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Lateral diffusion contributes to FRET from lanthanide-tagged membrane proteins

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Abstract

Diffusion can enhance Förster resonance energy transfer (FRET) when donors or acceptors diffuse distances that are similar to the distances separating them during the donor's excited state lifetime. Lanthanide donors remain in the excited state for milliseconds, which makes them useful for time-resolved FRET applications but also allows time for diffusion to enhance energy transfer. Here we show that diffusion dramatically enhances FRET between membrane proteins labeled with lanthanide donors. This phenomenon complicates interpretation of experiments that use long-lived donors to infer association or proximity of mobile membrane proteins, but also offers a method of monitoring diffusion in membrane domains in real time in living cells.

Keywords

GPI-anchored proteins; FRET; GPCR; diffusion; HTRF; TR-FRET

1. Introduction

Förster resonance energy transfer (FRET) between donor and acceptor fluorophores is frequently used to study association of macromolecules. Energy transfer requires only that donors and acceptors be in close proximity and in a permissive orientation. Therefore, FRET can take place between molecules that are in close proximity by chance as well as between molecules that are associated with each other. The former mechanism produces nonspecific or “bystander” energy transfer that must be distinguished from specific energy transfer in studies of molecular association. This is an especially important consideration for membrane proteins, which are concentrated by virtue of their association with a 2-dimensional surface.

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Conflict of interest
None declared.

Lanthanide ions (Tb^{3+} and Eu^{3+}) are unique FRET donors because they remain in the excited state for much longer time periods than typical organic fluorophores ($\sim 10^{-3}$ seconds versus 10^{-9} - 10^{-8} seconds). This property allows donor and acceptor emission to be monitored in a time-gated fashion, after background autofluorescence and acceptor emission due to direct excitation have decayed, thus greatly facilitating detection of FRET. The recent introduction of improved chelates and labeling methods has led to an increase in the use of lanthanide donors in time-resolved FRET (TR-FRET) assays in living cells. For example, lanthanide donors have been widely used to study association or oligomerization of plasma membrane proteins in cells [1,2]. However, a potential disadvantage of lanthanide donors is that their long excited state lifetimes may allow diffusion to bring initially distant donors and acceptors into close proximity, thereby enhancing bystander FRET. This generally does not occur with conventional organic fluorophores, as processes such as diffusion do not significantly change the distance between macromolecules in 10^{-8} seconds. It has previously been shown that diffusion greatly enhances FRET from lanthanide-labeled molecules in solution, allowing efficient energy transfer to occur when acceptors are present at low concentrations [3]. Given the density of proteins in the plasma membrane and the rate at which they diffuse, it has been suggested that diffusion should also significantly enhance FRET from membrane proteins labeled with lanthanide donors [4]. Despite the widespread use of lanthanide donors in studies of membrane proteins this prediction has not been experimentally verified, and the possibility that diffusion contributes to FRET between membrane proteins has not been considered. Here we show that diffusion greatly enhances FRET from membrane proteins labeled with lanthanide donors, and discuss the implications of this phenomenon for studies of membrane protein oligomerization and nanoscale organization.

2. Materials and methods

2.1 Plasmid DNA constructs

SNAP- β_2 AR was obtained from New England Biolabs (Ipswich, MA). A fragment incorporating a cleavable signal sequence, SNAP- β_2 AR and *Bam*HI and *Xho*I restriction sites was amplified and subcloned into pcDNA5/FRT/TO. SNAP-GPI was constructed by replacing YFP in YFP-GL-GPI (provided by Dr. Anne Kenworthy, Vanderbilt University, Nashville, TN) with a cleavable signal sequence and the SNAP tag using an adaptation of the QuikChange (Agilent Technologies, Santa Clara, CA) mutagenesis protocol. SNAP-GPI was subcloned into pcDNA5/FRT/TO with *Hind*III and *Xho*I. Both constructs were verified by automated sequencing.

