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REVIEW

Functional membrane diffusion of G-protein coupled receptors

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Abstract G-protein-coupled receptor function involves interactions between the receptor, G-proteins and effectors in the cell plasma membrane. The main biochemical processes have been individually identified but the mechanisms governing the successive protein–protein interactions of this complex multi-molecular machinery have yet to be established. We discuss advances in understanding the functional dynamics of the receptor resulting from diffusion measurements, and in the context of the plasma membrane organization.

Keywords Signal transduction · Protein interactions · Membrane domain · Cytoskeleton · FRAP · SPT

Abbreviations

GPCR	G-protein-coupled receptor
FRAP	Fluorescence recovery after photobleaching
vrFRAP	Variable radius FRAP
SPT	Single particle tracking
SMT	Single molecule tracking
FCS	Fluorescence correlation spectroscopy

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Introduction

G-protein-coupled receptors (GPCRs) represent the largest receptor family involved in the communication of a cell with its external environment (Bockaert and Pin 1999). This signal transduction across cell membranes is initiated by the activation of GPCRs on binding extracellular agonist ligands (Fig. 1). Agonist binding is followed by a cascade of transient and successive molecular interactions in the cytoplasm leaflet of the cell plasma membrane (Kristiansen 2004). The process is started by the recruitment and the activation of the heterotrimeric G-proteins, stimulating GDP-GTP exchange (Gilman 1987) and inducing the activation of GTP-bound G_{α}-subunit and $G_{\beta\gamma}$-dimer. (Fig. 1) (Bockaert and Pin 1999; Gales et al. 2006; Kenakin 2002; Rebois et al. 2006). All GPCRs couple to at least two classes of G_{α} proteins, which, like the $G_{\beta\gamma}$-dimer proteins, induce distinct biological functions after activating their specific secondary effectors (Gilman 1987) (Fig. 1). This process is amplified through repetitive coupling between various G-proteins and the ligand-bound GPCR (Neubig 1994), and also between the activated G-protein subunits and their effectors. These sequential encounters suggest that the heterotrimeric G-protein complex dissociates after it is activated for signalling (Digby et al. 2006); however, results obtained for several GPCR instead indicate that the complex remains stable throughout (Gales et al. 2005). Data also suggest the existence of receptor-G-protein-effector complexes during transduction (Hur and Kim 2002). In any case, the membrane mobility of the activated receptor on the cell surface together with that of the G-proteins and the effectors constitute the main determinant in their encounters.

A GPCR deactivation process involving two main mechanisms occurs in parallel with signal transduction:

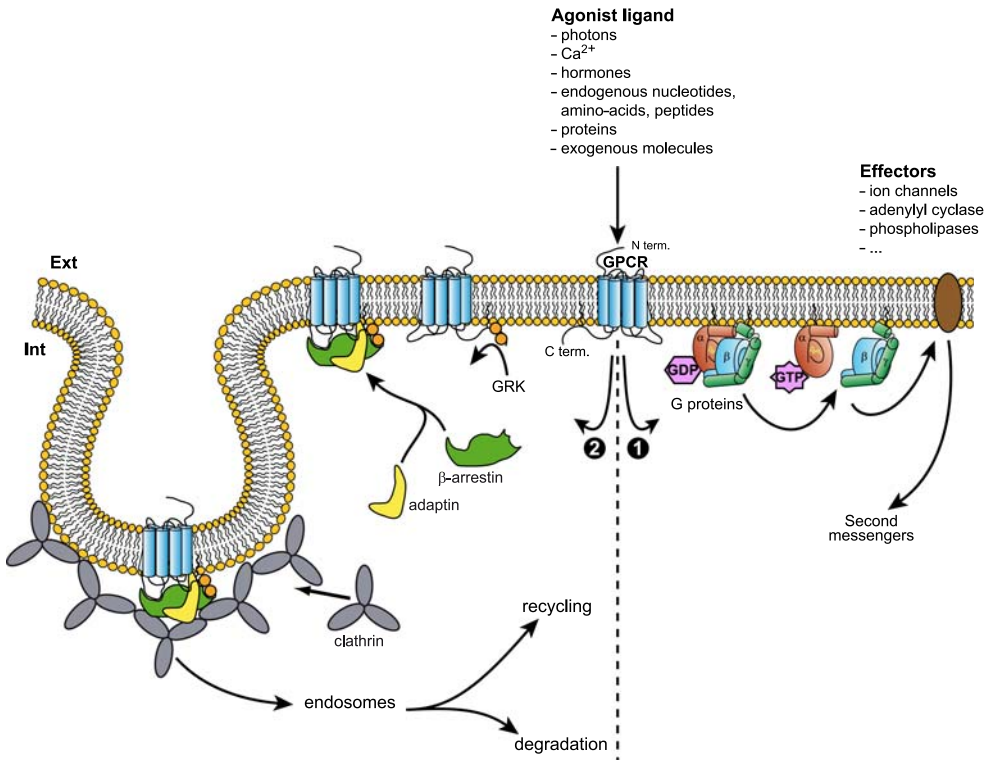

Fig. 1 Main events consecutive to an agonist binding by a GPCR: signal transduction (*1*) and receptor internalisation (*2*). *1* Stimulated receptor interacts with the GDP- bound heterotrimeric G protein, promoting a GDP-GTP exchange. The activated Gα and G$\beta\gamma$ subunits are then able to interact with effectors inducing the cellular responses. *2* A phosphorylation at GPCR C terminus is accomplished by G-protein receptor kinase (*GRK*) followed by the β-arrestin binding and subsequently adaptin leading to the formation of clathrin-coated pits further internalised

(1) unbinding of the agonist and (2) endocytosis of the receptor, followed by either its recycling or degradation (Fig. 1). Endocytosis of the receptor follows GPCR phosphorylation, induced by G-protein receptor kinase (GRKs) after agonist binding. β-arrestin subsequently forms a complex with the phosphorylated GPCR and adaptins, and this complex is targeted and collected to clathrin coated pits (Carman et al. 1998).

