G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there?

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Abstract

This review describes the advances in our understanding of the role of G-protein coupled receptor (GPCR) localisation in membrane microdomains known as lipid rafts and caveolae. The growing interest in these specialised regions is due to the recognition that they are involved in the regulation of a number of cell functions, including the fine-tuning of various signalling molecules. As a number of GPCRs have been found to be enriched in lipid rafts and/or caveolae by means of different experimental approaches, we first discuss the pitfalls and uncertainties related to the use of these different procedures. We then analyse the addressing signals that drive and/or stabilise GPCRs in lipid rafts and caveolae, and explore the role of rafts/caveolae in regulating GPCR trafficking, particularly in receptor exo- and endocytosis. Finally, we review the growing evidence that lipid rafts and caveolae participate in the regulation of GPCR signalling by affecting both signalling selectivity and coupling efficacy.

Journal of Molecular Endocrinology (2004) **32**, 325–338

Introduction

Lipid rafts and caveolae have been found to be involved in the regulation of various cell functions, including the homeostasis of cholesterol (Ikonen & Parton 2000), the intracellular sorting of proteins and lipids (Sprong *et al.* 2001), the establishment of cell polarity (Manes *et al.* 2003), a number of vesicular transport processes such as transcytosis, endocytosis and potocytosis (Johannes & Lamaze 2002, Conner & Schmid 2003, Nabi & Le 2003) and, finally, the fine-tuning of components of the cell signalling machinery located on the cell surface (Simons & Ikonen 1997, Okamoto *et al.* 1998, Simons & Toomre 2000). A number of receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), G-proteins, kinases and phosphatases have been located in lipid rafts and/or caveolae, and the efforts to define them have recently culminated in the proteomic identification of lipid raft signalling components (Foster *et al.* 2003).

However, although the role of lipid rafts in regulating some transduction pathways is now well established, such as the assembly of the various components of the immune receptor signalling

cascade (Dykstra *et al.* 2001, Sedwick & Altman 2002, Werlen & Palmer 2002), unravelling the role of rafts and caveolae in the functional regulation of other signalling components is still at its very beginning.

We here review the growing evidence that lipid rafts and caveolae actively participate in regulating the signalling and trafficking of a number of GPCRs.

GPCRs in lipid rafts/caveolae (a matter of definitions?)

Lipid rafts are planar domains of cell membranes enriched in specific lipids and proteins. In particular, they are characterised by a high glycosphingolipid and cholesterol content in the outer leaflet of the lipid bilayer that gives them a gel-like liquid-ordered (Lo) organisation in comparison with the surrounding phospholipid-rich disordered membrane (Brown & London 1998, Vereb *et al.* 2003). Because of their particular biochemical and biophysical nature, lipid rafts are resistant to low-temperature solubilisation by

non-ionic detergents such as Triton X-100, a property that allows their separation by means of differential flotation after density-gradient centrifugation (London & Brown 2000). When whole-cell detergent lysates are loaded on the bottom of density gradients and centrifuged, non-solubilised membrane 'islets' including lipid rafts float toward the lighter surface fractions, and are thus separated from the bulk of the soluble cellular lipids and proteins remaining in the bottom loading zone. Low-buoyancy density fractions have been variously called detergent-insoluble glycolipid-enriched membrane domains, glycosphingolipid-enriched membranes or detergent-resistant membranes (DRMs). Although the exact correlation between DRMs and lipid rafts in living cells is still unknown, the partitioning of a protein in DRMs suggests its localisation in lipid rafts. However, it must be remembered that fractionation procedures are certainly not devoid of pitfalls (Shogomori & Brown 2003) as it has been shown that the degree of lipid raft/caveolae solubilisation depends upon the temperature, the kind of detergent and the extraction conditions. The importance of each of these variables means that a given protein may be identified as being associated with DRMs or not, thus explaining the conflicting results obtained in different laboratories (Schuck *et al.* 2003).

Furthermore, the morphological identification of lipid rafts is still elusive; they are probably too small and/or too dynamic to be detected by means of conventional microscopy, and so a number of new biophysical, microscopic and imaging techniques are being developed in the hope of being able to see them in living cells (reviewed in Lai 2003, Ritchie *et al.* 2003, Vereb *et al.* 2003).

On the contrary, caveolae were first defined on the basis of their electron microscopy morphological appearance as 50–100 nm diameter flask-shaped invaginations located at or near the plasma membrane of some, but not all cells (Palade 1953, Yamada 1955). The shape and structural organisation of caveolae are due to the presence of a specific set of proteins (caveolin-1, -2 and -3) that self-assemble in high-mass oligomers to form a cytoplasmic coat on the membrane invaginations.

