate cyclase may be involved. However, this mechanism cannot be invoked for the $\beta$-adrenergic uncoupling observed in our study since treatment with 8-bromo-cGMP did not alter cAMP production in response to isoproterenol.

The observation that SIN-1 did not affect basal cAMP production is consistent with a number of previous reports (4, 46, 47) that fail to detect changes in basal adenylyl cyclase activity upon NO treatment. However, an increase in basal cAMP production has been observed in kidney and anal sphincter cells following NO stimulation (48, 49). The discrepancy among these studies may be explained by the finding that NO can directly stimulate the GTPase of both G$\alpha_s$ and G$\alpha_i$ (50). The relative contribution of these two G proteins to the basal adenylyl cyclase activity in a specific cell type may therefore determine the ultimate effect of NO on this activity. In any case, no effect of NO donor was observed on the basal cAMP accumulation in any of the cell types examined in the present study.

The inhibitory effect of NO on the $\beta$-adrenergic signaling efficacy was also illustrated by the reduced $\beta$-adrenergic-stimulated palmitoylation of G$\alpha_s$ observed upon SIN-1 treatment. This contrasted with the absence of effect of NO on either basal or cholera toxin-stimulated palmitate incorporation indicating that neither the intrinsic activity of G$_s$ nor the palmitoylation process itself were affected by the gaseous second messenger.

As for G proteins, the palmitoylation state of the $\beta_2$-adrenergic receptor has been shown to be regulated by agonist occupancy and activation (13, 15). Therefore, the inhibitory effect of NO on the agonist-stimulated level of receptor palmitoylation could also be a reflection of the decreased ability of the receptor to be activated and thus a consequence of the blunted $\beta$AR responsiveness. However, in contrast to what was observed for G$\alpha_s$, the basal palmitoylation level of the $\beta_2$AR was also found to be affected by NO. This raises the possibility of a direct action of NO on the palmitoylation status of the receptor. A direct effect of NO on the palmitoylation state has been previously suggested. Palmitoylation of SNAP-25 (a synaptic protein) and GAP-43 (a growth cone protein) was shown to be inhibited by NO through a cGMP-independent pathway(s) in PC12 cells and dorsal root ganglion neurons (13). In that study, the inhibition of protein acylation was accompanied by slower neurite growth, growth cone collapse, and retraction of dorsal root ganglion neurons suggesting that NO-mediated regulation

of palmitoylation may have had functional consequences.

NO-mediated depalmitoylation of the $\beta_2$AR could also have functional consequences. Inhibition of signaling occurs for several G protein-coupled receptors, including the endothelin A and B (51, 52), $D_1$-dopamine (53), and m2 muscarinic receptors (54), by mutation of their palmitoylation sites. Likewise for the $\beta_2$AR, inhibition of receptor palmitoylation by site-directed mutagenesis of its unique palmitoylation site (cysteine 341) significantly decreases the ability of the receptor to interact with $G_s$ and to stimulate adenylyl cyclase activity (16, 39, 40). Our hypothesis that NO-promoted depalmitoylation of the $\beta_2$AR leads to receptor uncoupling and decreased efficacy of $\beta$-adrenergic signaling is strengthened by our findings that SIN-1 had no effect on signaling through the nonpalmitoylated $Gly^{341}\beta_2$AR. Although one cannot exclude that NO may be able to act at several steps in G protein signaling, its major effect in this system is likely to be the result of depalmitoylation of the receptor.

The identity of the active species that mediate biological actions promoted by NO production is always a delicate issue. Indeed, NO can react with oxygen in aqueous solution to generate at least seven nitrogen oxide species that can exist simultaneously (NO, $^-$OONO, $NO_2$, $(NO)_2$, $N_2O_3$, $NO_2^-$, and $NO_3^-$) (55). Depending on the relative concentrations of reactants and targets, reactive intermediates can cross-react with each other and with a number of biological molecules, leading to an array of possible reaction pathways and products. The use of specific NO donors can favor the formation of a subset of possible nitrogen oxide species. For example, SIN-1 releases NO and SIN-1C but also superoxide anion ($O_2^-$) that can react with NO to accelerate the formation of peroxynitrite ($O_2^-$ + NO = $^-$OONO) (55) that could be toxic. However, the toxicity that could result from the formation of peroxynitrite, when using SIN-1, is most likely not responsible for the effects observed in this study since both endogenous NO production and another NO donor, GEA, that do not promote superoxide anion formation (56) had similar effects on $\beta$-adrenergic responsiveness.

In summary, our study demonstrates that NO inhibits $\beta$-adrenergic-stimulated cAMP production through functional uncoupling of the receptor from $G_s$. This attenuated $\beta$-adrenergic responsiveness was accompanied by an inhibition of receptor palmitoylation and was only seen for the wild type receptor that undergoes palmitoylation. Our data suggest that the action of NO to regulate $\beta$-adrenergic signaling occurs through modulation of receptor palmitoylation. More studies are needed to determine whether NO has similar effects on other receptors and whether other agents act as physiologic regulators of palmitoylation. We may find that changes in protein palmitoylation is a common means for cross-talk among signaling pathways.

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# 3. Discussion

Les travaux réalisés au cours de cette thèse ont permis 1) de démontrer que la palmitylation du $\beta_2AR$ est une modification post-traductionnelle dynamique modulée par l'état d'activation du récepteur, 2) de mettre en évidence une régulation réciproque entre la palmitylation et la phosphorylation du $\beta_2AR$ et 3) de révéler l'existence d'un lien de régulation croisée entre la production de NO et la réactivité $\beta$-adrénergique. La modulation de l'état de palmitylation du récepteur par le NO serait responsable de la diminution de l'activité fonctionnelle du récepteur.

## 3.1 La dynamique de palmitylation du $\beta_2AR$

Dans le cadre de cette thèse, nous avons mis en évidence que le renouvellement du palmitate sur le $\beta_2AR$ est un processus relativement rapide. De plus, la ½ vie du palmitate est significativement plus courte que celle du récepteur suggérant que cette modification soit indépendante de la synthèse *de novo* du récepteur. Des observations similaires ont également été rapportées concernant la palmitylation, en autres, de la rhodopsine (O'Brien and Zatz, 1984), de p21[N-ras] (Magee et al., 1987), du récepteur de la transférrine (Adam et al., 1988) et de la protéine $G\alpha s$ (Wedegaertner and Bourne, 1994).

Ainsi, la palmitylation du $\beta_2AR$ correspond à une modification post-traductionnelle. Cela signifie que cette modification du récepteur pourra être sujette à une modulation potentielle. Nous avons donc proposé qu'il existe un équilibre entre des formes palmitylées et non palmitylées du récepteur. Cet équilibre dynamique de l'état de palmitylation a été observé dans deux systèmes d'expression (hétérologue et

homologue) suggérant qu'il s'agit d'un phénomène général. Les termes hétérologue et homologue sont utilisés en référence à l'utilisation de cellules d'insectes (Sf9) (hétérologue) ou de mammifères (HEK293) (homologue) pour exprimer le $\beta_2$AR humain.

Une distinction importante entre ces deux systèmes d'expression se situe au niveau de la vitesse de renouvellement du palmitate sur le $\beta_2$AR. À ce propos, le renouvellement du palmitate associé au récepteur est cinquante-sept fois plus rapide lorsque le $\beta_2$AR est exprimé dans les cellules d'insectes. Cette courte ½ vie du palmitate (9 minutes) associé au récepteur peut s'expliquer, en partie, par le fait que le métabolisme des cellules d'insectes infectées est beaucoup plus rapide que celui des cellules de mammifères. Cela se reflète aussi sur la ½ vie du récepteur, celle-ci est 14 fois plus courte dans les cellules d'insectes comparativement aux cellules de mammifères (articles 2 et 3). Outre ces considérations, ce système d'expression hétérologue a été utilisé par différents groupes pour confirmer la palmitylation de sept RCPG (pour la liste de ces récepteurs, voir le tableau IV). Parmi ces récepteurs, il y a seulement le récepteur muscarinique M2 pour lequel une étude de cinétique d'incorporation du palmitate tritié a été rapportée (Hayashi and Haga, 1997). Les résultats de cette étude suggèrent, comme pour le $\beta_2$AR, un renouvellement rapide du palmitate associé à ce récepteur. En effet, en moins de quinze minutes, une incorporation maximale du palmitate tritié au niveau du récepteur muscarinique M2 a été observée. Le profil d'incorporation rapide du palmitate tritié au niveau de ce récepteur n'atteint jamais un équilibre (plateau). Cette observation découle probablement du fait que dans les cellules d'insectes, le pool de palmitate tritié n'est jamais à l'équilibre contrairement au système d'expression homologue où nous avons un équilibre du palmitate tritié durant une heure.

À la lumière de ce qui précède, l'utilisation des cellules d'insectes pour étudier la dynamique de palmitylation du $\beta_2AR$ comporte des limites. D'une part, un métabolisme de base rapide se reflète sur la ½ vie du palmitate et du récepteur. D'autre part, un pool de palmitate tritié n'atteint jamais l'équilibre. Dans ces conditions, l'incorporation du palmitate au niveau du $\beta_2AR$ n'est pas le reflet exclusif de la dynamique de palmitylation mais aussi de la bio-disponibilité du palmitate tritié dans les cellules d'insectes. De plus, nous devons insister sur le fait que l'infection des cellules d'insectes entraîne la diminution de la synthèse protéique endogène au profit des protéines virales. Après vingt-quatre heures d'infection, il y a arrêt presque total de la synthèse protéique endogène (O'Reilly et al., 1992). D'une manière générale, une infection d'une durée de quarante-huit heures est nécessaire pour obtenir un bon niveau d'expression de la protéine d'intérêt (O'Reilly et al., 1992). Dans ce contexte, l'utilisation des cellules d'insectes pour étudier la cinétique des modifications post-traductionnelles d'une protéine demeure peu recommandée, puisque les résultats peuvent, entre autre, refléter des changements d'expression des protéines endogènes impliquées dans la régulation de ces modifications rendant ainsi l'interprétation des résultats ardue. Cette hypothèse est appuyée par l'étude suivante: l'expression de la protéine p-53 dans les cellules d'insectes démontre qu'il y a une baisse de son niveau de phosphorylation quarante-huit heures après l'infection des cellules comparativement à vingt-quatre heures (O'Reilly et al., 1992). Une chute de l'expression des protéines kinases endogènes pourrait expliquer cette différence de phosphorylation de la protéine p-53. Par ailleurs, pour étudier certains aspects des modifications post-traductionnelles, les cellules d'insectes qui peuvent exprimer à un niveau élevé la protéine d'intérêt sont un choix judicieux.

En tout état de cause, la palmitylation du $\beta_2AR$ est une modification post-traductionnelle indépendamment du système d'expression utilisé (hétérologue ou homologue). Des changements de l'état de palmitylation du récepteur pourraient influencer l'activité fonctionnelle du $\beta_2AR$. Dans cet ordre d'idées, il a déjà été proposé que la palmitylation puisse modifier certaines fonctions des RCPG.

Avant d'élaborer sur les différents rôles proposés pour la palmitylation RCPG, signalons que la stratégie communément utilisée pour étudier les rôles fonctionnels de la palmitylation consiste à abolir les sites de palmitylation par mutagenèse dirigée. Dans ces conditions, il devient difficile de distinguer entre un effet attribuable à la perte de la palmitylation et/ou à une modification de la structure primaire de la protéine. Pour pallier à ce problème, l'utilisation d'inhibiteurs de la palmitylation serait à privilégier. Sur ce point, il a été suggéré que la tunicamycine pourrait jouer un rôle d'inhibiteur de la palmitylation (Patterson and Skene, 1994). Cependant, la tunicamycine est très peu utilisée puisque c'est un antibiotique qui inhibe aussi la N-glycosylation des protéines. Pour l'instant, aucun inhibiteur spécifique de la palmitylation n'est disponible. De toute évidence, les approches pour étudier les rôles de la palmitylation sont très limitées.

Compte tenu de ce qui précède, la mutagenèse dirigée demeure un des seuls outils pour étudier les rôles fonctionnels de la palmitylation. Cette approche a permis de mettre en évidence qu'au moins trois fonctions des RCPG peuvent être affectées à la suite de la mutation des sites de palmitylation. Une diminution du ciblage à la membrane plasmique a été observée pour les récepteurs de la vasopressine V2 (Sadeghi et al., 1997) et de la thyrotropine (Tanaka et al., 1998). Une augmentation de l'internalisation et de la régulation négative a été démontrée pour le récepteur de

la choriogonadotropine (Kawate and Menon, 1994). Une absence de régulation négative a été observée pour le récepteur adrénergique $\alpha_{2A}$ (Eason et al., 1994). Finalement, une diminution de la capacité des récepteurs non palmitylés à se coupler avec la protéine G a été démontrée pour les récepteurs muscariniques $M_2$ (Hayashi and Haga, 1997), endothéline B (Okamoto et al., 1997) et A (Horstmeyer et al., 1996), dopaminergique $D_1$ (Jensen et al., 1995) et adrénergique $\beta_2$ (O'Dowd et al., 1989). À la suite de ces nombreuses études, il semble évident que la palmitylation ne joue pas un rôle unique aux seins des RCPG. Pourquoi y a-t-il une diversité d'effets de cette modification post-traductionnelle sur les RCPG ? L'état de palmitylation pourrait-il être spécifique (voir intrinsèque) à chaque récepteur de façon à influencer une fonction particulière ? Dans cet ordre d'idées, un parallèle pourrait se faire avec un autre type de modification post-traductionnelle, la phosphorylation. Il est bien connu que l'état de phosphorylation du $\beta_2 AR$ peut moduler sa capacité d'interagir avec certaines protéines. Parmi celles-ci, l'interaction de la $\beta$-arrestine avec le récepteur demeure la mieux caractérisée. Une des conséquences de cette interaction est de provoquer un découplage fonctionnel du récepteur avec la protéine G. Dans ces conditions, la $\beta$-arrestine associée au récepteur est responsable de l'internalisation du récepteur via les vésicules tapissées de clathrine (Zhang et al., 1997). Cependant, ce mécanisme d'internalisation ne peut pas être généralisé pour tous les RCPG. Par exemple, l'internalisation du récepteur de l'angiotensine de type 1A est indépendante de la $\beta$-arrestine. Un mutant négatif de la $\beta$-arrestine inhibe l'internalisation du $\beta_2 AR$ sans affecter l'internalisation du récepteur de l'angiotensine de type 1A (Zhang et al., 1996). Les auteurs de cette étude suggèrent que des déterminants propres à chaque RCPG pourraient, en partie, dicter les mécanismes de désensibilisation telle que l'internalisation. Un concept similaire pourrait être envisagé pour la palmitylation des RCPG. Ainsi, la palmitylation pourrait réguler l'interaction de certaines protéines avec les récepteurs. La spécificité de ces interactions serait responsable d'influencer

des fonctions particulières des RCPG. À ce sujet, une étude récemment publiée a démontré que l'absence de la palmitylation du récepteur de l'endothéline A (Horstmeyer et al., 1996) provoque un découplage avec la protéine Gq mais pas avec la protéine Gs. C'est la première fois qu'une étude propose que la palmitylation puisse sélectivement moduler une voie de signalisation plutôt qu'une autre.

Compte tenu du nombre d'études démontrant que la palmitylation affecte certaines fonctions des RCPG, l'état de palmitylation des récepteurs doit potentiellement être régulé. Dans la prochaine section, nous allons discuter de l'effet de l'activation du $\beta_2AR$ sur sa dynamique de palmitylation.

## 3.2    La régulation de la palmitylation du $\beta_2AR$

Nous avions émis l'hypothèse que la palmitylation du $\beta_2AR$ pourrait être modulée à la suite de l'activation du récepteur. Nos résultats ont confirmé que la présence d'agonistes influence grandement l'état de palmitylation du récepteur indépendamment du système d'expression utilisé. Nous avons démontré qu'une stimulation à court terme du récepteur par un agoniste provoque une augmentation de l'incorporation du palmitate tritié (articles 2 et 3). Au moins deux explications peuvent être proposées pour rendre compte d'une telle observation : la stœchiométrie de palmitylation et/ou la vitesse de renouvellement du palmitate sur le récepteur (palmitylation/dépalmitylation) pourraient être modifiées. Ainsi, une augmentation de la stœchiométrie de palmitylation du $\beta_2AR$ pourrait être initiée au cours de l'activation du récepteur. Cependant, il est impossible de vérifier une telle hypothèse puisque dans nos conditions expérimentales, le pool de palmitate tritié dans la cellule n'est pas à l'équilibre. Afin de contourner ce problème, la spectrométrie de masse

pourrait être une stratégie envisageable. Cette technologie a déjà été utilisée pour évaluer la stœchiométrie de palmitylation de la rhodopsine (Papac et al., 1992). Les auteurs de cette étude se sont limités à confirmer l'existence de deux sites de palmitylation (cystéines 323 et 322 ) dans la portion C-terminale du récepteur. Récemment, la palmitylation du récepteur de la bradykinine a été confirmée par cette technique (Soskic et al., 1999). Cette approche est encore peu répandue mais réserve un grand potentiel pour étudier les modifications post-traductionnelles telle que la palmitylation.

