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Palmitoylation of G-protein-coupled receptors: a dynamic modification with functional consequences

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Introduction

In recent years, covalent modification of proteins by lipids has been found to be a more frequent modification than originally anticipated [1]. Three major classes of such modifications have been particularly well characterized. These are: (a) the N-terminal myristoylation of glycine residues through amide linkage; (b) the prenylation of cysteine residues, located in C-terminal domains, via the formation of thioether links to farnesyl or geranylgeranyl moieties; and (c) the palmitoylation which occurs through the thioesterification of cysteine residues, located in various domains of proteins, with palmitic acid. In contrast to myristylation and prenylation, which typically occur cotranslationally, palmitoylation is a genuine post-translational modification which can undergo dynamic regulation and which, in some cases, can be modulated by external stimuli [2–4]. This modification has been found to be particularly prevalent for proteins involved in processes such as cell adhesion, cell growth and signal transduction, raising the intriguing possibility that it could play regulatory roles in these processes. Palmitoylated proteins include $p21^{ras}$ [5], GAP-43 [6], several tyrosine kinases belonging to the $p60^{src}$ family such as $p56^{lck}$ [7], $p59^{fyn}$, $p55^{fgr}$ and $p56^{hck}$ [8], as well as many G-protein α-subunits [9]. For these proteins, the attachment of the 16-carbon-long fatty acid has been proposed to be essential for their targeting to the inner face of the plasma membrane, the site of their biological functions [10–13]. However, the observation that integral membrane proteins, which are anchored in the membranes through one or more transmembrane domains, can also be palmitoylated has led to speculation that such modifications may also serve other purposes. Evidence has been found supporting the involvement of fatty acylation in protein–protein interactions [14], protein trafficking [4,15] and protein phosphorylation [16]. Among these, the palmitoylation of G-protein-coupled receptors (GPCRs) has attracted considerable attention [17–20].

Palmitoylation of G-protein-coupled receptors

Rhodopsin was the first GPCR for which covalent palmitoylation was demonstrated. As early as 1984, it was observed that bovine rhodopsin could incorporate one to two molecules of palmitate per receptor following incubation of rod outer segments with [9,10-^{3}H]palmitate [21]. Four years later, physicochemical analysis of a C-terminal peptidic fragment derived from bovine rhodopsin led to the identification of Cys-322 and Cys-323 as the main palmitoylation sites of this protein [22]. Primary sequence comparison among GPCRs reveals that at least one of these two cysteine residues is conserved in a similar position within the C-terminus, in the majority of these receptors. Figure 1 illustrates this conservation for selected members of this protein family. This high level of conservation led us to propose that palmitoylation could be a general process with potentially important functional consequences for this class of receptors. To test this hypothesis, metabolic labelling experiments using [^{3}H]palmitate were carried out to determine whether or not the human β_2-adrenergic receptor (β_2AR), which possesses a cysteine residue (Cys-341) in an equivalent position to that of Cys-322 and Cys-323 of

Abbreviations used: β_2AR, β_2-adrenergic receptor; GPCR, G-protein-coupled receptor; 5HT, 5-hydroxytryptamine.

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Figure 1

Partial sequence alignment for selected GPCRs

The alignment starts with the first amino acid predicted to come out of the seventh transmembrane domain (TM7). The underlined cysteine residues represent potential palmitoylation sites. ★ indicates that the palmitoylation of these receptors has been confirmed experimentally.

