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Membrane Organization and Dynamics of the G-Protein-Coupled Serotonin$_{1A}$ Receptor Monitored Using Fluorescence-Based Approaches

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The G-protein-coupled receptor (GPCR) superfamily represents one of the largest classes of molecules involved in signal transduction across the plasma membrane. Fluorescence-based approaches have provided valuable insights into GPCR functions such as receptor–receptor and receptor–ligand interactions, real-time assessment of signal transduction, receptor dynamics on the plasma membrane, and intracellular trafficking of receptors. This has largely been possible with the use of fluorescent probes such as the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* and its variants. We discuss the potential of fluorescence-based approaches in providing novel information on the membrane organization and dynamics of the G-protein-coupled serotonin$_{1A}$ receptor tagged to the enhanced yellow fluorescent protein (EYFP).

KEY WORDS: G-protein-coupled receptor; serotonin$_{1A}$ receptors; green fluorescent protein; enhanced yellow fluorescent protein; membrane domains; cell surface dynamics.

INTRODUCTION

The G-protein-coupled receptor (GPCR) superfamily represents the largest class of molecules involved in signal transduction across the plasma membrane [1]. This class of membrane proteins includes about 1000–2000 members and represents ~1% of the human genome [2]. G-protein-coupled receptors respond to a variety of ligands as diverse as biogenic amines, peptides, glycoproteins, lipids, nucleotides, and even photons, thereby mediating multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, and inflammatory and immune responses. As a consequence, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas [3]. Disruption of GPCR signaling is linked to disease conditions such as retinal degeneration and nephrogenic diabetes insipidus [4,5]. It is estimated that up to 50% of clinically prescribed drugs act as either agonists or antagonists at GPCRs with several ligands of GPCRs among the top 100 globally selling drugs, which points out their immense therapeutic potential [5–7].

The common structural features of G-protein-coupled receptors include seven transmembrane domains with an extracellular N-terminus and a cytoplasmic C-terminus. They are broadly categorized into three subfamilies based on certain key sequences [8,9]. Rhodopsin-type receptors (type A) are by far the largest and most extensively investigated group. Receptors belonging to this group are activated by a large variety of stimuli including odorants, photons, and small biogenic amines such as serotonin, to large peptide hormones. The type B receptors (calcitonin type) have a relatively large N-terminal extracellular domain with six conserved cysteine residues. This group includes receptors for peptides such as secretin and glucagon. Metabotropic neurotransmitter receptors constitute type C receptors, which have an exceptionally long N-terminus and include the metabotropic glutamate and

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$GABA_B$ receptors. G-protein-coupled receptors transmit signals across the plasma membrane via their interactions with heterotrimeric G-proteins present on the cytoplasmic side of the cell membrane [10,11]. The G-protein heterotrimer, composed of α, β, and γ subunits, is maintained in an inactive state by mutual association in a complex, with the α subunit bound to a GDP moiety. Binding of specific ligands activates GPCRs by inducing or stabilizing a conformational state of the receptor, which can in turn activate heterotrimeric G-proteins. This results in exchange of GDP for GTP on the α subunit and dissociation/reorganization of the complex, which facilitates transduction of signals to effector molecules such as adenylate cyclase, phospholipases, and ion channels [10]. The multiple components of GPCR signal transduction such as different types of receptors and G-protein subunits provide cells with the much-needed versatility with which to customize their responses to a diverse array of ligands, which include hormones, neurotransmitters, and pharmacological agonists. Importantly, the spatiotemporal organization and dynamic confinement of receptors and effector molecules in the plasma membrane microdomains has given rise to new challenges and complexities in receptor signaling [12,13].

New technologies to analyze GPCR function in an intact cellular environment are predicted to have a major impact on GPCR research [3]. Such technologies currently involve fluorescence-based approaches to gain insight into GPCR functions such as receptor–receptor and receptor–ligand interactions, real-time assessment of signal transduction, receptor dynamics in the plasma membrane, and intracellular trafficking of receptors. Fluorescence-based approaches in general are considered superior over other existing molecular detection technologies due to their enhanced sensitivity, minimal perturbation, multiplicity of measurable parameters, and suitable time scales that allow the analysis of several biologically relevant molecular processes [14,15]. In this article, we provide a brief overview on fluorescence-based approaches using autofluorescent proteins such as green fluorescent protein to monitor membrane organization and dynamics of the $serotonin_{1A}$ receptor, an important member of the GPCR superfamily. The following sections are by no means an exhaustive review on the development and applications of fluorescent probes and proteins to monitor protein function in cells, for which several excellent articles are available [16–19]. This review aims to highlight the potential of fluorescence imaging and fluorescence recovery after photobleaching (FRAP) to yield novel information on the membrane organization and dynamics of GPCRs using the $serotonin_{1A}$ receptor as a representative member of this class of proteins.

