

REVIEW

G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats

Bice Chini and Marco Parenti¹

Cellular and Molecular Pharmacology Section, CNR Institute of Neuroscience, Via Vanvitelli 32, 20129 Milan, Italy

¹Department of Experimental Medicine, University of Milano-Bicocca, Monza, Italy

(Correspondence should be addressed to B Chini; Email: b.chini@in.cnr.it)

Abstract

G-protein-coupled receptors (GPCRs) are integral membrane proteins, hence it is not surprising that a number of their structural and functional features are modulated by both proteins and lipids. The impact of interacting proteins and lipids on the assembly and signalling of GPCRs has been extensively investigated over the last 20–30 years, and a further impetus has been given by the proposal that GPCRs and/or their immediate signalling partners (G proteins) can partition within plasma membrane domains, termed rafts and caveolae, enriched in glycosphingolipids and cholesterol. The high content of these specific lipids, in particular of cholesterol, in the vicinity of GPCR transmembranes can affect GPCR structure and/or function. In addition, most GPCRs are post-translationally modified with one or more palmitic acid(s), a 16-carbon saturated fatty acid, covalently bound to cysteine(s) localised in the carboxyl-terminal cytoplasmic tail. The insertion of palmitate into the cytoplasmic leaflet of the plasma membrane can create a fourth loop, thus profoundly affecting GPCR structure and hence the interactions with intracellular partner proteins. This review briefly highlights how lipids of the membrane and the receptor themselves can influence GPCR organisation and functioning.

Journal of Molecular Endocrinology (2009) **42**, 371–379

G-protein-coupled receptors–cholesterol interactions

Eighty-ninety per cent of whole-cell cholesterol is contained within the plasma membrane, where it accounts for 20–25% of lipid molecules (Lange 1991, Simons & Ikonen 2000). Cholesterol, which is amphiphilic and has a small –OH head group and a rigid, planar four-ring backbone, influences the functional properties of the plasma membrane by interacting with other resident lipids (Ohvo-Rekila *et al.* 2002, Ikonen 2008) and proteins (Lee 2004). In particular, it may act on the conformation of membrane proteins: i) by alterations in the physico-chemical properties of the bilayer; ii) through specific and localised molecular interactions; or iii) by a combination of both.

Alterations in the physico-chemical properties of the bilayer

The effects of cholesterol on the fluidity and rigidity of the plasma membrane are well known: a cholesterol molecule can span approximately half a bilayer, preferentially interacting with the saturated fatty chains

of phospholipids. By increasing the packing, and hence the lateral assembly of phospholipids, cholesterol reduces membrane fluidity and increases rigidity (Mouritsen & Zuckermann 2004). The ‘rafts/caveolae signalling hypothesis’ suggests that plasma membrane domains enriched in cholesterol and sphingolipids compartmentalise the proteins involved in specific signalling tasks. By regulating lateral mobility and compartmentalisation, cholesterol affects the probability with which receptors interact with their downstream signalling partners, thus optimising their spatial-temporal interactions, hence signalling efficiency and regulation (Simons & Toomre 2000). The trafficking of receptors between regions with different lipid compositions is still unclear, but an interesting hypothesis has recently been advanced for the human δ -opioid receptor based on results obtained with plasmon-waveguide resonance spectroscopy experiments on solid-supported bilayers (Alves *et al.* 2005). It has been found that the unbound inactive receptor is enriched in phosphatidylcholine bilayers, whereas the activated receptor is concentrated in sphingomyelin bilayers, characterised by a greater thickness. Since it is known that receptor activation involves changes in the

orientation of transmembrane helices with an increase in receptor vertical length (i.e. perpendicular to the plane of the plasma membrane; Salamon *et al.* 2000), it is hypothesised that the driving force for receptor redistribution comes from the increased hydrophobic match of the elongated receptor for the thicker sphingomyelin-enriched bilayer, a sorting mechanism that has previously been observed in other subcellular compartments (Jensen & Mouritsen 2004, Lee 2004).

Specific and localised molecular interactions

Some membrane proteins directly bind cholesterol, as firstly shown for caveolin-1 (Murata *et al.* 1995), whose cholesterol-binding domain has not yet been precisely mapped, even if its scaffolding domain, originally considered to be a protein interaction domain, is now thought to play a role in cholesterol binding (Parton *et al.* 2006). Some membrane proteins involved in the transport of cholesterol possess a conserved five-transmembrane helix domain called the sterol-sensing domain, which bind cholesterol with a measured affinity of around 100 nM (Radhakrishnan *et al.* 2004). Others bind cholesterol via a conserved hydrophobic binding pocket, such as OSBP (Suchanek *et al.* 2007) and START proteins (Alpy & Tomasetto 2005), or short cholesterol-binding motifs (Li & Papadopoulos 1998). The fact that cholesterol plays a role in stabilising the conformation of specific G-protein-coupled receptors (GPCRs) has become clear after the first attempts to purify them for pharmacological studies or X-ray diffraction analysis (Schertler & Hargrave 2000, Albert & Boesze-Battaglia 2005). As first noted for rhodopsin, cholesterol greatly improves crystal quality and quantity; furthermore, in the resolved structure of metarhodopsin I, it was found that a cholesterol molecule sits in a pocket formed between adjacent helices of a receptor dimer, thus suggesting that it may help to form or stabilise receptor dimers (Ruprecht *et al.* 2004). As further discussed below, it has also been observed that cholesterol contributes to dimer formation in the recently resolved structures of the β_2 -adrenergic receptor, thus further supporting its direct role in regulating GPCR conformation (Cherezov *et al.* 2007).