The main biochemical processes have been individually identified, but the molecular structures, the dynamics of each partner, and the successive protein–protein interactions that govern these multi-molecular events remain to be established. Random collision-coupling, the prevailing long-standing model for these interactions, was proposed by Levitzki et al. on the basis of kinetic analysis of the coupling between β-adrenergic receptors and adenylate cyclase effectors; this model suggests that lateral mobility of the signalling partners controls the sequential interaction process (Tolkovsky and Levitzki 1978). This model accounts well for transducine activation being dependent on light-activated rhodopsin diffusion rates (Calvert et al. 2001). However, it fails to explain the specificity and rapidity of the signalling pathway, as shown by biochemical and pharmacological studies. More recent models propose a compartmentalisation of the signalling partners favouring their encounters (Chidiac 1998; Hur and Kim 2002; Neubig 1994). A concept has been developed involving particular membrane platforms: transducisomes, a term describing all protein machineries requisite for a given biological function (Burack and Shaw 2000; Tsunoda et al. 1997). This view is corroborated by recent advances in understanding the dynamic organisation of neuronal synapses (Choquet and Triller 2003). This question must be considered in the context of the lateral organisation of the plasma membrane. The plasma membrane is characterised by complex dynamic heterogeneous distributions of lipids and proteins, which are believed to have functional implications (Engelman 2005; Marguet et al. 2006).

A full understanding of GPCR signal transduction mechanisms requires a refined description of the dynamic organisation of the signalling partners, which can be achieved by investigating their membrane diffusion. In this mini-review, we discuss advances in the understanding of receptor diffusion behaviour, which have been permitted by the recent progress made in molecular biology and biophysics instrumentation.

Techniques for membrane diffusion measurements

Current methods to measure the lateral mobility of proteins (or lipids) in the plasma membrane use optical microscopy, and most often fluorescence. Fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) provide average measurements, whereas single molecule or particle tracking (SMT or SPT) acquires the trajectories of single molecules (Table 1). The literature is already rich in reviews dealing with these techniques (Chen et al. 2006). Therefore, we will restrict ourselves to an overview of their respective ability to distinguish several populations and characterise confined diffusion.

First, let us examine two important steps preceding any diffusion measurements: the strategy for the necessary labelling of the receptor and the choice of the expression cell line. The choice of the label depends on the envisaged technique. FCS and SMT require particularly photostable fluorophores, whereas FRAP takes advantage of fluorescence fading. SPT makes use of functionalised nanoparticles, which can be fluorescent or not. Using fluorescent ligands limits the analysis to monitoring receptors in a bound state, and antibodies directed against the native receptors are not always available or of sufficiently high specificity and affinity. Thus, the study of tagged receptors has been favoured ever since the start of molecular biological engineering. The most exploited constructs consist of a direct fusion with the receptor of intrinsically fluorescent proteins, such as the green fluorescent protein (GFP) and its mutant variants (Kallal and Benovic 2000; White and Stelzer 1999). Alternative routes, which allow for the labelling of proteins at the cell surface, are fusions with small tags (T7, Myc or His tags); these are recognized by antibodies bearing the probe or with new tags (based on proteins covalently labelled with chemically diverse compounds (George et al. 2004; Tirat et al. 2006).

Besides classical limitations, such as the adherence and the ability to grow on glass coverslips, which are common to all optical microscopy-based techniques, the possibility of transfection and expression at the plasma membrane for the study of G-protein coupled receptors imposes additional requirements in the choice of the cell line. There are multiple protein partners involved in the signalling process of the membrane. Therefore in terms of biological relevance, it seems quite reasonable that the dynamic behaviour of a given G-protein coupled receptor should be investigated in the environment in which it is naturally expressed: neuronal cells for neurotransmitter and opioid receptors (Choquet and Triller 2003; Sauliere et al. 2006), and immune cells for chemokine receptors, and so on. The expression levels and the pharmacological properties of the tagged transfected receptor are also important. Ideally, these conditions should be as similar to those of the untagged receptor. Monoclonal stable transfected cell lines, in contrast to transient transfections, offer a cell-to-cell quasi-invariant number of receptors and constant pharmacological properties, but cannot be envisaged for all cells (neurons, for example). Conserving pharmacological properties of the wild-type receptor may restrict the choice of large tags, such as GFP-fused proteins. Large tags introduced at the C terminus can hamper the interaction of the receptor with cytosolic protein partners. However, large N-terminal tags may obstruct the ligand binding site on the receptor or may cause receptor misfolding in the plasma membrane. All conditions can rarely be brought together, and thus most studies result from experiments carried out in eukaryote heterologous expression systems and/or on transiently transfected cells.

