3 Membrane Organization and Dynamics of the Serotonin\textsubscript{1A} Receptor Monitored Using Fluorescence Microscopic Approaches

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RELEVANCE OF MEMBRANE ORGANIZATION AND DYNAMICS TO PROTEIN FUNCTION

Biological membranes are complex noncovalent assemblies of a diverse variety of lipids and proteins. From the original proposal of membranes containing lipids by Overton in 1895 to Gorter and Grendel’s concept of a membrane bilayer, to Singer and Nicolson’s fluid mosaic model, it has been a long journey of evolution in ideas on the organization of biological membranes (see [1] for a historical perspective). Subsequent developments have greatly refined this model. The current understanding of the structure of biological membranes will be described in this section in the context of its implications in the functioning of membrane proteins.

Because a significant portion of integral membrane proteins remains in contact with the membrane [2] and reaction centers in them are often buried within the membrane, the function of membrane proteins depends on the surrounding membrane environment. Lipid–protein interaction in membranes has attracted much attention in relation to its role in assembly, stability, and function of membrane proteins [2–4]. These effects have been attributed either to specific interactions of lipids with residues in proteins or to bulk properties of membranes. Considering the diverse array of membrane lipids, and a large repertoire of membrane proteins, it is believed that physiologically relevant processes occurring in membranes involve an intense coordination of multiple lipid–protein interactions. Since the organization and dynamics of membranes have considerable impact on membrane protein structure and function [5,6], the development and characterization of experimental tools to analyze these aspects of membranes assume significance.

The fluid mosaic model for cell membranes [7] envisages a largely fluid membrane bilayer in which proteins are embedded. This model proposes a dynamic bilayer with free translational diffusion of lipids and proteins and possible interactions between them, and a restricted movement of the membrane components across the bilayer which would preserve asymmetry of the bilayer. Some of the tenets put forward by this model were soon modified to accommodate experimental observations emerging from several laboratories, both in model membrane systems and in biological membranes, which favored a nonrandom organization of lipids and proteins in the form of domains [8]. The current understanding of membrane organization incorporates the idea of membrane domains, which are enriched in specific lipids and proteins, and which facilitate processes such as trafficking, sorting, and signal transduction [9–11]. Several forms of membrane domains such as caveolae, lipid rafts, and glycolipid-enriched domains, which could have overlapping characteristics in terms of composition and physical properties, have been proposed [9,11,12].

The implication of membrane organization on the signaling functions of membrane proteins in general, and on G-protein coupled receptors (GPCR) in particular, is an interesting and emerging area. The relevance of membrane organization to GPCRs arises from the fact that they represent the largest class of molecules involved in signal transduction across the plasma membrane [13]. Indeed, genomewide analysis of integral membrane proteins indicates a larger representation of proteins with seven transmembrane domains than others in the human genome [14]. GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members which together constitute ~2% of the human genome [15]. They respond to a variety of ligands and mediate multiple physiological processes and have therefore emerged as major targets for the development of novel drug candidates in all clinical areas [16,17]. The classical view of receptor-G-protein function in cells proposes free diffusion of molecules on the cell surface such that the probability of such interaction would depend on random collisions [18]. However, the specific and rapid signaling responses, characteristic of GPCR activation, cannot be explained solely based on a uniform distribution of receptors, G-proteins and effectors, one or more of which could even be in low abundance, on the cell surface [19,20]. This leads to the possibility that receptor-G-protein interactions may be dependent on their organization in membranes and not solely on the binding sites present on the interacting proteins. Spatiotemporal regulation of interactions between receptor, G-proteins, and effectors on the cell surface by the restriction imposed on their mobility along with selectivity of receptors to specific G-protein subunits and effectors is now believed to be an important determinant in GPCR signaling [18–21].

New technologies to analyze GPCR function in an intact cellular environment are predicted to have a major impact on GPCR research [16]. 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 organization and 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 [22,23]. This chapter aims to provide experimental guidelines to the successful application of fluorescence-based approaches such as quantitative fluorescence imaging and fluorescence recovery after photobleaching (FRAP) to yield novel information regarding the organization and dynamics of the serotonin_{1A} receptor, a representative member of the GPCR superfamily.