GREEN FLUORESCENT PROTEINS AS SUITABLE FLUORESCENT PROBES

Green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* and its variants have become popular reporter molecules for monitoring protein expression, localization, and dynamics of various membrane and cytoplasmic proteins [16]. Importantly, tagging GPCRs with GFP has allowed direct visualization of signaling and real-time trafficking in living cells [17,18]. They represent a convenient alternative to other fluorescent probes for visualizing proteins due to the advantage of tagging such proteins at the genetic level. This allows investigators to visualize proteins for a longer duration of time in an intact cellular environment than is possible with the use of other extrinsic fluorescent probes. The GFP in *Aequoria* is a protein of 238 amino acids with a molecular mass of ~27 kDa, whose natural function is to convert the blue chemiluminescence of the Ca^{2+}-sensitive photoprotein aqueorin into green light [20]. The formation of the chromophore in GFP involves an intrinsic autocatalytic process that does not require additional enzymes or cofactors. This results in reconstitution of its fluorescence in virtually all organisms. The chromophore is *p*-hydroxybenzylideneimidazolidinone that is formed posttranslationally after the protein adopts a native-like 11-stranded β-barrel structure [21] by a complex set of reactions that primarily involve three residues in the protein, SYG (single-letter amino acid codes) at positions 65–67.

The GFP in *Aequoria* has the most complex spectra of all GFPs. The protein has a predominant absorption maximum at 395 nm at low pH and at 475 nm at high pH [22,23]. Based on pH titration experiments that lead to interconversion of the two species, the simplest interpretation is that these species represent molecules in which the phenol group in the tyrosine residue at position 66 has different ionization states. The 395 nm peak corresponds to molecules containing a neutral phenol group while the 475 nm peak corresponds to molecules with an anionic phenolate group [24]. Although GFP is excited at two different wavelengths, only one emission peak is observed under normal conditions. This is attributed to excited state proton transfer reactions causing GFP molecules to adopt a conformation that emits fluorescence with a peak centered at 507 nm [25]. The presence of such ground state heterogeneity in natural GFPs, with a predominant species excitable at 395 nm and a minor one excitable at 475 nm, has few disadvantages in regular cell biological applications. The most apparent among them being the requirement of UV light for efficient excitation of GFP that can damage biological samples upon prolonged exposure.

In addition, use of such low wavelengths can contribute to scattering effects and autofluorescence. Excitation of GFP with 475 nm wavelength could alleviate this problem. However, this results in excitation of a relatively small (∼15%) fraction of molecules [16].

Site-directed mutagenesis has generated GFP variants that display relatively simple absorption spectra and red-shifted spectral characteristics. The enhanced green fluorescent protein (EGFP) [26], also referred to as GFP-mut1 [27], is one such example, which bears two mutations, F64L and S65T. The S65T mutation suppresses the 395 nm peak arising due to the neutral phenol group in the tyrosine at position 66, and the 475 nm peak contributed by the anionic phenolate group is enhanced 5–6 fold in amplitude. In addition, the absorption maximum is shifted to 489 nm with a maximum in fluorescence emission centered at 509 nm. Under normal cellular conditions, the F64L mutation effectively leads to enhancement in net fluorescence by allowing chromophore formation to occur at physiological temperatures [16,28]. The combined result of these mutations is a GFP variant with a 5–6-fold enhancement in fluorescence emission, excitable in the visible wavelength range and with improved thermal stability [28] relative to the wild-type GFP.

The enhanced yellow fluorescent protein (EYFP), which displays enhanced brightness and a more red-shifted fluorescence emission, represents another popular and well-characterized variant of GFP. This variant was generated by site-directed mutation of GFP through a rational-design approach [21]. The threonine residue at position 203 in the GFP S65T mutant was replaced with a tyrosine based on the proposal that placing an aromatic ring next to the phenolate ion of the chromophore would result in π-stacking interactions between the chromophore and the highly polarizable phenol group of the tyrosine residue. These interactions would reduce the energy required for exciting the chromophore resulting in a GFP variant with more red-shifted spectral properties than GFP S65T [16,21,29]. Along with a few other mutations such as V68L and S72A, this resulted in a yellow–green variant of GFP with an excitation and emission maxima at 514 and 527 nm, respectively. This variant of GFP, referred to as EYFP (previously known as GFP 10C, see [21]), has a high molar extinction coefficient (ε) of 84,000 M^{-1} cm^{-1} and a quantum yield of 0.61, thus making it the brightest among the popular GFP variants [30]. The absorption in the visible range with enhanced brightness and other photophysical properties of EYFP have resulted in its use as a probe to monitor protein expression, localization, and dynamics in cells. EYFP–protein fusions have been used to monitor protein expression and distribution, in addition to monitoring near-neighbor interactions using fluorescence resonance energy transfer (FRET) with other variants of GFP such as the enhanced cyan fluorescent protein (ECFP), in a wide variety of cell types [16]. The fluorescence properties of EYFP are highly sensitive to pH of the environment resulting in its use as a noninvasive pH indicator for intracellular organelles and cytoplasm [31]. The fluorescence intensity of EYFP reduces with a drop in pH (apparent $pK_a = 6.5–7.1$) [30,31] and is attributed to a reduction in the population of molecules that absorb and emit at 514 and 527 nm, respectively, with a concomitant increase in a nonfluorescent population that absorbs at 390 nm.