GPCR palmitoylation: sites and dynamics

Glycosylation and phosphorylation are certainly the best known post-translational modifications affecting GPCR functions, but lipids are also post-translationally added to most GPCRs in the form of the 16-carbon saturated fatty acid, palmitic acid. One more 'exotic' lipidation has been reported for the human prostacyclin receptor, which, in addition to being palmitoylated,

has a farnesyl isoprenoid chain attached to the cytoplasmic carboxyl terminal (Miggin *et al.* 2003).

Cysteines are by far the most frequent acceptor sites of palmitoylation, which occurs on the thiol side chain through a thioester bond. However, it must be kept in mind that, although not yet reported for GPCRs, the addition of palmitic acid to proteins can also occur onto the terminally exposed amides of cysteine (Pepinsky *et al.* 1998) and glycine (Kleuss & Krause 2003), the hydroxyl group of threonine (Branton *et al.* 1993) and serine (Jing & Trowbridge 1990, Percherancier *et al.* 2001), and the ϵ -amino group of lysine via an amide bond (Stanley *et al.* 1998). In addition, other fatty acids, such as palmitoleate, stearate or oleate, can, much less commonly, bind to cysteines (Liang *et al.* 2004).

Rhodopsin was the first GPCR found to be palmitoylated at two adjacent cysteines in the carboxyl-terminal cytoplasmic tail (Ovchinnikov *et al.* 1988) but, since then, many other GPCRs have been experimentally shown to be mono-, bis- or tris-palmitoylated at conserved carboxyl-terminal cysteine residues (see Qanbar & Bouvier 2003 for a review), and yet other GPCRs containing the canonical cysteines are presumably palmitoylated. Interestingly, the μ -opioid receptor is not palmitoylated at the two conserved carboxyl-terminal cysteines, and cysteine-170 in the second intracellular loop (the only other intracellular cysteine) can be the palmitoylation site; however, as its substitution by alanine generates a mutant with very low expression levels, it has not been possible to measure [3 H]palmitate incorporation (Chen *et al.* 1998).

Some GPCRs are not modified by palmitic acid, including the α -isoform of the thromboxane A2 receptor, which lacks any cysteines in its carboxyl terminal (Reid & Kinsella 2007), and the GnRH receptor, which is unique among class A GPCRs as its cytoplasmic carboxyl terminal tail is only one to two amino acids long and therefore lacks both palmitoylation and phosphorylation sites (Navratil *et al.* 2006).

The thioester bond linking palmitate to cysteine is labile, thus making the attachment of palmitate to proteins reversible and the turnover rate of the bound fatty acid generally shorter than that of the protein. The addition and removal of palmitate are respectively enzymatically catalysed by palmitoyltransferases (PATs) and thioesterases. Identifying these enzymes proved to be a very challenging task, and it is only recently that a family of proteins with PAT activity has been identified in yeast by means of genetic screening. They all share a common motif referred to as 'DHHC', which is enriched in cysteines and contains a conserved aspartate-histidine-histidine-cysteine signature (see Mitchell *et al.* 2006 for a review). So far, one enzyme (APT1) has been purified from rat liver cytosol that removes palmitate from certain proteins on the cytosolic surface of membranes (Duncan & Gilman

1998). The non-enzymatic palmitoylation of proteins has also been reported, including membrane-bound rhodopsin (Veit *et al.* 1998).

As it is a dynamic process, the extent of palmitoylation is subject to regulation. In the case of GPCRs, this often occurs as a result of agonist-induced activation: e.g. isoproterenol stimulation of the β_2 -adrenergic receptor increases [^3H]palmitate incorporation (Mouillac *et al.* 1992). This increased receptor labelling can be interpreted in two ways: i) agonist binding increases the proportion of receptor being palmitoylated, or ii) agonist binding increases the turnover rate of receptor-linked palmitate, hence it favours the exchange of unlabelled for tritiated palmitate. The latter explanation, which implies an agonist-induced acceleration of depalmitoylation, has been experimentally supported in relation to the β_2 -adrenergic receptor (Loisel *et al.* 1999) and other GPCRs, including dopamine D1 (Ng *et al.* 1994), $\alpha_1\text{B}$ - (Stevens *et al.* 2001) and $\alpha_2\text{A}$ -adrenergic (Kennedy & Limbird 1994), serotonin 4A (Ponimaskin *et al.* 2002) and δ -opioid receptors (Petaja-Repo *et al.* 2006). However, it is not true for every GPCR: e.g. the palmitoylation of serotonin 1A receptors is not at all affected by agonist exposure (Papoucheva *et al.* 2004).

Activation by an agonist is not the only way in which the GPCR palmitoylation rate can be affected. Adam *et al.* (1999) have shown that high nitric oxide levels reduce both the basal and the isoproterenol-induced incorporation of [^3H]palmitate into β_2 -adrenergic receptors.

How to study cholesterol–GPCR interactions and palmitoylation: a few technical tips

Assaying the effects of cholesterol has proved to be relatively easy since the introduction of cholesterol-depleting agents, such as cyclodextrins (Christian *et al.* 1997, Zidovetzki & Levitan 2007), and cholesterol synthesis inhibitors, such as statins (Brown & Goldstein 1980). However, such procedures always have both direct and indirect effects, due to the alterations of the physico-chemical properties of the plasma membrane and of membrane domain organisation. On the contrary, measuring direct cholesterol binding to membrane proteins, in general (and GPCRs in particular), is an extremely daunting task. Tritiated steroids can be used to measure cholesterol affinity directly in saturation and/or competition assays usually involving purified proteins, as can be seen from the very elegant work recently done to characterise cholesterol binding to the binding domain of SCAP (Radhakrishnan *et al.* 2004) and NCP1 (Infante *et al.* 2008a,b). Photo-activatable cholesterol can also be used to map

protein–cholesterol interactions, as originally described in Thiele *et al.* (2000); in this case, binding can be performed *in vivo* but, after labelling, an immunoprecipitation or a pull-down step is necessary to detect the incorporation of the reactive steroid in the protein of interest. Finally, the proximity of cholesterol to the membrane nicotinic acetylcholine receptor was deduced from a series of experiments designed to quench the fluorescence of tryptophan residues in the receptor using spin-labelled cholesterol and phospholipid analogues (Jones & McNamee 1988).