As caveolae membranes are as highly enriched in cholesterol and glycosphingolipids as rafts, caveolae are considered to be a non-planar subfamily of lipid rafts. Upon detergent extraction and density-gradient centrifugation, caveolae membranes and

their associated proteins end up in the same subcellular fractions (DRMs) as other lipid rafts. DRMs thus contain a heterogeneous mixture of lipid domains (in the simplest case, rafts plus caveolae), which means that it should not be concluded that a GPCR is localised in caveolae simply on the basis of its partitioning in caveolin-containing DRMs. In order to characterise the signalling molecules, including GPCRs (see Table 1), present in caveolae, detergent-free extraction methods have been developed (Smart *et al.* 1995, Song *et al.* 1996). However, it must be kept in mind that membrane fractions separated by these procedures elude the definition of rafts/caveolae based on detergent insolubility. Most importantly, the lipid and protein composition of these fractions may differ from those of DRMs.

As caveolae can be properly defined by their morphology, the only incontrovertible evidence for GPCR localisation in caveolae is provided by electron microscopy. Furthermore, recent advances in electron microscopy of intact plasma membrane sheets allowed the localisation of a particular GPCR, the angiotensin (AT)₁ receptor, in regions of the plasma membrane not corresponding to caveolae (Wyse *et al.* 2003).

What about other experimental techniques? Confocal imaging is limited by the fact that its intrinsic spatial resolution power (0.2 µm) is more than the estimated diameters of lipid rafts (25–100 nm) or caveolae (50–100 nm); however, it can be very useful when investigating the intracellular trafficking of GPCRs: e.g. to dissect the pathways of internalisation (via caveolae vs clathrin-coated pits (CCPs)), or to study their movements to endosomes, lysosomes and other intracellular locations, especially in combination with markers of the different intracellular compartments. Finally, the fluorescence resonance energy transfer (FRET) technique seems to be promising because it allows GPCR localisation to be determined in the microdomains of living cells by detecting their close interaction (<100 nm) with established domain-resident proteins (e.g. caveolin) (Zacharias *et al.* 2002).

Co-immunoprecipitation of GPCRs and caveolin has also been widely used to support receptor localisation in caveolae. However, as caveolin localisation is not restricted to the plasma membrane and an important intracellular pool of caveolin exists in several cell types, receptor–

caveolin interactions may not only occur on the cell surface, but also in intracellular compartments and particularly along the exocytic pathway, as has been recently demonstrated in the case of the AT1 receptor (Wyse *et al.* 2003). AT1 receptor is located in fractions enriched with caveolin-1 and immunoprecipitates with caveolin, but it is not located in plasma membrane caveolae. In order to demonstrate receptor–caveolin interactions on the cell surface, co-immunoprecipitation experiments should be performed on purified plasma membrane fractions in which the vast majority of caveolin (>90%) is located in caveolae (van Deurs *et al.* 2003), thus supporting receptor localisation in these structures.

In this review, a GPCR will be referred to as located in lipid rafts when a biochemical procedure was used, and the term 'localisation in caveolae' will be reserved for the receptors whose presence in caveolae has been demonstrated by localisation at the electron microscopy level and/or by means of co-immunoprecipitation from plasma membrane fractions.

How do GPCRs go to lipid rafts/caveolae? (GPCR localisation signals)

Under basal conditions, some GPCRs are almost exclusively located in DRMs (more than 90% of the gonadotrophin-releasing hormone (GnRH) receptor) (Navratil *et al.* 2003), whereas others are present in a small amount (<10% of the oxytocin receptor (OTR)) (Gimpl & Fahrenholz 2000, Guzzi *et al.* 2002). There must therefore be one or more molecular determinants affecting the 'avidity' of certain GPCRs for lipid rafts/caveolae. But what are these targeting signals, and where do they reside within the receptor molecule? Furthermore, as some GPCRs can move inside or outside lipid rafts/caveolae upon activation, these determinants must be subject to dynamic modulation by one or more effectors.

In principle, targeting signals may be located in different regions of the GPCR molecule (Fig. 1).

They may be located in the extracellular part of the GPCRs, where they would interact with lipids and/or proteins on the outer leaflet of the lipid bilayer. In the epidermal growth factor receptor (EGFR), a single transmembrane protein shuttling inside/outside lipid rafts (Mineo *et al.* 1999, Roepstorff *et al.* 2002), it has been shown that the

interactions between the extracellular receptor region and GM1 gangliosides participate in targeting the protein to lipid rafts (Miljan *et al.* 2002). However, no similar evidence has yet been found to support the participation of the extracellular regions of GPCRs.