VISUALIZING G-PROTEIN-COUPLED RECEPTORS USING GFP FLUORESCENCE

Visualization of GPCRs using fluorescence-based approaches has tremendously improved our understanding of their dynamic behavior in a cellular milieu. Information regarding cellular distribution and trafficking of GPCRs upon activation have predominantly been obtained using fluorescence-based approaches [32]. G-protein-coupled receptors have been visualized using fluorescent ligands, fluorescently labeled antibodies against epitope-tagged GPCRs, and using GFPs [17,18,32]. The use of fluorescent reporter proteins such as GFP has advantages over fluorescently labeled ligands and antibodies to visualize receptors for the following reasons: (i) the stoichiometry of the receptor and fluorescent protein is well defined as the latter is covalently attached to the receptor at the genetic level, (ii) complications encountered while using fluorescent ligands such as ligand dissociation, or while using fluorescent antibodies such as nonspecific binding are avoided, (iii) this approach allows analysis of the unliganded states of the receptor (not possible with fluorescent ligands), (iv) the possibility of perturbation induced by bulky fluorescent groups to small endogenous ligands such as biogenic amines limits their applicability, and (v) cellular biosynthesis ensures the presence of receptors attached to fluorescent proteins in cells and eliminates the necessity of labeling receptors with fluorescent ligands and/or antibodies before each experiment. Although GFPs were initially used for rather low-end applications such as transfection markers, their true potential as fluorescent reporters was soon realized by their fusion with various proteins to monitor the subcellular localization and trafficking of proteins [17]. The original use of GFP as a fluorescent reporter for proteins was to monitor gene expression [33]. One of the first applications of the GFP-fusion technology to GPCRs was with the β_2-adrenergic receptor to monitor their cellular

distribution, ligand-dependent trafficking, and dynamics on the plasma membrane [34]. The increasing use of GPCR–GFP fusions to monitor receptor dynamics, trafficking, and oligomerization has established the applicability of this technology since then [17,18]. The following sections will review specific application of the GPCR–GFP fusion technology to monitor novel aspects in GPCR research such as their distribution in the membrane and G-protein-dependent cell surface dynamics using the serotonin$_{1A}$ receptor as an important representative of the GPCR family of proteins.

THE SEROTONIN$_{1A}$ RECEPTOR: AN IMPORTANT G-PROTEIN-COUPLED RECEPTOR

The serotonin$_{1A}$ receptor belongs to the type A class of GPCRs [9] and is one of the first GPCRs for which the gene was cloned [35,36]. The gene is intronless and exhibits ~50% amino acid homology with the β2-adrenergic receptor in the transmembrane domains [35]. The corresponding mRNA is expressed mainly in the brain, spleen, neonatal kidney, and gut [35,36]. The human gene for the receptor encodes a protein of 422 amino acids with a core molecular mass of ~46 kDa. The serotonin$_{1A}$ receptor is not yet purified and structural details at atomic resolution are not known [37]. However, hydropathy plots of the amino acid sequences predict the presence of seven putative transmembrane domains, each of ~25 residues in length, which are thought to represent membrane-spanning α-helices (reviewed in [38,39]). Based on the three consensus N-linked glycosylation sites in the amino terminus, and its homology to the β_2-adrenergic receptor, the serotonin$_{1A}$ receptor is predicted to have a topology where the amino terminus is oriented facing the extracellular space. The hydrophilic sequences connecting the transmembrane helices would form three intracellular and extracellular loops in the protein with respect to the plasma membrane. Receptors that bind to biogenic amines (such as the serotonin$_{1A}$ receptor) are known to possess ligand binding sites within the transmembrane regions [40]. Ligands that act as agonists, bind to this pocket and induce a conformational change in the transmembrane helices. This change acts as a switch to activate G-proteins bound to the second and third intracellular loops of the receptor. Other structural features of the serotonin$_{1A}$ receptor include a fourth cytoplasmic loop that is palmitoylated posttranslationally at the two conserved cysteine residues at the carboxy terminus of the receptor.