The conventional approach to assessing the palmitoylation of proteins (including GPCRs) involves the metabolic labelling of cultured cells expressing the protein of interest with [^3H]palmitate, followed by immunoprecipitation, SDS gel electrophoresis and fluorography. The latter is necessary in order to increase the low detection limit of [^3H], which takes a very long time (weeks or even months) of gel exposure on films. Maximum sensitivity can be obtained by soaking the gels in 2,5-diphenyloxazole, according to the procedure described by Bonner & Laskey (1974), and the signal can be further enhanced by shrinking the gels in polyethylene glycol 2000 or 4000 (Magee *et al.* 1995).

Palmitoylation sites can be identified by replacing predicted cysteines with alanines, glycines or serines using site-directed mutagenesis. However, serine is not ideal because its use as an alternative palmitoylation site via an ester linkage has been documented in membrane-associated proteins such as the human transferrin receptor (Jing & Trowbridge 1990) and even a GPCR, the CCR5 receptor (Percherancier *et al.* 2001).

Some studies designed to clarify the functional consequences of GPCR palmitoylation have used 2-bromopalmitate to inhibit the reaction (see for example Petaja-Repo *et al.* 2006), as initially suggested by Webb *et al.* (2000). However, caution is necessary because inhibition by 2-bromopalmitate extends to all palmitoylated proteins (including the immediate α -protein α -subunit partners of GPCRs), and thus it may well affect their functions as well.

Cholesterol regulation of GPCR function

Cholesterol depletion experiments have shown that it is very difficult to distinguish the direct and indirect effects of cholesterol on receptor function. Although the effects of cholesterol depletion on several functions have been reported for many GPCRs (Chini & Parenti 2004, Pucadyil & Chattopadhyay 2006, Patel *et al.* 2008), here we describe only a few GPCRs for which direct and specific effects of cholesterol have been clearly documented.

In the case of the human oxytocin receptor (OTR), it has been shown that high-affinity agonist (but not antagonist) binding is highly dependent on the cholesterol content of the plasma membrane (Klein *et al.* 1995). The specificity of cholesterol was determined by means of structure–activity analyses of a large number of steroids replacing cholesterol and, as only some were capable of restoring the affinity of OTR in cholesterol-depleted membranes, the authors concluded that indirect effects on the physico-chemical properties of the membrane could be excluded (Gimpl *et al.* 1997). Modulation of ligand binding affinity has also been shown in the case of the galanin receptor, the specificity of which was again supported by experiments in which only a limited number of steroids could restore agonist affinity to cholesterol-depleted membranes (Pang *et al.* 1999). Interestingly, biochemical and fluorescence recovery after photobleaching experiments have shown that only a small fraction of OTRs seem to be located in lipid rafts (Gimpl & Fahrenholz 2000, Reversi *et al.* 2006); as this receptor fraction is capable of activating specific signalling events (the stimulation of cell growth), cholesterol may be involved in modulating very selective receptor-induced signalling events (Guzzi *et al.* 2002, Rimoldi *et al.* 2003). However, molecular modelling and mutagenesis studies have thus far failed to map any directed binding sites in the transmembrane regions (Politowska *et al.* 2001), and the cholesterol-binding region of receptor has not yet been identified.

Functional consequences of palmitoylation

It has been shown that all of the properties of GPCRs are affected by the absence of palmitoylation, but the effects depend on the type of receptor.

Receptor maturation and membrane delivery

It has been reported that the lack of palmitoylation reduces the number of receptors reaching the cell surface in various GPCRs, including dopamine D1 (Ng *et al.* 1994), vasopressin V2 (Schulein *et al.* 1996, Sadeghi *et al.* 1997), TSH (Tanaka *et al.* 1998), chemokine CCR5 (Percherancier *et al.* 2001), histamine H2 (Fukushima *et al.* 2001) and δ -opioid receptors (Petaja-Repo *et al.* 2006). This suggests that early palmitate binding, presumably at the level of the endoplasmic reticulum, is required for the correct processing of a number of immature and mature GPCRs, as well as for receptor progression along the biosynthetic route from synthesis to plasma membrane localisation.

Compartmentalisation of the serotonin 1A receptor within sphingolipid/cholesterol-rich membrane

domains (lipid rafts and caveolae; see Chini & Parenti 2004 for a review), measured as the association with Triton X100-insoluble floating fractions (TIFFs), also depends on palmitoylation (Renner *et al.* 2007). However, this is not a general rule because the GnRH receptor, which lacks palmitoylation sites (see above), is constitutively associated with TIFFs (Navratil *et al.* 2006). Interestingly, fusion of the palmitoylated carboxyl terminal of the LH receptor (normally excluded from lipid rafts) to the GnRH receptor removes the chimeric GnRH/LH receptor from TIFFs (Navratil *et al.* 2006), whereas fusion of the palmitoylated carboxyl terminal from non-mammalian GnRH receptors preserves the TIFF localisation, thus suggesting that neither the extension of the carboxyl terminal extension nor palmitoylation are signatures for raft targeting.