The transmembrane regions of GPCRs, which are embedded in the lipid bilayer, can dictate domain localisation by interacting with the lipid and/or protein components of rafts/caveolae. In particular, cholesterol (which is specifically enriched in lipid microdomains) has attracted attention as a possible determinant of GPCR localisation in lipid rafts. It has been known for some time that the affinity of some GPCRs can be modulated by cholesterol (Gimpl *et al.* 1997): for example, the human OTR localisation in lipid rafts positively correlates with an increased affinity for the agonist (Gimpl *et al.* 2000, Guzzi *et al.* 2002), a finding that has also very recently been demonstrated in the case of *Drosophila* mGluR, which directly binds to cholesterol (Eroglu *et al.* 2003). However, as some GPCRs do not change their agonist affinities on the basis of cholesterol content, this is probably not a general phenomenon (Gimpl *et al.* 1997). Molecular modelling attempts to dock cholesterol on GPCRs have also been carried out and have indicated the participation of alpha-helices in the formation of cholesterol-binding sites (Politowska *et al.* 2001). As the conformation of alpha-helices depends on the activation state of GPCRs, it is tempting to speculate that agonist binding may affect their localisation in lipid rafts by means of the same molecular mechanisms that lead to receptor activation: i.e. transmembrane rearrangements. Similarly, receptor oligomerisation also affects the orientation of the transmembranes helices and may thus modulate cholesterol binding and/or raft localisation.

Finally, the intracellular loops and carboxyl-terminal tail may all be involved in receptor targeting to lipid rafts by means of different addressing signals: fatty acylation and protein–protein interactions.

Fatty acylation

One possible mechanism for targeting proteins into rafts is their modification by the covalent attachment of myristic and palmitic fatty acids. It is expected that long-chain saturated fatty acids pack well in the Lo phase and increase protein

Table 1 List of GPCR localised to lipid rafts and/or caveolae

Receptor	Enrichment in DRMs	Enrichment in 'caveolar fractions'	Enrichment change upon agonist application	Interaction with caveolin (CoIP)	Localisation in caveolae (ME)	Internalisation via rafts/caveolae	References
Adenosine A1	—	Variable results	Variable results	—	Yes	Yes	Lasley <i>et al.</i> (2000), Gines <i>et al.</i> (2001), Escrache <i>et al.</i> (2003)
α_1 -Adrenergic	—	Variable results	—	Yes	—	—	Fujita <i>et al.</i> (2001), Dreja <i>et al.</i> (2002)
β_1 -Adrenergic	—	Variable results	No	Variable results	—	Yes	Schwencke <i>et al.</i> (1999), Ostrom <i>et al.</i> (2000, 2001, 2002), Xiang <i>et al.</i> (2002), Rapacciuolo <i>et al.</i> (2003)
β_2 -Adrenergic	—	Variable results	Decrease	Variable results	—	—	Schwencke <i>et al.</i> (1999), Rybin <i>et al.</i> (2000), Ostrom <i>et al.</i> (2001, 2002), Xiang <i>et al.</i> (2002)
Angiotensin AT1	Yes <10%	Yes <10%	Variable results	Yes	Not located in caveolae	—	Ishizaka <i>et al.</i> (1998), Ushimo-Fukai <i>et al.</i> (2001), Leclerc <i>et al.</i> (2002), Wyse <i>et al.</i> (2003)
Bradykinin B1	—	No	Variable results	—	—	—	Lamb <i>et al.</i> (2002), Sabourin <i>et al.</i> (2002)
Bradykinin B2	—	Yes	Variable results	—	—	Yes	de Weerd & Leeb-Lundberg (1997), Haasemann <i>et al.</i> (1998), Ju <i>et al.</i> (2000), Lamb <i>et al.</i> (2002)
Ca sensing Chemokine CCR5 and CXCR4	Yes CCR5 yes, CXCR4 no	— CCR5 no	—	Yes	—	—	Kifor <i>et al.</i> (1998, 2003), Kozak <i>et al.</i> (1999), Manes <i>et al.</i> (1999), Mueller <i>et al.</i> (2002), Nguyen & Taub (2002), Percherancier <i>et al.</i> (2003), van Buul <i>et al.</i> (2003), Venkatesan <i>et al.</i> (2003)
Cholecystokinin CCK	Yes	—	—	—	—	Yes	Roetger <i>et al.</i> (1995)
Sphingosine EDG-1	—	Yes	Increase	Yes	—	—	Igarashi & Michel (2000)
Endothelins ETA _A	Yes	—	—	Yes	—	Yes	Chun <i>et al.</i> (1994), Okamoto <i>et al.</i> (2000)
Endothelins ET _B	Yes	Yes	—	Yes	—	—	Teixeira <i>et al.</i> (1999), Yamaguchi <i>et al.</i> (2003)