Fluorescence recovery after photo-bleaching (FRAP)

Fluorescence recovery after photobleaching has been the most extensively used approach for diffusion analysis in

Table 1 Qualitative comparisons of the different light microscopy-based techniques for single cell measurements of the lateral diffusion of membrane lipids and proteins

Technique	Spatial (δx) and temporal (δt) resolutions	Advantages	Disadvantages
Fluorescence recovery after photobleaching	$\delta x \sim 200$ nm $\delta t \sim 1$ ms	Rapid acquisition of an average measurement Identification and characterization of domains by variable radius or waist	Dependence of D and M with observation radius (FRAP) or beam waist (FCS) Modelisation is required for interpreting the data Modelisation is required for interpreting the data
Fluorescence correlation spectroscopy	$\delta x \sim 200$ nm (using nano-apertures can lower this limit) $\delta t \sim 50$ µs		
Single particle tracking	$\delta x \sim 1$–10 nm $\delta t \sim 25$ µs-40 ms	Detailed information on the displacement modes and direct determination of the distribution between sub-populations of the tracked molecules	Size of the particle Possible selectivity of the labeling of the tracked molecules
Single molecule tracking	$\delta x \sim 10-40$ nm $\delta t \sim$ a few ms		Short duration of the trajectories Possible cell damage due to the illumination

membranes, since its emergence 30 years ago. In a FRAP experiment, the intensity of fluorescence is monitored in a defined zone after photo-bleaching fluorophores with a short pulse. The analysis of the fluorescence recovery curve, due to the diffusion of the unbleached molecules from outside of this zone, leads to the determination of the diffusion coefficient and the fraction of mobile molecules. However, the simplicity of this principle masks major concerns in interpreting observations or comparing data from various labs. Indeed, for molecules not homogeneously distributed, which is true for most complex molecular assemblies such as membranes, the measured diffusion coefficient is an apparent value and is dependent on the geometry of the bleached zone.

Interestingly, FRAP has given rise to a powerful approach that allows the identification and characterization of domains: FRAP at variable spot radius (vrFRAP). We expect a decrease in the mobile fraction, as a function of the observation area, in the range of observation radii (R) larger than the domain size (L). For a compartmentalised membrane organised with domains that are side by side, the mobile fraction has been shown to increase linearly with $1/R$, as $M = M_p + 0.63\ L/R$, where $M_p = 0$ for closed domains and has positive values for permeable domains. The slope of $M = f(1/R)$ allows the calculation of L. In addition, the real diffusion coefficient inside the domains can then be calculated from the apparent measured diffusion coefficient (Salome et al. 1998). If the domains are not joined, an additional contribution to the mobile fraction extrapolated at infinite R (or large scale) can arise. In this case, the population of molecules which diffuses over a long distance would therefore experience various environments. This population would have a distinct diffusion coefficient, and any analysis should consider the contribution of two populations with two diffusion coefficients. Such an approach has been successfully applied in the study of the NK2 and CCR5 receptor (Baker et al. 2007; Cezanne et al. 2004). It is important to note that, these experiments need the development of dedicated devices to be performed rigorously. The equipment implemented in our laboratory has a range of observation radii between 1 and 5 μm, giving access to domain sizes as small as 150 nm (Salome et al. 1998).

Fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy developed simultaneously with FRAP; however, this method has yet to be widely exploited for membrane diffusion measurements. Recent developments have seen it used similarly to vrFRAP, and the possibility of varying the observation volume size makes it a promising tool (Wawrezinieck et al. 2005). FCS analyses the fluctuations of the fluorescence intensity produced by a small number of molecules. These molecules diffuse in and out of an observation volume defined by a focused laser. The diffusion coefficient (D) of the molecule is deduced from the autocorrelation function of the fluorescence signal. The variation of D with w, the waist of the focused laser beam, allows the user to discriminate between isolated and joint domains of sizes down to 30 nm when measurements are performed across metallic nanoapertures (Wenger et al. 2006).

Single particle or molecule tracking (SPT or SMT)

Single particle or molecule tracking techniques use videomicroscopy coupled to image analysis to monitor the movements of individual molecules with nanometric spatial resolution (Bates et al. 2006; Sako and Yanagida 2003). The probes used are either single fluorescent molecules or more frequently nanometre-sized particles (latex beads, nanocrystals or gold colloids), which compensate for the disadvantage of large size by a capacity for longer periods of imaging. The particle is attached to the membrane molecule through antibodies, which have been adsorbed onto the particle surface. Ideally, the number of antibody molecules should be one per particle to minimize potential multiple attachment of targeted molecules to the particle (Daumas et al. 2002). Fab fragments should be favoured but they cannot be used systematically due to a low affinity constant. Also, particular care is needed to check for labelling specificity, which should not be lower than 80%. The spatial resolution of the trajectories ranges between 10 and 40 nm, whereas the image acquisition time can be decreased down to the millisecond range [exceptionally, data have been published at a rate of 25 μs/frame (Fujiwara et al. 2002)]. Trajectory analysis aims at determining the type of movement or diffusion mode of the tracked molecule and its characteristic parameters (e.g. diffusion coefficients, domain size, velocity). This analysis involves a simple calculation of the mean square displacement for increasing time intervals, whose analytical expression is known for elementary diffusion modes and can be used to distinguish efficiently between random diffusion, directed diffusion combining a directed motion to a pure diffusion, and confined diffusion (see Fig. 2). There are inherent fluctuations that characterise the trajectories; therefore, more complex behaviour, such as transient confinement or other combinations of simple modes, require a thorough statistical analysis for extracting the contained invaluable information. Recently, powerful new algorithms have been developed for detecting various forms of transient behaviour: confinement and jumps between adjacent domains (Meilhac et al. 2006; Destainville and Salome 2006), directed motion (Bouzigues and Dahan 2007) and multi-type motion (Huet et al. 2006).