Comme nous l'avons mentionné précédemment, en plus de la stœchiométrie de palmitylation, l'activation du récepteur pourrait influencer la vitesse de renouvellement du palmitate sur le récepteur. Consistant avec cette hypothèse, nos résultats suggèrent que l'activation du récepteur favorise une augmentation de la vitesse de dépalmitylation. Cela se traduit par une diminution de la ½ vie du palmitate associé au récepteur activé. Des résultats similaires ont été rapportés concernant la dynamique de palmitylation du récepteur adrénergique $\alpha_{2A}$ (Kennedy and Limbird, 1994). L'activation de ce récepteur par un agoniste provoque une diminution de la ½ vie du palmitate (13 heures à 8.9 heures) sans modifier la ½ vie du récepteur. L'interprétation des résultats demeure limitée puisque à l'état basal la ½ vie du palmitate (13 heures) est supérieure à celle du récepteur (9.5 heures). Ces résultats sont très surprenants puisque jusqu'à maintenant, aucune étude n'a rapporté une telle observation (½ vie du palmitate associé à la protéine plus longue que la ½ vie de la protéine) suggérant ainsi une erreur expérimentale dans la détermination de la ½ du palmitate associé au récepteur ou du récepteur. De plus, il est inattendu qu'aucune régulation négative n'ait été observée suite à l'activation du récepteur (½ vie du récepteur inchangée). À cet égard, une étude récente a démontré qu'en mutant

le résidu (cystéine 442) responsable de la palmitylation, on provoquait une diminution significative de la régulation négative du récepteur adrénergique $\alpha_{2A}$ (Eason et al., 1994). Dans l'ensemble, les résultats de cette étude demeurent litigieux.

La régulation négative est observée pour plusieurs RCPG dont le $\beta_2AR$. Ce phénomène ne doit pas être négligé au moment d'évaluer la ½ vie du palmitate associé au récepteur puisque dans des conditions d'activation par un agoniste, une diminution de la ½ vie du récepteur peut également être observée. Si tel est le cas, il faut distinguer l'effet de l'agoniste sur la ½ vie du palmitate associé au récepteur versus la ½ vie de la protéine. Nous avons démontré que dans les cellules de mammifères, la ½ vie du palmitate et celle du récepteur sont toutes les deux affectées à la suite de l'activation du récepteur par un agoniste $\beta$-adrénergique. Dans nos conditions expérimentales, nous avons observé qu'une perte de 50% du palmitate est accompagnée d'une dégradation de 21% des récepteurs. Consistant avec ces résultats, les valeurs des pentes des expériences de "pulse-chase" ($^3$H et $^{35}$S) confirment que la palmitylation est influencée plus rapidement à la suite de l'activation du récepteur qu'à sa dégradation (article 3). En éliminant le pourcentage de dégradation du récepteur, il est clair que la ½ vie nette du palmitate associé au récepteur activé est bien inférieure à celle du récepteur. En somme, la modulation de la palmitylation du récepteur activé est indépendante du mécanisme de régulation négative. Cette interprétation des résultats est cohérente avec les observations provenant du système d'expression hétérologue. Dans ce système, l'activation du récepteur provoquant une diminution de la ½ vie du palmitate est indépendante de la dégradation du récepteur. En effet, dans les cellules d'insectes, la régulation négative est très faiblement détectée (Loisel et al., 1996). Ainsi, il est légitime de proposer qu'un changement de conformation structurale du récepteur à la suite de son activation puisse réguler son

interaction avec certaines protéines régulatrices permettant une dépalmitylation accélérée (diminution de la ½ vie du palmitate). Cette hypothèse est corroborée par le fait que la modulation de la palmitylation du $\beta_2$AR est indépendante d'une augmentation de la concentration d'AMPc cellulaire (articles 2 et 3) suggérant fortement que l'activation du récepteur soit déterminante. Il faut toutefois mentionner que les protéines potentiellement impliquées dans la régulation de la palmitylation des RCPG demeurent à être identifiées et caractérisées (voir section dans l'introduction: Enzymologie de la palmitylation).

La palmitylation n'a pas été observée uniquement pour les RCPG. En effet, plusieurs protéines cytosoliques sont également palmitylées. Un nombre restreint d'études a été réalisé pour caractériser la dynamique de palmitylation de ces protéines. Parmi celles-ci, aux moins six groupes ont évalué les ½ vies du palmitate associé à des protéines cytosoliques. Telle qu'illustré au tableau V, la ½ vie du palmitate de ces protéines, N-RAS (Magee et al., 1987), H-RAS (Lu and Hofmann, 1995), GAP-43 (Baker and Storm, 1997), p56$^{lck}$ (Paige et al., 1993), G$\alpha$s (Wedegaertner and Bourne, 1994), NO synthase endothéliale (Robinson and Michel, 1995) et ankyrine (Staufenbiel, 1987), est toujours inférieure à celle des protéines. Ces résultats sont consistants avec nos observations concernant la palmitylation du $\beta_2$AR. De plus, l'aspect régulation de la palmitylation pour ces protéines cytosoliques a intéressé aux moins deux groupes. Le groupe du Dr Michel a démontré que l'activation de la NO synthase endothéliale provoque une diminution de la ½ vie du palmitate sans modifier la ½ vie de la protéine (Robinson and Michel, 1995).

**Tableau V**     **Protéines palmitylées pour lesquelles le taux de renouvellement du palmitate a été rapporté.**

|  | Système d'expression | ½ vie du palmitate (non-activée activée) | ½ vie de la protéine non-activée activée) |
|---|---|---|---|
| **_A- Protéines transmembranaires:_** | | | |
| 1- Récepteur de la transférrine | homologue | 10 h / N.D. | 60 h / N.D. |
| 2- Récepteur $\alpha_2$-adrénergique | homologue | 13 h / 8.9 h | 9.5 h / 9.5h |
| 3- Récepteur $\beta_2$-adrénergique | homologue | 8.8 h / 3.7 h | 23 h / 9 h |
| 4- Récepteur $\beta_2$-adrénergique | hétérologue | 9 min / 5.4 min | 100 min / 100 min |
| **_B- Protéines cytosoliques:_** | | | |
| 5- Neuromoduline (GAP-43) | homologue* | 5 h / N.D. | 48 h / N.D. |
| 6- N-Ras | homologue | 20 min / N.D. | 24 h / N.D. |
| 7- H-Ras | homologue | 90 min / N.D. | 22 h / 22 h |
| 8- p56$^{lck}$ | homologue | < 1 h / N.D. | 25 h / N.D. |
| 9- sous-unité G$\alpha$s | homologue | 50-90 min / 2-10 min | 22 h / 22 h |
| 10- NO synthase (NOSe) | homologue | 40 min / 10 min | 20 h / 20 h |
| 11- Ankyrine | homologue | 50 min / N.D. | > 20 h/ ND |

N.D. : Non Disponible     * : protéine endogène

Le groupe du Dr Bourne a observé un phénomène similaire de la dynamique de palmitylation de Gαs suite à son activation (Wedegaertner and Bourne, 1994). L'activation de cette dernière directement (en présence de AlF$_4^-$) ou par le β$_2$AR induit une diminution de la ½ vie du palmitate associé à la protéine Gαs (Mumby, 1997; Wedegaertner and Bourne, 1994). Finalement, comme pour la palmitylation du β$_2$AR, cette modification des protéines cytosoliques est modulable en fonction de leur état d'activation.

Malgré des similitudes avec la dynamique de palmitylation entre les protéines cytosoliques et transmembranaires, une différence notable se situe au niveau des valeurs de ½ vie du palmitate (50-90 minutes vs 8.8 heures). Comment expliquer cette différence ? Il est difficile de répondre à cette question puisque nos connaissances actuelles concernant la régulation de la palmitylation sont encore très limitées. Toutefois, il a été proposé que l'ancrage des protéines à la membrane plasmique pourrait être un facteur déterminant de la vitesse de renouvellement du palmitate (Lu and Hofmann, 1995). Les auteurs de cette étude ont démontré, en utilisant différents mutants de la protéine H-RAS, que la ½ vie du palmitate était inversement proportionnelle à l'ancrage de la protéine à la membrane plasmique. Cette protéine de type sauvage possède deux sites de palmitylation (cystéines 181 et 184) et un site pour la prénylation (cystéine 186) dans sa portion C-terminale. Par exemple, une ½ vie du palmitate associé à la protéine plus courte est observée lorsqu'un de ces deux sites de palmitylation est aboli (15 minutes vs 90 minutes). Dans une telle hypothèse, l'activation du β$_2$AR par l'isoprotérénol module sa propre palmitylation mais également celle de la protéine Gαs, pour un même stimuli. La ½ vie du palmitate pour la plupart de ces protéines est inversement proportionnelle à leur ancrage à la membrane (Tableau V).

Il est certain que les rôles respectifs joués par la palmitylation pour les protéines transmembranaires et cytosoliques sont différents. Pour les protéines cytosoliques, il a été proposé que la palmitylation favoriserait leur attachement à la membrane où ces protéines pourraient interagir avec d'autres protéines importantes pour leur fonction. Pour les RCPG déjà ancrés à la membrane plasmique par sept segments transmembranaires, leur site de palmitylation localisé dans la portion C-terminale pourrait réguler le rapprochement de cette région du récepteur avec la membrane plasmique et ainsi, imposer une certaine conformation à la queue C-terminale. Pour plusieurs RCPG dont le $\beta_2$AR, cette portion du récepteur possède des sites consensus pour des protéines kinases (PKA, PKC et GRK). Cette interaction récepteur-kinases joue un rôle déterminant dans l'activité fonctionnelle des récepteurs. Dans ce contexte, la palmitylation des RCPG pourrait-elle influencer les interactions de ces protéines kinases avec la portion C-terminale du récepteur ? Dans la prochaine section, nous allons discuter de la possibilité que la palmitylation du $\beta_2$AR, cette modification régulée par l'état d'activation du récepteur, puisse influencer l'interaction de la PKA avec le récepteur en modifiant l'accessibilité de ces sites de phosphorylation.

## 3.3    Relation entre la palmitylation et la phosphorylation du $\beta_2$AR

Dans la portion C-terminale du $\beta_2$AR, il y a plusieurs sites de phosphorylation pour la GRK et un site commun pour la PKC et la PKA. Le site consensus de cette dernière (sérines 345-346) est à quatre acides aminés en aval de la cystéine 341 responsable de la palmitylation du récepteur. Nous avons mis en évidence qu'il existe une interaction entre ce site de phosphorylation pour la PKA et la palmitylation. En

effet, nos résultats ont démontré que la phosphorylation du site de PKA (sérines 345-346) n'est pas favorisée lorsque le récepteur est palmitylé. Comme nous l'avons déjà mentionné, par la nature lipophilique de l'acide palmitique, il a été suggéré que le palmitate du $\beta_2AR$ soit ancré dans la membrane plasmique. Cette hypothèse est corroborée par l'étude de la palmitylation de la rhodopsine utilisant un analogue fluorescent du palmitate (Moench et al., 1994). Pour le $\beta_2AR$, la formation de la quatrième boucle intracellulaire causée par l'ancrage du palmitate dans la membrane plasmique pourrait, en plus, être stabilisée par la présence de résidus hydrophobes ($^H$) ou chargés positivement ($^+$) situés de part et d'autre de la cystéine palmitylée 341 du récepteur: F $^H$ R$^+$I $^H$ A $^H$ F $^H$ Q E L $^H$ L $^H$ C$^{341}$L $^H$ R$^+$R$^+$S S K$^+$. Selon ce modèle, le site de phosphorylation de la PKA (sérines 345 et 346) est situé très près de la membrane plasmique lorsque le récepteur est palmitylé. L'accessibilité du site de PKA serait diminuée en raison de sa proximité avec la membrane plasmique. Cette hypothèse est appuyée par les résultats d'expériences de phosphorylation *in vitro* avec la PKA purifiée démontrant que le récepteur non palmitylé peut davantage être phosphorylé que le récepteur de type sauvage (Article 1). Des résultats similaires ont été rapportés concernant le récepteur glutamate R6 (Pickering et al., 1995). En effet, une augmentation de la phosphorylation par la PKC aux sites consensus (sérines 815 et 837) a été rapportée lorsque les cystéines 827 et 840 responsables de la palmitylation sont mutées. Ainsi, la palmitylation des récepteurs pourrait moduler l'accessibilité des sites de phosphorylation à proximité pour des kinases. Dans cet ordre d'idées, l'expression du $\beta_2AR$ non palmitylé dans les cellules d'insectes ou de mammifères démontre une diminution de sa capacité à stimuler correctement l'adénylyl cyclase (Moffett et al., 1993). Il a été démontré que le niveau de phosphorylation à l'état basal du récepteur non palmitylé est bien supérieur à celui d'un récepteur de type sauvage. L'amplitude de la phosphorylation de ce mutant se compare à un récepteur désensibilisé suite à un traitement avec un agoniste. Ainsi, la perte de couplage de ce

mutant peut être expliquée par son niveau de phosphorylation élevé. Il est important de préciser que la phosphorylation du site de PKA dans la portion C-terminale semble jouer un rôle déterminant dans le profil de phosphorylation élevé du récepteur non palmitylé. En effet, en mutant le site de phosphorylation pour la PKA dans la portion C-terminale du récepteur non palmitylé, le niveau de phosphorylation de ce récepteur mutant est similaire au récepteur de type sauvage. De plus, ce récepteur mutant peut de nouveau stimuler l'adénylyl cyclase (article 1). Dans l'ensemble, nos résultats suggèrent fortement que la palmitylation du $\beta_2AR$ module l'accessibilité du site de phosphorylation par la PKA situé dans la portion C-terminale du récepteur. Ainsi, la palmitylation du récepteur en modulant le profil de phosphorylation pourrait jouer un rôle déterminant sur l'activité fonctionnelle du $\beta_2AR$.

Jusqu'ici, nous nous sommes limités à mettre en évidence l'influence de la palmitylation sur le site de phosphorylation de la PKA du $\beta_2AR$. Nous allons maintenant discuter de l'effet de la phosphorylation par la PKA des sérines 345-346 sur la dynamique de palmitylation du récepteur. Par mutagenèse dirigée, nous avons démontré que ce site de phosphorylation par la PKA modifie la dynamique de palmitylation du $\beta_2AR$ indépendamment du système d'expression utilisé (hétérologue ou homologue). En effet, le niveau d'incorporation du palmitate tritié demeure inchangé pour ce récepteur mutant (A345S/A346S) à la suite de son activation. Comme nous l'avons mentionné dans la section précédente, l'incorporation du palmitate tritié au sein d'une protéine semble être inversement proportionnelle à son ancrage. Nous croyons que la phosphorylation des sérines 345 et 346 pourrait perturber la stabilité de l'ancrage du palmitate dans la membrane. La répulsion entre

les charges négatives des phospholipides de la membrane et celles des sérines 345 et 346 serait responsable de l'augmentation de l'incorporation du palmitate tritié au niveau du récepteur de type sauvage. Si on retient cette hypothèse, l'incorporation du palmitate tritié au niveau d'un récepteur dont le site de phosphorylation est absent ne devrait pas être influencée à la suite de l'activation de ce $\beta_2AR$ mutant. Nos résultats de palmitylation avec ce récepteur mutant corrobore une telle hypothèse (dans les cellules d'insectes et de mammifères). Dans ce contexte, il serait fortement recommandé de réaliser des expériences de "pulse-chase" avec ce récepteur mutant afin de comparer les ½ vies du palmitate associé au récepteur mutant avec celle du récepteur de type sauvage. Nous croyons que ces expériences démontreront que la ½ vie du palmitate associé à ce récepteur mutant sera moins affectée en présence de l'isoprotérénol qu'à celle du récepteur de type sauvage (Tableau V). Nous croyons pouvoir affirmer que la palmitylation du $\beta_2AR$ est influencée par la phosphorylation du site de PKA dans la portion C-terminale du récepteur.