Continued

Table 1 Continued

Receptor	Enrichment				Interaction with caveolin (ColP)	Localisation in caveolae (ME)	Internalisation via rafts/caveolae	References
	Enrichment in DRMs	Enrichment in 'caveolar fractions'	change upon agonist application					
δ-aminobutyric acid GABA _b	Yes	—	—	—	—	—	—	Becher <i>et al.</i> (2001)
Gonadotropin releasing hormone GnRH	Yes	Yes	No	—	—	—	Yes	Navratil <i>et al.</i> (2003), Pawson <i>et al.</i> (2003)
Muscarinic M2	—	Variable amounts	Increase	Yes	—	—	Yes	Feron <i>et al.</i> (2000), Dessy <i>et al.</i> (2000), Rybin <i>et al.</i> (2000)
Glutamate Metabotropic mGLUR	Variable results	—	—	—	—	—	—	Becher <i>et al.</i> (2001), Eroglu <i>et al.</i> (2003)
Oxytocin OTR	Yes <10%	Yes <10%	No	—	—	—	—	Gimpl <i>et al.</i> (2000), Guzzi <i>et al.</i> (2002)
Somatostatin SST2	—	Yes	—	—	Yes	Yes	Yes	Krisch <i>et al.</i> (1998), Mentlein <i>et al.</i> (2001)
Serotonin 5HT2	—	Yes	—	—	—	—	—	Dreja <i>et al.</i> (2002)
Thyrotrophin TRH	—	Yes	Increase	—	—	—	—	Drnota <i>et al.</i> (1999)

Localisation of GPCR in lipid rafts often gives rise to variable results. As discussed in the text, this may be due to several reasons. First, the localisation of GPCR in lipid rafts/caveolae may depend on the biochemical procedure used. When detergent-based extraction procedures are used to obtain DRMs, different results can be obtained depending on the type of detergent, the extraction conditions, the cell type analysed and the metabolic state of the cells. Furthermore, 'caveolar fractions' obtained by means of detergent-free methods elude the definition of rafts/caveolae based on detergent-insolubility and the lipid and protein composition of these fractions may differ from those of DRMs. Secondly, movements of receptors in/out of lipid rafts may depend on the times chosen in the experimental setting. Finally, receptor internalisation via rafts/caveolae is often dependent on the cell type and receptor routing through this pathway may be modulated by several factors, as further discussed in the text. ColP refers to co-immunoprecipitation of caveolin(s) and GPCR. ME refers to immunolocalisation of GPCR in caveolae by electron microscopy.

Figure 1 GPCR localisation signals to rafts/caveolae. Addressing signals to lipid rafts and caveolae are potentially located in several GPCR regions: in the extracellular receptor part, where they may interact with lipid and protein enriched in the membrane outer leaflet, in the transmembrane regions and finally, in the intracellular portions of the receptor molecule. Lipid rafts/caveolae domains enriched in gangliosides, cholesterol and caveolins are shown as white regions of the plasma membrane.

avidity for sphingolipid/cholesterol-enriched domains (Melkonian *et al.* 1999). Accordingly, a number of palmitoylated proteins or proteins carrying both kinds of modification (myristoylation per se is not sufficient), including α -subunits of heterotrimeric G-proteins ($G\alpha$ -subunits), non-receptor Src-like tyrosine kinases (NRTKs), and endothelial nitric oxide synthase (eNOS), mainly partition into DRMs (Okamoto *et al.* 1998). On the other hand, mutagenesis of myristoylation and/or palmitoylation sites in the amino-terminal sequences of $G\alpha$ -subunits, NRTKs and eNOS leads to the loss of the association of proteins with DRMs (Shenoy-Scaria *et al.* 1994, Shaul *et al.* 1996, Song *et al.* 1997, Moffett *et al.* 2000). However, the direct fusion of short peptides containing consensus sequences for fatty acylation relocates soluble proteins, such as the green fluorescent protein (GFP), to DRMs (Galbiati *et al.* 1999); this *in vitro* result has also been confirmed by FRET studies of living cells showing that fatty acylated GFPs co-localise with caveolin-1 (Zacharias *et al.* 2002).

It has therefore been proposed that fatty acylation, particularly palmitoylation, can act as a targeting signal for partitioning proteins into rafts/caveolae. However, although this may well be true for peripheral membrane proteins, it cannot be automatically generalised to integral palmitoylated

membrane proteins, because GPCRs, whose carboxyl-terminal intracellular tails are generally palmitoylated, are not all associated with DRMs (see above). Furthermore, caveolin-1 partitioning in DRMs is not at all affected by the mutation of all three palmitoylatable cysteines located in its cytosolic carboxyl-terminus (Dietzen *et al.* 1995). This suggests that the presence of transmembrane helices in a protein may somehow overcome the preference of palmitoyl moieties for lipid domains, as has been directly demonstrated by the finding that synthetic transmembrane peptides are excluded from model membranes mimicking Lo domains, regardless of whether they are palmitoylated or not (van Duyl *et al.* 2002).