THE SEROTONIN_{1A} RECEPTOR

The serotonin_{1A} receptor binds the neurotransmitter serotonin and signals across the membrane through its interaction with heterotrimeric G-proteins which are membrane-associated signaling molecules on the cytoplasmic side of the membrane. Among ~14 different subtypes of serotonin receptors, the serotonin_{1A} receptor is one of the most extensively studied for a number of reasons. These include its important role in neuronal physiology and the availability of a selective ligand 8-OH-DPAT (8-hydroxy-2-(di-N-propylamino)tetrinal) allowing extensive biochemical, physiological, and pharmacological characterization of the receptor [24–26]. The serotonin_{1A} receptor has been shown to have a role in neural development [27], and protection of stressed neuronal cells undergoing degeneration and apoptosis [28]. Treatment using agonists for the serotonin_{1A} receptor constitutes a potentially useful approach in case of children with developmental disorders [29]. The serotonin_{1A} receptor agonists and antagonists represent a major class of molecules with potential therapeutic effects in anxiety- or stress-related disorders [25,26]. As a result, the
serotonin<sub>1A</sub> receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. Interestingly, mutant (knockout) mice lacking the serotonin<sub>1A</sub> receptor exhibit enhanced anxiety-related behavior [30], and represent an excellent model system to understand anxiety-related behavior in higher animals [31]. On the clinical front, serotonin<sub>1A</sub> receptor levels have been shown to be altered in schizophrenia, and in patients suffering from major depression [25]. Interestingly, a recent observation has associated genetic polymorphisms at the upstream repressor region of the serotonin<sub>1A</sub> receptor gene to major depression and suicide in humans [32] linking its expression status to these clinical syndromes. The selective serotonin<sub>1A</sub> receptor agonist 8-OH-DPAT has recently been shown to inhibit growth of *Plasmodium falciparum* (reviewed in [33]) opening novel possibilities in antimalarial drug research. Besides, serotonin<sub>1A</sub> receptors are implicated in feeding, regulation of blood pressure, temperature, and working memory [25]. Taken together, these reports highlight the key role played by the serotonin<sub>1A</sub> receptor in a multitude of physiological processes, and point toward the significance of serotonin<sub>1A</sub> receptors in human health and disease.

**MEMBRANE ORGANIZATION OF THE SEROTONIN<sub>1A</sub> RECEPTOR MONITORED USING DETERGENT INSOLUBILITY**

**RATIONALE**

In the context of the membrane environment being an important modulator of protein function, membrane domains could serve as platforms for signaling by concentrating certain lipids (such as cholesterol and sphingolipids) and proteins while excluding others [20,34,35]. 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 [9,36–38]. Insolubility of membrane components in non-ionic detergents such as Triton X-100 at low temperature has been a widely utilized biochemical tool to identify and characterize membrane domains [12,39]. 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 [40,41]. 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. Several GPI-anchored proteins, a few transmembrane proteins and certain G-proteins have been found to reside in detergent resistant membrane domains, popularly referred to as DRMs [34,39,42]. In spite of concerns on the possibility of membrane perturbation due to the use of detergents, resistance to detergent extraction continues to be a principal tool to study membrane domains since the need for relatively simple and straightforward biochemical methods for detecting membrane domains persists. Further, 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 [43,44].

The pharmacological and signaling functions of the serotonin<sub>1A</sub> receptor have been well addressed since the receptor has been cloned and heterologously expressed [reviewed in 25]. However, molecular details of the membrane organization and dynamics of the serotonin<sub>1A</sub> receptor have been relatively unexplored and are only beginning to be addressed now. The application of detergent insolubility as a biochemical means to understand membrane organization of the serotonin<sub>1A</sub> receptor will be described in the following section.