		TM 7
★	HUM β_2	^{329}PDFRIAFQELLCLRRSSLKAYGNGYSSNGNGEQSGYHVEKENKLLCEDL ...
	HUM β_1	^{388}DFRKAFQGLLCCARRAARRRHTATHGDRPPRASGCLARPGPPPSPGAASDD ...
	HAM α_{1B}	^{351}SSKEFKRAFMRILGCQCRSGRRRRRRRRLGGCAYTYRRWTRGGS ...
	HUM α_{1A}	^{351}SREFKRAFLRLLRCQCRRRRRRLWSLRPPLASLDRRRATRLRPQFSHRSR ...
	BOV α_{1C}	^{316}SQEFKKAFQMVLRIQCLRRKQSSKHTLGYTLHAPSHVLEGQHK ...
★	HUM α_{2A}	^{430}NHDFRRAFKKILCRGDRKRIV
★	HUM D_1	^{334}NADFRKAFSTLLGCYRLCPATNNAIETVSINNNGAAMFSSHHEPRGSISKE ...
	RAT D_2	^{311}IEFRKAFMKILHC
	HUM $5HT_{1A}$	^{404}NKDFQNAFKKIIKCNFCRQ
★	HUM $5HT_{1B}$	^{374}EDFKQAFHKLIRFKCTS
	RAT $5HT_{1C}$	^{373}NKIYRRAFSKYLRCDYKPDKKPPVRQIPRVAATZLSGRELNVNIYRHTNER ...
	RAT $5HT_2$	^{361}NKTYRSAFSRYIQCQYKENRKPLQLILVNTIPALAYKSSQMQVGQKKNSQE ...
★	HUM Rho	^{310}NKQFRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVAPA ...
	HUM PAF	^{297}TKKFRKHLTEKFYSMRSSRKCSRATTDTVTEVVVPFNQIPGNSLKN ...
	MOU PGE_{EP2}	^{360}LRKTVLSKAIEKIKCLFCRIGGSGRDSSAQHCSESRRTSSAMSGHSRSFLA ...
	RAT NK_3	^{346}NKRFRAGFKRAFRWCPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESVT ...
	HUM THR	^{375}SECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT
	HUM M1ACh	^{415}NKAFRDTFRLLLLCRWDKRRWRKIPKRPGSVHRTPSKRC
	HUM M2ACh	^{440}NATFKKTFKHLLMCHYKNIGATR
	HUM M3ACh	^{456}NATFKKTFRHLLLCQYRNIGTAR
	HUM M4ACh	^{547}NTFRTTFKTLLLCQCDKRKRRKQQYQQRQSVIFHKRVPEQAL

rhodopsin, could be palmitoylated. Using various heterologous expression systems, the covalent incorporation of palmitate into the β_2AR was clearly demonstrated [16,17,23]. Furthermore, the sensitivity of the palmitate labelling to reducing agents and to neutral hydroxylamine treatment confirmed that the fatty acid is attached through a thioester link. Finally, it was shown that the substitution of a glycine residue for the Cys-341 of the β_2AR completely prevents the incorporation of [^{3}H]palmitate in the receptor, thus confirming that this cysteine is the main palmitoylation site.

Following this first demonstration that an hormonal receptor belonging to the GPCR family could be palmitoylated, the palmitoylation of three other members of this family has been shown experimentally. These are the α_{2A}-adrenergic [18], the D_1-dopaminergic [20] and the 5-hydroxytryptamine type 1B (5-HT_{1B}) serotonergic [19] receptors which all have a cysteine residue in the juxtamembranous domain of their C-terminal tail (Figure 1). The palmitoylation of GPCRs thus appears to be a general phenomenon, at least for those receptors with cysteine residues in the appropriate position in their C-terminal tail. For receptors lacking such cysteine residues it will be of interest to determine whether palmitoylation site(s) are located in different cystoplasmic domains.

Because of the hydrophobic nature of the palmitate residues, Ovchinnikov and collaborators [22] proposed that palmitoylation of the Cys-322 and Cys-323 of rhodopsin would favour the interaction of this domain with the plasma membrane through the insertion of the fatty-acid chains in the

lipid bilayer. This type of interaction, which would lead to the creation of a fourth intracytoplasmic loop, has recently been experimentally confirmed, for bovine rhodopsin, using fluorescent analogues of palmitic acid [24]. A similar model proposed for the human β_2AR is shown in Figure 2.