Protein–protein interactions

A number of studies support the hypothesis that caveolin oligomers provide a scaffold for the sequestration of resident caveolae proteins in caveolae domains. There is evidence that caveolin-1 directly interacts with a number of signalling proteins, including $G\alpha$ -subunits, Src-like NRTKs, eNOS, and protein kinase A (PKA) and C (Okamoto *et al.* 1998, Razani *et al.* 1999). The ‘caveolin scaffolding domain’ has been mapped to a stretch of 41 amino acids (residues 61–101) in the

cytoplasmic amino-terminal tail of caveolin-1. Interestingly, a peptide consisting of residues 82–101 of caveolin-1 is capable of switching off the activities of G α -subunits, Src-like NRTKs, eNOS and PKA, thus suggesting that the caveolin scaffold does not merely play a passive role, but actively keeps the bound signalling molecules silent (Liu *et al.* 2002).

Using the 'caveolin scaffolding domain' as a bait to screen a library of synthetic peptides, two related caveolin-binding motifs have been identified containing regularly spaced aromatic amino acids (Φ X Φ XXXX Φ and Φ XXXX Φ XX Φ , where Φ =Trp, Phe or Tyr, and X=any amino acid) (Couet *et al.* 1997). These motifs are present in all known caveolae-associated polypeptides, including some GPCRs (e.g. β -adrenergic receptor (β -AR), endothelin (ET)_A and muscarinic receptors), but it still has to be demonstrated that the detection of these motifs in a protein sequence unequivocally predicts its association with caveolae domains. The association of some GPCRs with rafts/caveolae is therefore due to a still unidentified mechanism.

When do GPCRs go to lipid rafts/caveolae? (role of lipid rafts/caveolae in regulating GPCR trafficking)

It has been demonstrated that rafts are important in the regulation of GPCRs at all of the stages of their lifecycle: (i) in the exocytic pathway; (ii) in the plasma membrane; and (iii) in the endocytic pathway.

GPCRs are synthesised in the endoplasmic reticulum, in which the first steps of glycosylation take place and where oligomerisation may occur (Terrillon *et al.* 2003); full glycosylation is achieved in the Golgi apparatus, and the receptors are then sorted to the plasma membrane. A role for caveolin as a molecular chaperone involved in GPCR routing to the plasma membrane has been recently reported (Leclerc *et al.* 2002, Wyse *et al.* 2003). Interestingly, mutations in the putative 'caveolin-binding domain' of the AT1 receptor did not affect its localisation in lipid rafts, but reduced its cell surface expression. Furthermore, the AT1 receptor has been shown to be located in lipid rafts but not caveolae at the plasma membrane, despite positive receptor/caveolin co-immunoprecipitation. In cells

lacking caveolin, it is trapped in intracellular compartments and, in caveolin-1 null mice, a deficit in kidney AT1 receptor expression has been reported (Wyse *et al.* 2003). The AT1 receptor–caveolin interactions taking place in the exocytic pathway are therefore crucial for receptor sorting to the plasma membrane but not for localisation in caveolae.

It has been suggested that lipid rafts at the cell surface may be involved in the regulation of receptor stability, as in the case of ET_B and Ca²⁺ sensing receptors. The ET_B receptor is responsible for clearing ET from the extracellular space by binding it in an almost irreversible manner (Fukuroda *et al.* 1994); after binding, the receptor–ET complex is then internalised, sorted to lysosomes and degraded (Bremnes *et al.* 2000). Interestingly, the ET_B receptor is characterised by a very high constitutive and agonist-induced internalisation rate, and it has been hypothesised that the small fraction (10%) of ET_B receptor located in lipid rafts becomes more resistant to rapid constitutive internalisation, thus allowing it to couple to specific signalling pathways (Yamaguchi *et al.* 2003). Lipid rafts are also involved in the regulation of the stability of the Ca²⁺ sensing receptor by m-calpain, a proteolytic enzyme that cleaves and inactivates the receptor, and whose presence and activity in lipid rafts is dependent on calcium concentration (Kifor *et al.* 1998, 2003).

It has been found that a number of GPCRs shuttle inside/outside lipid rafts upon agonist binding, but some methodological remarks must be made before discussing the physiological role of these 'translocations'. First of all, the methods used to detect such movements vary: biochemical fractionation and binding, imaging techniques that follow the receptor at the cell surface and in endocytic compartments and electron microscopy analyses of receptor internalisation in specifically coated or uncoated vesicles.