**EXPERIMENTAL METHODOLOGY**

Detection of proteins in detergent resistant membranes (DRMs) is usually performed either by immunoblotting or ligand binding studies. However, these methods are not suitable in detecting proteins (e.g., in the case of the serotonin<sub>1A</sub> receptor) when the availability of antibodies with high specificity is limited [45] and/or ligand binding is compromised in presence of the detergent [46]. Membrane proteins tagged with the green fluorescent protein (GFP) provide an alternative which can overcome these difficulties. GFP from the jellyfish *Aequorea victoria* and its variants have become popular reporter molecules for monitoring protein expression, localization, and dynamics of various membrane and cytoplasmic proteins [47]. More specifically, tagging of GPCRs with GFP has allowed direct visualization of signaling and their real-time trafficking in living cells [48]. The use of fluorescent reporter proteins has its advantages over fluorescently labeled ligands to visualize receptors because (1) the stoichiometry of the receptor and fluorescent protein is well defined as the latter is covalently attached to the receptor at the DNA level, (2) complications encountered while using fluorescent ligands such as ligand dissociation are avoided, (3) this approach allows analysis of the unliganded states of the receptor (not possible with bulky fluorescent groups to small endogenous ligands such as biogenic amines is eliminated, and (5) cellular biosynthesis ensures the presence of receptors attached to fluorescent proteins in cells and eliminates the necessity of labeling receptors with fluorescent ligands before each experiment.

The serotonin<sub>1A</sub> receptor has been fused to the enhanced yellow fluorescent protein (EYFP), a variant of the GFP, in order to visualize the receptor [49]. The EYFP in particular displays enhanced brightness and a more red-shifted fluorescence emission compared to the GFP. A schematic diagram indicating the site of the EYFP tag on the serotonin<sub>1A</sub> receptor, and its typical fluorescence distribution when stably expressed in Chinese hamster ovary (CHO) cells are shown in Figure 3.1. The serotonin<sub>1A</sub>-EYFP receptor was found to be essentially similar to the native receptor in pharmacological assays and therefore can be used to reliably explore aspects such as cellular distribution and dynamics on account of its intrinsic fluorescence [49]. We have employed this fusion protein to directly determine detergent insolubility of the serotonin<sub>1A</sub> receptor by a GFP-fluorescence-based approach [50]. This method is based on quantitating fluorescence of the membrane protein in cells before and after detergent treatment. It is important to compare the fluorescence of the same group of cells before and after detergent treatment at 4°C in order to obtain an unambiguous estimate of the fraction of receptors which are detergent insoluble.
the coverslip during this wash, it is advisable to perform the wash gently by allowing small volumes of cold buffer to pass over the coverslip. This should be repeated a few times to ensure substantial removal of the detergent.

5. Fluorescence images of the same group of cells acquired before and after extraction with detergent are analyzed using the Meridian DASY Master Program v4.19. Sections of cells largely representing the plasma membrane are selected and projected together resulting in a single combined image of the chosen sections. Outlines of each cell (or a small group of cells) are drawn, and integrated fluorescence intensities within these outlines are determined using the Meridian DASY Master Program. The extent of detergent insolubility of the receptor is estimated by comparing the integrated fluorescence intensities before and after detergent extraction of cells.

6. In order to validate this fluorescence-based approach, lipid and protein markers, whose insolubility in nonionic detergents such as Triton X-100 has been well described in literature, have been employed. For this, CHO-K1 cells labeled with membrane domain-specific fluorescent lipid probes, DiIC<sub>6</sub> and FAST Dil, or fluorescently labeled transferrin (see below) are used. Stock solutions of the DiI probes are made in absolute ethanol and diluted in HEPES-Hanks buffer to prepare the labeling solutions while ensuring that the residual ethanol concentration was always low (<1%, v/v). Stock solutions of Texas-red-labeled transferrin are prepared in PBS. Cells grown for 2 days are washed twice in cold HEPES-Hanks buffer (pH 7.4) before labeling them with either of these reagents. Cells are labeled either with DiIC<sub>6</sub>(8 μM for 75 min), or FAST Dil (14 μM for 35 min) at 4°C, or with Texas-red-labeled transferrin (100 μg/ml for 30 min) at 37°C, in HEPES-Hanks buffer. Labeled cells are washed three times in cold HEPES-Hanks buffer before performing detergent extractions as described above.