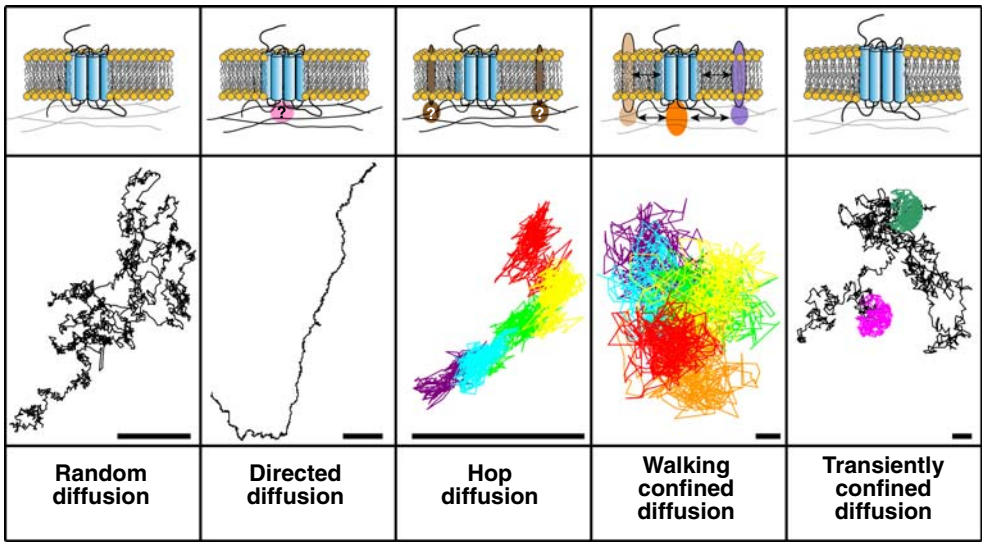

Fig. 2 Various putative GPCR membrane dynamic organisations: models and associated trajectories. Random diffusion: in the absence of interactions a free Brownian motion is expected. Directed diffusion: a protein link with cytoskeleton filaments drives a directional motion. Hop diffusion: in the "Anchored protein-Pickets and Skeleton Fences model" (Fujiwara et al. 2002) the diffusion proceeds by jumps across the barriers delineating the juxtaposed domains. Walking confined diffusion: a Brownian model of protein–protein attractive interactions predicts a rapid confined diffusion into domains with slower diffusion (Daumas et al. 2003) Transiently confined diffusion: an affinity with lipid micro-domains is supposed to correlate with transient confinement zones (Dietrich et al. 2002). Except transient confinement trajectory which is a simulated one, all trajectories represented come from MOP tracking (Daumas et al. 2003) (Saulière, unpublished data). Scale bar 200 nm

Diffusion behaviour of free GPCRs

Recently, there has been a significant increase in the number of studies relating to membrane diffusion of G-protein coupled receptors. These studies offer a wealth of data on various receptors and cellular expression systems (Table 2). Most of the studies, that use conventional FRAP, report systematically long-range diffusion properties for a large mobile fraction of the receptors, in addition to an immobile fraction. However, these studies do not provide detailed information relating to dynamic membrane organisation of the receptors. To date, few data are available by FCS at constant waist. However, the studies by vrFRAP and SPT are more informative, revealing two prevailing types of movement.

In general, the vrFRAP and SPT approaches showed a confinement, or partial confinement, in receptor diffusion. Our analysis of NK2, hMOP and CCR5 receptor diffusion using vrFRAP, supported a total or partial compartmentalization of the receptors in domains of sizes ranging from 400 nm to 1.5 µm. The NK2 (Cezanne et al. 2004) and CCR5 (Baker et al. 2007) receptors were partially confined in domains, whereas the remainder of the receptors, 70 and 25%, respectively, had long distance diffusion. By contrast, hMOP receptors (in neuroblastoma) were found to have diffusion totally restricted to permeable domains (Sauliere et al. 2006). The first SPT study dedicated to a G-protein coupled receptor was carried out by Choquet's laboratory and involved the neurotransmitter receptor, mGluR5 (Serge et al. 2002). Receptor trajectories, tracked at the surface of neurons, displayed alternating periods of fast random diffusion and slow confined diffusion; the latter had slower long-range superimposed diffusion. We analysed the hMOP receptor at the surface of NRK cells by SPT. Ninety percent of the hMOP receptors displayed permanent behaviour similar to the precedent slow period, including a short-term confined diffusion and a long-term random walk. A quadratic increase in the diffusion coefficient (D) with domain size (L), found to be between 30 and 550 nm, could not be explained by the "skeleton picket and fence model" (Fujiwara et al. 2002). Instead of hop diffusion, hMOP receptors consistently displayed short-term diffusion confined to a domain, which also diffuses. This is termed a "walking confined diffusion" (Fig. 2). We statistically analysed the trajectories, and concluded that the confinement was most probably due to attractive inter-protein interactions; thus, we proposed a simple Brownian model that was consistent with our experimental results (Daumas et al. 2003; Meilhac et al. 2006). Another group subsequently obtained contradictory results on the same receptor expressed transiently in similar cells (Suzuki et al. 2005). Suzuki et al. (2005), using single fluorescent molecules and high-speed single particle tracking, found that rat MOP undergoes rapid hop diffusion over a double-mesh-sized (about 200 and 700 nm) network, according to the "skeleton picket and fence model" (Fig. 2). By contrast, two other GPCRs were found