Serotonergic signaling is initiated by binding of the intrinsically fluorescent [41] neurotransmitter serotonin to distinct cell surface receptors. These receptors have been classified into ~14 subtypes on the basis of their pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways [42]. Such signaling pathways play key roles in the generation and modulation of various cognitive and behavioral functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, and learning [39,43,44]. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive-compulsive disorder [45,46]. In addition, serotonin receptors play a crucial role in brain development processes such as neurogenesis and axonal branching [47,48]. The serotonin$_{1A}$ receptor is the most extensively studied of the serotonin receptors for a number of reasons that include the availability of a selective ligand 8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin) allowing extensive biochemical, physiological, and pharmacological characterization of the receptor, and its importance in neuronal physiology [39]. As a result, the serotonin$_{1A}$ receptor represents an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. Importantly, the serotonin$_{1A}$ receptor knockout mice exhibit enhanced anxiety-related behavior [49–51], and represent an excellent model system to understand such behavior in higher animals [52]. These reports point toward the significance of serotonin$_{1A}$ receptors in human health and disease.

While considerable information is available regarding the pharmacology and neurobiology of the serotonin$_{1A}$ receptors, molecular details regarding their organization and dynamics in the membrane remain largely unexplored [37,39]. To address this issue, we have visualized serotonin$_{1A}$ receptors stably expressed in mammalian cells by its fusion to EYFP [53]. A schematic diagram indicating the site of the EYFP tag on the serotonin$_{1A}$ receptor, and its typical fluorescence distribution when stably expressed in Chinese hamster ovary (CHO) cells are shown in Fig. 1. This fusion protein was found to be essentially similar to the native receptor in pharmacological assays and therefore can be used to reliably explore aspects of receptor biology such as cellular distribution and dynamics on account of its intrinsic fluorescent properties [53]. The following sections describe the use of serotonin$_{1A}$ receptors fused to EYFP in fluorescence-based approaches to analyze their organization and dynamics in the plasma membrane.

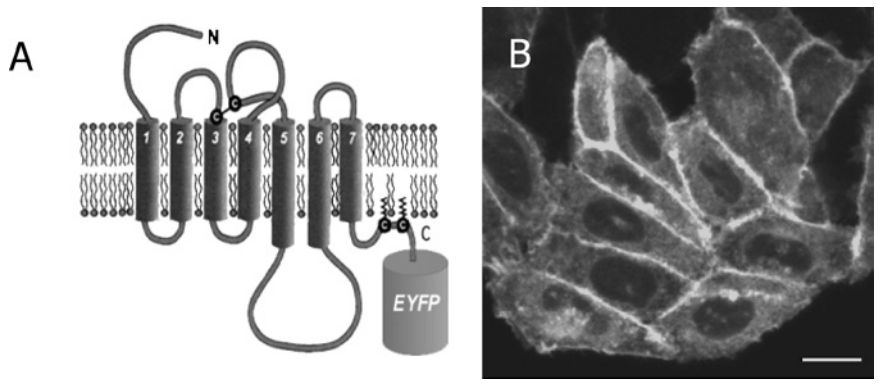

Fig. 1. Visualization of the serotonin$_{1A}$ receptor in cells using EYFP fluorescence. (A) A schematic diagram indicating the overall topology of the serotonin$_{1A}$ receptor with the site of the EYFP tag on the receptor. (B) Typical fluorescence distribution of the serotonin$_{1A}$ receptor-EYFP fusion protein stably expressed in Chinese hamster ovary (CHO) cells. The image represents a midplane section of this group of cells acquired on a Zeiss LSM 510 Meta confocal microscope. The scale bar represents 10 μm.

MEMBRANE ORGANIZATION OF THE SEROTONIN$_{1A}$ RECEPTOR MONITORED USING GFP FLUORESCENCE

The role of organization of lipids and proteins in membranes and its relevance in membrane function [54–56] assumes significance in the context of the membrane environment being an important modulator of membrane protein function [57,58]. Increasing evidence favors the concept of membranes being organized into domains with defined lipid and protein compositions. These domains, sometimes referred to as "rafts," are believed to serve as platforms for signaling by concentrating certain lipids (such as cholesterol and sphingolipids) and proteins while excluding others [55,56,59]. Such an organization of membranes assumes importance due to its potential role in a number of processes such as membrane trafficking, sorting, signal transduction, and pathogen entry [60–63].

Resistance to solubilization by mild nonionic detergents such as Triton X-100 at low temperature represents an extensively used biochemical criterion to identify, isolate, and characterize certain types of membrane domains [64–67]. Evidence from model membrane studies shows that enrichment with lipids such as sphingolipids (with high melting temperature) and cholesterol serves as an important determinant for the phenomenon of detergent resistance [68,69]. The tight acyl chain packing in cholesterol-sphingolipid rich membrane regions is thought to confer detergent resistance to membrane regions enriched in these lipids and to the proteins which reside in them. In spite of concerns over the use of membrane-perturbing agents such as Triton X-100 to understand membrane organization [70,71], detergent insolubility continues to be a principal tool to study membrane domains due to its wide applicability [72]. Information obtained from this extensively used biochemical approach can often form the basis for a more detailed analysis of membrane domains utilizing other specialized techniques.