**Detergent Insolubility of the serotonin<sub>1A</sub> Receptor**

A typical fluorescence distribution of the serotonin<sub>1A</sub>-EYFP receptor upon detergent extraction is shown in Figure 3.2. Utilizing the approach described above, >26% of fluorescence of the serotonin<sub>1A</sub>-EYFP receptor is found to be retained upon extraction with 0.05% (w/v) Triton X-100 [50]. This represents the fraction of serotonin<sub>1A</sub> receptors which are resistant to detergent treatment under these conditions. In order to validate this fluorescence microscopic approach toward determination of detergent insolubility of membrane components, specific lipid (DiIC<sub>6</sub> and FAST Dil) and protein (transferrin receptor) markers were utilized, whose organization in membranes and ability to be extracted by cold, nonionic detergents have been well documented. The dialkyl-lyso-phosphatidylcholine (DiL) series of lipid analogues 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 [52–54]. For example, DiIC<sub>6</sub> with its two 16-carbon saturated alkyl chains preferentially partitions into relatively rigid (highly ordered) domains, whereas FAST Dil which has two 18-carbon chains with...
MEMBRANE DYNAMICS OF THE SEROTONIN_{1A} RECEPTOR

RATIONALE

As mentioned earlier, the lateral organization and dynamics of GPCRs in membranes have significant implications in the manner in which cellular signaling processes involving GPCRs are regulated. The significance of receptor lateral diffusion on the plasma membrane in the signaling functions of GPCRs forms the basis of the mobile receptor hypothesis [57]. This model proposes that receptor–effector interactions at the plasma membrane are controlled by lateral mobility of the interacting components. Evidence for this comes from reports that correlate receptor signaling to membrane dynamics of individual components involved in such signaling. These include experimental evidences such as (1) the dependence of the vasopressin V₂ receptor to activate adenylyl cyclase through G-proteins on the fraction of receptors that are mobile on the cell surface [58], (2) dependence of the agonist-stimulated adenylyl cyclase signal transduction process on the mobile fractions of proteins in reticulocyte plasma membranes [59], (3) the correlation between lateral diffusion of rhodopsin in the membrane and light-stimulated G-protein activation [60], and (4) theoretical calculations in simulated models where the efficacy of cellular signaling could be modeled more accurately based on the diffusion limited collisional encounter of receptors and G-proteins rather than mere density of receptors and G-protein in a given membrane [61]. This model has evolved taking into consideration more recent observations on the nature and specificity of GPCR signal transduction events along with the current understanding of the organization of cell membranes. Recent evidence indicates a spatiotemporally organized system of receptors and G-proteins in membrane domains (such as caveolae or non-ionic detergent-insoluble membrane microdomains such as lipid rafts, see previous text) rather than a freely diffusible system that is responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [18,21]. For example, the efficient interaction of β₁- and β₂-adrenergic receptors with adenylyl cyclase (compared to prostanlagnin E₂ receptors) appears to correlate with the localization of β₁- and β₂-adrenergic receptors and adenylyl cyclase (and absence of prostanlagnin E₂ receptors) together in caveolae [62]. Overexpression of adenylyl cyclase selectively enhances β-adrenergic receptor-mediated stimulation of adenylyl cyclase activity, but not that stimulation mediated by prostanlagnin E₂ receptors. Furthermore, β₂-adrenergic receptors are found to stimulate adenylyl cyclase more efficiently than β₁-adrenergic receptors. Although both β₁- and β₂-adrenergic receptors are initially localized in caveolae along with adenylyl cyclase, the latter signal to adenylyl cyclase with lower efficiency due to their translocation out of caveolae upon agonist-stimulation [63]. In addition, constitutive localization of the gonadotropin-releasing hormone (GnRH) receptor into low-density, non-ionic detergent-insoluble membrane fractions appears to be necessary for its signaling functions, namely activation of the extracellular signal-related kinase (ERK) [64]. Interestingly, stimulation of the GnRH receptor by its agonist has earlier been reported to reduce its lateral diffusion in the membrane [65], and induce homodimerization [66]. On the other hand, targeting of the oxytocin receptor, which is predominantly excluded from caveolae [67], to such membrane...
microdomains by its fusion with caveolin can turn the receptor-mediated inhibition of cell growth into a proliferative response [68]. Taken together, the spatiotemporal segregation of GPCRs and their effectors into microdomains has given rise to new challenges and complexities in receptor signaling since signaling now has to be understood in context of the three dimensional organization of various signaling components which include receptors and G-proteins.