Dynamic regulation of receptor palmitoylation

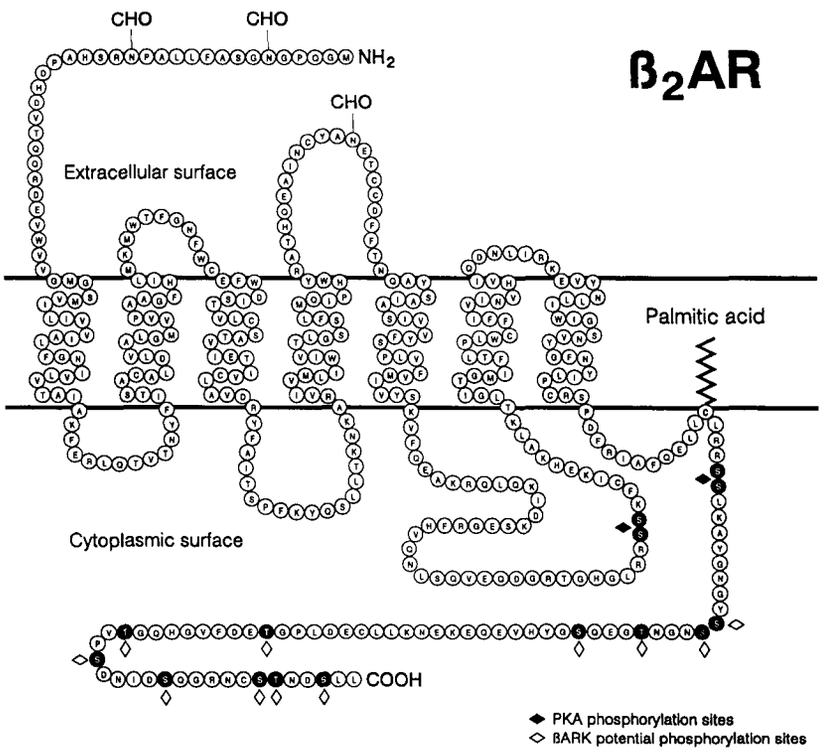
As previously mentioned, the palmitoylation of several proteins has been shown to be independent of protein synthesis and also to be modulated by various extracellular stimuli [6,25–28]. Recent studies on the β_2AR suggest that dynamic regulation of the palmitoylation state of GPCR could be part of the activation process. Indeed, stimulation of the β_2AR with the agonist isoprenaline increases the incorporation of [^{3}H]palmitate into the receptor [23]. Under the experimental conditions used, such an increase could result either from: (a) an increase in the proportion of the β_2AR being palmitoylated; or (b) an elevation of the turnover rate of the palmitate bound to the receptor, thus favouring the exchange of unlabelled palmitate for [^{3}H]palmitate. Results obtained from kinetic and pulse-chase experiments support the second hypothesis. Such regulation of the palmitoylation state of a receptor through its biological activation is not limited to the β_2AR. Indeed, we recently reported that dopamine favours the incorporation of [^{3}H]palmitate into the D_1-dopaminergic receptor and that this increased incorporation is inhibited by an antagonist [20]. Activation of GPCRs thus appears to modulate their palmitoylation/depalmitoylation cycle. Based on the largely reciprocal nature of biological systems, this could suggest that the palmitoylation state of the receptors could influence their biological activity.

A dynamic regulation of the palmitoylation state of membrane receptor presupposes the existence of cytosolic or membranous enzyme(s) which can catalyse the palmitoylation (palmitoyl transferase) and the depalmitoylation (palmitoyl thioesterase) reactions. Until now, no such enzyme acting on GPCRs has been identified. However, a palmitoyl transferase activity, which can catalyse the palmitoylation of GAP-43, is found in membrane fractions derived from neural growth cone [29,30]. Moreover, a palmitoyl thioesterase, which can cleave the palmitic acid covalently attached to

Figure 2

Primary sequence and schematic representation of the topographic organization of the β_2AR in the plasma membrane

The palmitoylation site and the major phosphorylation sites are indicated.



p21$^{H\text{-}ras}$, has recently been purified and characterized [31].

Functional role of receptor palmitoylation

GPCRs are believed to be inserted in the plasma membrane through seven α-helical transmembrane domains and thus their association with the plasma membrane cannot be attributed to their palmitoylation. However, the fact that palmitoylation of the C-terminal tail favours the interaction of this domain with the membrane [24] will undoubtedly influence the three-dimensional organization of this part of the receptor. Given the important role attributed to the cytoplasmic tail of many receptors in modulating their interaction with their cognate G-proteins, such changes could greatly influence receptor function.