Table 2 Diffusion characteristics of G-protein-coupled receptors found in literature data

GPCR family	Cell type	Techniques	Conditions	Diffusion type	L (nm)	$M^{R(\mu m)}(\%)$	Behaviour origin	References
Neurokinine								
NK1-R	HEK293	SMT	Agonist × t = 0	Conf.	500 and 88		–	(Lill et al. 2005)
			Agonist × t = 1 s		↓			
NK2[a]-R	HEK293	vrFRAP	Free	Partially conf.	420	∼L/R	–	(Cezanne et al. 2004)
	HEK293		Agonist	Partially conf.	170	∼L/R	Coated pits	
Adrenergic								
β_2AR	Chang liver cell	FRAP	Antagonist[a]	–		$20^{2.25}$	Clustering	(Henis et al. 1982)
β_2AR*	HEK293	cFRAP	Free	–		$40^{1.6}$	–	(Barak et al. 1997)
β_2AR	Neuron, epithelial cell	FCS	Agonist[a]	–			Coated pits	(Hegener et al. 2004)
Opioid								
hMOR[a]	NRK	SPT	Free	Conf.	200		Inter-protein int.	(Daumas et al. 1982)
MOR[a]	NRK	SPT(25 μs)	Free	Conf.	210		Actin	(Suzuki et al. 2005)
	NRK	SPT(25 μs)	Latrunculin B	Conf.	320		–	
hMOR[a]	SH-SY5Y	vrFRAP	Free	Conf.	700	∼L/R	–	(Sauliere et al. 2006)
Neurotransmitter								
mGluR5[a]	Ptk2, neuron	SPT	Free	Transiently conf.	ND		Scaffolding protein	(Serge et al. 2002)
	Ptk2, neuron	SPT	Agonist	Transiently conf.	ND		Protein int. (G-protein)	
	Ptk2, neuron	SPT	Homer	Transiently conf.	ND		–	
mGlur5[a]	NIH3T3, neuron	SPT	Free	Directed			Actin, microtube	(Serge et al. 2003)
	NIH3T3, neuron	SPT	Taxol	Directed			–	
	NIH3T3, neuron	SPT	Nocodazole + cyto. D	Random			–	
$5HT_{1A}$-R[a]	CHO	cFRAP	Free	–		$70^{1.4}$	–	(Pucadyil et al. 2004)
	CHO	cFRAP	Antagonist	–		→		
	CHO	cFRAP	Agonist	–		→	Protein int. (G-protein)	
	CHO	cFRAP	G-protein decoupling	–		→		
$5HT_{1A}$-R[a]	CHO	vrcFRAP	Free	Random		75	–	(Pucadyil and chattopadhyay 2007)
	CHO	vrcFRAP	MβCD	Conf.	ND	∼L\R	–	
Hormone								
LH-R	Luteal cell	FRAP	Agonists[a]	–		$\approx 30^{1}$	–	(Niswender et al. 1985)
LH-R[a]	CHO	cFRAP	Free	–		$50^{0.4}$	–	(Horvat et al. 1999)
	CHO	cFRAP	Agonists	–		↓	–	
LH-R	HEK293	fpFRAP	Agonists[a](hCG and LH)	–		70 and 45	Report int. + actin	(Roess et al. 2000)
	HEK293	fpFRAP	hCG[a] + cyto D	–		↑	–	
	HEK293	fpFRAP	LH[a] + cyto D	–		↓	–	

Table 2 continued

GPCR family	Cell type	Techniques	Conditions	Diffusion type	L (nm)	$M^{R(\mu m)}$(%)	Behaviour origin	References
LH-R[a]	CHO	SPT	Free	Conf.	115		–	(Smith et al. 2006)
	CHO	SPT	Agonist (hgc)	Conf.	43		Cholesterol	
	CHO	SPT	MβCD + agonist	Conf.	75		–	
V2-R	LLC-PK$_1$	mpFMP	Agonist[a]	–		90^{2}	–	(Jans et al. 1989)
V2-R	LLC-PK$_1$	mpFMP	Agonist[a]	–		↓	Coated pits	(Jans et al. 1990a)
V1-R	A7R5	mpFMP	Agonist[a]	–		36^{2}	–	(Jans et al. 1990b)
GnRH-R[a]	CHO, αT3	cFRAP	Free	–		$80^{0.4}$	–	(Nelson et al. 1999)
	CHO, αT3	cFRAP	Agonist	–		↓	Protein int	
	CHO, αT3	cFRAP	Antagonist	–		→	–	
Chemokine								
CCR5[a]	CHO, HEK 293	cFRAP	Free	–		100^{ND}	Cholesterol	(Steffens and Hope 2004)
	HeLa, HOS	cFRAP	MβCD	–		↓	–	
CCR5[a]	HEK293	vrFRAP	Free	Conf.	1500	~L/R	–	(Baker et al. Submitted)
	HEK293	vrFRAP	co-receptor CD4	Partially conf.	340	~L/R	Protein int. (CD4)	
CXCR1[a]	HEK293	cFRAP	Free	–		100^{ND}	–	(Jiao et al. 2005)
	HEK293	cFRAP	Agonist	–		↓	–	
Miscellaneous								
OR17-40-R[a]	HEK293	SMT	Free	Partially conf.	150 and 350		Coated pits	(Jacquier et al. 2006)
	HEK293	SMT	Agonist	Partially conf.	↓		Coated pits	
		SMT	Antagonist	Partially conf.	↓		Coated pits	
GRP-R[a]	KNRK	cFRAP	Free	–	–	$80^{2.6}$	–	(Young et al. 2001)
	KNRK	cFRAP	Agonist	–	–	↓	Protein int.	
	KNRK	cFRAP	c-Src inhibitor + agonist	–	–	→	–	
CCK-R	CHO,	FRAP	Partial agonista	–	–	$88^{0.5}$	Coated pits	(Roettger et al. 1999)
	Pancereatic acini					$58^{0.5}$		