Fluorescence recovery after photobleaching (FRAP) is a widely used approach to quantitatively estimate diffusion properties of molecules in cells. This approach provides information on the diffusion behavior of an ensemble of molecules, as the area monitored is large (in the order of micrometers) [69,70]. Fluorescence recovery after photobleaching involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the sample region. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from unbleached regions in the membrane is an indicator of mobility of fluorophores in the membrane. The recovery of fluorescence into the bleached spot in FRAP experiments is described by two parameters, an apparent diffusion coefficient (D) and mobile fraction (Mf). Thus, the rate of fluorescence recovery provides an estimate of the apparent D of molecules, whereas the extent of fluorescence recovery provides an estimate of Mf of diffusing molecules. It must be kept in mind that Mf is only an estimate of the fraction of molecules mobile in the time scale of the FRAP experiment.

We have analyzed the diffusion characteristics of serotoninα, EYFP receptors using FRAP to monitor the role of receptor-G-protein interaction in determining its membrane dynamics [49,71]. The following sections describe experimental details involved in a typical FRAP experiment on cells stably expressing the serotoninα, EYFP receptor.

**Experimental Methodology**

FRAP experiments are performed on an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany), with a 63x, 1.2 NA water-immersion objective using the 514 nm line of an argon laser. Fluorescence emission is collected using the 535-590 nm band pass filter. Images are recorded using a 225 μm pinhole thereby providing a resolution in the z-axis of 1.7 μm. Since temperature has a direct influence on the diffusion of molecules, FRAP experiments described here are performed on cells which have been acclimatized to a particular temperature by incubating them in a temperature-controlled chamber. Before imaging, glass coverslips with attached cells on them are washed with HEPES-Hanks buffer and mounted on an PCS2 closed temperature-controlled Biopuchs chamber (Butler, PA). The chamber is gently perfused with buffer and allowed to attain the required temperature. Importantly, cells are maintained under these conditions for a period of ~10 min before performing FRAP experiments.

1. The rule of thumb for any FRAP experiment is to achieve significant photobleaching within the shortest time possible. Thus, a short bleach event with high laser strength is preferable to a long event with low laser strength. This is because the bleach duration should in principle be

... infinitesimally small compared to the characteristic diffusion time (τD), or the time it takes for fluorescence recovery after photobleaching to reach its half-maximal value. A bleach duration comparable to or longer than the characteristic diffusion time of the fluorophore in a particular system can induce formation of a "corona" around the bleached region. This occurs due to repeated photobleaching of fluorophores adjacent to the bleach region on account of their diffusion into the bleach region effectively increasing the bleached spot size [72,73]. A consequence of this is an underestimation of the diffusion coefficient. To avoid this, our experiments are typically performed under conditions where the illuminating light intensity (laser strength) is set to its maximum to achieve the shortest possible bleach period. Consequently, optimization of fluorescence imaging parameters is necessary to avoid photobleaching of the sample during routine monitoring of fluorescence.