Nous avons également constaté que la stimulation à long terme avec un agoniste $\beta$-adrénergique semble favoriser une forme non-palmitylée du récepteur de type sauvage. En utilisant le système d'expression hétérologue, nous avons constaté que le $\beta_2AR$ ne semble plus apte à incorporer du palmitate tritié suite à une activation de plus de quinze minutes (article 2). Avec les cellules Sf9, le récepteur ne peut pas être déphosphorylé, comme nous l'avons déjà mentionné, le mécanisme d'internalisation est presque inexistant dans ce type de cellules (Loisel et al., 1996). Ainsi, le récepteur demeure à la membrane plasmique hautement phosphorylé. Nous croyons que le récepteur doit être déphosphorylé pour être de nouveau palmitylé. En

considération de ce qui précède, dans les cellules de mammifères où le mécanisme d'internalisation est présent, l'augmentation soutenue de l'incorporation du palmitate tritié observée suite à l'activation à long terme du $\beta_2AR$ serait le reflet du recyclage permanent du récepteur (article 3). Le modèle que nous proposons est résumé à la figure 6. Afin de vérifier une telle hypothèse, il faudrait bloquer l'internalisation du $\beta_2AR$ et vérifier son état de palmitylation à la suite de son activation. On pourrait s'attendre à une cinétique d'incorporation du palmitate similaire à celle observée dans les cellules d'insectes, c'est à dire à une absence de l'augmentation du palmitate tritié au niveau du récepteur. Une stratégie complémentaire pour vérifier l'influence de la phosphorylation sur la palmitylation serait de mimer le site de phosphorylation de la PKA à proximité de la palmitylation par la présence de résidus acides tel que l'acide glutamique.

Plusieurs constatations de notre étude suggèrent qu'il existe une régulation croisée entre la palmitylation et la phosphorylation du $\beta_2AR$. Les résultats d'une étude récente proposent également une relation entre la palmitylation et la phosphorylation du récepteur de la bradykinine (Soskic et al., 1999). En utilisant une technique de spectroscopie de masse, les auteurs ont mis en évidence le fait que la cystéine 356 peut être palmitylé seulement lorsque la tyrosine 352 n'est pas phosphorylée. Ainsi, la phosphorylation de la tyrosine 352 pourrait jouer un rôle similaire au site de phosphorylation de la PKA à proximité de la cystéine palmitylée 341 au niveau du $\beta_2AR$.

**Figure 6**      **Modèle schématique proposé de l'interaction entre la palmitylation et la phosphorylation du récepteur $\beta_2$-adrénergique.**

# A) Équilibre dynamique de l'état de palmitylation du $\beta_2$-AR
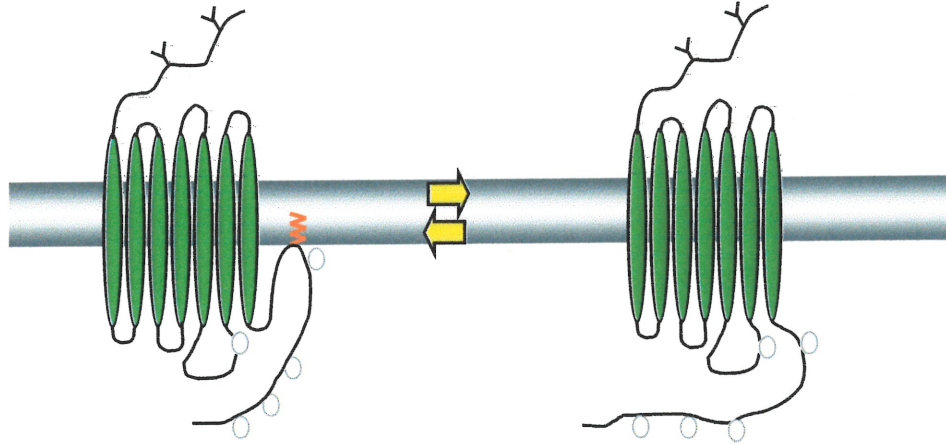


# B) Activation du $\beta_2$-AR
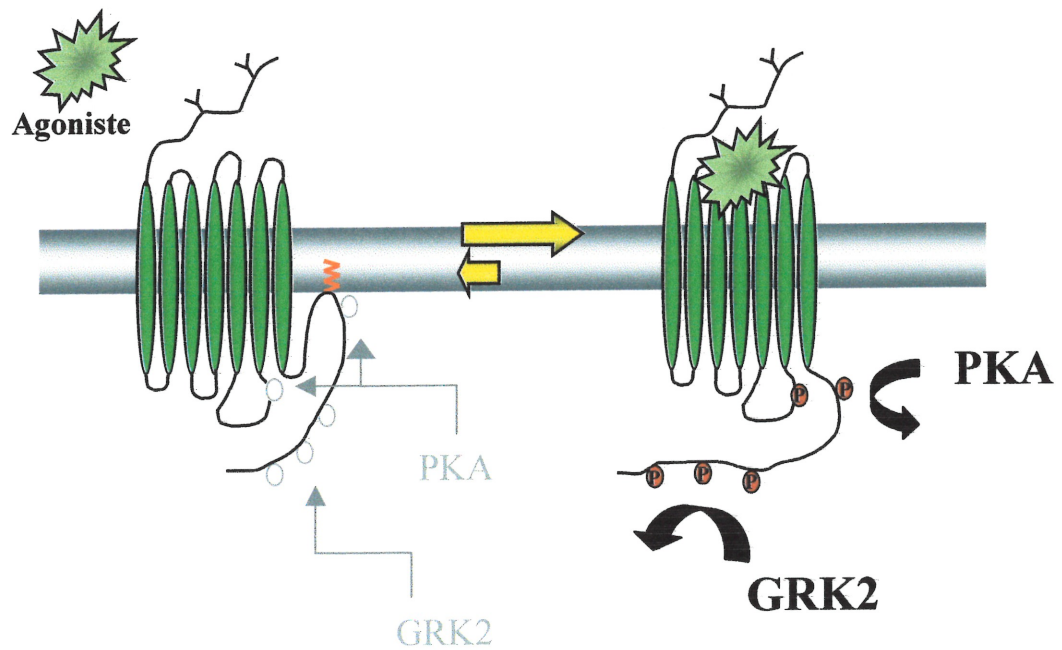
# -C- Dynamique de la palmitylation du β₂AR: cellules d'insectes (Sf9)
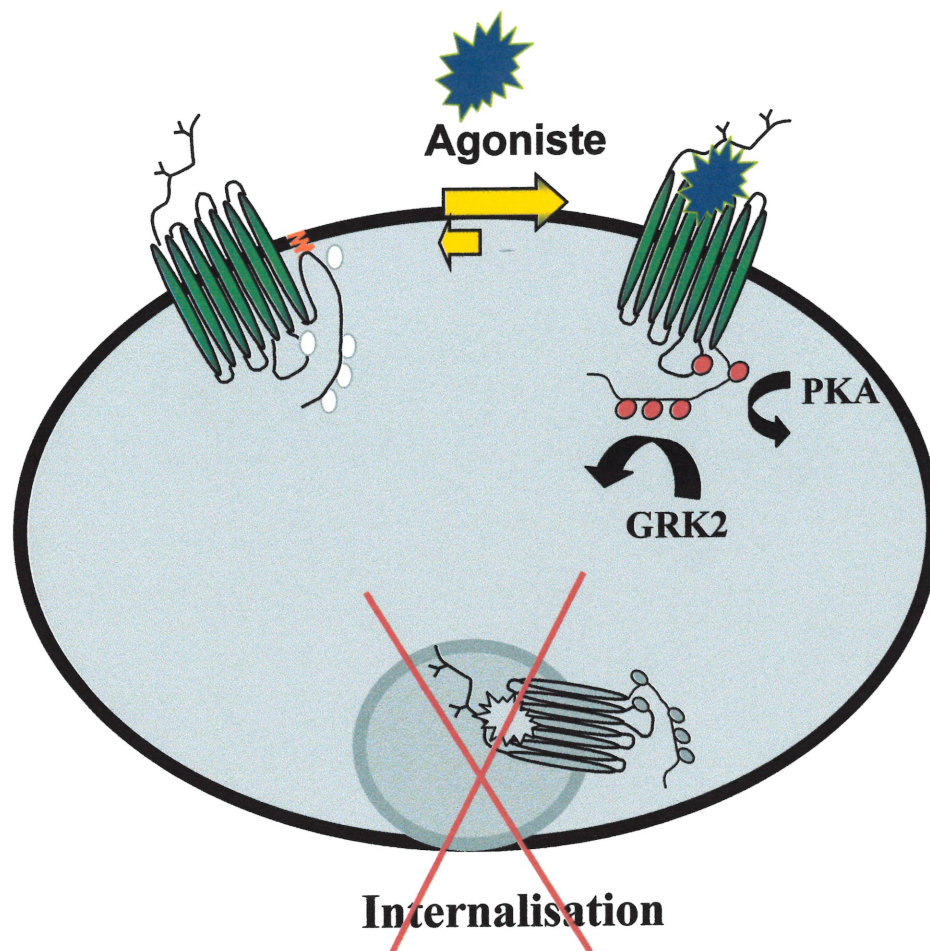


**1- Activation**

**2- Phosphorylation**

**3- Dépalmitylation**

# D) Expression du β₂-AR:
## Cellules de mammifères (HEK293)
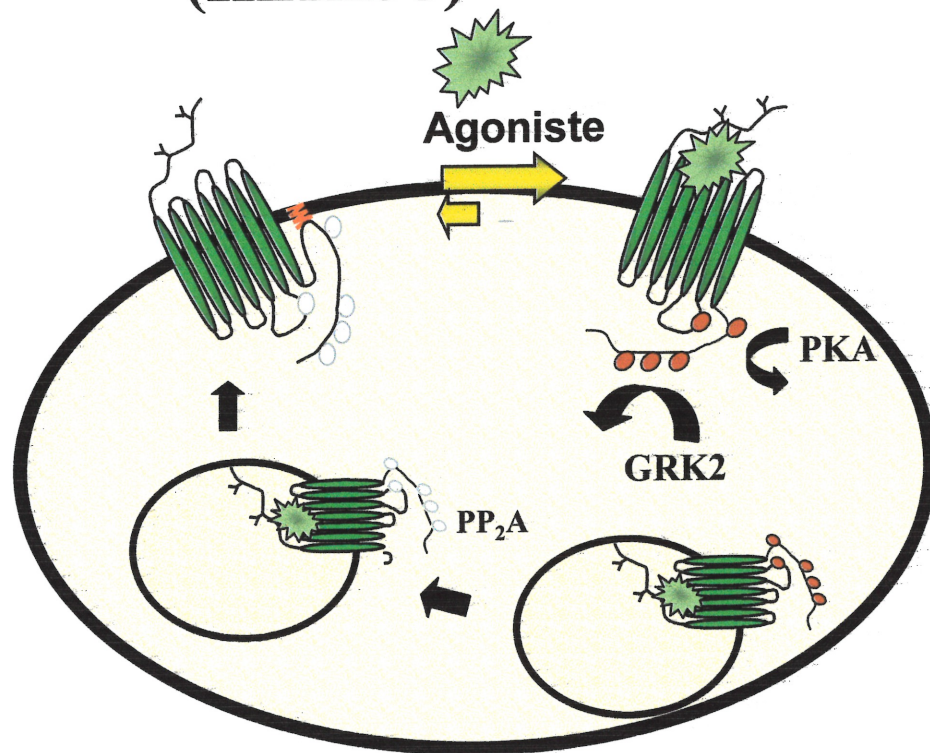


**1- Activation du récepteur**

**2- Phosphorylation**

**2-1 *Dépalmitylation***

**3- Internalisation**

**4- Déphosphorylation**

**5- Resensibilisation**

## 3.4 Modulation de la palmitylation du $\beta_2$AR par le monoxyde d'azote

Nos travaux nous ont aussi permis de mettre en évidence un autre mécanisme de régulation de la palmitylation qui est indépendant de l'état d'activation du récepteur. En effet, nous avons démontré que la palmitylation du $\beta_2$AR peut être modulée par la présence de monoxyde d'azote (NO) (section résultat: article 4). Une telle modulation du NO sur la palmitylation de GAP-43 et SNAP-25 a également été rapportée (Hess et al., 1993). Ces deux protéines sont impliquées dans l'allongement des cônes de croissance neuronales ("neuronal growth cone"). À la suite d'analyses vidéo, il a été observé que le NO bloque l'élongation de ces cellules neuronales. Cet effet du NO est réversible. En effet, l'allongement des cellules peut être de nouveau observé après plusieurs lavages pour éliminer le NO. Les auteurs de cette étude ont proposé que la diminution de l'incorporation du palmitate tritié au niveau de SNAP-25 et GAP-43, attribuable à la présence de NO, puisse être responsable, en partie, de la régulation de cette mobilité des cellules neuronales. Bien que cette étude le suggère indirectement, une régulation de l'état de palmitylation de GAP-43 et SNAP-25 par le NO pourrait influencer leurs fonctions.

À l'instar de cette étude, nos résultats suggèrent que la régulation de l'état de palmitylation du $\beta_2$AR par le NO puisse aussi affecter la fonction du récepteur. En effet, une diminution de la capacité du récepteur à stimuler l'adénylyl cyclase a été observée à la suite d'un traitement avec un donneur de NO. Ce résultat peut sans doute s'expliquer par une diminution du couplage entre le récepteur et la protéine G puisque nos études pharmacologiques ont démontré une diminution de l'affinité de l'isoprotérénol pour le complexe récepteur-protéine G (site de haute affinité du

récepteur). Un phénomène similaire du NO sur le découplage du récepteur de la bradykinine (Miyamoto et al., 1997) a aussi été observé. Les auteurs de cette étude ont démontré qu'en utilisant un donneur de NO, il y avait une diminution du couplage entre le récepteur et les protéines Gq et Gi. De plus, la présence d'un analogue du GMPc (dibutyryl-cGMP) mimait l'effet du NO. Dans ces conditions, le GMPc produit à la suite de l'activation de la guanylate cyclase par le NO est impliqué dans la modulation de l'activité du récepteur de la bradykinine. Ce mécanisme d'action du NO ne peut être évoqué concernant nos résultats puisque la présence d'un analogue du GMPc ne modifie pas la capacité du $\beta_2AR$ à stimuler l'adénylyl cyclase.

Selon nos résultats, le NO module directement, sans intermédiaire, la voie $\beta$-adrénergique en régulant l'état de palmitylation du $\beta_2AR$. En effet, la présence de NO n'affecte pas la capacité d'un récepteur mutant non-palmitylé (G341C) à se coupler à la protéine G. Il y aurait donc un lien entre la régulation de la palmitylation par le NO et son effet sur l'activité fonctionnelle du récepteur. Dans ces circonstances, le NO pourrait interagir directement avec la cystéine 341 responsable de la palmitylation du récepteur formant ainsi une liaison de type S-nitrosylation (cystéine[341]-S-NO). Le récepteur nitrosylé ne pourrait plus incorporer de palmitate. Cela expliquerait la diminution de l'incorporation de l'acide gras tritié observée à la suite d'un traitement avec un donneur de NO. La capacité du NO à former une liaison de type S-nitrosylation a été proposée pour un certain nombre de protéines (Stamler et al., 1992; Lei et al., 1992; Hess et al., 1994; Lander et al., 1993; Gopalakrishna et al., 1993; Stamler, 1994; Hess et al., 1993; Rossig et al., 1999). Chacune de ces études, a énoncé qu'une liaison de type S-nitrosylation pourrait affecter les fonctions des protéines. Parmi ces exemples, l'étude la plus exhaustive a été réalisée avec la protéine p21[ras] (Lander et al., 1995; Lander et al., 1996). Dans un premier temps

(Lander et al., 1995), les auteurs ont démontré par des essais *in vitro* que le NO influençait l'activité de la protéine p21$^{ras}$ en augmentant l'échange GDP/GTP. L'effet du NO sur la protéine p21$^{ras}$ était comparable aux protéines régulatrices permettant l'échange des nucléotides ("guanine nucleotide exchange factor"). Dans un deuxième temps (Lander et al., 1996), le même groupe a démontré par mutagenèse dirigée que le NO interagissait directement avec la cystéine 118. Cette interaction spécifique du NO sur cette cystéine 118 a été confirmée en utilisant la spectrométrie de masse ("electrospray-ionization mass spectrometry"). L'effet du NO sur la protéine p21$^{ras}$ est abolie lorsque la cystéine 118 est mutée. Les auteurs de ces études ont donc proposé qu'un changement de conformation attribuable à la formation de la liaison S-nitrosylation entre le NO et la cystéine 118 est responsable de la modification de l'activité de la protéine p21$^{ras}$. Nous croyons qu'une telle hypothèse pourrait être proposée concernant l'effet du NO sur l'activité du β$_2$AR. Le β$_2$AR nitrosylé ne serait plus palmitylé et par le fait même, un changement de conformation lui serait imposé. Comme nous l'avons mentionné précédemment dans la discussion, le récepteur non palmitylé peut davantage être phosphorylé que le récepteur de type sauvage (section résultat: article 1). Dans ce contexte, le profil de phosphorylation du récepteur modifié par le NO serait plus élevé. Cela expliquerait le découplage du récepteur avec la protéine G.