2.2 Generation of Flp-In T-REx 293 cells expressing SNAP- β_2 AR and SNAP-GPI

Flp-In T-REx HEK 293 cells (Invitrogen, Carlsbad, CA) were maintained in growth medium (minimum essential medium supplemented with 10% FBS, 4 mM glutamine, and 1% penicillin/streptomycin mixture at 5% CO_2 , 37 °C) supplemented with $15 \mu g ml^{-1}$ blasticidin and $100 \mu g ml^{-1}$ zeocin, and were transfected with a 1:9 ratio of SNAP- β_2 AR /FRT/TO or SNAP-GPI/FRT/TO and pOG44 in growth medium using linear polyethyleneimine (MW 25,000; Polysciences Inc., Warrington, PA) at an N/P ratio of 20. 24 hours after transfection, cells were reseeded in medium supplemented with $100 \mu g ml^{-1}$

hygromycin B and 15 $\mu\text{g ml}^{-1}$ blasticidin but without zeocin to select stably transfected cells. Positive cell colonies were collected as a pool and screened by fluorescence microscopy. Expression of SNAP- $\beta_2\text{AR}$ and SNAP-GPI was induced by incubating with tetracycline (0-0.1 $\mu\text{g ml}^{-1}$) for 24 hours prior to experiments.

2.3 Radioligand binding

Flp-In T-REx SNAP- $\beta_2\text{AR}$ cells were seeded onto 24-well plates at 2×10^5 cells per well. Expression was induced by incubating with tetracycline (0-0.1 $\mu\text{g ml}^{-1}$) for 24 hours. After induction cells were incubated in MEM containing 30 nM [^3H]-CGP12177 for 90 minutes at room temperature. Cells were washed twice with ice cold MEM, and surface-bound ligand was extracted with 0.5 ml of 1 M NaOH for 2 hours. Radioactivity was counted by liquid scintillation counting in 3.5 ml of Ecoscint A (National Diagnostics, Atlanta, GA). Nonspecific binding was determined in the presence of 20 μM alprenolol. Binding sites per cell was calculated by comparing the specific radioactivity recovered from each well to the radioactivity of a known input (0.6 pmol), and dividing by the number of cells in each well. Receptor density was calculated using the surface area of HEK 293 cells ($670 \mu\text{m}^2$) measured in a previous study. For SNAP- $\beta_2\text{AR}$ this calculation yielded 2,090 receptors μm^{-2} . The density of SNAP-GPI was estimated by multiplying this value by the ratio of donor emission values measured from an equivalent number of SNAP-GPI- and SNAP- $\beta_2\text{AR}$ -expressing cells labeled with donor only, yielding a density of 3,340 SNAP-GPI μm^{-2} .

2.4 Cell labeling and FRET spectroscopy

Cells expressing SNAP- $\beta_2\text{AR}$ or SNAP-GPI were labeled with 20 nM Lumi4-Tb and 80 nM SNAP-red (Cisbio, Codolet, France) for 2 hours at 37°C in complete growth medium. Cells were washed three times in PBS, harvested by trituration, and transferred to white opaque 96-well plates. Time-resolved FRET was measured using a Biotek (Winooski, VT) Synergy 2 plate reader using a xenon flash lamp. Filters used for excitation, donor emission and acceptor emission were 330/80 nm, 620/10 nm and 665/7 nm, respectively, and emission was integrated for 10 ms starting 150 μs after each excitation flash. Background and donor bleedthrough were negligible at this 665 nm, and direct acceptor excitation was undetectable after 150 μs , therefore Em 665 corresponds essentially to sensitized emission.

2.5 Measuring FRET efficiency

In order to measure FRET efficiency (E) cells expressing high levels of SNAP- $\beta_2\text{AR}$ or SNAP-GPI were incubated for 5 minutes with 20 nM Lumi4-Tb only to label a fraction of the cell surface SNAP tags. Donor-labeled cells were washed with PBS and used to measure donor intensity in the absence of the acceptor (I_D). The same cells were then incubated with 80 nM SNAP-red for 2 hours and used to measure (quenched) donor intensity in the presence of the acceptor (I_{DA}). Finally, the same cells were fixed by adding PFA/GA and used to measure partially dequenched donor emission. FRET efficiency was calculated as: $E = 1 - (I_{DA}/I_D)$. To control for possible changes in donor emission due to time or photobleaching parallel experiments were performed with 20 μM benzylguanine (a non-

fluorescent SNAP substrate) substituted for SNAP-red. These controls indicated that I_D was stable in the absence of an acceptor.

2.6 Calculating expected FRET efficiency

Expected FRET efficiency was calculated using the analytical approximation of Wolber and Hudson [5]. Reduced acceptor density (C_A) was calculated using the estimated densities of SNAP- β_2 AR or SNAP-GPI multiplied by 0.8 (assuming stoichiometric labeling of donor and acceptor) and a Förster distance (R_0) of 58 Å. A distance of closest approach (R_e) of 0 Å was assumed. This assumption maximizes the estimated FRET efficiency, and is consistent with exposure of covalently-linked benzyl-linker-fluorophore substrates on the surface of the alkyltransferase SNAP-tag (PDB: 3L00). Monte Carlo simulations were carried out using the same parameters and ExiFRET (www.exifret.com) [6].