When a biochemical approach is used alone, it is difficult to identify where the receptor really resides during the period of agonist stimulation. The stimulation times (1, 5, 15 and 30 min) are generally compatible with internalisation and the transient presence of receptor in vesicles that disappear after fusion with endosomal compartments may be particularly difficult to detect. As a consequence, a receptor moving from the DRMs after 15–30 min of agonist stimulation may

correspond to a situation in which the receptors have internalised via rafts/caveolae, but the vesicles have already fused with the endosomal compartments. One possible example of this is adenosine A1 receptors, which 'biochemically' move out from cardiac myocyte lipid rafts in 15 min (Lasley & Smart 2001), but internalise via caveolae in smooth muscle cells, the co-localisation of caveolin and the internalised receptor is lost 20 min after agonist application (Escrive *et al.* 2003). The combination of biochemistry with other methods, such as the use of membrane permeable or impermeable receptor ligands, as in the case of muscarinic receptors (Dessy *et al.* 2000), may better discriminate plasma membrane and intracellular receptors.

Secondly, although the internalisation pathway mediated by lipid rafts and/or caveolae is still being intensely investigated (Johannes & Lamaze 2002, Conner & Schmid 2003, Nabi & Le 2003), various methods can be used to determine whether a receptor is internalised via a classic CCP or rafts/caveolae. In particular, endocytosis via classical caveolae seems to be a rather slow process (Pelkmans *et al.* 2001), whereas the internalisation of interleukin-2 receptors via other lipid rafts seems to proceed as rapidly as classic CCP-mediated endocytosis (Lamaze *et al.* 2001). Both processes depend on dynamin (Henley *et al.* 1998, Oh *et al.* 1998), but only CCP formation requires Eps15 (Lamaze *et al.* 2001), a component of the clathrin machinery (Salcini *et al.* 1999). The agonist-induced internalisation that still takes place in cells transfected with dominant-negative mutants of Eps15 may thus be considered indicative of a non-CCP-mediated endocytic process. Cholesterol depletion with filipin or cyclodextrins have been used to demonstrate the involvement of rafts and caveolae in receptor internalisation but, as these treatments also alter the formation of CCPs under some conditions (Rodal *et al.* 1999, Subtil *et al.* 1999), CCP endocytosis should always be checked in control experiments. Confocal and *in vivo* imaging of receptor/fluorescent ligands combined with markers of the different endocytic pathways may also help to elucidate the fate of the internalised receptor. Finally, electron microscopy might unequivocally identify the type(s) of vesicle involved.

Given the complexity and still undefined state of the art of raft/caveolae-mediated endocytosis for many of the GPCRs that have been localised in lipid rafts, the precise relationships between

endocytosis and receptor shuttling inside/outside lipid rafts remain to be clarified. A number of paradigms have recently emerged (Fig. 2), and an example is given of each: (a) the receptor moves into lipid rafts/caveolae upon agonist binding and, like the somatostatin receptor sst2, is internalised via this pathway (Krisch *et al.* 1998, Mentlein *et al.* 2001); (b) the receptor moves into lipid rafts after agonist binding in order to activate specific signalling events, but eventually moves out to be internalised via CCPs, as in the case of the AT1 receptor (Ishizaka *et al.* 1998, Wyse *et al.* 2003); (c) the receptor is mainly in lipid rafts and enters cells via this pathway by default, such as the ET_A receptor (Chun *et al.* 1994, Okamoto *et al.* 2000); and (d) the receptor is in lipid rafts but leaves after agonist binding to be internalised via CCPs, such as the β_2 -AR (Schwencke *et al.* 1999, Rybin *et al.* 2000).

These different paradigms may be related to the different roles played by lipid rafts in regulating the signalling and trafficking of any particular GPCR. Furthermore, published data support the concept that the shuttling and/or the endocytic pathways chosen by GPCRs may depend on cell-specific factors. It has long been known that GPCRs can be internalised by different pathways in different cells (Tsao *et al.* 2001), but the physiological significance of these differences has only recently begun to emerge. For example, it has been shown that, in the case of cholecystokinin (CCK) receptor type A, the CCP-dependent pathway leads to lysosomal compartments, whereas non-CCP-mediated endocytosis does not (Roettger *et al.* 1995). However, switching the internalisation pathway of the ET_A receptor from rafts/caveolae to CCP does not alter its final lysosomal destination (Okamoto *et al.* 2000). It is very interesting to note that ET_A internalisation via CCP can be induced by cholesterol oxidation, thus indicating that the metabolic state of the cells may have a considerable effect on receptor internalisation within the same cellular context (Okamoto *et al.* 2000). Finally, the molecular basis of this internalisation switch has been elucidated in the case of the β_1 -AR expressed in Hek293 cells, which enters via both rafts/caveolae and CCPs, with each pathway contributing half of the internalised receptor pool. In these cells, it has been shown that PKA phosphorylation directs the receptor to a CCP, whereas G-protein coupled receptor kinase (GRK) phosphorylation directs it to raft/caveolae-mediated endocytosis