Dans l'ensemble, nos travaux ont révélé l'existence d'un lien de régulation croisé entre la production de NO et la réactivité β-adrénergique. La modulation de l'état de palmitylation du récepteur par le NO serait responsable de la diminution de l'activité fonctionnelle du récepteur. Un tel effet sur l'activité du récepteur a été observé non seulement avec du NO généré par un donneur de NO, mais aussi avec du NO endogène provenant de la stimulation de la NOSi exprimée dans les macrophages. Le rôle du NO sur la voie β-adrénergique des macrophages est peu caractérisé. Par

ailleurs, il est bien connu que le NO endogène joue un rôle important au niveau du système cardio-vasculaire (Calver et al., 1993). En effet, le NO est un puissant agent vasodilatateur (Crépel and Lemaire, 1995). Au cours du développement de certains troubles pathologiques causant une augmentation importante du NO, une diminution de l'effet des catécholamines a été observée au niveau cardiaque (Han et al., 1994). Cette diminution de la réponse aux catécholamines a été suggérée pour expliquer, en partie, la défaillance myocardique causée par un choc septique où le NO est généré en très grande quantité. À cet égard, une étude récente (Joe et al., 1998) a démontré que la présence de NO endogène produit par la NOSi des macrophages provoquait une baisse de la production d'AMPc à la suite de la stimulation des récepteurs β-adrénergiques des myocites cardiaques. L'effet observé sur la voie β-adrénergique était partiellement indépendant de la production de GMPc. Nous croyons, sous toutes réserves, qu'un des mécanismes potentiels du NO endogène sur la production d'AMPc pourrait être la régulation de l'état de palmitylation des récepteurs β-adrénergiques des myocites cardiaques.

Figure 7     Modèle schématique proposé de la régulation
             de la palmitylation du récepteur
             $\beta_2$-adrénergique par le monoxyde d'azote.

-E- Mécanisme de régulation suggéré du NO sur l'état de palmitylation du β₂AR (HEK293): la S-nitrosylation
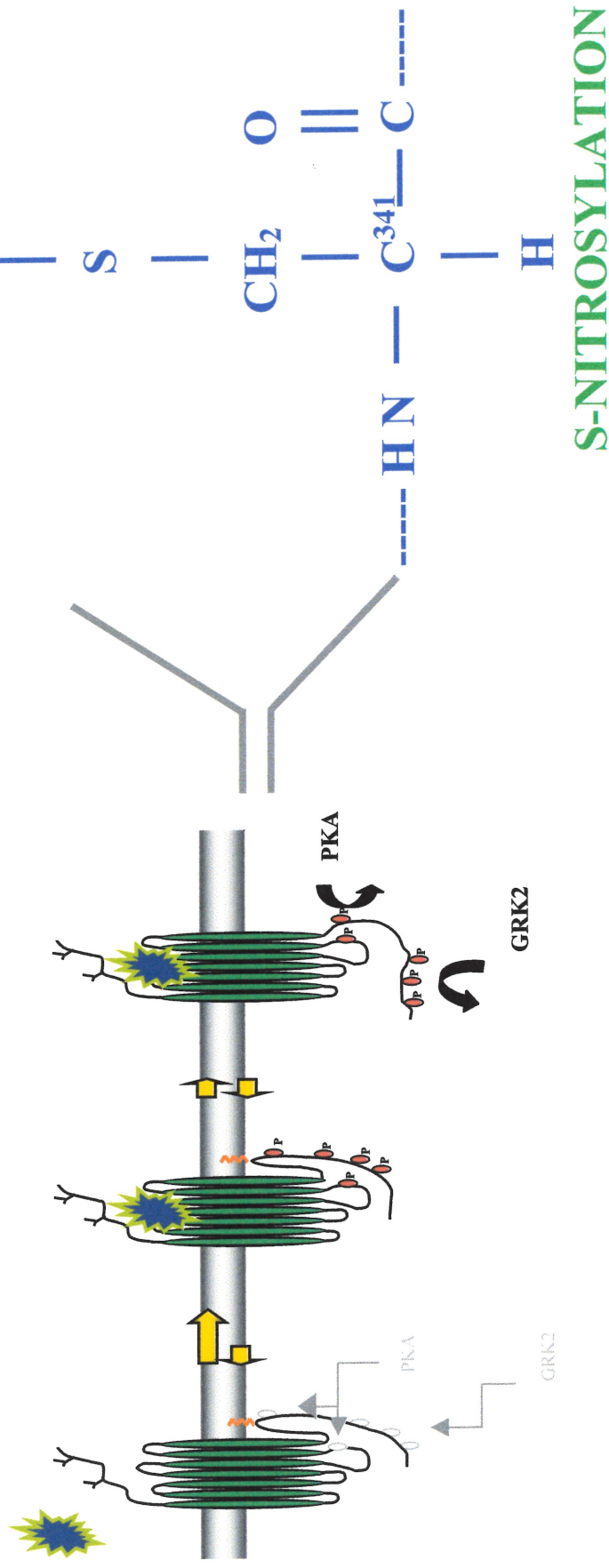
## 1.13 Conclusion

Il ressort de l'ensemble de ces travaux que la palmitylation du $\beta_2 AR$ est une modification post-traductionnelle réversible durant la vie du récepteur. Une telle observation a été faite en utilisant des cellules d'insectes et des cellules de mammifères suggérant que cette modification soit indépendante du système d'expression utilisé. De plus, la palmitylation du $\beta_2 AR$ est grandement influencée à la suite de l'activation du récepteur. Dans ce contexte d'activation, nous avons mis en évidence que le site de phosphorylation de la PKA dans la portion C-terminale du récepteur est un facteur déterminant de la dynamique de palmitylation. Finalement, la phosphorylation du récepteur favoriserait une forme non palmitylée du $\beta_2 AR$. La déphosphorylation serait nécessaire pour permettre au récepteur d'être de nouveau palmitylé. Cette interaction entre la phosphorylation et la palmitylation du récepteur a été observée indépendamment du système d'expression utilisé. Par ailleurs, une différence importante existe entre les deux systèmes d'expression (cellules d'insectes et cellules de mammifères) en ce qui à trait à la vitesse de renouvellement du palmitate associé au récepteur. Le ou les rôles de la palmitylation du $\beta_2 AR$ en fonction du système d'expression utilisé devront être approfondis.

Nous avons également démontré que la palmitylation du $\beta_2 AR$ pouvait être régulée indépendamment de l'activation du récepteur. En effet, nous avons mis en évidence que le monoxyde d'azote peut diminuer la palmitylation du $\beta_2 AR$. Cet effet du monoxyde d'azote est accompagné par une diminution de la capacité du récepteur à stimuler l'effecteur, l'adénylyl cyclase. Selon nos résultats, le monoxyde d'azote module directement la voie $\beta$-adrénergique en régulant l'état de palmitylation du $\beta_2 AR$.

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# 5.    Annexes

## 5.1 Article A-1: Functional Rescue of a Constitutively Desensitized β₂AR Through Receptor Dimerizatrion.

# Functional rescue of a constitutively desensitized $\beta_2$AR through receptor dimerization

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We have recently demonstrated that wild-type $\beta_2$-adrenergic receptors ($\beta_2$AR) form homodimers and that disruption of receptor dimerization inhibits signalling via $G_s$ [Hebert, Moffett, Morello, Loisel, Bichet, Barret and Bouvier (1996) J. Biol. Chem. **271**, 16384–16392]. Here taking advantage of the altered functional properties of a non-palmitoylated, constitutively desensitized mutant $\beta_2$AR (C341G$\beta_2$AR), we sought to study whether physical interactions between mutant and wild-type $\beta_2$AR expressed in Sf9 cells could occur and have functional consequences. Using metabolic labelling with [$^3$H]palmitate and co-immunoprecipitation we demonstrated the existence of heterodimerization between wild-type and C341G$\beta_2$AR. Furthermore, we show that, in co-expression experiments, wild-type receptors have a dominant positive effect resulting in the functional complementation of C341G$\beta_2$AR. Indeed, when

expressed alone, the mutant C341G receptor displays altered functional characteristics in that (1) the response of the receptor to agonist is reduced as compared to the wild-type receptor and (2) the desensitization of the receptor in response to prolonged exposure to agonist is minimal. In contrast, when C341G and the wild-type $\beta_2$AR were expressed together, both the response to agonist and subsequent desensitization (at a constant level of total receptor) were equivalent to the wild-type $\beta_2$AR expressed alone. This dominant positive effect was also seen when C341G was co-expressed with a second receptor mutant in which the two protein kinase A phosphorylation sites (S261, 262, 345, 346A $\beta_2$AR) were mutated. Taken together these data suggest that intermolecular interactions between receptors may have both functional and structural implications for G-protein-mediated signalling.

## INTRODUCTION

The $\beta_2$-adrenergic receptor ($\beta_2$AR) is perhaps the best characterized member of the family of G-protein-linked seven transmembrane segment receptors [1,2]. This receptor activates $G_s$, which subsequently activates adenylate cyclase. The stoichiometry of the interaction between the receptor, G protein and the effector molecule is assumed to be 1:1:1 [3]. Indeed, this is one of the assumptions of the ternary model classically used to describe this interaction [4]. However, evidence from several lines of inquiry suggests that this picture may be oversimplified. For example, studies on the cooperativity in binding patterns of agonists at G-protein-coupled receptors (GPCRs) have suggested that both the receptors and their cognate G proteins may be part of oligomeric arrays [5–14]. Structural studies, including photoaffinity labelling of muscarinic receptors [15], radiation inactivation of $\alpha$- and $\beta$-AR [16], cross-linking of glucagon receptors [17] and hydrodynamic properties of cardiac muscarinic receptors [18] also lend credence to the idea that GPCRs can form dimers.

A number of recent functional studies have also lent support to the notion that GPCRs may form dimers. Co-expression of two binding defective angiotensin II receptor mutants was demonstrated to rescue ligand binding [19]. Maggio et al. [20] also demonstrated using co-transfection of chimaeric $\alpha_2$-adrenergic/m3-muscarinic receptors that intermolecular interactions occurs between receptors. Indeed, two chimaeras composed of the first five transmembrane domains of one receptor linked to the last two of the other could only form functional receptors with the normal binding properties of each parent receptor when co-transfected, while no binding was seen when each chimaeric receptor was expressed alone.

The role that such oligomeric GPCRs play in normal signal transduction remains poorly characterized. We have demonstrated previously that the $\beta_2$AR can form homodimers that are involved in the activity of the receptor [21]. Using a peptide derived from TM VI of the receptor we also show that this region of the receptor may form part of the dimer interface. Functionally, this peptide specifically inhibits receptor-mediated adenylate cyclase stimulation [21]. Whether the peptide inhibited signalling by preventing receptor dimerization or else led to the formation of an inactive receptor by binding to monomer thus forming 'pseudodimers' remains an open question. In many instances where protein dimerization is an integral part of biological function, both dominant negative and positive effects of mutation can be observed when mutant proteins are co-expressed with their wild-type parents. If such functional complementation can be demonstrated for the $\beta_2$AR it would suggest that dimerization is important for receptor-mediated signalling. Here, we take advantage of a previously characterized mutant (C341G; [22,23]) which, unlike wild-type $\beta_2$AR, fails to incorporate [$^3$H]palmitate and signals less efficiently than the wild type receptor. We use co-expression and immunoprecipitation of epitope-tagged wild-type and mutant $\beta_2$ARs expressed in Sf9 cells using the baculovirus system to assess the possibility of physical and functional interactions between receptors.

## EXPERIMENTAL

### Materials

Radiolabelled compounds were obtained from DuPont–NEN. Tissue-culture reagents and goat anti-mouse alkaline

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Abbreviations used: $\beta_2$AR, $\beta_2$-adrenergic receptor; GPCR, G-protein-coupled receptor; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HA, influenza haemagglutinin; MOI, multiplicity of infection; CYP, [$^{125}$I]cyanopindolol.
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phosphatase-coupled second antibody were obtained from GIBCO-BRL. Centriprep and Centricon cartridges were purchased from Amicon. The enhanced chemiluminescence detection system was purchased from Amersham. All other chemicals were obtained from either Sigma or BioRad.

## Construction of recombinant baculoviruses

The recombinant baculoviruses containing either HA- or c-*myc*-tagged wild-type $\beta_2AR$ (HA$\beta_2AR$, c-*myc* $\beta_2AR$) or c-*myc*-tagged C341G $\beta_2AR$ (c-*myc* C341G) were constructed as described [24]. Briefly, HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) and c-*myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) tags containing initiator methionine residues were introduced into the receptor cDNAs immediately before their initiator methionines by subcloning the corresponding double-stranded oligonucleotides. These tagged receptor coding sequences were inserted into the Sf9/baculovirus recombination plasmid pJV-c-*myc* $\beta_2AR$ as described previously [24]. Additional constructs, encoding mutant forms of the receptor, used in this study (pBC12BI- $\beta_2AR$-C341G [23], pTZ18R-$\beta_2AR$-S261, 262, 345, 346A [25], were subsequently inserted into the tagged vector construct. Briefly, purified plasmids were digested by *NcoI*–*Ppu*M1 (NEB). The fragments coding for either C341G $\beta_2AR$ or S261, 262, 345, 346A $\beta_2AR$ were inserted in the pJV-c-*myc* fragment by cohesive end ligation. Positive clones were confirmed by dideoxy sequencing [26]. Recombination of the receptor-bearing plasmids into the baculovirus genome was achieved by co-transfection into Sf9 cells and subsequent screening, purification and amplification of viral plaques [27]. Confirmation of $\beta_2AR$ mutant protein expression was assayed by whole cell radio-ligand binding as previously described [24].

## Sf9 cell culture

Sf9 cells were maintained at 27 °C in serum-supplemented [10% (v/v) fetal bovine serum (FBS)] Grace's insect medium. Cells were grown either as monolayers in T-flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell tearing due to agitation. Cells were infected at exponential phase at a density of $1 \times 10^6$–$2 \times 10^6$ cells per ml for 48 h with multiplicities of infection (MOI) from 2 to 6.

## Membrane preparation

Membranes were prepared as described previously [21]. Briefly, cells were washed twice with ice-cold PBS. They were then disrupted by homogenization with a Polytron in 10 ml of ice-cold buffer containing 5 mM Tris/HCl, pH 7.4, 2 mM EDTA, 5 $\mu$g/ml leupeptin, 10 $\mu$g/ml benzamidine and 5 $\mu$g/ml soybean trypsin inhibitor. Lysates were centrifuged at 500 $g$ for 5 min at 4 °C, the pellet homogenized and centrifuged as before, then supernatants from both centrifugations pooled. The supernatant was then centrifuged at 45000 $g$ for 20 min and the pellet washed twice in the same buffer. This membrane preparation was then immediately used for adenylate cyclase and binding assays or processed further as described below.

## Metabolic labelling of cells with [³H]palmitate or [³²P]phosphate

Metabolic labelling with tritiated palmitate was performed as described previously [28,29]. Briefly, at 30 h post-infection, cells were transferred to serum-free Grace's medium. At 47 h cells were counted and adjusted to 1% FBS at a density of approximately 50 million cells/20 ml. At 48 h post-infection,

[9,10-³H]palmitic acid (60 Ci/mmol) dissolved in 50 $\mu$l of DMSO was added to the culture at a final concentration of 0.2 mCi/ml for 60 min. Metabolic labelling with [³²P]P$_i$ was performed as described [23] with the following modifications; 2 h prior to labelling cells were incubated in phosphate-free Grace's medium. Cells were then incubated with 5 mCi of [³²P]P$_i$ in Grace's medium for 2 h. In either experiment, labelling was terminated by centrifugation at 500 $g$ for 5 min at 4 °C followed by two washes with ice-cold PBS. The cells were then disrupted by sonication (two 5 s bursts), membranes prepared and receptors purified by alprenolol–Sepharose affinity chromatography or by immunoprecipitation as described below.