Detection of proteins in detergent-resistant membranes (DRMs) is usually performed either by immunoblotting or ligand binding. However, these methods are not suitable in cases where ligand binding of the protein in question is compromised in the presence of the detergent [73,74] and/or is limited by availability of antibodies with high specificity [75]. Membrane proteins tagged with GFP provide an alternative, which can overcome these difficulties. We have recently described a GFP-fluorescence based approach to directly determine detergent insolubility of the serotonin$_{1A}$ receptor [74]. This method is based on quantitating fluorescence of the membrane protein before and after detergent treatment. Utilizing this approach, $\sim$26% of fluorescence of the serotonin$_{1A}$ receptor–EYFP fusion protein is shown to be retained upon extraction with 0.05% (w/v) Triton X-100 [74]. This represents the fraction of the serotonin$_{1A}$ receptor, which is resistant to detergent treatment under these conditions. A typical fluorescence distribution of the serotonin$_{1A}$ receptor fused to EYFP upon detergent extraction is shown in Fig. 2.

In order to validate this fluorescence microscopic approach toward determination of detergent insolubility of membrane components, specific lipid (DiIC$_{16}$ and *FAST*

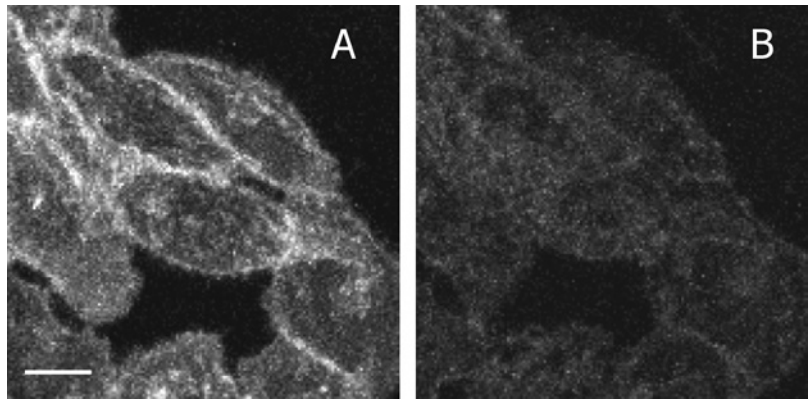

Fig. 2. Visualization of detergent insolubility of the serotonin$_{1A}$ receptor in CHO cells using EYFP fluorescence. Cells expressing the serotonin$_{1A}$ receptor-EYFP fusion protein are shown (A) before and (B) after treatment with 0.05% (w/v) cold Triton X-100 for 10 min. The images represent combined midplane confocal sections of the same group of cells before and after detergent extraction (from Ref. [74]). The scale bar represents 10 μm.

DiI) and protein (transferrin receptor) markers were utilized, whose organization in membranes and the ability to be extracted by cold nonionic detergents have been well documented. The dialkylindocarbocyanine (DiI) series of lipid analogs have been shown to exhibit preferential phase partitioning into biological and model membranes of varying degrees of order (fluidity) depending on the relative headgroup to tail cross-sectional areas and the chain length [76–78]. For example, DiIC$_{16}$ with its two 16-carbon saturated alkyl chains preferentially partitions into relatively rigid (highly ordered) domains, whereas *FAST* DiI which has two 18-carbon chains with two *cis* double bonds in each chain preferentially partitions into fluid domains in membranes [78] since packing of this probe would not be very efficient in an ordered phase. Ordered membrane domains and the proteins that partition into them are known to be resistant to detergents [55]. Accordingly, when cells labeled with either of these probes were extracted with Triton X-100, DiIC$_{16}$ was found to be insoluble in detergent to a greater extent than *FAST* DiI [74]. These results show that this method is capable of distinguishing ordered domains labeled by DiIC$_{16}$ from the fluid regions of the membrane characterized by *FAST* DiI labeling. This fluorescence-based approach for determining detergent insolubility of membrane components has been further tested by monitoring detergent insolubility of a protein marker, the transmembrane protein transferrin receptor. Several reports have earlier shown this receptor to be soluble in Triton X-100 and therefore it is often used as one of the controls for detergent insolubility experiments [79,80]. In agreement with these reports, transferrin receptor is found to be largely soluble in Triton X-100 by the GFP-based fluorescence approach to monitor detergent insolubility of the serotonin$_{1A}$ receptor [74].