Experimental data supporting the existence of a functional role for receptor palmitoylation came from site-directed mutagenesis studies. In addition to preventing palmitoylation, substitution of a glycine residue for the Cys-341 of the β_2AR (Gly341β_2AR) dramatically decreased the ability of the receptor to productively interact with G_s and to stimulate the activity of the adenylyl cyclase [16,17]. This reduction in the transducing capacity of the β_2AR is accompanied by a significant increase in the basal phosphorylation level of the receptor [16]. This constitutively elevated level of phosphorylation could be responsible for its functional uncoupling. Indeed, many studies have documented the role played by phosphorylation in the functional uncoupling which accompanies agonist-promoted desensitization (for a review, see [32]). In particular, phosphorylation sites for the cyclic AMP-dependant protein kinase and the β-adrenergic receptor kinase, which are located in the C-terminal tail of the β_2AR, have been shown to play crucial roles in this process [33,34]. Similarly, the functional uncoupling of Gly341β_2AR could result from increases in the phosphorylation of some of these sites. Interestingly, a number of regulatory phosphorylation sites of the C-terminal tail are located reasonably close to the palmitoylated Cys-341 (Figure 2) and might not be readily accessible to the kinases because of their proximity to the plasma membrane. It follows that the detachment of the C-terminal tail from the membrane, which would result from the mutation of Cys-341, could favour the phosphorylation of these sites. Consistent with this model is our recent observation that substitution of alanine residues for Ser-345 and Ser-346 of Gly341β_2AR restored both the phosphorylation and the effector activation to levels observed with wild-type β_2AR. These observations raise the intriguing possibility that a dynamic regulation of the receptor palmitoylation state, upon agonist stimulation, could govern the accessibility of phosphorylation sites to regulatory kinases and thus could contribute to the development of agonist-promoted desensitization.

Few data are available concerning the potential role that palmitoylation plays for other GPCRs. Substitution of an alanine or a serine for the palmitoylated Cys-442 of the α_{2A}-adrenergic receptor is without apparent effect on the ability of the receptor to interact with G_i and to inhibit adenylyl cyclase activity [18]. However, it is noteworthy that the C-terminal tail of the α_{2A}AR is relatively short and lacks any potential phosphorylation sites (Figure 1). For rhodopsin, chemical depalmitoylation has been reported to increase its ability to stimulate transducin [35], whereas the substitution of serine residues for Cys-322 and Cys-323 was reportedly without effect on the function of the photo-receptor [36]. Finally, the substitution of glycine residues for Cys-335 and Cys-337 of the mouse thyrotropin-releasing hormone receptor did not affect the ability of the receptor to stimulate the production of Ins(1,4,5)P_3, but significantly blocked its rapid agonist-promoted internalization [37]. It is clear from the above observations that further studies will be required before a general model concerning the functional role of GPCR palmitoylation can be proposed.

Palmitoylation of G-proteins

Recent studies have demonstrated that in addition to the receptors, the α subunits of all the G-proteins tested are palmitoylated [9,11,38]. For subunits such as α_s and α_q, which are not myristylated, palmitoylation has been shown to be essential for their targeting to the plasma membrane. However, palmitoylation is not limited to the non-myristylated subunits, as the myristylated α_z, α_{i1}, α_{i2}, α_{i3} and α_o subunits have also been shown to be thioesterified by palmitic acid [9].

As with the receptors, the palmitoylation state of the G_α subunit has recently been shown to be dynamically regulated [39,40]. Indeed, in metabolic labelling experiments, the activation of G_s, by NaF or through the stimulation of the β_2AR, leads to an increased incorporation of [^{3}H]palmitate in G_αs. Such dynamic regulation of the palmitoylation state could have dramatic consequences on the subcellular localization and the function of the α subunits.

Conclusion

The observations summarized in the present paper show that at least two components of the G-protein-coupled signalling pathway, namely the receptor and the G_α subunit, can be palmitoylated. Although the functional consequences of these modifications are not completely understood, it is already clear that they play an important role in the regulation of signal transduction. Furthermore, the dynamic modulation of the palmitoylation state observed both for the receptors and the G-proteins indicated that concerted enzymatic processes are involved in the control of this post-translational modification. Further studies should lead to the elucidation of the enzymatic reactions involved in palmitoylation as well as of the functional implications of this covalent modification.

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