## Immunoprecipitation of $\beta_2AR$

Epitope-tagged $\beta_2AR$ was immunoprecipitated with a mouse anti-c-*myc* monoclonal antibody (9E10 [30]) or a mouse anti-haemagglutinin monoclonal antibody (12CA5) as described previously [24]. Briefly, receptors were solubilized in 2% digitonin for 2 h at 4 °C with gentle agitation. Removal of digitonin and concentration of the solubilized receptor was performed by dialysis using Centriprep cartridges against an ice-cold solution (Buffer A) containing 100 mM NaCl, 10 mM Tris/HCl, pH 7.4, and 2 mM EDTA (including the protease inhibitors described above) until the digitonin concentration was reduced below $\approx 0.05$%. Purified 9E10 or 12CA5 antibodies (1:1000 dilution) were added to the concentrate and gently agitated for 2 h at 4 °C. Anti-mouse IgG–agarose (at an 11:1 molar ratio of secondary to primary antibodies) was then added. The reaction was allowed to proceed overnight at 4 °C with gentle agitation. The immuno-precipitate was centrifuged at 12000 rev./min in a microcentrifuge for 10 min at 4 °C. The pellet was washed three times in buffer A and finally resuspended in 200 $\mu$l of non-reducing SDS/PAGE loading buffer for 30 min, sonicated and centrifuged at 12000 rev./min. The supernatant was then subjected to SDS/PAGE as described below. The efficiency of our immunoprecipitation protocol was determined by soluble [¹²⁵I]cyanopindolol (CYP) binding before and after specific immunoprecipitation as previously described [24,33] and was $75 \pm 8$%.

## Affinity purification of $\beta_2AR$

Receptor solubilized as above was purified using alprenolol–Sepharose chromatography [31] and the affinity purified preparations were concentrated using Centriprep and Centricon cartridges. The amount of receptor was determined in soluble CYP radioligand-binding assays as described [24,33].

## SDS/PAGE and Western blotting

Membranes or purified $\beta_2AR$ were prepared for non-reducing SDS/PAGE on 10% slab gels as described [32]. Samples were dissolved in 10% glycerol/8% SDS/0.005% Bromophenol Blue, 25 mM Tris, pH 6.5 and heated to 75 °C for 10 min. For labelling studies, equal amounts of receptor as determined by soluble binding assays using CYP as radioligand were loaded in each lane. Following metabolic labelling with [³²P]P$_i$, gels were fixed, dried and exposed to Kodak XAR-5 film at $-70$ °C for several days. In experiments where receptors were metabolically labelled with [³H]palmitate gels were fixed, treated with Enlightning (DuPont), dried and exposed to Kodak XAR-5 film at room temperature for several weeks. For Western blotting, proteins were transferred to nitrocellulose and blotted with either a mouse anti-c-*myc* monoclonal antibody (9E10) or a mouse anti-HA monoclonal (12CA5) antibody at dilutions of 1:1000.

Immunoblots were revealed with a goat anti-mouse alkaline phosphatase-coupled second antibody (Gibco–BRL) or using the ECL system (Amersham).

### Receptor quantification and adenylate cyclase assay

Membranes were prepared and washed as described above. Total receptor number was calculated from binding experiments using CYP as the radioligand [33]. Briefly, 10 $\mu$l of an Sf9 membrane preparation (3–5 $\mu$g of protein) in a total volume of 0.5 ml was labelled with 250 pmol of CYP which is at a near saturating concentration. To determine the efficiency of immuno-precipitation or to calculate amounts of affinity-purified $\beta_2$AR soluble receptor binding assays were performed as described previously [24,33]. In all cases non-specific binding was defined using 10 $\mu$M alprenolol. Adenylate cyclase activity was assayed by the method of Salomon et al. [34]. Enzyme activities were determined in the presence of 1 nM–100 $\mu$M isoproterenol. Data were calculated as pmol cAMP produced per min/mg protein and were analysed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

### RESULTS

#### Characterization of C341G mutant $\beta_2$AR in Sf9 cells

In previous studies, we have shown that wild-type $\beta_2$AR expressed in the Sf9/baculovirus system has properties that are similar to receptors expressed in mammalian cells [21,24,25,35]. In order to study co-expression of the wild-type $\beta_2$AR with the C341G mutant in Sf9 cells, we first wanted to confirm that this mutant exhibits similar properties when expressed in Sf9 cells as when expressed in mammalian cells. As shown in Figure 1, the previously described reduction in signalling efficacy of C341G [22,23] was also observed in Sf9 cells expressing this receptor. Indeed, at all levels of receptor expression, maximal iso-proterenol-stimulated adenylate cyclase activity was higher for the wild-type than for the mutant receptor (Figure 1B). Furthermore, the mutant was found to have constitutively higher basal level of phosphorylation (in the absence of agonist) than the wild-type receptor (Figure 1A, inset) and to be resistant to agonist-promoted desensitization (Table 1) as previously reported in mammalian cells [23,36].

In Figure 1(A) (inset) phosphorylated receptors appeared as a single molecular species of approx. 46–54 kDa when purified by alprenolol–Sepharose affinity chromatography. However, when receptors were immunoprecipitated, phosphorylated molecular forms corresponding to monomeric and dimeric species were detected (Figure 2). These results are consistent with a previous report indicating that receptor antagonists (such as alprenolol used in the affinity purification scheme) reduced the level of the dimeric species [21]. This also indicates that receptor dimers can either act as substrates for regulatory protein kinases or that phosphorylated monomeric receptors can form dimers as the level of phosphorylation increases upon stimulation.

#### Functional interaction between different $\beta_2$ARs

To determine if wild-type and C341G $\beta_2$ARs interacted with each other in a functional manner, c-myc C341G$\beta_2$AR and the HA$\beta_2$AR were either expressed individually or co-expressed in Sf9 cells. Figure 3(A) shows dose–response curves from cells expressing each receptor alone or in combination at a total receptor density of $\sim$ 4 pmol of receptor per mg of membrane protein. The isoproterenol-stimulated adenylyl cyclase activity
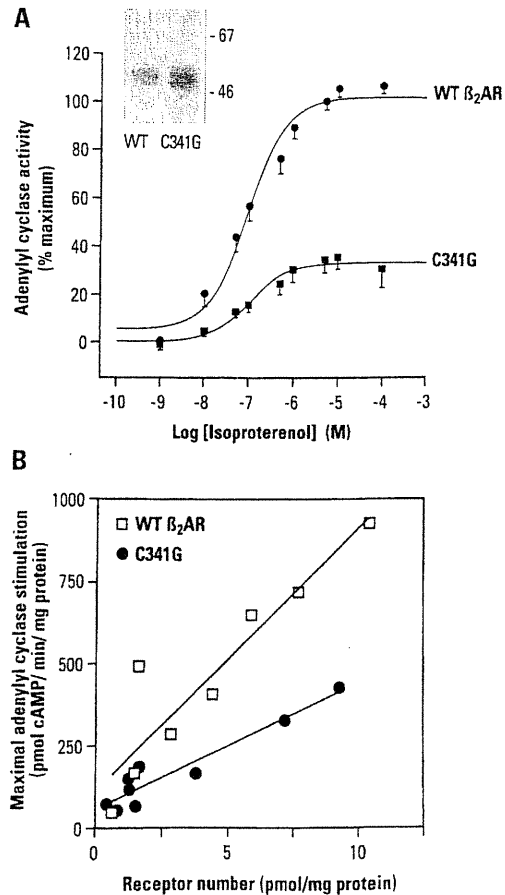


**A**

**B**

**Figure 1    Expression of wild-type $\beta_2$AR and C341G in Sf9 cells**

Sf9 cells were infected with recombinant baculoviruses encoding either wild-type (WT) or C341G $\beta_2$AR at different MOI (varying from 2–4 for wild-type and 3–6 for C341G). Membranes were prepared from these cells 48 h post-infection and adenylate cyclase assays were performed. (**a**) Isoproterenol-stimulated adenylate (adenylyl) cyclase activity was determined in membranes from wild-type (5.6 $\pm$ 1.3 pmol/mg membrane protein, closed circles, $n = 11$) or C341G (3.2 $\pm$ 1.4 pmol/mg membrane protein, closed squares, $n = 3$). Values are expressed as % maximum of wild-type receptor stimulation. Inset: basal phosphorylation level of wild-type and C341G $\beta_2$AR. Cells expressing either receptor were metabolically labelled with [$^{32}$P]P$_i$. Solubilized receptors were purified by affinity chromatography and subjected to 10% SDS/PAGE. 100 fmol of receptor were loaded into each lane. (**b**) Net (maximal-basal) adenylate cyclase stimulation (pmol cAMP $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ membrane protein) by isoproterenol is plotted versus receptor number. Receptor number was determined by saturation binding with CYP as radioligand.

measured in cells infected with C341G alone was 19 % of that observed in cells expressing a similar number of the wild-type receptor (Figure 3A). Surprisingly, cells co-infected with equal amounts of each virus showed a similar level of stimulation as cells expressing the wild-type $\beta_2$AR alone. Assuming no functional interactions between the two receptors and that each receptor represented half of the total receptor population, an intermediate phenotype of adenylyl cyclase stimulation would have been expected.

Based on the observed phenotype for each receptor expressed alone (Figure 1B) we calculated a relation between receptor number and net (maximal-basal) adenylate cyclase stimulation. From Figure 3(A), at 4 pmol of receptor the net stimulation (maximal-basal) by C341G is 76 pmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ and for the WT receptor it is 398 pmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$. By linear regression of the data in Figure 1(B), we calculated that the net activity ob-

## Table 1  Desensitization of wild-type and mutant $\beta_2$AR

Prior exposure of Sf9 cells expressing various $\beta_2$AR to agonist (1 $\mu$M isoproterenol for 30 min) leads to a reduction of response to ligand subsequently applied to membranes prepared from these cells for measurement of adenylate cyclase stimulation.

| | % Desensitization* (maximal) | % Desensitization† (at $10^{-8}$ M ISO) |
|---|---|---|
| Wild-type | $30 \pm 4.5$ | $21 \pm 2.7$ |
| C341G | $10 \pm 5.2$ | $9 \pm 1.5$ |
| Wild-type/C341G | $27.5 \pm 6.1$ | $23.6 \pm 3.2$ |

\* The extent of desensitization is expressed as the % reduction in the maximal isoproterenol-stimulated adenylate cyclase activity induced by pretreatment relative to control. Maximal reduction of response relative to control is plotted ($\pm$ S.E.M., $n = 3$) for wild-type, C341G, and co-expressed C341G/wild-type.

† The extent of desensitization is expressed as the % reduction in the adenylyl cyclase activity stimulated by $10^{-8}$ M isoproterenol (ISO) after pretreatment with $10^{-6}$ M isoproterenol relative to control. The isoproterenol stimulation was determined using full dose–response curves of isoproterenol-stimulated adenylate cyclase activity. The concentration of total receptor was $\approx$ 5 pmol/mg membrane protein for all experiments. Adenylate cyclase activity is measured as described in the Experimental section.
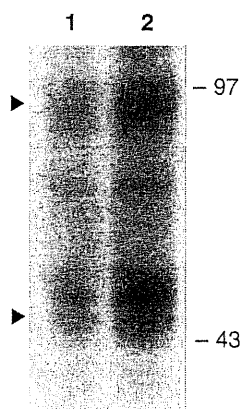


## Figure 2  Phosphorylation of monomeric and dimeric $\beta_2$AR

c-*myc* $\beta_2$AR were immunoprecipitated from cells metabolically labelled with [$^{32}$P]P$_i$ were treated with vehicle (lane 1) or 1 $\mu$M isoproterenol (lane 2) for 30 min prior to cell harvesting. After SDS/PAGE, incorporation of label was determined by exposing dried gels to Kodak XAR film at $-70$ °C. Positions of the monomeric and dimeric species are denoted by arrows and molecular-mass markers are shown on the right. Data are representative of three independent experiments.

tained at an expression level of 2 pmol receptor (i.e. when the two receptors are co-expressed at equivalent levels) should be 0.62 and 0.65-fold of that found at 4 pmol of receptor for WT and C341G expressed alone, respectively. It follows that, if the receptors are not interacting, co-expression should lead to a net activity of 297 pmol·min$^{-1}$·mg$^{-1}$ [$(0.62 \times 398) + (0.65 \times 76)$]. The measured value in Figure 3(A) for the co-expressed receptors was 425 pmol cAMP·min$^{-1}$·mg$^{-1}$ protein thus suggesting a dominant-positive effect of the wild-type receptor.

Further supporting the notion that functional interactions between the two receptor species occurred, we found that the maximal isoproterenol-stimulated adenylate cyclase activity measured in co-expression experiments in which the relative stoichiometries of the two viruses were varied (up to 2:1,

C341G:wild-type) was always higher than the predicted simple summation of wild-type and mutant activity alone (data not shown).

To confirm that infection of cells with equal titres of viruses led to the expression of the two proteins, Western blot analysis was carried out. Crude membranes from cells infected with HA$\beta_2$AR and c-*myc* C341G$\beta_2$AR were immunoblotted with either anti-HA or anti-c-*myc* antibodies. As shown in the inset of Figure 3(A), both receptors were detected and thus co-expression was confirmed. Unfortunately, direct quantitative comparison is prevented by the use of different primary antibodies.

To determine if the apparent dominant positive effect of the $\beta_2$AR over the C341G mutant was a unique feature of the wild-type receptor, we co-expressed C341G with another receptor mutant displaying a distinct phenotype. Toward this end we used S261, 262, 345, 346A $\beta_2$AR, which has two consensus protein kinase sites mutated and thus is somewhat resistant to agonist-promoted desensitization but stimulates adenylate cyclase with an efficacy that is similar to that of the wild-type $\beta_2$AR (cf. Figures 3A and 3B; [25,37]). As for the wild-type receptor, the phenotype of the S261, 262, 345, 346A $\beta_2$AR mutant dominated over that of C341G. Indeed, when the two mutants were co-expressed, the agonist-stimulated adenylate cyclase activity measured was similar to that of the S261, 262, 345, 346A $\beta_2$AR expressed alone (Figure 3B). These data indicate that both the wild-type $\beta_2$AR and S261, 262, 345, 346A $\beta_2$AR acted in a dominant positive fashion, complementing the C341G phenotype.

In order to assess if the dominant positive effect extended to other properties of the receptor, the profiles of agonist-promoted desensitization were studied in cells infected with wild-type and C341G receptors. Prolonged exposure to agonist leads to a desensitization of the $\beta_2$AR which is associated with an increased phosphorylation of the receptor [38]. As described above, the C341G $\beta_2$AR is constitutively desensitized and agonist-stimulation results in little additional agonist-promoted desensitization (Table 1). As demonstrated for the efficacy of signalling, co-expression of receptors led to a dominant effect on the desensitization profile. The extent of desensitization observed in the co-expression experiments of C341G with wild-type $\beta_2$AR was similar to those measured when wild-type $\beta_2$AR was expressed alone (Table 1) confirming the dominant-positive effect of the wild-type receptor.

## Physical interactions between $\beta_2$ARs

In a previous study we demonstrated using an immunoblotting approach that wild-type $\beta_2$AR can form homodimers [21]. To determine if C341G$\beta_2$AR can also form homodimers, membranes prepared from cells expressing c-*myc* C341G $\beta_2$AR or c-*myc* $\beta_2$AR were subjected to 10% non-reducing SDS/PAGE, transferred to nitrocellulose and blotted with the anti-c-*myc* antibody. Figure 4 shows that molecular species representing monomeric and dimeric c-*myc*-tagged wild-type $\beta_2$AR (lane 2) and c-*myc* C341G$\beta_2$AR (lane 1) were detected. As we have demonstrated previously for the wild-type $\beta_2$AR [21] higher molecular mass forms of the C341G receptor were detected as well suggesting the possibility of higher order structures such as receptor tetramers. However, these structures were not seen consistently in all preparations, in contrast to the monomeric and dimeric forms.