Taken together, results obtained using the lipid (DiI) and protein (transferrin receptor) markers validated the observation of detergent insolubility of the serotonin$_{1A}$ receptor in particular, and the GFP fluorescence-based approach in general. Importantly, this method of analysis of detergent insolubility could be potentially useful in exploring membrane localization of other G-protein-coupled receptors. In addition, this approach is free from possible artifacts induced by antibodies in immunoblotting experiments. Thus, this GFP fluorescence-based approach represents a useful application of GPCR–GFP fusion proteins to explore membrane organization of G-protein-coupled receptors. This approach has the potential to be used in large-scale screenings of detergent insolubility of membrane proteins by making fluorescent fusion proteins and testing for insolubility by an automated fluorescence imaging system capable of handling multiple samples.

MEMBRANE DYNAMICS OF THE SEROTONIN$_{1A}$ RECEPTOR MONITORED USING GFP FLUORESCENCE

The major paradigm in the G-protein-coupled receptor signal transduction process is that activation of GPCRs leads to the recruitment and activation of heterotrimeric GTP-binding proteins (G-proteins) [11]. The activation process stimulates the GDP–GTP exchange on the α-subunit of the G-protein leading to dissociation of the heterotrimer from the GPCR. These initial events are

fundamental to all types of GPCR signaling and occur at the plasma membrane *via* protein–protein interactions. Hence, the dynamics of the activated receptor on the cell surface represents an important determinant in its encounter with G-proteins and has a significant impact on the overall efficiency of the signal transduction process. This aspect forms the basis of the "mobile receptor" hypothesis in which the lateral mobility of the receptor on the cell surface is assumed to play an important role in receptor–effector interactions at the plasma membrane that determine efficiency of cellular signaling processes [81]. This model has evolved over time due to novel findings on G-protein-coupled signal transduction events. Recent evidence indicates that receptors and G-proteins are less dynamic than previously appreciated. A spatiotemporally organized system rather than a freely diffusible system has been suggested to be responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [12,82]. It has been proposed that GPCRs and their cognate G-proteins are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains [13]. This heterogenous distribution of GPCRs and G-proteins into domains has given rise to new challenges and complexities in receptor signaling since it now has to be understood in the context of the three-dimensional organization of various signaling components.

In light of the proposed significance of a spatiotemporally restricted environment in modulating receptor and G-protein interaction, we have utilized the intrinsic fluorescence of the human serotonin$_{1A}$ receptor tagged to the enhanced yellow fluorescent protein (EYFP) to analyze its cell surface dynamics (diffusion characteristics) using the technique of fluorescence recovery after photobleaching (FRAP) [53]. Fluorescence recovery after photobleaching involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the observation region. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from the unbleached regions in the membrane is an indicator of the mobility of the fluorophores in the membrane [83,84]. Fluorescence recovery after photobleaching is one of the most widely used approaches to quantitatively estimate diffusion characteristics of molecules in solution and cellular systems. However, obtaining consistent and reliable quantitative estimates of mobility of molecules using FRAP is hindered by the lack of appropriate standards for calibrating the FRAP setup (microscope configuration and data fitting algorithms) used in a given experiment. We have validated our approach to analyze the cell surface dynamics of the serotonin$_{1A}$ receptor by independent measurement of the mobility of a standard solution of EGFP in a viscous solution. Our experimentally determined diffusion coefficient of EGFP under such conditions is in excellent agreement with the value predicted for GFP in a solution of comparable viscosity as calculated using the Stokes–Einstein equation [53]. Fluorescence recovery after photobleaching analysis carried out in a similar manner on serotonin$_{1A}$ receptors fused to EYFP indicate that the mobility of the receptor is dependent on its interaction with G-proteins. Prior incubation with agents that activate G-proteins through receptor-dependent and -independent means increases receptor mobility on the plasma membrane. A typical FRAP measurement performed on cells expressing the serotonin$_{1A}$ receptor–EYFP fusion under optimized imaging conditions is shown in Fig. 3. The figure also shows the effect of aluminum fluoride (AlF_4^-), a receptor-independent activator of G-proteins in cells, on the kinetics of fluorescence recovery after photobleaching of the serotonin$_{1A}$ receptor–EYFP fusion. The G-protein heterotrimer is a large protein complex with an average molecular mass of ~88 kDa [85], which would be ~1.2 times the mass of the receptor tagged to EYFP. It is therefore possible that their association with the receptor would reduce its mobility. Receptor-dependent and -independent activation of G-proteins stimulates the exchange of a GTP for the existing GDP molecule at the Gα subunit of G-proteins, resulting in the dissociation of G-protein heterotrimer complex from the receptor. The proposal that the association of G-proteins to the receptor reduces its mobility is further validated by the observation that treatment of cells with pertussis toxin that reduces receptor and G-protein interaction also causes an increase in receptor mobility [53]. Importantly, these results for the first time provide convincing evidence that the cell surface dynamics of a GPCR is dependent on its interaction with G-proteins.