cFRAP confocal FRAP *mpFRAP* microphotolysis measurements, *fFPR* fringe fluorescence photobleaching recovery, *FCS* fluorescence correlation spectroscopy, *SPT* single particle tracking (at 40 ms time resolution except otherwise mentioned), *SMT* single molecule tracking, *MβCD* Methy-β- cyclodextrin, *conf.* confined, *int.* interaction, *ND* not determined. *L* confinement domain size, *M* mobile fraction, *R* observation radius in FRAP measurements

[a] Fluorescent labelled molecule

to exhibit similar diffusion behaviour as that reported in our study, including a short-term confined diffusion and a long-term long-range diffusion compatible with $D \sim L^2$: (1) the odorant receptor OR17-40 was analysed by single molecule tracking at the surface of HEK cells (Jacquier 2005) and (2) the luteneinizing hormone receptor LHR was monitored by single particle tracking at the surface of CHO cells (Smith et al. 2006). In the following sections, we will examine the diverse possible origins of confined diffusion.

G-protein-coupled receptors trajectories, in addition to compartmentalisation, display directed diffusion, combining random diffusion and displacement at a constant velocity in a preferred direction (Fig. 2). Such behaviour, for neurotransmitter receptors, has been shown to be due to interactions of the receptors with cytoskeleton actin filaments and microtubules. This behaviour has been detected for mGluR5 in cellular regions that have a highly dynamic cytoskeleton, including neuronal growth cones and fibroblast lamellipodia (Serge et al. 2003). This process may play a role in receptor targeting specific sites and in its lateral organisation. The directed diffusion observed for the opioid receptor hMOP was attributed to its entry into the internalisation pathway (Daumas et al. 2003); consistent with the expected membrane receptor recycling rate and the increase from 10 to 50% of this population for agonist bound receptors (Daumas et al., unpublished data).

There are few comparable studies that analyse the same receptor in distinct cells; however, they suggest a similar overall behaviour, but the major changes that are reported relate to the proportion of receptors with various types of movement (Daumas et al. 2003; Serge et al. 2002, 2003) and Saulière et al. unpublished results.

Temperature has a role

Although recommended for the maintenance of physiological conditions, some studies have not been performed at 37°C, but rather at room temperature to limit receptor endocytosis. This was particularly the case for receptor behaviour in the presence of its agonist ligand. Here, induced internalisation of the receptor disturbs its diffusion. However, sucrose, known to selectively inhibit the clathrin-mediated endocytosis, offers an interesting alternative for decreasing the temperature to avoid the internalisation of the receptors (Jacquier et al. 2006; Young et al. 2001). Nevertheless, lowering the temperature can have a much more complex effect than uniquely decreasing the thermal agitation temperature, which varies the diffusion coefficient according to the Arrhenius law. Indeed, the diffusion measurements by fluorescence microphotolysis (a variant of the FRAP technique) performed at various temperatures show different effects on two isoforms of the vasopressin receptor bound to their agonist ligand. V1R exhibits a decrease in the diffusion coefficient after decreasing the temperature from 37 to 22°C, with no effect on the mobile fraction. However, a similar decrease in the V2R diffusion coefficient is accompanied by a 30% loss in the mobile fraction (Jans et al. 1990b; Jans et al. 1989). More striking is the immobilisation of V2-R, observed after the temperature is lowered from 37 to 10°C. By contrast, the lipids remain in a highly fluid state, as demonstrated by the mobile fraction being close to 100%, and the diffusion coefficient decreasing by about factor 4. A temperature change can also differentially affect the various populations of a given receptor, with each having different diffusion behaviours. In our study of hMOR by SPT, the diffusion coefficient of the receptors with directed diffusion increased by a factor of about 10, after the temperature was raised from 22 to 37°C. At the same time, the diffusion coefficient of the receptors with short-term walking confined diffusion was lowered by a similar factor (and accompanied by a decrease of the domain size by a factor 2, in rough agreement with $D \sim L^2$) (Daumas et al. 2003). These opposing changes may compensate one another and may explain the absence of variation in the hMOR diffusion coefficient, as measured by FRAP for similar temperature values (Sauliere et al. 2006). Together, these results indicate that temperature has subtle effects on the dynamic organization of membrane constituents, probably due to thermally controlled biological processes. These processes make it difficult to assign the origins of observed behaviours.