3. The area to be monitored and photobleached in the sample (preferably a circular region of interest (ROI) of ~1-2 μm in radius) is then selected. We frequently perform FRAP on the basal surface of well spread cells in contact with the glass coverslip. This is because analysis of FRAP data based on the theoretical framework described below assumes that fluorescence recovery into the bleached spot is isotropic in the plane of the membrane, which would be more true when monitoring the uniformly fluorescent bottom surface of cells attached to the coverslip. Furthermore, the planar geometry of the uniformly fluorescent bottom surface of cells ensures that the dimensions of the circular ROI used for bleaching are not distorted in the actual sample.

4. Present-day confocal microscopes use an area bleaching protocol where a focused laser beam is used to bleach and monitor an ROI, as opposed to a point bleach protocol where the laser bleaches and monitors a single spot in the sample. The number of times the laser scans the ROI to achieve a significant extent of bleach then needs to be set. This number depends on intrinsic factors such as photostability and mobility of the fluorophore in a given system, and on extrinsic factors such as laser power and scan speed of the laser beam, and should typically reduce fluorescence in the ROI area by 70-80% of the initial value in order to observe appreciable
fluorescence recovery. Higher extents of bleach can induce artifacts in FRAP experiments since it could damage the sample or induce crosslinking of fluorophores.

5. The total number of scans to be acquired for the entire experiment using the time series dialog is then set. This refers to the number of scans determined in the optimization procedure before significant photobleaching of the sample becomes apparent. At the same time, the time interval between successive scans is specified. This value is dependent on the dynamics of the particular system being monitored. For a fast process, the time interval would be small whereas for a slow process, the time interval would be large. To ensure complete recovery of fluorescence after bleach, it is advisable to monitor fluorescence recovery for a period of ~4τp.

6. After performing a FRAP experiment based on the guidelines specified above, data representing the mean fluorescence intensity in the ROI as a function of time is background subtracted. Data for background fluorescence can be collected by performing a mock FRAP experiment on an area without cells (or fluorescence). This data is normalized to both the prebleach fluorescence intensity in the ROI and the time of bleach. The latter is achieved by subtracting the midpoint of the bleach duration from each data point on the time axis. This results in the first postbleach time point starting from a time t > 0.

7. Data on the change in the mean background-subtracted fluorescence intensity in the ROI (F(t)) vs. normalized time (t) is analyzed based on the uniform-disk illumination model [74] (according to Equation 3.1) which describes fluorescence recovery into a spot that is uniformly photobleached or which has a steplike intensity profile across the bleached spot (as is nearly the case in the present experiments).

\[
F(t) = [F(\infty) - F(0)][\exp(-2\tau_p)I(0)(2\tau_p/t) + I(2\tau_p/t)] + F(0) \tag{3.1}
\]

where \(F(\infty)\) is the recovered fluorescence at time t \(\rightarrow \infty\), \(F(0)\) is the bleached fluorescence intensity in the ROI immediately after bleach, and \(\tau_p\) is the characteristic diffusion time. \(I(0)\) and \(I(1)\) are modified Bessel functions. We routinely perform nonlinear curve fitting of the recovery data to Equation 3.1 using GraphPad Prism software version 4.00 (San Diego, CA). Diffusion coefficient (D) is determined from the equation:

\[
D = 0^2/4\tau_p \tag{3.2}
\]

where \(0^2\) is the radius of the ROI. Mobile fraction (M) is determined from the equation:

\[
M = [F(\infty) - F(0)]/[F(0) - F(0)] \tag{3.3}
\]

where \(F(0)\) is the mean background corrected and normalized prebleach fluorescence intensity.

8. It should be kept in mind that precise determination of the diffusion coefficient from a FRAP experiment depends to a large extent on how similar the dimensions of the set ROI (o) are to the actual bleached spot in the sample (see Equation 3.2). For routine experiments, one assumes that the actual size of the bleach spot is the same as the dimensions of the ROI. However, this may not be true for all experiments. Since the bleach duration is never very small compared to the characteristic diffusion time (\(\tau_p\)), especially for present day confocal microscopes with relatively low power lasers, the effective size of the bleach spot would depend on the duration of bleach. A long bleach duration in a sample with high mobility can broaden the bleach spot leading to an underestimation in D. These estimates can be corrected to a significant extent by analyzing the effective bleached spot size obtained in such FRAP experiments by relatively simple image analysis procedures previously described by us [75]. Obtaining consistent and reliable quantitative estimates of mobility of molecules using FRAP can be hindered by the lack of appropriate standards for calibrating the FRAP set-up (microscope configuration and data fitting algorithms) used in a given experiment [75]. We have validated our FRAP experiments performed on serotonin1A-EYFP receptors in cells by independent measurement of the mobility of a standard solution of EGFP in a viscous solution using a similar experimental procedure described above [75]. 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.