Diffusion behavior of several integral membrane proteins indicates that the cytoskeleton underlying the plasma membrane can act as a barrier to free diffusion of these proteins. This is thought to occur due to the steric hindrance imposed by the cytoskeleton on the cytoplasmic domains of these proteins. Treatment of cells with agents that disrupt the cytoskeleton [86], truncation of the cytoplasmic domains of transmembrane proteins [87], or a lack of interaction of membrane proteins with cytoplasmic effector molecules [88] tends to increase their mobility on the cell surface. Likewise, the presence of the bulky heterotrimeric G-protein complex associated with the receptor (since G-proteins, when bound to membrane receptors, could be considered as equivalent to cytoplasmic domains of membrane proteins) could further reduce (over the differences arising due to molecular mass of

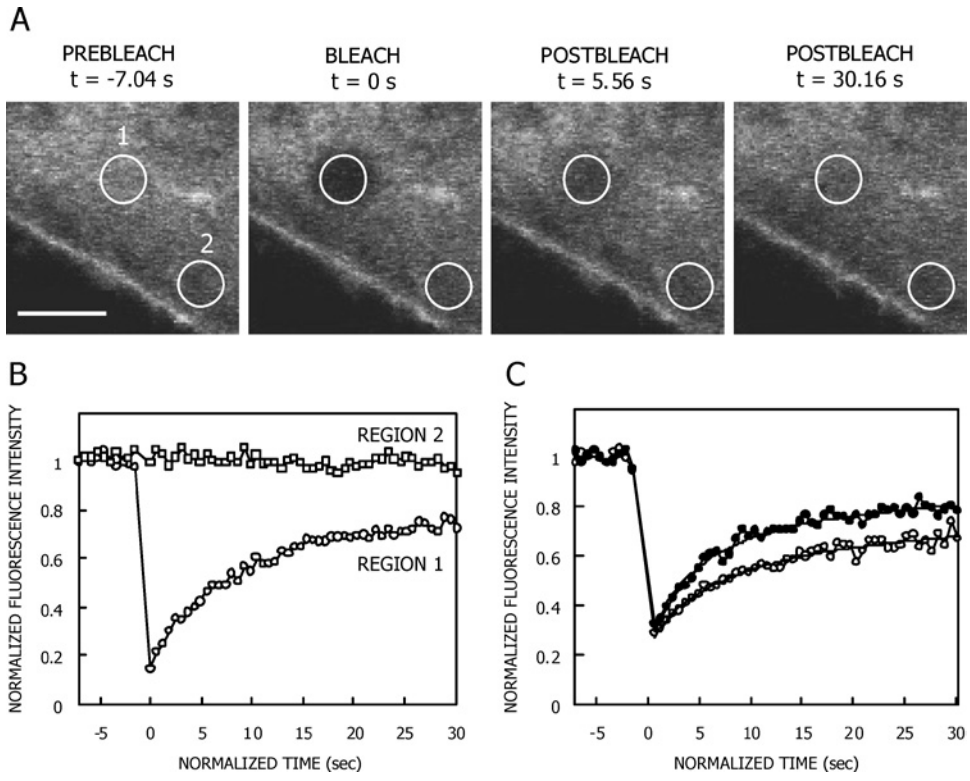

Fig. 3. Fluorescence recovery after photobleaching (FRAP) of serotonin$_{1A}$ receptors fused to EYFP in CHO cells. (A) Confocal fluorescence images corresponding to the base of the same cell are shown before and after photobleaching for the indicated duration of time. The prebleach image is shown at time $t < 0$ and the bleach event is shown at time $t = 0$. (B) Normalized fluorescence intensity in regions 1 (bleach region) and 2 (control region) of the images in panel A are shown for the entire duration of the FRAP experiment. The constant fluorescence intensity in region 2 indicates no significant photobleaching of the field due to repeated imaging. The prebleach intensities are shown at time, $t < 0$. (C) Typical fluorescence recovery plots of the serotonin$_{1A}$ receptor–EYFP fusion protein in cells in the absence (–○–) or presence (–●–) of aluminum fluoride (AlF_4^-), a receptor-independent activator of G-proteins. The curves are nonlinear regression fits to the model describing fluorescence recovery under uniform disk illumination condition. The scale bar represents 5 μm. The plot in (C) is adapted from Ref. [53].