Coupling to G protein and signalling

Palmitoylation can markedly influence receptor coupling to G proteins, and hence the intracellular strength of signalling, or have no effect. The agonist-induced stimulation of adenylyl cyclase by dopamine D1 (Jin *et al.* 1997) and serotonin 4A receptors (Ponimaskin *et al.* 2002) is not affected by mutated palmitoylation sites, whereas the isoproterenol stimulation of adenylyl cyclase mediated by the palmitoylation-negative C341G mutant of the β_2 -adrenergic receptor is greatly reduced. This and the decreased ability of C341G to form a guanyl nucleotide-sensitive, high-affinity state for agonists indicate that palmitate attachment is necessary for efficient coupling between receptor and G_s protein (O'Dowd *et al.* 1989). Later studies offered insights into the mechanism underlying such uncoupling by showing that C341 is constitutively highly phosphorylated, and thus behaves as a wild-type receptor desensitised by the agonist (Moffett *et al.* 1993). The increased phosphorylation is due to the greater accessibility of C341 to protein kinase A in comparison with the wild-type receptor (Moffett *et al.* 1996). These findings, together with the observation that the palmitoylation of cysteine-341 is dynamically regulated by the agonist (Loisel *et al.* 1999), highlight the key role played by the addition/removal of palmitate in regulating the phosphorylation of β_2 -adrenergic receptors and, hence, their signalling efficiency.

Palmitoylation can also 'direct' pleiotropic GPCRs to signal preferentially through a certain pathway. This has been shown in the case of endothelin receptor A, in which mutation of the palmitoylation sites does not affect the agonist stimulation of adenylyl cyclase but impairs phospholipase C activation, thus suggesting that the presence of palmitate regulates the coupling to G_q , but not to G_s (Horstmeyer *et al.* 1996). Likewise, the

V2 receptor-linked MAPK (but not the adenylyl cyclase) pathway is affected by the palmitoylation state of the receptor (Charest & Bouvier 2003).

Desensitisation and internalisation

As agonist stimulation usually promotes GPCR desensitisation and internalisation (as well as signalling), it is not surprising that palmitoylation also regulates these processes as mentioned above in the case of the β_2 -adrenergic receptor. However, different mechanisms apply to the various receptors. It has been reported that the palmitoylationless V2 receptor undergoes agonist-promoted internalisation less efficiently than the wild-type receptor (Charest & Bouvier 2003), and so the palmitoylation state modulates both signalling (MAPK) and endocytosis. The reduced internalisation positively correlates with less recruitment of β -arrestin2 to the non-palmitoylated receptor. Interestingly, the phosphorylation state of the mutant is not affected, thus suggesting that palmitoylation *per se* regulates the receptor's affinity for β -arrestin.

The picture is completely different in the case of the serotonin 4A receptor (Ponimaskin *et al.* 2005), which has a complex palmitoylation pattern at two adjacent cysteines (328 and 329) and a more distal cysteine-386. The combined substitution of cysteines 328 and 329 with serines generates a constitutively active mutant that is hyperphosphorylated and more effectively desensitised and internalised than the wild-type receptor through a β -arrestin2-dependent mechanism. On the contrary, the C386S and C328/329/386S mutants do not show any constitutive activity despite being as highly phosphorylated as the C328/329S mutant. In addition, the agonist-induced desensitisation and internalisation of single and triple mutants are less efficient than those of the double mutant. There is thus a complex interplay between site-dependent palmitoylation and phosphorylation and their related functional consequences, while not forgetting that the palmitoylation state of serotonin 4A receptor is dynamically regulated by agonist activation (Ponimaskin *et al.* 2001).

In essence, palmitoylation can do everything to GPCRs, and no predictions can be made concerning

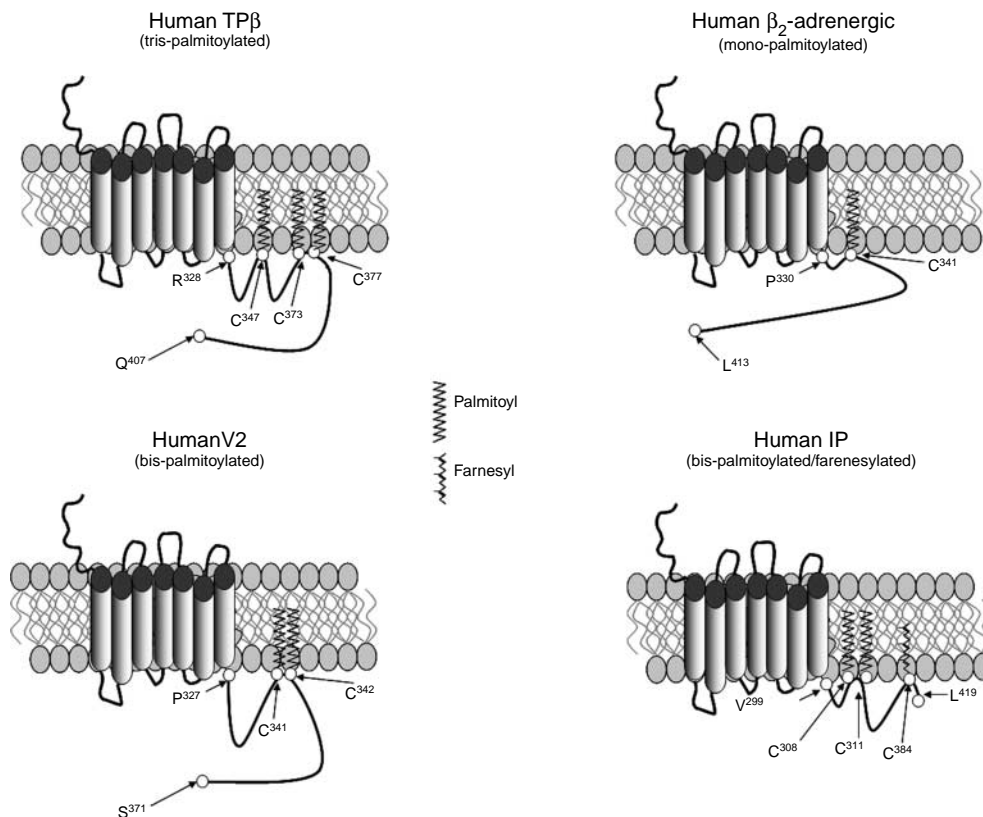


Figure 1 Some examples of lipidation patterns affecting the carboxyl terminals of GPCRs. The insertion of lipid anchors (palmitoyl and/or farnesyl) into the plasma membrane can cause the formation of one or more intracellular loops that change the topology of carboxyl terminals, thus affecting interactions with receptor partner proteins. Palmitate-dependent loops can be disrupted by agonist-promoted depalmitoylation, whereas an isoprenyl loop (IP receptor) is stable. TP β , the β -isoform of the thromboxane A2 receptor; V2, vasopressin V2 receptor; IP, prostacyclin receptor.