In order to determine if there was a physical correlate to the functional interactions between wild-type and C341G $\beta_2$AR, we specifically assessed whether heterodimers of wild-type and C341G receptors could form. Toward this end, we adopted a variation of the co-immunoprecipitation approach that we recently used to demonstrate $\beta_2$AR homodimerization [21]. We
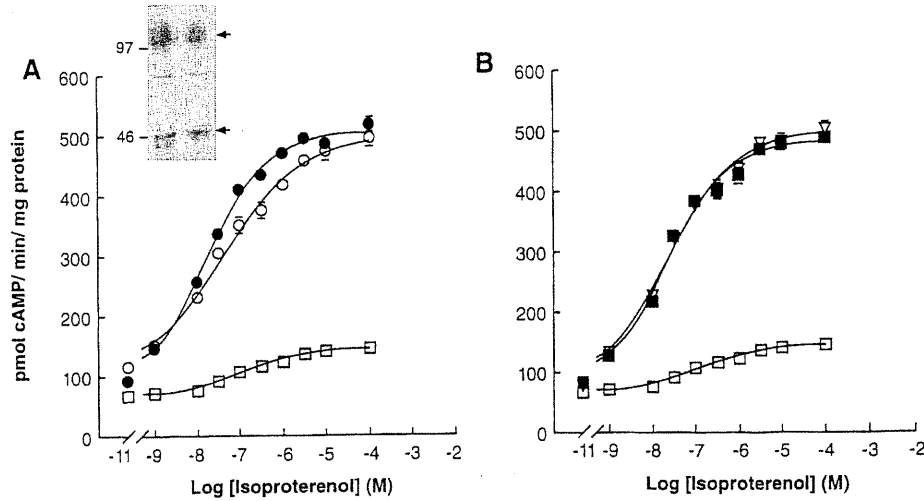
**Figure 3    Adenylate cyclase stimulation after co-expression of wild-type or S261, 262, 345, 346A $\beta_2$AR with C341G $\beta_2$AR**

Equivalent amounts of total receptor (approx. 4 pmol/mg membrane protein) were used in all curves shown. Receptor number was determined by saturation binding with CYP as radioligand. Stimulation of adenylate cyclase activity is measured as described in Experimental section. (a) Wild-type $\beta_2$AR (4.1 ± 0.2 pmol/mg membrane protein) is represented by open circles, C341G (4.2 ± 0.4 pmol/mg membrane protein) is represented by open squares and co-expressed wild-type $\beta_2$AR and C341G (3.8 ± 0.4 pmol/mg membrane protein) are represented by closed circles. Inset: crude membrane preparations (2 pmol receptor/mg membrane protein, 500 fmol/lane) of wild-type HA-$\beta_2$AR (lane 1) or c-myc C341G (lane 2) which were immunoblotted following SDS/PAGE with either the anti-HA monoclonal 12CA5 or the anti-c-myc monoclonal 9E10 antibodies, respectively. Arrows are used to denote monomeric and dimeric forms. (b) C341G is represented by open squares, S261, 262, 345, 346A $\beta_2$AR (4.3 ± 0.6 pmol/mg membrane protein) is represented by open triangles and co-expressed S261, 262, 345, 346A $\beta_2$AR and C341G (4.4 ± 0.5 pmol/mg membrane protein) receptors are represented by closed squares. Data are presented as means of five independent experiments ± S.E.M.
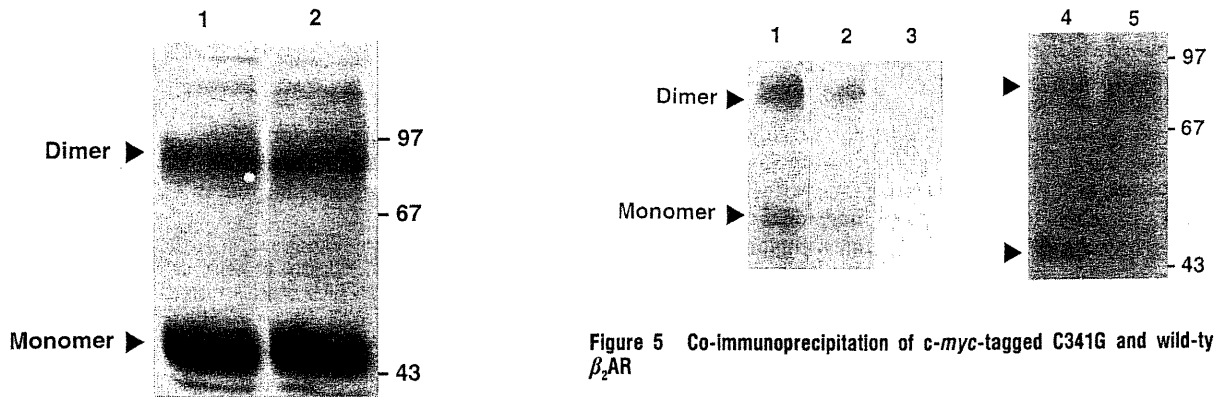


**Figure 4    Dimeric species are seen in both wild-type and C341G $\beta_2$AR**

Crude membrane preparations (2 pmol receptor/mg membrane protein) of wild-type c-myc $\beta_2$AR (lane 1) or c-myc C341G (lane 2) were immunoblotted following SDS/PAGE with the anti-c-myc monoclonal 9E10 antibody. The blots reveal immunoreactive bands corresponding to the expected monomeric form (43–50 kDa) and dimeric species (85–95 kDa) forms as well as possible higher molecular mass forms. Position of the monomeric and dimeric species are denoted by arrows and molecular-mass markers are shown on the right. Data are representative of three independent experiments.



**Figure 5    Co-immunoprecipitation of c-myc-tagged C341G and wild-type $\beta_2$AR**

Cells expressing either wild-type c-myc $\beta_2$AR (lane 1), c-myc C341G and untagged wild-type $\beta_2$AR together (lane 2) or c-myc C341G receptors alone (lane 3) were metabolically labelled with tritiated palmitate as described in the Experimental section. Receptors were solubilized from crude membrane preparations and immunoprecipitated with an anti-c-myc monoclonal antibody followed by SDS/PAGE (10%) under non-reducing conditions (1.5 pmol receptor were loaded in each lane). The gel was then fixed, dried and exposed to XAR film at −70 °C. Data are representative of three independent experiments. A parallel experiment using cells co-expressing HA-$\beta_2$AR and c-myc C341G was performed where solubilized receptors were immunoprecipitated with the anti-HA antibody and blotted with either anti-HA (lane 4) or anti-c-myc (lane 5). Data are representative of two independent experiments. Position of the receptor bands are denoted by arrows and molecular-mass markers are shown on the right.

co-expressed a c-myc-tagged C341G receptor with a non epitope-tagged wild-type $\beta_2$AR and metabolically labelled the cells with [³H]palmitate. Receptors were solubilized and immuno-precipitated with the anti-c-myc antibody and then subjected to SDS/PAGE and fluorography. Since it has been previously demonstrated that the C341G mutant does not incorporate [³H]palmitate [22,23], labelled palmitoylated wild-type receptor should only be detected if it is co-immunoprecipitated with the tagged-non-palmitoylated C341G mutant. Figure 5 (lane 1) shows

incorporation of labelled palmitate into the immunoprecipitated c-myc wild-type $\beta_2$AR when expressed alone. It also demonstrates the incorporation of labelled palmitate into immunoprecipitates obtained from cells expressing the c-myc C341G and a non-tagged wild-type $\beta_2$AR (lane 2). Noticeably, it is mainly the dimeric species which is immunoprecipitated consistent with the notion that the two molecular species were co-immuno-precipitated as part of a complex. When the c-myc-tagged C341G$\beta_2$AR was expressed alone, no [³H]palmitate labelling

could be immunoprecipitated, confirming that C341G cannot incorporate tritiated palmitate (Figure 5, lane 3). In a parallel experiment we also show that immunoprecipitation of HA-$\beta_2$AR results in a co-immunoprecipitation of c-*myc*-tagged C341G. When cells co-expressing the two receptors are solubilized and HA-$\beta_2$AR are immunoprecipitated with the anti-HA antibody both the monomeric and dimeric species are detected in Western blots using the anti-HA antibody (Figure 5, lane 4). When the same immunoprecipitate is blotted with the anti-c-*myc* antibody it is the dimeric species which again predominates (Figure 5, lane 5), confirming that the C341 $\beta_2$AR is associated with the HA-$\beta_2$AR as part of a heterodimeric complex.

## DISCUSSION

To study the functional and/or physical interactions between $\beta_2$ARs we have used co-expression and immunoprecipitation of receptors bearing epitope tags. We also took advantage of a mutated $\beta_2$AR (C341G) which cannot be palmitoylated and is impaired in its ability to couple to $G_s$ and to undergo desensitization. When C341G is co-expressed with the wild-type $\beta_2$AR at equal levels the wild-type receptor exhibits a dominant positive effect complementing the defects in the mutant. Indeed, both signalling and desensitization properties were rescued. Similar functional complementation was also seen between the S261, 262, 345, 346A $\beta_2$AR and C341G $\beta_2$AR as the signalling phenotype of the former dominated when the two receptors were co-expressed. The existence of heterodimeric receptor species was confirmed using a co-immunoprecipitation approach coupled with a differential palmitate labelling paradigm (Figure 5). The observation that C341G and wild-type receptors can be co-immunoprecipitated suggests that the functional complementation most likely is a manifestation of physical interactions between the two receptor species.

Receptor dimerization has long been recognized to be important for most families of signal transducing receptors. These include many growth factor receptors with tyrosine kinase activity, members of the growth hormone receptor family, bacterial sensory receptors and cytokine receptors (see [39,40] for review). However, until recently the oligomeric nature of GPCRs was not generally appreciated despite a significant amount of circumstantial evidence. The data reported in the present study brings additional support to recent reports suggesting that GPCRs also exist physically and functionally as oligomers (see Introduction). The idea that signal transduction through GPCR systems occurs via the formation of higher order structures is also supported by the recent isolation of oligomeric G protein and GPCR complexes [14,41–43] as well as by recent observations that structural cytoskeletal elements may be required for proper signal transduction by organizing such complexes ([44]; reviewed in [42]).

Whether or not the oligomeric receptor and receptor–G protein complexes represent the active signalling forms of these proteins or a passive organizational network remains an open question. In an recent study we demonstrated that a peptide corresponding to transmembrane domain VI of the $\beta_2$AR inhibits both receptor activation and dimerization arguing that dimerization plays a functional role rather than simply a structural role [21]. However, it was not clear from this study whether (1) the dimeric species of the receptor is the active form of the receptor and the peptide deactivates the receptor by inhibiting dimerization or (2) if the peptide causes the formation of a 'pseudodimer' which inactivates the monomeric form of the receptor. The results obtained here in our co-expression studies show that wild-type $\beta_2$AR and S261, 262, 345, 346A $\beta_2$AR can complement the functional

defects in C341G receptors, suggesting that the dimer is the active species of the receptor.

Also consistent with the notion that receptor dimers are important for receptor function is an elegant study demonstrating the rescue of ligand binding by co-expression of two different binding-defective angiotensin II receptor mutants [19]. Indeed, intermolecular interaction between the two receptors is the simplest explanation for the complementation of binding seen as genetic recombination events were carefully controlled for in this study.

Although our data are consistent with the idea that dimerization may be important for receptor activation, one could still posit a role for dimerization in subsequent regulatory events such as desensitization and down-regulation. If this were the case, one would expect the extent of dimerization to be altered in a mutant receptor which is constitutively desensitized such as C341G$\beta_2$AR. However, the data obtained suggest that dimerization and desensitization are independent phenomena, since C341G was found to form dimers to a similar extent as the wild type receptor (Figure 4). A role for dimerization in longer term regulatory processes such as sequestration and/or down-regulation cannot be ruled out by the present study.

Interestingly, our results also suggests that dimerization between a constitutively desensitized and a wild-type receptor yields a fully functional receptor and thus that phosphorylation of a single receptor polypeptide chain may not be sufficient to regulate receptor responsiveness. Further, our observations show that dimerization is not regulated by processes such as phosphorylation and palmitoylation, which are intimately involved in the regulation of receptor desensitization, since a non-palmitoylated receptor which is constitutively phosphorylated can form both homodimers as well as heterodimers with wild-type receptor.

Taken together the results presented here provide a link between complementation of receptor function and physical interaction between receptor moieties suggesting that dimerization plays an important role in GPCR signalling.

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## 5.2 Article A-2: Activation of the $\beta_2$-adrenergic receptor-G$\alpha_s$ complex leads to rapid depalmitoylation and inhibition of repalmitoylation of both the receptor and G$\alpha_s$.

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# Activation of the $\beta_2$-Adrenergic Receptor-G$\alpha_s$ Complex Leads to Rapid Depalmitoylation and Inhibition of Repalmitoylation of Both the Receptor and G$\alpha_s$*

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Palmitoylation is unique among lipid modifications in that it is reversible. In recent years, dynamic palmitoylation of G protein $\alpha$ subunits and of their cognate receptors has attracted considerable attention. However, very little is known concerning the acylation/deacylation cycle of the proteins in relation to their activity status. In particular, the relative contribution of the activation and desensitization of the signaling unit to the regulation of the receptors and G proteins palmitoylation state is unknown. To address this issue, we took advantage of the fact that a fusion protein composed of the stimulatory $\alpha$ subunit of trimeric G protein (G$\alpha_s$) covalently attached to the $\beta_2$-adrenergic receptor ($\beta_2$AR) as a carboxyl-terminal extension ($\beta_2$AR-G$\alpha_s$) can be stimulated by agonists but does not undergo rapid inactivation, desensitization, or internalization. When expressed in Sf9 cells, both the receptor and the G$\alpha_s$ moieties of the fusion protein were found to be palmitoylated via thioester linkage. Stimulation with the $\beta$-adrenergic agonist isoproterenol led to a rapid depalmitoylation of both the $\beta_2$AR and G$\alpha_s$ and inhibited repalmitoylation. The extent of depalmitoylation induced by a series of agonists was correlated (0.99) with their intrinsic efficacy to stimulate the adenylyl cyclase activity. However, forskolin-stimulated cAMP production did not affect the palmitoylation state of $\beta_2$AR-G$\alpha_s$, indicating that the agonist-promoted depalmitoylation is linked to conformational changes and not to second messenger generation. Given that, upon activation, the fusion protein mimics the activated receptor-G protein complex but cannot undergo desensitization, the data demonstrate that early steps in the activation process lead to the depalmitoylation of both receptor and G protein and that repalmitoylation requires later events that cannot be accommodated by the activated fusion protein.

Palmitoylation is a post-translational modification that is limited to a small subset of cellular proteins among which proteins involved in signal transduction are prevalent (1). This thioesterification of cysteine residues by palmitate distinguishes itself from other lipid modifications such as prenylation and myristoylation by its reversibility. Indeed, in contrast to myristoyl and prenyl moieties that are added co-translationally and generally remain attached to the proteins until the protein gets degraded, the protein-bound palmitate is added post-translationally and turns over more rapidly than the protein itself (2–4). Moreover, the palmitoylation state of several proteins has been shown to be dynamically regulated. In particular, biological regulation of the palmitoylation state of heterotrimeric G proteins and of their cognate receptors has been demonstrated (5–12).

Activation of G$\alpha_s$[1] through receptor stimulation, following direct activation with aluminum fluoride and cholera toxin or as a result of site-directed mutagenesis, has been shown to lead to an increased incorporation of [$^3$H]palmitate into G$\alpha_s$ during pulse labeling experiments. Because pulse-chase labeling experiments clearly indicated that stimulation increased the depalmitoylation rate, the enhanced incorporation was attributed to an accelerated turnover rate of the G$\alpha_s$-bound palmitate (10–12). Interestingly, Jones *et al.* (8) found that, despite the increased turnover rate, activation of G$\alpha_s$ did not significantly affect its stoichiometry of palmitoylation, thus challenging the notion that stimulation ultimately favors the depalmitoylation reaction (12). Agonist stimulation of the $\beta_2$-adrenergic receptor ($\beta_2$AR) has also been shown to increase the amount of covalently attached [$^3$H]palmitate (5) as a result of an increased turnover rate of the receptor-bound palmitate (7). A similar agonist-promoted increase in the turnover rate of receptor-bound palmitate was observed for the $\alpha_{2A}$AR (13), the D$_2$-dopamine receptor (14), and the m$_2$-muscarinic receptor (9).

Biologically regulated changes in the palmitoylation state of either receptors or G proteins may have important functional consequences. For example, mutations that prevent palmitoylation of various G$\alpha$ subunits have been found to inhibit their association with the plasma membrane and thus their signal-

ing function (15–18), suggesting that biological modulation of the G protein palmitoylation state could regulate their signaling properties. Palmitoylation of $G\alpha_s$ has also been reported to increase its affinity for $G\beta\gamma$ (19). For receptors, abolition of palmitoylation by site-directed mutagenesis has been shown to either decrease coupling to G proteins (9, 20–23), affect receptor internalization (24–26), or modulate receptor phosphorylation by regulatory kinases (5, 27, 28).