Ligand binding affects the receptor diffusion

Antagonist ligands represent a large proportion of therapeutic agents targeting GPCR, but the molecular events following their binding to a GPCR are poorly documented. The current view is that antagonist binding prevents further activation by an agonist and does not give rise to a pharmacological response. The same is true for diffusion studies, which only rarely compare the free and antagonist-bound receptor. However, interestingly we found various contrasting situations in the literature: no modification of the 5-HT_{1A} receptor diffusion (in contrast to the agonist) (Pucadyil et al. 2004), decreases in the domain size and diffusion coefficient inside the domains (similar to the agonist) for the odorant receptor OR17-40 mainly found in compartments (Jacquier et al. 2006) and, for GnRH-R, a decrease in the diffusion coefficient at constant mobile fraction (whereas this was lowered by the agonist) (Nelson et al. 1999). These contradictory results question of the effect of antagonist binding, and whether it may be more complicated than simply "blocking" the receptor. In turn, these results should encourage further

investigation into the biochemical and diffusional aspects of antagonist binding.

The agonist is responsible for activating the signalling cascade; therefore, changes in the receptor dynamics induced by an agonist received much more attention. In contrast to the antagonist, there is a remarkable convergence of the literature data by FRAP and SPT or SMT. In general, the diffusion coefficient, the domain size and/or the mobile fraction decreased. The first evidence of this was obtained by FRAP studies of hormone receptors. GnRH activation of gonadotropin-releasing hormone receptor decreased its mobile fraction from 80 to 50% and lowered its diffusion coefficient by a factor of 3 (Nelson et al. 1999). Two agonists of luteneizing hormone receptors led similarly to significant decreases from about 55–20% for the mobile fraction and by a factor of 2–100 for the diffusion coefficient (Horvat et al. 1999). We analysed the NK2 receptor and lipid diffusion by vrFRAP. The confined NK2 receptors are distributed in domains, whose size is reduced from 420 to 170 nm, in the presence of the agonist. At the same time, the diffusion coefficient inside the domains significantly decreased from 10^{-1} to 10^{-3} $\mu m^2/s$. Colocalization of the receptor with β-arrestin and transferrin receptor supported that the small domains corresponded to clathrin coated pits (Cezanne et al. 2004). It has been proposed that entry into the endocytotic process results in domain size and diffusion coefficient reduction for several other receptors. Clathrin recruitement of receptors was suggested to explain the more constrained diffusion of NK1-R as a function of time (down to about 175 nm) during the first 1,000 ms following binding of the agonist (Lill et al. 2005), and the partitioning of OR17-40 into smaller domains of 190 nm in the presence of agonist (Jacquier et al. 2006). The persistence of the decrease of lateral mobility of CCK receptor after agonist or partial agonist binding after acid-induced dissociation of the agonist underlined a role in this behaviour of the internalization of this receptor (Roettger et al. 1999). FCS measurements of ligand bound $\beta_2$adrenergic receptors revealed two populations of diffusing bound receptors—the low mobility population was consistent with internalization of β_2AR (Hegener et al. 2004; Meissner and Haberlein 2003).

Nevertheless, the slowing down, and even the immobilisation of the receptors after agonist binding has been also found to be independent of endocytosis. Young et al. (2001) observed the contradictory effects of various treatments blocking the bombesin/gastrin releasing-peptide (GRP) receptor internalization. Young et al. (2001) demonstrated that the agonist-induced decrease of the mobile fraction was dependent on c-Src activity. They further argued for a possible role of this MAPkinase in mediating the multi-protein complex responsible for GRP-R immobilisation (Young et al. 2001). However, the dissociation from internalisation remains questionable, as c-Src is known to interact with β-arrestin and dynamin involved in the clathrin-coated pits (Claing et al. 2002). Analysis of LH-R by SPT revealed that the receptors have confined diffusion into domains of 230 nm size, which is reduced to 85 nm in the presence of the hCG agonist with the calculated diffusion coefficient inside the domains decreasing by a factor of 10 (Smith et al. 2006). The effect of agonist addition was reversed by methyl-β cyclodextrin cholesterol depletion. This reversal correlated well with the inhibition of the agonist-dependent localisation of the receptors in the detergent-resistant membrane fractions of sucrose gradients. This putative promotion of ligand-induced partitioning of receptors with lipid rafts accompanied by a decrease in receptor mobility had been previously suggested for the human CXCR1 chemokine receptor (Jiao et al. 2005). Without discussing the controversial area of lipid rafts in membrane biology (Munro 2003; Pike 2003), which is largely beyond the scope of this review, we point out the extreme difficulty, as illustrated by the existence of contradictory results (Kwik et al. 2003; Vrljic et al. 2005), in assessing the presence of a molecule in raft domains from diffusion measurements in cholesterol depleted cells.

In contrast to the results that have preceded, neurotransmitter receptors exhibit an increase in the diffusion coefficient after agonist binding, probably due to the G-protein uncoupling consecutive to receptor activation. This was first proposed to explain the threefold increase in mGluR5 mobility (Serge et al. 2002), and it was later demonstrated to be at the origin of the moderate, but significant, mobility increase by agonist binding for $5HT_{1A}$-R (Pucadyil et al. 2004).