**G-protein Dependent Cell Surface Dynamics of the Serotonin1A Receptor**

In light of the proposed significance of a spatiotemporally restricted environment in modulating receptor and G-protein interaction, we have analyzed the effect of serotonin1A receptor activation on its cell surface dynamics (diffusion characteristics) using FRAP [49,71]. These experiments indicate that the mobility of the receptor is dependent on its interaction with G-proteins. For example, pre-incubation with agents that activate G-proteins through receptor-dependent and -independent means increases receptor mobility on the plasma membrane. A typical FRAP experiment performed on cells expressing serotonin1A-EYFP receptors under optimized imaging conditions is shown in Figure 3.3. The figure also shows the increase in fluorescence recovery kinetics of serotonin1A-EYFP receptors in presence of aluminum fluoride (AlF3), a receptor-independent activator of G-proteins in cells.

The G-protein heterotramer is a large protein complex with an average molecular mass of ~88 kDa [76], 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 Gs subunit of G-proteins, resulting in the dissociation of G-protein heterotrimer complex from the
FIGURE 3.3 (Color figure follows p. 110.) FRAP of serotonin_{A}EYFP receptors 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, red) and 2 (control region, blue) 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_{A}EYFP receptor in cells in the absence (blue) or presence (red) of aluminum fluoride (AlF_{3}), 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. Adapted and modified from Ref. [49].

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. Importantly, these results for the first time provide convincing evidence that the cell surface dynamics of a GPCR is dependent of 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 [77]. 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 [78], truncation of the cytoplasmic domains of transmembrane proteins [79], or a lack of interaction of membrane proteins with cytoplasmic effector molecules [80] 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 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 [81] and the cholecystokinin receptor [82], or their partitioning into or out of domains proposed to exist on the cell surface [20]. Incidentally, there is growing evidence on the compartmentalized localization of G-proteins in detergent-insoluble, cholesterol-rich membrane domains [19], which have been reported to diffuse as separate entities on the membrane [77,83]. Whether differences in the diffusion properties of the receptor upon agonist activation result from its movement into or out of such domains enriched in cholesterol represents an interesting possibility. In this regard, our recent report on the detergent insolubility of serotonin_{A}EYFP receptors under conditions of agonist stimulation suggests that this may not be the case [56]. The extent of detergent insolubility of serotonin_{A}EYFP receptors remains unchanged in the presence or absence of the agonist, although agonist treatment is found to enhance the diffusion coefficient of the receptor [49].

Our results on the G-protein dependent cell surface dynamics of the serotonin_{A} 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 be occurring for 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.

CONCLUSION

The fluorescence-based approaches and their application to the serotonin_{A} receptor described in this chapter provide novel insights into the membrane biology of the serotonin_{A} receptor, and represent promising strategies to understand membrane protein function under native-like conditions. In conjunction with recent advances on the role of the membrane lipid environment (especially cholesterol) on the ligand binding, G-protein coupling and membrane organization of the serotonin_{A} receptor (reviewed in 6,25,26), the methodologies described here provide significant insight into developing a comprehensive understanding of receptor function. This area of research assumes relevance considering the fact that membrane protein function and its dependence on the lipid environment are found to have enormous implications in health and disease [6].

ACKNOWLEDGMENTS

Work in A.C.'s laboratory is supported by the Council of Scientific and Industrial Research, Department of Biotechnology, Life Sciences Research Board, and the
REFERENCES


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