G-proteins) receptor diffusion, which would be partially relieved when the G-protein dissociates from the receptor. Another possibility could be that the increase in receptor diffusion could reflect changes in the oligomeric state of the receptor, as has been shown for the δ-opioid receptor [89] and the cholecystokinin receptor [90], or their partitioning into or out of domains proposed to exist on the cell surface [12,13]. There is growing evidence on the compartmentalized localization of G-proteins in detergent-insoluble, cholesterol-rich membrane domains [91], which have been reported to diffuse as separate entities on the membrane [92]. Importantly, we have earlier observed that the cell surface dynamics of the serotonin$_{1A}$ receptor is modulated by membrane cholesterol [93], an essential constituent of such domains [55,68]. Whether the differences in the diffusion properties of the receptor while being associated with or without G-proteins result from the movement of receptors into or out of such domains enriched in cholesterol represents an interesting possibility and is currently being addressed in our laboratory. Our results on the G-protein-dependent cell surface dynamics of the serotonin$_{1A}$ receptor provide novel insight into signal transduction involving this receptor. Due to similarity in the initial events of signal transduction involving GPCRs, it is possible that the increase in receptor mobility upon G-protein activation could take place in case of other GPCRs as well. Analysis of GPCR mobility therefore could be a sensitive and powerful approach to assess receptor/G-protein interaction in intact cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

The development of fluorescence-based approaches to monitor protein expression, distribution, and dynamics has tremendously contributed to the understanding of protein functions in an intact cellular environment. Techniques for visualizing proteins in cells have earlier relied on the use of fluorescently labeled antibodies and ligands. The GFP-fusion technology represents an important development in fluorescence imaging of cellular proteins. In combination with imaging technologies with improved signal-to-noise ratio and with enhanced spatial and temporal resolution, the use of autofluorescent proteins to monitor protein function in real time in cells appears poised for further growth.

However, the use of GFPs as fluorescent reporters has a few limitations. The early variants of GFPs have been reported to display a tendency to self-associate into dimers [16]. Such associations can induce significant artifacts in fluorescence resonance energy transfer experiments due to erroneous estimation of proximity of proteins tagged with GFP. The generation of nonoligomerizing mutants of GFP variants has provided an alternative to this problem [19]. The most notable limitation of the GFP family of proteins as fluorescent markers is their molecular mass ($\sim$27 kDa) that is large enough to induce perturbations upon fusion to relatively smaller proteins or proteins that function as part of large organized assemblies. For instance, the kinetics of the agonist-dependent internalization of the β_1- and β_2-adrenoceptors are slower for receptors tagged to GFP [94]. In addition, single-channel properties of the pentameric neuronal nicotinic acetylcholine receptor have been reported to be altered by fusion of its subunits with GFP [95].

Such limitations may be overcome with the use of smaller fluorescent probes. A promising alternative to GFP is the biarsenical-tetracysteine system, more specifically the fluorescein arsenical hairpin binder (FlAsH) [96–98]. FlAsH is fluorescent only when bound to a small tetracysteine motif, CCPGCC that can be introduced into proteins at the genetic level. The applicability of this technology is still limited due to partial reactivity of the fluorescent probe to proteins containing the tetracysteine motif in a cellular environment, in addition to high background staining that reduces its sensitivity compared to GFPs [19]. Nonetheless, a recent report describes the use of this fluorescent reporter to visualize a GPCR, the adenosine A_{2A} receptor, in intact cells [98]. Another constraint with GFP-like proteins is the complicated photophysics of such molecules that leads to phenomena such as reversible photobleaching. These characteristics are exhibited by several variants of GFPs and can induce significant artifacts in photobleaching experiments to determine molecular mobility and in resonance energy transfer experiments to determine proximity of molecules [99]. Such artifacts can be minimized with the use of fluorescent proteins such as EYFP that show minimal reversible photobleaching compared to other variants such as ECFP [99]. Alternatively, techniques that provide information on molecular mobility but are not based on fluorescence photobleaching, such as fluorescence correlation spectroscopy (FCS) [100], can be employed to measure diffusion rates of molecules. Importantly, discrete analysis of subpopulations of molecules diffusing at different rates can be conveniently carried out using fluorescence correlation spectroscopy. The application of this technique to detect the presence of multiple diffusing populations of the serotonin$_{1A}$ receptor and the relative abundance of these populations under conditions of receptor activation represents a useful means to analyze receptor function.

Notwithstanding these shortcomings, the GFP-fusion technology continues to be a powerful tool to monitor expression, localization, and mobility of cellular proteins in general, and GPCRs in particular, even after more than a decade since it was first used. Ongoing efforts to develop newer fluorescence-based probes, some of them generated by mutating existing GFP variants for enhanced brightness, more red-shifted spectral properties, reduced sensitivity to pH and halides, and the ability to be activated by light [19], combined with enhanced fluorescence detection technologies, would certainly contribute to a more refined understanding of the molecular aspects of cellular functions in the future.

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