Confinement of the receptors: origin and regulation

As suggested by the biochemical studies for GPCR signalling, GPCR diffusion analysis gives strong evidence for their compartmentalisation and definitively rules out the collision-coupling model. The various findings reported here emphasise the tremendous work that remains to be achieved for a clear view of the organization of these systems to emerge. Several sources of confinement, including the cytoskeleton, and the protein-protein and the protein-lipid interactions appear to be involved and understanding their origin and their mode of regulation presents a challenging question.

Besides its role in the intracellular and plasma membrane traffic of the receptors, the cytoskeleton is thought to act as a barrier to the diffusion of receptors, restricting their movements (Fig. 2) (Suzuki et al. 2005). This suggestion relies on the conjugated observations of hop diffusion and an increase in the compartment sizes after partial destabilisation

of the actin meshwork. Similar findings exist for a variety of proteins (Marguet et al. 2006) and lipids (Fujiwara et al. 2002). If undoubtedly, the cytoskeleton appears to compartmentalise membrane protein diffusion by a simple obstruction mechanism, more subtle effects are probably involved as the signalling partners encounter and interact. Nevertheless, the cytoskeleton is specifically involved in the confinement of the receptors after it is attached to scaffolding proteins recruited by (or recruiting) the receptors (Choquet and Triller 2003).

As found in a large part of the data, protein-protein interactions represent the predominant source of the compartmentalisation for receptors and their modulation. In this context, the diffusion coefficient being dependent on domain size is consistent with confinement due to protein–protein attractive interactions. Various groups have observed similar behaviour for several receptors and cellular systems independently providing encouragement for further work in this direction to determine the nature of the dominating interactions. We can reasonably suggest that these interactions take place between membrane proteins in the membrane. These interactions also take place in the cytoplasmic neighbourhood of the membrane, between cytosolic proteins coupled to the receptor and intracellular parts of other proteins (see Fig. 2). In this respect, the changes to the receptor diffusion observed in the presence of ligands or the disruption of interactions of the receptors with protein partners provide very useful information. The binding of the agonist, which more frequently reduces receptor mobility and confinement size and thus apparently reinforces the interaction, is accompanied by conformational changes. On one hand, these could result in a modulation of dipole–dipole interactions between helices of neighbouring proteins. On the other hand, owing to a possible resulting change in the hydrophobic length of the receptor (Salamon et al. 2002), it would modulate lipid-mediated attractive protein-protein interactions, as expected in conditions of hydrophobic mismatch. For such conjectures to be tested, there is a crucial need of experiments to be performed on membrane model systems, which offer the possibility of tuning the relevant factors. The role of cytosolic proteins in the confinement of the receptors is well established, especially in the extreme case of trapping the receptors in clathrin-coated pits. However, there are many other proteins, identified or not, which interact with GPCR after its activation (Bockaert and Pin 1999), thereby affecting receptor diffusion. This has been shown effectively in various elegant studies, in which the state of coupling of the G-protein was modulated (Pucadyil et al. 2004; Serge et al. 2002). The observed effects on the mobility of the receptors vary after agonist binding: an increase for neurotransmitter receptors (associated to G-protein decoupling) and a decrease for other receptors (attributed to endocytosis). These discrepancies could be only apparent and due to the dependence of the kinetics of the respective signalling and internalization events as a function of the receptors and/or cellular expression systems.

The eventual partitioning of the activated receptors in lipid microdomains, as suggested by Smith et al. and Jiao et al. (Jiao et al. 2005; Smith et al. 2006), would involve lipid–protein interactions. Indeed, these domains are the putative in vivo correlate of detergent-resistant membrane fractions enriched in cholesterol, sphingolipids and saturated glycerophospholipids (Pike 2006), which are characterised by a thickness larger than that of the surrounding membranes (Gandhavadi et al. 2002). The process driving the recruitment of receptors in these domains or the formation of such domains around the receptors would be to satisfy matching conditions between the hydrophobic cores of receptors and lipids (Fig. 2). The partitioning in liquid-ordered domains of agonist-bound delta opioid receptor in model membrane systems (Alves et al. 2004) supports this suggestion. Comparative diffusion analysis by SPT on model and cell plasma membranes suggests that lipid microdomains might be identified by transient confinement zones on the trajectories [Fig. 2, (Dietrich et al. 2002)]. However, detecting these lipid microdomains at the surface of living cells is still a delicate problem (Lagerholm et al. 2005). If partitioning of GPCRs in rafts is confirmed, it would be of particular interest to understand how this could be related to the effects of cholesterol content on the pharmacological properties of these receptors (Gimpl et al. 2002; Harikumar et al. 2005).

Perspectives

Only very few studies have been devoted to the diffusion analysis of GPCRs, in comparison to the extent that this receptor family has been studied and its major interest as a therapeutic molecule target. However, they demonstrate their potential for unravelling the molecular mechanisms of the GPCR signal transduction. Two major diffusion modes have been detected: directed diffusion probably involved in the redistribution of receptors at (or to) specific sites and confined diffusion presumably favouring the interactions with the signal transduction partners. The overall understanding of the machinery requires the investigation of the associated partners, G-proteins, but also effectors. There is even less data on these proteins and this does not enhance our understanding of the dynamic organisation of this complex system. Now, to further this challenging issue, there is an absolute need for the simultaneous analysis of the diffusion of the various signalling partners using emerging techniques for multicolour detection.

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