Despite these potentially important roles, very little is known concerning the mechanism that regulates palmitoylation of these proteins. Both enzymatic (29–32) and nonenzymatic (33, 34) acylation reactions have been proposed for $G\alpha$, whereas an enzyme that can catalyze the depalmitoylation of $G\alpha$ proteins has recently been identified (35). However, the mechanisms by which activation of the signaling pathway could control the acylation/deacylation cycle remain unknown. Analysis of the effects of stimulation on the palmitoylation status of receptors and G proteins is complicated by several factors (for a review, see Ref. 36). These include the fact that, following the initial conformational changes and protein-protein interactions that are promoted by receptor stimulation, multiple processes that limit the extent of the activation and contribute to signal termination come rapidly into play. It follows that it is difficult to temporally distinguish between the early events that lead to activation from the ones involved in rapid desensitization of the signaling system. This is an important problem because these two sets of events could theoretically have opposite effects on the palmitoylation reaction. Indeed, on a time scale that is virtually indistinguishable from that of the activation of the G proteins, stimulation of the receptors leads to their progressive functional inactivation. This desensitization results largely from agonist-promoted phosphorylation, uncoupling, and internalization of the receptors (37, 38). Internalization of the G proteins has also been suggested to contribute to desensitization of the signaling unit (39–43).

In an effort to distinguish between the effects of activation and desensitization on receptor and G protein palmitoylation, we took advantage of a $\beta_2AR\text{-}G\alpha_s$ fusion protein that can be activated but not desensitized, internalized, or down-regulated (44, 45). The pharmacological properties of such receptor-G protein fusion constructs has recently attracted considerable attention and many of their properties have been recently reviewed (46, 47). The agonist-bound $\beta_2AR\text{-}G\alpha_s$ fusion protein presumably mimics an early intermediate in the normal activation cycle. Also of interest to the present study is the fact that complete physical dissociation between the receptor and $G\alpha_s$, which normally follows the initial stimulatory interaction, is not permitted in the fusion protein. These features of the fusion protein allow study of the effects of early activation events on the palmitoylation state of the receptor and G protein independently of those resulting from the inactivation processes. Furthermore, the use of fusion protein restricts the analysis to those receptors and G proteins that did physically interact in the course of the experiment. We report that stimulation of $\beta_2AR\text{-}G\alpha_s$ with $\beta$-adrenergic agonists promotes rapid depalmitoylation and inhibits repalmitoylation of both the receptor and the $G\alpha$ subunit. This contrasts with the facilitated repalmitoylation that is observed when the two proteins are expressed individually and suggests that early events in the activation process lead to the depalmitoylation of the two proteins, whereas later deactivation mechanisms, that do not occur for the fusion protein, are required for the repalmitoylation reaction.

## EXPERIMENTAL PROCEDURES

*Materials*—Grace's insect medium, lactalbumin, yeastolate, penicillin, streptomycin, glutamine, fungizone, pluronic acid, and phosphate-buffered saline were from Life Technologies, Inc. Fetal bovine serum

was obtained from Immunocorp. [$^{125}$I]CYP, [$\alpha$-$^{32}$P]ATP, [$\gamma$-$^{32}$P]ATP, [$^3$H]cAMP, and (9,10)-[$^3$H]palmitate were purchased from Mandel. Alprenolol, isoproterenol, dichloroisoproterenol, ATP, GTP, cAMP, forskolin, isobutylmethylxanthine, phosphoenolpyruvate, bovine serum albumin, myokinase, cyanogen bromide, and anti-Flag™ M2 were obtained from Sigma. Pyruvate kinase and Geneticin were from Calbiochem. ICI118551 was from Tocris. Benzamidine, soybean trypsin inhibitor, leupeptin, and $n$-dodecyl-$\beta$-D-maltoside was from Alexis Corp.

*Recombinant Baculoviruses Construction*—The recombinant c-Myc-$\beta_2AR$ baculovirus was generated by subcloning the cDNA of a c-Myc-tagged human $\beta_2AR$ (5) into the pJVELTZ recombination plasmid (InVitrogen). The pBacPAK-pHIS-$\beta_2AR\text{-}G\alpha_s$ was constructed by inserting in phase the Klenow-filled *NcoI-SalI* cDNA fragment of the fusion protein $\beta_2AR\text{-}G\alpha_s$ (45, 51) into the Klenow-filled *BamHI-EcoRI* pBacPAK1-polyHIS vector (CLONTECH). The constructs were confirmed by DNA sequencing. The viruses were then produced by homologous recombination in Sf9 cells according to standard procedures (52). The recombinant baculovirus encoding the $\beta_2AR\text{-}Thr\text{-}G\alpha_s$ construct was generated as described previously (53). Following infection of Sf9 cells with the appropriate viruses, expression of $\beta_2AR$ and $\beta_2AR\text{-}G\alpha_s$ was assessed by radioligand binding assays and Western blot analysis.

*Cell Culture, Metabolic Labeling, and Membrane Preparations*—Sf9 cells were cultured in Grace's supplemented media containing 10% fetal bovine serum, 0.001% pluronic acid in spinner flasks (Bellco Glass) at 27 °C. Cells (2 × 10⁶/ml) were infected with the recombinant baculoviruses at a multiplicity of infection varying between 2 and 5 for 48 to 72 h. [$^3$H]Palmitate labeling was then carried out in cells expressing the $\beta_2AR$ or $\beta_2AR\text{-}G\alpha_s$ fusion proteins. Cells were harvested and placed in serum-free medium for 1 h prior to the start of metabolic labeling. [$^3$H]palmitate dissolved in a minimal volume of dimethyl sulfoxide was then added (100 $\mu$Ci/millions of cells), and the cells were incubated at 27 °C in the presence or absence of $\beta$-adrenergic ligands for various periods of time as described previously (7). In some experiments, labeling was allowed to proceed for 45 min before $\beta$-adrenergic ligands were added. Labeling was stopped by chilling the reaction on ice. Cells were centrifuged at 500 × *g* for 5 min at 4 °C, rinsed twice with ice-cold PBS and resuspended in 20 ml of an ice-cold lysis buffer containing 20 mM Tris-HCl, 5 mM EDTA, pH 7.4, and the following protease inhibitors: 5 $\mu$g/ml leupeptin, 5 $\mu$g/ml soybean trypsin inhibitor, and 10 $\mu$g/ml benzamidine. Cells were disrupted by sonication and the lysate was centrifuged 5 min at 500 × *g* at 4 °C. The supernatant was then centrifuged at 45,000 × *g* for 20 min at 4 °C. The pelleted membranes were then resuspended in 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, containing 0.3% $n$-dodecyl-$\beta$-D-maltoside and protease inhibitors (solubilization buffer). Solubilization was carried out for 90 min at 4 °C, and solubilized receptors were purified as described below.

*Receptor Affinity Purification*—Alprenolol-Sepharose affinity purification matrix was synthesized according to the method of Benovic *et al.* (54). This matrix was used to purify the Sf9-derived $\beta_2AR$, $\beta_2AR\text{-}G\alpha_s$, and $\beta_2AR\text{-}Thr\text{-}G\alpha_s$ as described previously (5). The affinity purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon), and the amount of $\beta_2AR$, $\beta_2AR\text{-}G\alpha_s$, or $\beta_2AR\text{-}Thr\text{-}G\alpha_s$ in each sample was determined by [$^{125}$I]CYP soluble radioligand binding assay as described elsewhere (55).

*Hydroxylamine Treatment, Chemical and Enzymatic Cleavages*—For hydroxylamine treatment, purified $\beta_2AR$ or $\beta_2AR\text{-}G\alpha_s$ was mixed to an equal volume of 1 M Tris, pH 7.0, containing or not 1 M NH$_2$OH and incubated overnight at 4 °C. Cyanogen bromide (CNBr) cleavage was carried out following a protocol described by Luo *et al.* (56). Briefly, affinity purified $\beta_2AR\text{-}G\alpha_s$ were separated by SDS-PAGE in nonreducing condition. The proteins were then transferred electrophoretically to nitrocellulose membrane. The band corresponding to $\beta_2AR\text{-}G\alpha_s$ was cut out and the strip submerged in 500 $\mu$l of 70% (v/v) formic acid. The cleavage reaction was started by adding 10 $\mu$l of 5 M CNBr in acetonitrile. The reaction was allowed to proceed for 180 min at room temperature in the dark. The thrombin cleavage of the $\beta_2AR\text{-}Thr\text{-}G\alpha_s$ construct was carried out on affinity purified fusion protein using 10 NIH units/ml of thrombin from human placenta in the solubilization buffer containing only 0.03% $n$-dodecyl-$\beta$-D-maltoside for 30 min at room temperature.

*SDS-PAGE and Western Blot Analysis*—Affinity purified $\beta_2AR$, $\beta_2AR\text{-}G\alpha_s$, $\beta_2AR\text{-}Thr\text{-}G\alpha_s$, $\beta_2AR\text{-}G\alpha_s$ cleaved with CNBr, or $\beta_2AR\text{-}Thr\text{-}G\alpha_s$ cleaved with thrombin were resolved on nonreducing (or mildly reducing, 10 mM dithiothreitol, when specified) 10–15% slab gels containing 6 M urea. The gels were then fixed, incubated in Enlightning (DuPont), or in 1 M salicylic acid, dried, and exposed to DuPont RE-FLECTION® films from 10 to 60 days at −80 °C. Fluorograms were scanned and digitized (Hewlett Packard laser scanner), and densitometric analysis was carried out using the NIH Image program. When
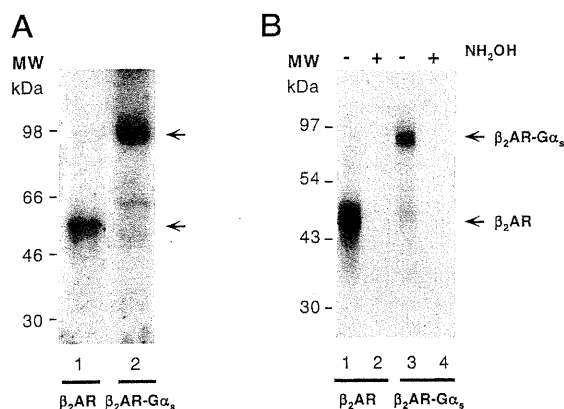
**FIG. 3. Palmitoylation of $\beta_2AR$ and $\beta_2AR$-$G\alpha_s$ via hydroxyla-mine-sensitive thioester linkage.** Sf9 cells infected with the $\beta_2AR$ or the $\beta_2AR$-$G\alpha_s$ recombinant baculoviruses were labeled for 45 min with [$^3H$]palmitate. Receptor and fusion proteins were then purified by al-prenolol-Sepharose chromatography and prepared for nonreducing SDS-PAGE. *Panel A*, 0.3 pmol of $\beta_2AR$ and $\beta_2AR$-$G\alpha_s$, as assessed by [$^{125}I$]CYP binding, were loaded in *lanes 1* and *2*, respectively. *Panel B*, the purified receptor and fusion protein were treated (+) or not (−) with $NH_2OH$ (1 M; pH 7.0) prior to SDS-PAGE. 2.2 pmol of $\beta_2AR$ were loaded in *lanes 1* and *2*, and 0.3 pmol of $\beta_2AR$-$G\alpha_s$ was loaded in *lanes 3* and *4*. The fluorogram shown is representative of three independent experiments.
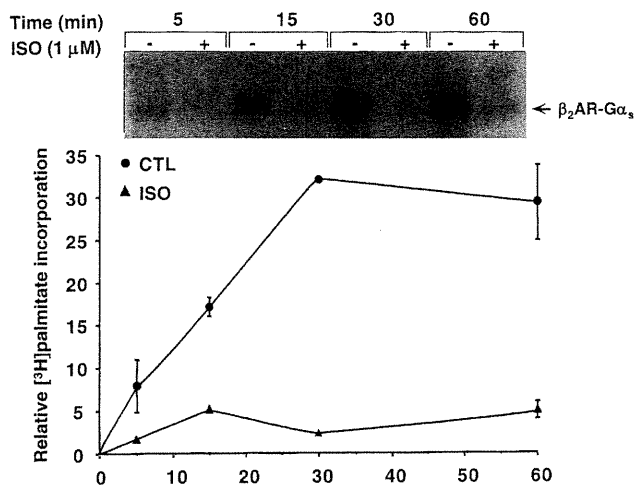


**FIG. 4. Effects of isoproterenol on the incorporation of [$^3H$]palmitate into $\beta_2AR$-$G\alpha_s$.** Sf9 cells infected with the $\beta_2AR$-$G\alpha_s$ recombinant baculovirus were labeled with [$^3H$]palmitate for the indi-cated times in presence of 1 $\mu M$ isoproterenol (*ISO* and +) or the vehicle alone (control) (*CTL* and −). The fusion protein was then purified by alprenolol-Sepharose chromatography and resolved by SDS-PAGE. Iden-tical numbers of $\beta_2AR$-$G\alpha_s$ (0.5 pmol), as assessed by [$^{125}I$]CYP binding, were loaded in each lane. Relative incorporation of [$^3H$]palmitate into $\beta_2AR$-$G\alpha_s$ was estimated by densitometric analysis of the fluorograms. The *graph* shown is the mean ± S.E. of three independent experiments.

the sensitivity of the labeling to hydroxylamine treatment, indi-cating that the [$^3H$]palmitate was covalently attached to both $\beta_2AR$ and $\beta_2AR$-$G\alpha_s$ via thioester bonds.

*Agonist-promoted Depalmitoylation of $\beta_2AR$-$G\alpha_s$*—To assess the effect of receptor stimulation on the dynamics of palmitoy-lation, pulse labeling experiments were carried out in the pres-ence or absence of agonists for periods varying between 5 and 60 min. In the absence of agonist, incorporation of palmitate into $\beta_2AR$-$G\alpha_s$ increased almost linearly for the first 30 min of labeling and remains stable thereafter (Fig. 4). The presence of isoproterenol during the labeling period greatly inhibited the incorporation of [$^3H$]palmitate in the fusion protein. This un-expected result contrasts sharply with the agonist-promoted increase in palmitate incorporation observed on $\beta_2AR$ and $G\alpha_s$
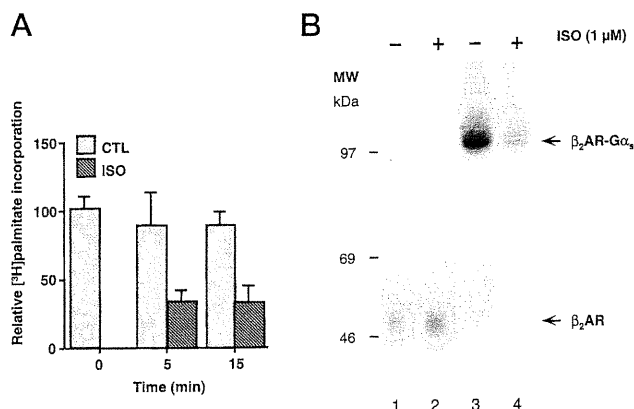


**FIG. 5. Isoproterenol-promoted depalmitoylation of $\beta_2AR$-$G\alpha_s$.** Sf9 cells infected with $\beta_2AR$ or $\beta_2AR$-$G\alpha_s$ recombinant baculovirus were labeled with [$^3H$]palmitate for 45 min. Cells were then treated with 1 $\mu M$ isoproterenol (*ISO* and +) or the vehicle (control) (*CTL* and −) alone, for 5 or 15 min in the continued presence of [$^3H$]palmitate. The fusion protein was then purified by alprenolol-Sepharose chromatography and resolved by SDS-PAGE. Identical numbers of receptor, as assessed by [$^{125}I$]CYP binding, were loaded in each lane, and the relative incorpo-ration of [$^3H$]palmitate into $\beta_2AR$-$G\alpha_s$ was estimated by densitomet-ric analysis of the fluorograms. The *bar graph* shown in *panel A* represents the mean ± S.E. of four independent experiments. The fluorogram in *panel B* shows the effect of a 5-min treatment with isoproterenol fol-lowing a 45-min pulse labeling on [$^3H$]palmitate incorporation into wild type $\beta_2AR$ (*lanes 1* and *2*) and into $\beta_2AR$-$G\alpha_s$ (*lanes 3* and *4*). Equivalent numbers of receptor (0.9 pmol) were loaded in each lane.
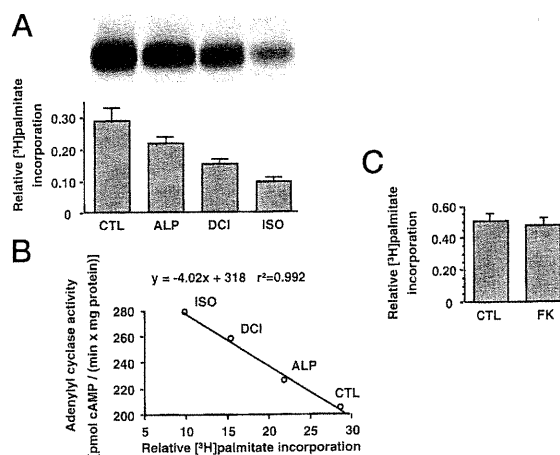


**FIG. 6. Effects of $\beta$-adrenergic ligands of various intrinsic ef-ficacy on the palmitoylation of $\beta_2AR$-$G\alpha_s$.** Sf9 cells infected with the $\beta_2AR$-$G\alpha_s$ recombinant baculovirus were labeled with [$^3H$]palmitate for 1 h in presence of alprenolol (*ALP*), dichloroisoproterenol (*DCI*), isoproterenol (*ISO*), forskolin (*FK*), or the vehicle (*CTL*). Affinity chro-matography purified $\beta_2AR$-$G\alpha_s$ was then resolved by SDS-PAGE. In each experiment, identical amount of fusion protein obtained from the experimental and control conditions was loaded. Relative incorporation of [$^3H$]palmitate into $\beta_2AR$-$G\alpha_s$ was estimated by densitometric analy-sis of the fluorograms. In *panels A* and *C*, the *bar graphs* shown are the mean ± S.E. of five independent experiments. *Panel B*, linear regres-sion between the extent of agonist-stimulated adenylyl cyclase activity observed for the various ligands in membranes derived from cells ex-pressing $\beta_2AR$-$G\alpha_s$ and the relative [$^3H$]palmitate incorporation ob-served into $\beta_2AR$-$G\alpha_s$ in the presence of the same ligands. Data repre-sent the mean of six independent experiments.

when these proteins are expressed individually (5, 7, 10–12).