Figure 2 Role played by rafts/caveolae in GPCRs trafficking. Even if the precise relationships between endocytosis and receptor shuttling inside/outside lipid rafts remain to be clarified, a number of paradigms have recently emerged: (a) the receptor moves into lipid rafts/caveolae upon agonist binding; (b) the receptor moves into lipid rafts after agonist binding in order to activate specific signalling events, but eventually moves out to be internalised via CCPs; (c) the receptor is mainly in lipid rafts and enters cells via this pathway by default; (d) the receptor is in lipid rafts but leaves after agonist binding to be internalised via CCPs.

(Rapacciuolo *et al.* 2003). These findings may explain how different levels of β -arrestins or GRKs in different cells determine the different endocytosis pathway of specific GPCRs, as has recently been shown in the case of the GnRH receptor (Pawson *et al.* 2003). Finally, because of the close interconnections between receptor trafficking and signalling, the choice of alternative endocytosis pathways within the same cells could be critically important in determining the final outcome of receptor stimulation.

Why do GPCRs go to lipid rafts/caveolae? (role of lipid rafts/caveolae in regulating GPCR signalling)

As the main role of GPCRs is to elicit and/or modulate specific cell responses by activating second messenger systems, a key question is whether and how GPCR localisation in lipid rafts/caveolae affects their signalling properties.

One current view is that rafts/caveolae are 'stations' in which GPCRs can accomplish

specific signalling tasks by meeting a selected set of signalling molecules (Simons & Ikonen 1997, Okamoto *et al.* 1998, Simons & Toomre 2000), including their first downstream partners, the ethero-trimeric G-proteins (Fig. 3). As different G-proteins may be differently targeted to lipid rafts and caveolae by means of acylation of the α -subunit, selective receptor–G-protein coupling may only be efficient under conditions in which the receptor and G-protein meet in the same compartment. For example, it has been shown that $G\alpha_q$ interacts with caveolin, whereas $G\alpha_i$ and $G\alpha_{12}$ do not and may therefore be targeted to other lipid rafts (Oh & Schnitzer 2001). The $G\alpha_q$ - and $G\alpha_i$ -coupled human OTR is one example of a GPCR whose localisation in lipid rafts modulates the specificity of $G\alpha$ coupling (Rimoldi *et al.* 2003). When activated outside lipid rafts, it inhibits cell growth via a pertussis toxin (PTX)-sensitive pathway but, when activated in lipid rafts, it stimulates cell growth via a PTX-independent pathway; receptor coupling to $G\alpha_i$ therefore only seems to play a role in the signalling events generated when the receptor is excluded from lipid rafts.

Figure 3 Regulation of GPCR signalling and stability by rafts/caveolae. (a) Rafts/caveolae act as 'stations' in which GPCRs can accomplish specific signalling tasks by meeting a selected set of signalling molecules; (b) receptor targeting to lipid rafts/caveolae may protect receptors from rapid constitutive and/or agonist-induced internalisation, thus allowing their coupling to specific signalling pathways, as in the case of the ET_B receptor previously discussed in the text; (c) the endocytic pathways chosen by GPCRs may depend on cell-specific factors and switching the internalisation pathway from rafts/caveolae to CCPs may alter the final receptor destination. Furthermore, because of the close interconnections between receptor trafficking and signalling, the choice of alternative endocytosis pathways could be critically important in determining the final outcome of receptor stimulation.

Other signalling molecules enriched in rafts/caveolae that are involved in GPCR signalling include c-Src, EGFR and Raf, and it is not surprising that lipid raft integrity is crucial for EGFR transactivation and/or MAPK activation by AT1 receptor (Ushio-Fukai *et al.* 2001), ET_A and ET_B (Teixeira *et al.* 1999, Hua *et al.* 2003), GnRH (Navratil *et al.* 2003) and bradykinin B2 receptors (Ju *et al.* 2000). As EGFR and MAPK can be transactivated by GPCRs located inside or outside lipid rafts, an important question is whether the localisation of the initial signalling steps plays any role in the final outcome of receptor stimulation. In the case of the human OTR, it has been shown that receptor activation inside or outside lipid rafts may lead to a different EGFR and MAPK time course: stimulation inside lipid rafts leads to transient EGFR and ERK1/2 activation and cell proliferation, whereas stimulation outside lipid rafts is accompanied by the long-lasting activation of these signalling intermediates and inhibits cell growth (Rimoldi *et al.* 2003).