2.7 Manipulating diffusion

Most experiments were performed at room temperature (RT; $\sim 25^\circ\text{C}$). In some experiments labeled cells were first incubated at 37°C or on ice ($\sim 0^\circ\text{C}$), then immediately transferred to 96-well plates for continuous measurement of FRET as the sample temperature was allowed to return to RT. The actual temperature of the samples at the time of the first measurement was unknown due to the time required for cell transfer and the thermal density of the 96-well plate. Therefore, we refer to the starting temperatures of these experiments as “nominally” 37°C or 0°C . Control experiments measuring temperature with a thermistor indicated that samples started nominally at 0°C approached room temperature with a time constant of 74 seconds ($n=2$), which was similar to the time course of FRET changes. SNAP- β_2 AR or SNAP-GPI were immobilized by fixation with a final concentration of 4% paraformaldehyde and 0.2% glutaraldehyde (PFA/GA) in PBS, or by biotin-avidin crosslinking. For biotin-avidin crosslinking cells were incubated with 0.5 mg ml^{-1} NHS-sulfo-LC-LC-biotin for 15 minutes, washed, then incubated with 0.1 mg ml^{-1} immunopure avidin (Thermo Fisher Scientific Pierce, Waltham, MA).

2.8 Confocal microscopy

In order to determine the effect of aldehyde fixation on acceptor emission cells expressing SNAP- β_2 AR were grown on glass coverslips and were labeled with SNAP-red (80 nM) only for 2 hours at 37°C in complete growth medium. Cells were washed with PBS, and imaged using a Leica (Wetzlar, Germany) SP2 scanning confocal microscope and a 63X, 1.4 NA objective. SNAP-red was excited at 633 nm, and emission was collected at 640-800 nm. After a 2 minute control period PFA/GA was added, and images were collected for an additional 15 minutes.

3. Results

In order to study the possible oligomerization of β_2 adrenergic receptors we produced a stable HEK293 cell line that expressed SNAP-tagged receptors (SNAP- β_2 AR) under the control of a tetracycline-inducible promoter. Intact cell radioligand binding studies showed that cells exposed to increasing concentrations of tetracycline expressed between 2.6×10^4 and 1.4×10^6 SNAP- β_2 ARs per cell, or between 40 and 2,000 per μm^2 of surface area.

Extracellular SNAP tags were labeled with cell-impermeant substrates conjugated to a Tb³⁺ cryptate donor and a red acceptor fluorophore at a 1:4 molar ratio (see **Materials and methods**). Donor- and acceptor-labeled SNAP-β₂AR produced robust FRET, with an acceptor/donor FRET ratio (Em 665/620) that increased as receptor expression increased (*n*=3; Fig. 1A).

Density-dependent FRET between SNAP-β₂AR protomers suggested that at least a fraction of the labeled protomers might exist in a monomer-dimer equilibrium. We reasoned that if this was the case, then increasing the temperature might promote dimer dissociation and thus decrease FRET, whereas decreasing the temperature might promote association and increase FRET. Surprisingly, we observed exactly the opposite changes: increasing the temperature increased FRET, whereas decreasing the temperature decreased FRET (Fig. 1B). These changes were completely reversible, as FRET recovered as the samples were allowed to return to room temperature (*n*=3; Fig. 1B).

In light of these results we considered the possibility that the temperature-dependence of FRET might reflect the temperature-dependence of diffusion [4]. To test this idea we fixed cells with mixed aldehydes, which covalently crosslinks and immobilizes membrane proteins. Aldehyde fixation dramatically decreased FRET between SNAP-β₂AR protomers (*n*=5; Fig. 2A, B). The apparent decrease in FRET was not due to a direct effect of aldehydes on either the donor or acceptor, as fixation had no effect on donor emission (105 ± 8% of control, *n*=3) or acceptor emission after direct excitation (98 ± 8% of control, *n*=3) in cells that were labeled with donor only or acceptor only. As a further test of this idea we employed cell surface biotinylation and avidin crosslinking as an alternative method of immobilizing transmembrane proteins [7]. Avidin crosslinking was slightly less effective than fixation, but also drastically decreased FRET between SNAP-β₂AR protomers (Fig. 2A, B). The effects of avidin crosslinking and aldehyde fixation were not additive, and FRET was no longer sensitive to decreased temperature after immobilization by either method (Fig. 2C), consistent with the idea that all three manipulations decreased FRET by a common mechanism.