its functional relevance for a particular GPCR until experimental proof is obtained. The logical question to ask is how the attachment of palmitate(s) to the same domain of GPCRs can subserve so many different roles. Is the impact of palmitoylation influenced in any way by the length of the fourth intracellular loop formed upon the membrane insertion of palmitate and/or by the presence of other post-translational modification(s) (i.e. phosphorylation) within the loop? Does the presence of one or more palmitate(s) make any difference, by forming one or more loop(s) or making the loop(s) more or less flexible? These questions need to be addressed in a systematic way, although the task is extremely challenging given the number of different variables to take into account.

What is certain is the fact that, as was suggested by Miggin *et al.* (2003), 'looping' of the carboxyl terminal (Fig. 1) is important in the case of the prostacyclin receptor. Its tail can form two loops, one proximal to the seventh α -helix and reversibly anchored to the membrane by palmitates bound to cysteines 308 and 311, and a more distal one formed by the stable membrane insertion of a farnesyl isoprenoid bound to cysteine-383. Depending on which loop is present, the receptor signals through different effectors, i.e. adenylyl cyclase and/or phospholipase C.

Palmitoylation and cholesterol: filling a structural gap?

Moench *et al.* (1994) used fluorescent derivatives of palmitate incorporated into rhodopsin to show that the two fatty acyl chains reside within the membrane and are not held in the protein core (Moench *et al.* 1994). This finding induced them to hypothesise that the insertion of palmitate into the lipid bilayer creates a fourth intracellular loop between the cytoplasmic end of α -helix 7 and fatty acid anchors. The X-ray crystal structure of rhodopsin has indeed confirmed that there is a fourth intracellular loop consisting of an α -helix (α -helix 8) that lies parallel to the plane of the membrane and is kept in position by the palmitate anchor which thus plays an important structural role.

In addition to a structural role inside monomeric receptors, palmitate anchors may play an important role in forming/stabilising receptor oligomers; as in the recently resolved X-ray structures of β_2 -adrenergic receptors, it has been found that mono-palmitoylated receptor moieties are assembled in supramolecular complexes by interactions occurring via their lipidic anchors (Cherezov *et al.* 2007). Notably, two cholesterol molecules were intercalated within the palmitic residues, and contribute to filling the adjacent protomer interfaces (Fig. 2). On the basis of these observations, it seems that GPCR dimerisation essen-

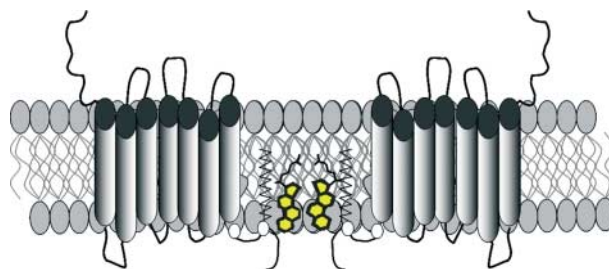


Figure 2 Schematic of palmitate- and cholesterol-mediated interfaces deduced from β_2 -adrenergic receptor crystals. In the recently resolved X-ray structures of β_2 -adrenergic receptors (Cherezov *et al.* 2007), receptor dimers appear to be organised in complexes whose major interactions are mediated by palmitate residues, bridged by cholesterol molecules; here, depicted in yellow.

tially takes place as a result of lipid-mediated interactions. Although these findings may not correspond to the *in vivo* situation (cholesterol is added during the purification procedure to help the crystallisation process), it is clear that the role of palmitoylation and cholesterol in oligomerisation is an intriguing question that needs to be carefully examined.

Thus far, the effect of palmitoylation on oligomerisation has only been investigated in serotonin 1A receptors by Kobe *et al.* (2008), who used the fluorescence resonance energy transfer (FRET) technique in murine N1E-115 neuroblastoma cells transiently co-transfected with receptor chimeras fused to cyan and yellow fluorescent proteins. They observed that the agonist decreased FRET intensity in wild-type receptors, but increased it in palmitoylation-negative receptors. These shifts from negative to positive FRET have been interpreted as changes in the way in which palmitoylated or non-palmitoylated receptors are spatially oriented within oligomers. As palmitoylation positively regulates the number of serotonin 1A receptors residing within rafts (Renner *et al.* 2007), it can also be suggested that raft oligomers differ from non-raft oligomers, but the functional implications of these findings have still to be ascertained.

Final remarks

Despite years of effort, a number of key aspects of GPCRs are still a matter of investigation. As the active and inactive states of heterotrimeric complex structures have now been solved, the G-protein cycle is fairly well documented and understood; however, there are still no conclusive data concerning the dynamics of the association between GPCRs and the G-protein α subunits, and their physical dissociation during the activation process is still a matter of debate. Similarly, the specificity of receptor coupling to different G proteins has not been definitely mapped. There is also

controversy concerning the monomeric/oligomeric GPCR state that may best accomplish G-protein docking and subsequent activation. Palmitoylation and/or cholesterol binding may play a more significant role than previously thought in all of these events, and more exhaustive studies of GPCR–lipid interactions may well contribute to exciting insights and advances in these fields.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by grants from the Italian Association for Cancer Research (AIRC 2006) and Fondazione Cariplo (grant 2006.0882) to B Chini, and a FIRB grant (RBIN04CKYN) from the Ministry for Universities and Research (MIUR) to M Parenti.