Another crucial signalling molecule enriched in caveolae is eNOS. The stimulation of Gq/phospholipase C coupled receptors leads to

sustained increases in Ca^{2+} and promotes the dissociation of caveolin from eNOS, thus allowing enzyme activation and NO production. It is very interesting to note that a fraction of the muscarinic M2 receptors in cardiac myocytes move into caveolae upon agonist binding, and it is likely that this sub-population is responsible for a further regulatory step in the eNOS/caveolin cycle by inactivating eNOS and contributing to switch off NO production. A caveolar localisation thus seems to have the dual function of promoting both the activation and inactivation of agonist-induced eNOS activity (Feron & Kelly 2001). Another point that needs to be stressed here is that the very specific function of eNOS inactivation is accomplished by only a small fraction of muscarinic receptors (Feron *et al.* 1999), and similar findings have been reported for AT1 receptors, only a small fraction of which move to caveolar fractions to transactivate EGFR (Ishizaka *et al.* 1988, Ushio-Fukai *et al.* 2001). Regulating the trafficking of a restricted pool of receptors might be a very efficient way of regulating specific signalling events.

A further level of signalling complexity may be generated by the simultaneous presence on the

same cells of receptor subtypes that are activated by the same ligand but whose raft/caveolae trafficking is different, such as β -AR and bradykinin receptors. Neonatal cardiac myocytes, in which the physiological effects of catecholamines are mediated by the predominant β 1-AR, express both β 1- and β 2-ARs in a ratio of 4:1 and, although the two receptors are coupled to the same signalling pathway (G_s /cAMP), a number of studies suggest that they may play different roles in regulating cardiac functions. Interestingly, β 1- and β 2-ARs are differently distributed in lipid rafts (see Table 1): some controversy still remains concerning the β 1-AR, but it has been shown that the β 2-AR is concentrated in lipid rafts, from which it moves out upon agonist binding; furthermore, filipin treatment disrupts β 2-AR but not β 1-AR signalling, thus indicating the prevalent role of rafts in regulating the β 2-AR (Rybin *et al.* 2000, Xiang *et al.* 2002). Lipid rafts are also enriched in specific adenylyl cyclases, and receptor localisation in lipid rafts is a means of regulating the coupling efficacy of β 1- and β 2-ARs to their effectors (Ostrom *et al.* 2001, 2002). Similarly, bradykinin B1 and B2 receptors variably translocate to lipid rafts upon agonist binding (Haasemann *et al.* 1998, Lamb *et al.* 2002, Sabourin *et al.* 2002); however, agonist activation desensitises and internalises B2, whereas B1 remains in caveolar domains. One interesting hypothesis is that the more persistent caveolar permanence of the B1 receptor may lead to its coupling to specific signalling pathways (Ostrom 2002).

Two final aspects deserve mention. The first is that the same receptor may be differently located in different cell types: for example, the β 2-AR is enriched in the lipid rafts of cardiac myocytes but excluded from the lipid rafts in vascular smooth muscle cells, thus indicating that compartmentalisation may be cell specific (Ostrom *et al.* 2002). The second is that protein distribution in lipid rafts may depend on factors such as age, drug use and ongoing diseases, thus offering a new perspective for the investigation of receptor signalling in normal and pathological conditions (Simons & Ehehalt 2002).

Concluding remarks

Despite the large number of published observations, there are still many open questions

concerning the relationship between GPCRs and lipid rafts/caveolae. These reflect the fog of uncertainty that pervades the field of membrane domains, and is mainly due to the intrinsic limitations of current methodological approaches. Nevertheless, the view that recruitment into lipid rafts/caveolae may affect multiple stages of the GPCR lifecycle (by regulating their intracellular trafficking and signalling properties) is arousing increasing interest. However, the inherent complexity of the topic must be taken into account insofar as each GPCR seems to possess a 'personal signature' specifying its trafficking to and/or association relationship(s) with rafts/caveolae. Furthermore, intradomain compartmentalisation seems to be both receptor- and cell-specific, thus making it difficult to compare the results of studies of endogenously or heterologously expressed receptors in different cell systems. Finally, lipid rafts/caveolae are potentially capable of regulating GPCRs in different ways depending on the cell's metabolic state, differentiation and stage of growth. As a result, it is difficult to make any generalisations and unravelling the exact role played by rafts and caveolae in different physiological and pathological conditions represents a real challenge for the future.

Acknowledgements

This work was supported by Cofin grants for 2000 (No. MM05242257-002) and 2002 (No. 2002055453), and a FIRB grant (No. RBAU01 XWS4) from the Italian Ministry for Universities and Research (MIUR).

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Received 12 January 2004

Accepted 15 January 2004

Made available online as an

Accepted Preprint 23 January 2004