These results suggested that much of the FRET signal between SNAP-β₂AR protomers was bystander FRET that depended on diffusion rather than specific FRET due to oligomerization. In order to explore this phenomenon further we constructed a model membrane protein that consisted only of a SNAP tag anchored to the extracellular surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and generated a cell line that expressed this protein (SNAP-GPI) under the control of a tetracycline-inducible promoter. Calibration using samples of SNAP-β₂AR (expressing a known number of cell surface receptors) and SNAP-GPI labeled with donor only indicated that SNAP-GPI density reached 3,340 μm⁻² at the highest level of expression. GPI-anchored proteins diffuse more rapidly than transmembrane proteins (*D* ~0.5 and ~0.1 μ² sec⁻¹, respectively) [8], thus we predicted that diffusion would have a large effect on FRET between SNAP-GPI molecules. Consistent with this prediction, SNAP-GPI produced very efficient FRET (Fig. 2D), with Em 665/620 values that were 4-5 fold greater than those produced by SNAP-β₂AR at comparable levels of expression (Fig. 2E). Moreover, low temperature, aldehyde fixation and biotin-avidin crosslinking all decreased FRET between SNAP-GPI in a mutually-

occlusive manner, as was the case with SNAP- β_2 AR ($n=3-5$; Fig. 2D-F). Since GPI-anchored proteins are thought to cluster weakly in cholesterol-dependent membrane domains (lipid rafts) [9,10], we also tested the effect of cholesterol depletion with methyl- β -cyclodextrin (M β CD, 5 mM) on FRET between SNAP-GPI. M β CD decreased Em 665/620 from 1.12 ± 0.29 to 0.58 ± 0.26 ($n=5$), consistent with previous reports of M β CD effects on the organization and mobility of GPI-anchored proteins [8,9].

A hallmark of diffusion-enhanced FRET with long-lived donors is FRET efficiency (E) that is greater than that expected for comparable short-lived donors [3]. We measured E in cells expressing SNAP- β_2 AR or SNAP-GPI by monitoring quenching of the donor upon labeling with the acceptor. Observed E values were 0.30 ± 0.06 ($n=4$) for SNAP- β_2 AR and 0.70 ± 0.03 ($n=4$) for SNAP-GPI at room temperature at the highest levels of expression. We then used the analytical approximation of Wolber and Hudson [5] and Monte Carlo simulations [11] to calculate the expected E for randomly distributed proteins at the same densities labeled with short-lived but otherwise identical donors (see **Materials and methods**). Both approaches yielded identical E values of 0.19 for SNAP- β_2 AR and 0.28 for SNAP-GPI. These values were considered to be upper limits, because both calculations were made using a distance of closest approach of 0 Å. The observed FRET efficiencies for both proteins were therefore substantially greater than expected for short-lived donors. E could be greater than expected because of diffusion, or because these proteins are not distributed randomly in the plasma membrane. Indeed, β_2 AR is widely thought to oligomerize in cells [12], and GPI-anchored proteins are thought to cluster in small, dense nanoscopic domains [9,10]. However, E decreased to values close to those predicted for randomly-distributed monomers after aldehyde fixation (0.16 ± 0.04 for SNAP- β_2 AR and 0.34 ± 0.04 for SNAP-GPI), suggesting that a significant portion of the discrepancy was due to diffusion.

4. Discussion

Membrane proteins with diffusion coefficients of $0.1-1.0 \mu\text{m}^2 \text{sec}^{-1}$ will be displaced 30-90 nm from their starting point in 2.2 milliseconds, the decay time constant of an excited Tb²⁺. At a density of 100-1,000 molecules μm^{-2} membrane-associated donors and acceptors will on average be separated from their nearest neighbors by 50-15 nm. Therefore, it is not surprising that diffusion enhances FRET between Tb³⁺-labeled membrane donors and acceptors at these densities. Indeed, diffusion-mediated enhancement of FRET between lanthanide-labeled membrane proteins was explicitly predicted by Thomas and Stryer in 1978 [4]. This phenomenon has several potentially important implications for studies of membrane proteins.