For the $\beta_2AR$ and $G\alpha_s$ expressed separately, the increase in palmitate turnover was linked to a faster rate of depalmitoy-lation upon agonist stimulation (7, 12). The apparent increase in [$^3H$]palmitate incorporation was thus attributed to a con-comitant acceleration of the repalmitoylation reaction. It fol-lows that the agonist-promoted reduction of [$^3H$]palmitate in-corporation into the $\beta_2AR$-$G\alpha_s$ fusion protein could result from
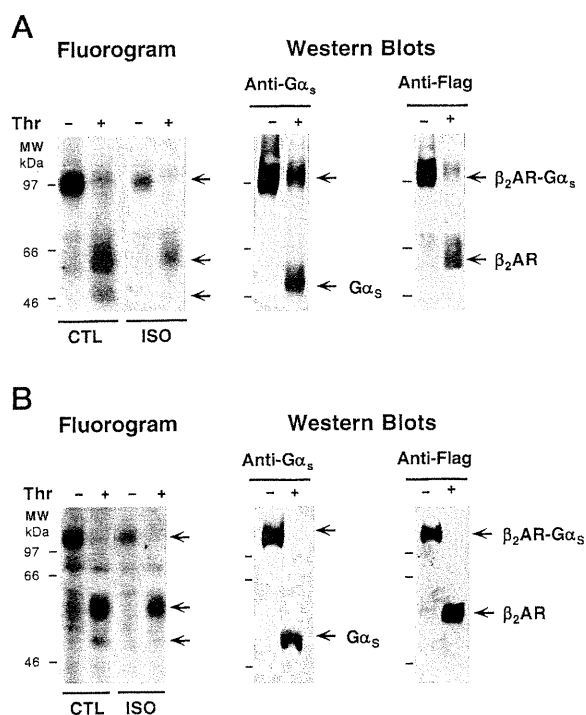
**A**

**Fluorogram**     **Western Blots**

**B**

**Fluorogram**     **Western Blots**

FIG. 7. **Thrombin cleavage of $\beta_2$AR-Thr-G$\alpha_s$.** Sf9 cells were infected with a baculovirus encoding the $\beta_2$AR-Thr-G$\alpha_s$ fusion protein. Metabolic labeling with [$^3$H]palmitate was then carried out for 45 min before adding 1 $\mu$M isoproterenol (*ISO*) or not (control) (*CTL*) for an additional 15 min. The fusion protein was purified by alprenolol-Sepharose affinity chromatography and treated (+) or not (−) with thrombin (*Thr*, 10 NIH units/ml) for 30 min. The reactions were resolved under nonreducing conditions (*panel A*) or mildly reducing (10 mM dithiothreitol) conditions (*panel B*) by SDS-PAGE containing 6 M urea. 1.2 pmol of receptor, as assessed by [$^{125}$I]CYP soluble radioligand binding, were loaded in each lane in both *panels A* and *B*. Relative incorporation of [$^3$H]palmitate into the fusion $\beta_2$AR-Thr-G$\alpha_s$ or the $\beta_2$AR and the G$\alpha$s moieties were estimated by densitometric analysis of the fluorograms. The fluorograms shown are representative of four independent experiments. In each case, parallel SDS-PAGE were carried out for Western blot analysis, as described under "Experimental Procedures," to confirm the identity of each protein.

a slower depalmitoylation or reflect an inhibition of the repalmitoylation reaction. To distinguish between these two hypotheses, cells were metabolically labeled with [$^3$H]palmitate in the absence of agonist. Following a 45-min pulse period, corresponding to the period required to attain steady state labeling, isoproterenol was added or not in the continued presence of [$^3$H]palmitate and incubated for an additional 5 or 15 min. As seen in Fig. 5, incubation with isoproterenol rapidly reduced the extent of $\beta_2$AR-G$\alpha_s$ palmitoylation, thus suggesting that agonist stimulation promotes its rapid depalmitoylation and that repalmitoylation of the active complex cannot occur. This is in sharp contrast with the increased repalmitoylation that is observed when identical treatment is carried out in cells expressing the wild type $\beta_2$AR as an individual protein (Fig. 5*B*).

Because the $\beta_2$AR-G$\alpha_2$ fusion protein can be activated but that later processes of inactivation such as G protein dissociation, desensitization, or internalization do not occur, it could be hypothesized that early events leading to activation of the receptor-G protein complex promote depalmitoylation but that later processes are required for repalmitoylation. The fact that the depalmitoylation and repalmitoylation reactions may occur with very similar kinetics when the $\beta_2$AR and G$\alpha_s$ are expressed as individual proteins may explain why Jones *et al.* (8) did not observe any change in the stoichiometry of palmitoylation of G$\alpha_s$ upon activation despite the universally observed increase in the turnover rate of the G$\alpha_s$-bound palmitate. The
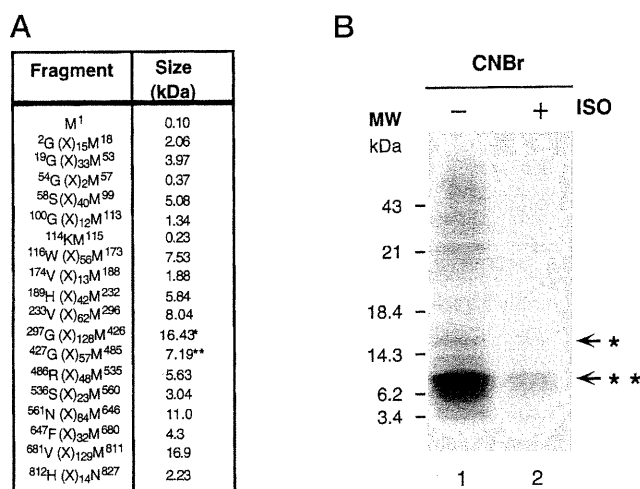
| Fragment | Size (kDa) |
|---|---|
| M$^1$ | 0.10 |
| $^2$G (X)$_{15}$M$^{18}$ | 2.06 |
| $^{19}$G (X)$_{33}$M$^{53}$ | 3.97 |
| $^{54}$G (X)$_2$M$^{57}$ | 0.37 |
| $^{58}$S(X)$_{40}$M$^{99}$ | 5.08 |
| $^{100}$G (X)$_{12}$M$^{113}$ | 1.34 |
| $^{114}$KM$^{115}$ | 0.23 |
| $^{116}$W (X)$_{56}$M$^{173}$ | 7.53 |
| $^{174}$V (X)$_{13}$M$^{188}$ | 1.88 |
| $^{189}$H (X)$_{42}$M$^{232}$ | 5.84 |
| $^{233}$V (X)$_{62}$M$^{296}$ | 8.04 |
| $^{297}$G (X)$_{128}$M$^{426}$ | 16.43* |
| $^{427}$G (X)$_{57}$M$^{485}$ | 7.19** |
| $^{486}$R (X)$_{48}$M$^{535}$ | 5.63 |
| $^{536}$S(X)$_{23}$M$^{560}$ | 3.04 |
| $^{561}$N (X)$_{84}$M$^{646}$ | 11.0 |
| $^{647}$F (X)$_{32}$M$^{680}$ | 4.3 |
| $^{681}$V (X)$_{129}$M$^{811}$ | 16.9 |
| $^{812}$H (X)$_{14}$N$^{827}$ | 2.23 |

**B**

**CNBr**

FIG. 8. **CNBr cleavage of $\beta_2$AR-G$\alpha_s$.** *Panel A*, expected sizes of the 19 fragments obtained after complete CNBr cleavage of the fusion protein, using the program PROLYSIS. Because the two palmitoylation sites are located on the carboxyl-terminal portion of the $\beta_2$AR moiety and the amino-terminal portion of the G$\alpha_s$ moiety, two distinct palmitoylated fragments of 16.4 and 7.2 kDa and corresponding to the $\beta_2$AR- (*) and the G$\alpha_s$ (**)-derived peptides, respectively, should be produced. *Panel B*, infected Sf9 cells expressing $\beta_2$AR-G$\alpha_s$ were labeled for 45 min with [$^3$H]palmitate and then treated with the vehicle (CTL, *lane 1*) or with isoproterenol (ISO, *lane 2*) for 15 min in the continued presence of labeled palmitate. The radiolabeled fusion protein was purified, resolved by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Bands corresponding to the fusion protein were then treated with CNBr for 3 h. Generated peptides were separated by SDS-PAGE. The fluorogram shown is representative of two independent experiments.

stabilization of the activated receptor-G protein complex using $\beta_2$AR-G$\alpha_s$ allowed isolation of the effects that resulted solely from the activation process.

The effect of $\beta$-adrenergic ligands of various levels of intrinsic activity was then assessed on the palmitoylation of $\beta_2$AR-G$\alpha_s$. As shown in Fig. 6, the addition of all ligands caused a significant reduction in the incorporation of the labeled fatty acid into $\beta_2$AR-G$\alpha_s$. Interestingly, the extent of the decrease in labeling was directly correlated ($r^2 = 0.992$) to the intrinsic activity of the compounds toward $\beta_2$AR-G$\alpha_s$ as assessed in a membrane adenylyl cyclase assay (Fig. 6*B*). However, direct stimulation of cAMP production by forskolin did not affect the palmitoylation of $\beta_2$AR-G$\alpha_s$ (Fig. 6*C*), thus suggesting that the agonist-promoted depalmitoylation is linked to conformational changes imposed by the agonists and not to second messenger generation.

*[$^3$H]Palmitate Incorporation into the $\beta_2$AR and G$\alpha_s$ Moieties of the Fusion Protein*—Cysteine 341 of $\beta_2$AR and cysteine 3 of G$\alpha_s$, corresponding to position 358 and 428 in $\beta_2$AR-G$\alpha_s$, respectively, represent the confirmed palmitoylation sites of these two proteins (20, 29). In the experiments described above, palmitoylation of $\beta_2$AR-G$\alpha_s$ was studied as a whole with no specific consideration of the individual palmitoylation sites. To determine whether the two sites were indeed palmitoylated and to assess if agonist treatment had similar effects on the palmitoylation state of the two proteins, we took advantage of another fusion protein construct in which a thrombin cleavage site was engineered between the receptor and G$\alpha_s$ ($\beta_2$AR-Thr-G$\alpha_s$; see Fig. 1). As a control, thrombin treatment was performed on wild type $\beta_2$AR without any effect on the palmitoylation state nor the integrity of the receptor (data not shown). Fig. 7*A* shows that thrombin treatment of the purified fusion protein, following metabolic labeling, generated two labeled proteins corresponding to the expected mobility for $\beta_2$AR and G$\alpha_s$, indicating that the two proteins were palmitoylated within the fusion construct. The identity of the cleaved frag-

ments was further confirmed by Western blot analysis using the anti-$\alpha_s$ antibody to detect $G\alpha_s$ and the anti-Flag M2 antibody to detect the Flag epitope-bearing $\beta_2AR$. The apparently higher [$^3$H]palmitate incorporation observed into the $\beta_2AR$ band when compared with $G\alpha_s$ most likely reflects the presence of some background labeling observed in this region of the gel even in the absence of thrombin.

As previously observed for $\beta_2AR$-$G\alpha_s$, the presence of isoproterenol during the metabolic labeling induced a significant reduction of the [$^3$H]palmitate incorporation into $\beta_2AR$-Thr-$G\alpha_s$. Thrombin cleavage revealed that the overall decrease in the radiolabeling of the fusion protein was the consequence of a reduction of [$^3$H]palmitate incorporation into both the receptor and $G\alpha_s$. The agonist-induced $G\alpha_s$ depalmitoylation that we observed could be mediated, in part, by the receptor-promoted dissociation of $\beta\gamma$ subunits from the activated fusion protein. Indeed, as reported by Iiri *et al.* (19), $\beta\gamma$ did protect GDP-bound $\alpha_s$ but not $\alpha_s$-GTP[$\gamma$S] from depalmitoylation by a recombinant esterase. Because nonreducing SDS-PAGE conditions could lead to aggregation of some proteins, including the receptor, reducing conditions were also used. As shown in Fig. 7*B*, identical results were obtained when receptor and $G\alpha_s$ were resolved under mildly reducing conditions (10 mM dithiothreitol) that diminished aggregation and promoted only partial chemical depalmitoylation.

Palmitoylation of both receptor and $G\alpha_s$ and the effect of isoproterenol on the two proteins was further confirmed using CNBr hydrolysis of the $^3$H-palmitoylated $\beta_2AR$-$G\alpha_s$ construct. The primary sequence of $\beta_2AR$-$G\alpha_s$ containing 18 methionines, complete cleavage should generate 19 fragments (Fig. 8*A*). Given that the two palmitoylation sites are located on two distinct fragments, two peptides distinguishable by their size are expected to be $^3$H-palmitoylated. Calculated masses for the expected palmitoylated fragments are 16.4 and 7.2 kDa corresponding to the $\beta_2AR$- and the $G\alpha_s$-derived peptides, respectively. As shown in Fig. 8*B*, CNBr treatment yielded two peptides of the expected electrophoretic mobility, confirming that both the receptor and $G\alpha_s$ were palmitoylated within the fusion protein. The difference in the labeling intensity of the two bands most likely results from quantitatively different elution and recovery of the two fragments from the nitrocellulose membrane during the hydrolysis. Weakly labeled bands at 21 and 44 kDa represent partial cleavage products. Addition of isoproterenol following a 45-min pulse labeling with [$^3$H]palmitate promoted the depalmitoylation of both the 16.4 and 7.2 kDa fragments, confirming once more that agonist activation favored the depalmitoylation of both the receptor and $G\alpha_s$.

Taken together, the data presented in this study show that the initial events leading to activation of the $\beta_2AR$-$G\alpha_s$ complex promote the rapid depalmitoylation of both receptor and $G\alpha_s$ and that sustained activation prevents repalmitoylation occurring. The extent of depalmitoylation is directly proportional to the intrinsic activity of the agonist and most likely depends on conformational changes and protein-protein interactions that are stabilized by the activating ligands. Also, this study demonstrates for the first time that depalmitoylation and repalmitoylation occur during distinct phases of the receptor-G protein activation/inactivation cycle. Further studies are now required to determine how receptor activation leads to depalmitoylation of the receptor-G protein complex and why deactivation is required for repalmitoylation to occur. In particular, the role played by the newly characterized acyl-protein thioesterase (35) in the activation driven depalmitoylation will need to be assessed.

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