References

- Adam L, Bouvier M & Jones TL 1999 Nitric oxide modulates beta(2)-adrenergic receptor palmitoylation and signaling. *Journal of Biological Chemistry* **274** 26337–26343.
- Albert AD & Boesze-Battaglia K 2005 The role of cholesterol in rod outer segment membranes. *Progress in Lipid Research* **44** 99–124.
- Alpy F & Tomasetto C 2005 Give lipids a START: the StAR-related lipid transfer (START) domain in mammals. *Journal of Cell Science* **118** 2791–2801.
- Alves ID, Salamon Z, Hruby VJ & Tollin G 2005 Ligand modulation of lateral segregation of a G-protein-coupled receptor into lipid microdomains in sphingomyelin/phosphatidylcholine solid-supported bilayers. *Biochemistry* **44** 9168–9178.
- Bonner WM & Laskey RA 1974 A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *European Journal of Biochemistry* **46** 83–88.
- Branton WD, Rudnick MS, Zhou Y, Eccleston ED, Fields GB & Bowers LD 1993 Fatty acylated toxin structure. *Nature* **365** 496–497.
- Brown MS & Goldstein JL 1980 Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *Journal of Lipid Research* **21** 505–517.
- Charest PG & Bouvier M 2003 Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *Journal of Biological Chemistry* **278** 41541–41551.
- Chen C, Shahabi V, Xu W & Liu-Chen LY 1998 Palmitoylation of the rat mu opioid receptor. *FEBS Letters* **441** 148–152.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK *et al.* 2007 High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318** 1258–1265.
- Chini B & Parenti M 2004 G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *Journal of Molecular Endocrinology* **32** 325–328.
- Christian AE, Haynes MP, Phillips MC & Rothblat GH 1997 Use of cyclodextrins for manipulating cellular cholesterol content. *Journal of Lipid Research* **38** 2264–2272.
- Duncan JA & Gilman AG 1998 A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). *Journal of Biological Chemistry* **273** 15830–15837.
- Fukushima Y, Saitoh T, Anai M, Ogihara T, Inukai K, Funaki M, Sakoda H, Onishi Y, Ono H, Fujishiro M *et al.* 2001 Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochimica et Biophysica Acta* **1539** 181–191.
- Gimpl G & Fahrenholz F 2000 Human oxytocin receptors in cholesterol-rich vs. cholesterol-poor microdomains of the plasma membrane. *European Journal of Biochemistry* **267** 2483–2497.
- Gimpl G, Burger K & Fahrenholz F 1997 Cholesterol as modulator of receptor function. *Biochemistry* **36** 10959–10974.
- Guzzi F, Zanchetta D, Cassoni P, Guzzi V, Francolini M, Parenti M & Chini B 2002 Localisation of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response. *Oncogene* **21** 1658–1667.
- Horstmeyer A, Cramer H, Sauer T, Muller-Esterl W & Schroeder C 1996 Palmitoylation of endothelin receptor A. Differential modulation of signal transduction activity by post-translational modification. *Journal of Biological Chemistry* **271** 20811–20819.
- Ikonen E 2008 Cellular cholesterol trafficking and compartmentalization. *Nature Reviews. Molecular Cell Biology* **9** 125–138.
- Infante RE, Abi-Mosleh L, Radhakrishnan A, Dale JD, Brown MS & Goldstein JL 2008a Purified NPC1 protein. I. Binding of cholesterol and oxysterols to a 1278-amino acid membrane protein. *Journal of Biological Chemistry* **283** 1052–1063.
- Infante RE, Radhakrishnan A, Abi-Mosleh L, Kinch LN, Wang ML, Grishin NV, Goldstein JL & Brown MS 2008b Purified NPC1 protein: II. Localization of sterol binding to a 240-amino acid soluble luminal loop. *Journal of Biological Chemistry* **283** 1064–1075.
- Jensen MO & Mouritsen OG 2004 Lipids do influence protein function – the hydrophobic matching hypothesis revisited. *Biochimica et Biophysica Acta* **1666** 205–226.
- Jin H, Zastawny R, George SR & O'Dowd BF 1997 Elimination of palmitoylation sites in the human dopamine D1 receptor does not affect receptor-G protein interaction. *European Journal of Pharmacology* **324** 109–116.
- Jing SQ & Trowbridge IS 1990 Nonacylated human transferrin receptors are rapidly internalized and mediate iron uptake. *Journal of Biological Chemistry* **265** 11555–11559.
- Jones OT & McNamee MG 1988 Annular and nonannular binding sites for cholesterol associated with the nicotinic acetylcholine receptor. *Biochemistry* **27** 2364–2374.
- Kennedy ME & Limbird LE 1994 Palmitoylation of the alpha 2A-adrenergic receptor. Analysis of the sequence requirements for and the dynamic properties of alpha 2A-adrenergic receptor palmitoylation. *Journal of Biological Chemistry* **269** 31915–31922.
- Klein U, Gimpl G & Fahrenholz F 1995 Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry* **34** 13784–13793.
- Kleuss C & Krause E 2003 Galpha(s) is palmitoylated at the N-terminal glycine. *EMBO Journal* **22** 826–832.
- Kobe F, Renner U, Woehler A, Wlodarczyk J, Papisheva E, Bao G, Zeug A, Richter DW, Neher E & Ponimaskin E 2008 Stimulation- and palmitoylation-dependent changes in oligomeric conformation of serotonin 5-HT1A receptors. *Biochimica et Biophysica Acta* **1783** 1503–1516.
- Lange Y 1991 Disposition of intracellular cholesterol in human fibroblasts. *Journal of Lipid Research* **32** 329–339.
- Lee AG 2004 How lipids affect the activities of integral membrane proteins. *Biochimica et Biophysica Acta* **1666** 62–87.
- Li H & Papadopoulos V 1998 Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. *Endocrinology* **139** 4991–4997.
- Liang X, Lu Y, Wilkes M, Neubert TA & Resh MD 2004 The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation

- and heterogeneous fatty acylation: role in membrane targeting, cell adhesion, and spreading. *Journal of Biological Chemistry* **279** 8133–8139.
- Loisel TP, Ansanay H, Adam L, Marullo S, Seifert R, Lagace M & Bouvier M 1999 Activation of the beta(2)-adrenergic receptor-Galpha(s) complex leads to rapid depalmitoylation and inhibition of repalmitoylation of both the receptor and Galpha(s). *Journal of Biological Chemistry* **274** 31014–31019.
- Magée AI, Wootton J & de Bony J 1995 Detecting radiolabeled lipid-modified proteins in polyacrylamide gels. *Methods in Enzymology* **250** 330–336.
- Miggin SM, Lawler OA & Kinsella BT 2003 Palmitoylation of the human prostacyclin receptor. Functional implications of palmitoylation and isoprenylation. *Journal of Biological Chemistry* **278** 6947–6958.
- Mitchell DA, Vasudevan A, Linder ME & Deschenes RJ 2006 Protein palmitoylation by a family of DHHC protein S-acyltransferases. *Journal of Lipid Research* **47** 1118–1127.
- Moench SJ, Moreland J, Stewart DH & Dewey TG 1994 Fluorescence studies of the location and membrane accessibility of the palmitoylation sites of rhodopsin. *Biochemistry* **33** 5791–5796.
- Moffett S, Mouillac B, Bonin H & Bouvier M 1993 Altered phosphorylation and desensitization patterns of a human beta 2-adrenergic receptor lacking the palmitoylated Cys341. *EMBO Journal* **12** 349–356.
- Moffett S, Adam L, Bonin H, Loisel TP, Bouvier M & Mouillac B 1996 Palmitoylated cysteine 341 modulates phosphorylation of the beta2-adrenergic receptor by the cAMP-dependent protein kinase. *Journal of Biological Chemistry* **271** 21490–21497.
- Mouillac B, Caron M, Bonin H, Dennis M & Bouvier M 1992 Agonist-modulated palmitoylation of beta 2-adrenergic receptor in Sf9 cells. *Journal of Biological Chemistry* **267** 21733–21737.
- Mouritsen OG & Zuckermann MJ 2004 What's so special about cholesterol? *Lipids* **39** 1101–1113.
- Murata M, Peranen J, Schreiner R, Wieland F, Kurzchalia TV & Simons K 1995 VIP21/caveolin is a cholesterol-binding protein. *PNAS* **92** 10339–10343.
- Navratil AM, Farmerie TA, Bogerd J, Nett TM & Clay CM 2006 Differential impact of intracellular carboxyl terminal domains on lipid raft localization of the murine gonadotropin-releasing hormone receptor. *Biology of Reproduction* **74** 788–797.
- Ng GY, Mouillac B, George SR, Caron M, Dennis M, Bouvier M & O'Dowd BF 1994 Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. *European Journal of Pharmacology* **267** 7–19.
- O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ & Bouvier M 1989 Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *Journal of Biological Chemistry* **264** 7564–7569.
- Ohvo-Rekila H, Ramstedt B, Leppimäki P & Slotte JP 2002 Cholesterol interactions with phospholipids in membranes. *Progress in Lipid Research* **41** 66–97.
- Ovchinnikov YuA, Abdulaev NG & Bogachuk AS 1988 Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated. *FEBS Letters* **230** 1–5.
- Pang L, Graziano M & Wang S 1999 Membrane cholesterol modulates galanin-GalR2 interaction. *Biochemistry* **38** 12003–12011.
- Papoucheva E, Dumuis A, Sebben M, Richter DW & Ponimaskin EG 2004 The 5-hydroxytryptamine (1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *Journal of Biological Chemistry* **279** 3280–3291.
- Parton RG, Hanzal-Bayer M & Hancock JF 2006 Biogenesis of caveolae: a structural model for caveolin-induced domain formation. *Journal of Cell Science* **119** 787–796.
- Patel HH, Murray F & Insel PA 2008 G-protein-coupled receptor-signaling components in membrane raft and caveolae microdomains. *Handbook of Experimental Pharmacology* **186** 167–184.
- Pepinsky RB, Zeng C, Wen D, Rayhorn P, Baker DP, Williams KP, Bixler SA, Ambrose CM, Garber EA, Miatkowski K *et al.* 1998 Identification of a palmitic acid – modified form of human Sonic hedgehog. *Journal of Biological Chemistry* **273** 14037–14045.
- Percherancier Y, Planchenault T, Valenzuela-Fernandez A, Virelizier JL, Arenzana-Seisdedos F & Bachelier F 2001 Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *Journal of Biological Chemistry* **276** 31936–31944.
- Petaja-Repo UE, Hogue M, Leskela TT, Markkanen PM, Tuusa JT & Bouvier M 2006 Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *Journal of Biological Chemistry* **281** 15780–15789.
- Politowska E, Kazmierkiewicz R, Wiegand V, Fahrenholz F & Ciarkowski J 2001 Molecular modelling study of the role of cholesterol in the stimulation of the oxytocin receptor. *Acta Biochimica Polonica* **48** 83–93.
- Ponimaskin EG, Schmidt MF, Heine M, Bickmeyer U & Richter DW 2001 5-Hydroxytryptamine 4(a) receptor expressed in Sf9 cells is palmitoylated in an agonist-dependent manner. *Biochemical Journal* **353** 627–634.
- Ponimaskin EG, Heine M, Joubert L, Sebben M, Bickmeyer U, Richter DW & Dumuis A 2002 The 5-hydroxytryptamine(4a) receptor is palmitoylated at two different sites, and acylation is critically involved in regulation of receptor constitutive activity. *Journal of Biological Chemistry* **277** 2534–2546.
- Ponimaskin E, Dumuis A, Gaven F, Barthet G, Heine M, Glebov K, Richter DW & Oppermann M 2005 Palmitoylation of the 5-hydroxytryptamine4a receptor regulates receptor phosphorylation, desensitization, and beta-arrestin-mediated endocytosis. *Molecular Pharmacology* **67** 1434–1443.
- Pucadyil TJ & Chattopadhyay A 2006 Role of cholesterol in the function and organization of G-protein coupled receptors. *Progress in Lipid Research* **45** 295–333.
- Qanbar R & Bouvier M 2003 Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacology and Therapeutics* **97** 1–33.
- Radhakrishnan A, Sun LP, Kwon HJ, Brown MS & Goldstein JL 2004 Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. *Molecular Cell* **15** 259–268.
- Reid HM & Kinsella BT 2007 Palmitoylation of the TPbeta isoform of the human thromboxane A2 receptor. Modulation of G protein: effector coupling and modes of receptor internalization. *Cellular Signalling* **19** 1056–1070.
- Renner U, Glebov K, Lang T, Papisheva E, Balakrishnan S, Keller B, Richter DW, Jahn R & Ponimaskin E 2007 Localization of the mouse 5-hydroxytryptamine (1A) receptor in lipid microdomains depends on its palmitoylation and is involved in receptor-mediated signaling. *Molecular Pharmacology* **72** 502–513.
- Reversi A, Rimoldi V, Brambillasca S & Chini B 2006 Effects of cholesterol manipulation on the signaling of the human oxytocin receptor. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **291** R861–R869.
- Rimoldi V, Reversi A, Taverna E, Rosa P, Francolini M, Cassoni P, Parenti M & Chini B 2003 Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains. *Oncogene* **22** 6054–6060.
- Ruprecht JJ, Mielke T, Vogel R, Villa C & Schertler GF 2004 Electron crystallography reveals the structure of metarhodopsin I. *EMBO Journal* **23** 3609–3620.
- Sadeghi HM, Innamorati G, Dagarag M & Birnbaumer M 1997 Palmitoylation of the V2 vasopressin receptor. *Molecular Pharmacology* **52** 21–29.
- Salamon Z, Cowell S, Varga E, Yamamura HI, Hruba VJ & Tollin G 2000 Plasmon resonance studies of agonist/antagonist binding to the human delta-opioid receptor: new structural insights into receptor-ligand interactions. *Biophysical Journal* **79** 2463–2474.