Diffusion will significantly affect interpretation of experiments that use lanthanide donors to assess membrane protein oligomerization. Efficient FRET between membrane proteins labeled with acceptors and lanthanide donors cannot be taken as evidence that the two are constitutively in close proximity due to oligomerization (or any other mechanism). In fact, if diffusion enhances FRET then this implies that the labeled molecules that produce this component of the signal are not part of the same oligomer. It is important to note, however, that the presence of diffusion-dependent FRET also does not rule out the existence of oligomers. For example, the diffusion-enhanced FRET that we observed between SNAP-

β_2 AR protomers could have arisen between monomers, between incompletely-labeled dimers, or between completely labeled dimers with a structure that does not permit efficient FRET within a dimer. Our results suggest that lanthanide-based FRET cannot be used to measure proximity or association of membrane proteins without first accounting for or preventing FRET due to diffusion. Lanthanide-based FRET has been used extensively to study oligomerization of G protein-coupled receptors (GPCRs) and other membrane proteins in model cells and in native tissue [1,2], but to our knowledge the possibility that FRET between GPCRs might depend largely on diffusion rather than (or in addition to) oligomerization has not been considered. It is possible that much of the FRET signal observed in these studies was due to diffusion, rather than oligomerization.

In a similar way diffusion complicates interpretation of lanthanide-based FRET between membrane proteins that are organized into nanoscale domains. We observed exceptionally efficient FRET between SNAP-GPI molecules. Although comparison between experiments is not straightforward, the FRET efficiency that we observed with SNAP-GPI was more than twice that observed in previous studies of GPI-anchored proteins that used comparable conventional fluorophores [9,13]. The robust diffusion-dependent FRET that we observed between SNAP-GPI molecules is consistent with the suggestion that a fraction of these molecules is clustered in such domains [9], but it is not clear if diffusion enhances FRET between SNAP-GPI molecules that reside inside or outside of domains (or both). The decrease in FRET that we observed after cholesterol depletion with M β CD might be due to disruption of these domains, but could also be due to slowed diffusion of SNAP-GPI within or between domains [8].

On a more positive note, lanthanide-based FRET could prove useful for monitoring membrane protein diffusion in real time. Below the rapid-diffusion limit FRET can be used to determine the diffusion coefficient, D [3,14]. Even if D is not explicitly determined, FRET will be sensitive to small changes in diffusion, even if these occur within domains that are too small to resolve by imaging methods such as fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy (FCS). For example, in the present study FRET increased monotonically for both SNAP- β_2 AR and SNAP-GPI as temperature increased (Fig. 2C and F). This suggests that the plasma membrane domains containing these proteins do not undergo a miscibility phase transition across the temperature range we studied, in agreement with a recent FCS study in live cells [15].

In summary, we find that diffusion is a predictable but previously-overlooked contributor to FRET between membrane proteins labeled with lanthanide donors. The contribution of diffusion to FRET should be considered in studies of membrane protein oligomerization and organization, and lanthanide-based FRET may prove useful for monitoring membrane protein diffusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Diffusion enhances TR-FRET from membrane proteins labeled with lanthanide donors.
- Diffusion-dependent FRET can overshadow FRET due to oligomerization or clustering.
- FRET studies using lanthanide-tagged membrane proteins should consider diffusion.
- FRET from lanthanide donors can be used to monitor membrane protein diffusion.

Figure 1. FRET between SNAP- β_2 AR protomers

(A), Time-resolved FRET (sensitized emission/donor emission; Em 665/620) increases with receptor density. Receptor density is increased by increasing the concentration of tetracycline, and is measured with radioligand binding. (B) FRET increases with increasing temperature and decreases with decreasing temperature. Both changes reverse over time as samples nominally started at the indicated temperature are allowed recovered to room temperature (RT). Mean $\pm$ SD, $n=3$.

Figure 2. Immobilization decreases FRET between membrane proteins

(A and B) Avidin crosslinking and fixation with paraformaldehyde and glutaraldehyde (PFA/GA) decreases sensitized acceptor emission (Em 665) and the FRET ratio (Em 665/620) between SNAP- β_2 AR protomers. (C) Immobilization largely occludes the increase in FRET between SNAP- β_2 AR protomers that occurs when samples nominally started at 0°C are allowed to warm to room temperature. (D-F) Similar experiments to panels A-C, but with SNAP-GPI. Bars in panels B and E represent mean $\pm$ SD, $n=3-5$.