- Schertler GF & Hargrave PA 2000 Preparation and analysis of two-dimensional crystals of rhodopsin. *Methods in Enzymology* **315** 91–107.
- Schulein R, Liebenhoff U, Muller H, Birnbaumer M & Rosenthal W 1996 Properties of the human arginine vasopressin V2 receptor after site-directed mutagenesis of its putative palmitoylation site. *Biochemical Journal* **313** 611–616.
- Simons K & Ikonen E 2000 How cells handle cholesterol. *Science* **290** 1721–1726.
- Simons K & Toomre D 2000 Lipid rafts and signal transduction. *Nature Reviews. Molecular Cell Biology* **1** 31–39.
- Stanley P, Koronakis V & Hughes C 1998 Acylation of *Escherichia coli* hemolysin: a unique protein lipidation mechanism underlying toxin function. *Microbiology and Molecular Biology Reviews* **62** 309–333.
- Stevens PA, Pediani J, Carrillo JJ & Milligan G 2001 Coordinated agonist regulation of receptor and G protein palmitoylation and functional rescue of palmitoylation-deficient mutants of the G protein G11alpha following fusion to the alpha1b-adrenoreceptor: palmitoylation of G11alpha is not required for interaction with beta*gamma complex. *Journal of Biological Chemistry* **276** 35883–35890.
- Suchanek M, Hynynen R, Wohlfahrt G, Lehto M, Johansson M, Saarinen H, Radzikowska A, Thiele C & Olkkonen VM 2007 The mammalian oxysterol-binding protein-related proteins (ORPs) bind 25-hydroxycholesterol in an evolutionarily conserved pocket. *Biochemical Journal* **405** 473–480.
- Tanaka K, Nagayama Y, Nishihara E, Namba H, Yamashita S & Niwa M 1998 Palmitoylation of human thyrotropin receptor: slower intracellular trafficking of the palmitoylation-defective mutant. *Endocrinology* **139** 803–806.
- Thiele C, Hannah MJ, Fahrenholz F & Huttner WB 2000 Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nature Cell Biology* **2** 42–49.
- Veit M, Sachs K, Heckelmann M, Maretzki D, Hofmann KP & Schmidt MF 1998 Palmitoylation of rhodopsin with S-protein acyltransferase: enzyme catalyzed reaction versus autocatalytic acylation. *Biochimica et Biophysica Acta* **1394** 90–98.
- Webb Y, Hermida-Matsumoto L & Resh MD 2000 Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *Journal of Biological Chemistry* **275** 261–270.
- Zidovetzki R & Levitan I 2007 Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochimica et Biophysica Acta* **1768** 1311–1324.

Received in final form 17 November 2008

Accepted 8 January 2009

Made available online as an Accepted Preprint 8 January 2009