Organization of Higher-Order Oligomers of the Serotonin$_{1A}$ Receptor Explored Utilizing Homo-FRET in Live Cells

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ABSTRACT The serotonin$_{1A}$ receptor is a representative member of the GPCR superfamily and serves as an important drug target. The possible role of GPCR oligomerization in receptor function is an active area of research. We monitored the oligomerization state of serotonin$_{1A}$ receptors using homo-FRET and fluorescence lifetime measurements. Homo-FRET is estimated by a reduction in fluorescence anisotropy and provides a superior approach for exploring oligomerization. In addition, homo-FRET offers the possibility of detecting higher-order oligomers. On the basis of an observed increase in fluorescence anisotropy upon progressive photobleaching and analysis of the difference between the extrapolated anisotropy and the predicted anisotropy of an immobile monomer, we propose the presence of constitutive oligomers of the serotonin$_{1A}$ receptor. To the best of our knowledge, these results constitute the first report of higher-order oligomers for the serotonin$_{1A}$ receptor. We further show that cholesterol depletion and antagonist treatment result in a reduced population of higher-order oligomers. In contrast, agonist stimulation and destabilization of the actin cytoskeleton lead to an increased contribution from higher oligomers. These results provide novel insight into the oligomerization status of the serotonin$_{1A}$ receptor that could enhance the ability to design better therapeutic strategies to combat diseases related to malfunctioning of GPCRs.

INTRODUCTION

The GPCR superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes (1). Cellular signaling by GPCRs requires their activation by ligands present in the extracellular environment and the subsequent transduction of signals to the interior of the cell through concerted changes in their transmembrane domain structure. GPCRs mediate multiple physiological processes, including neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, and inflammatory and immune responses. It is therefore only natural that GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas, and account for ~50% of current drug targets (2). The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important member of the GPCR superfamily and is the most extensively studied among the serotonin receptors, for a variety of reasons (3). The serotonin$_{1A}$ receptor agonists and antagonists have been shown to have potential therapeutic effects in anxiety or stress-related disorders (3). As a result, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression.

Aggregation and oligomerization have often been challenging yet exciting aspects in the study of membrane proteins. An emerging area is the possible role of oligomerization in GPCR organization and signaling (4–8). The potential implications of such oligomerization are far-reaching, especially considering the role of GPCRs as major drug targets (9). Fluorescence-based resonance energy transfer methods such as hetero-FRET and bioluminescence resonance energy transfer have been used to study GPCR oligomerization in live cell membranes (8). However, these techniques are often associated with a number of inherent complications arising from the use of receptors conjugated to two different probes, and a lack of control in their relative expression levels (10,11). Hetero-FRET measurements are performed utilizing using two different fluorophores with sufficient spectral overlap. In the case of heterologously expressed proteins, the expression levels of the tagged proteins may vary, making intensity-based hetero-FRET measurements difficult to interpret. These factors have limited the usefulness of these approaches for providing information about GPCR biology. In comparison with hetero-FRET, homo-FRET represents a superior approach. Homo-FRET is a simpler variant of energy transfer because it takes place between like fluorophores and therefore requires only a single type of fluorophore. Homo-FRET (FRET between two identical fluorophores) depends on the inverse sixth power of separation between interacting fluorophores on the nanometer scale and thus is sensitive to protein oligomerization. The excitation and emission spectra of fluorophores exhibiting homo-FRET should have considerable

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Abbreviations used: 5-HT, 5-hydroxytryptamine (serotonin); 5-HT$_{1A}$R-EYFP, 5-hydroxytryptamine$_{1A}$ receptor tagged to enhanced yellow fluorescent protein; CD, cytochalasin D; DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GPCR, G-protein coupled receptor; MβCD, methyl-β-cyclodextrin; *p*-MPPI, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine.

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overlap. In other words, fluorophores with a relatively small Stokes shift will have a greater probability of undergoing homo-FRET. In addition, homo-FRET is manifested by a reduction in fluorescence anisotropy, a parameter that is largely independent of the concentration of fluorophores (12). Another serious limitation of hetero-FRET measurements is the lack of ability to distinguish dimers from higher-order oligomers. This is often crucial, particularly in a microheterogeneous environment such as the membrane, where multiple types of oligomeric clusters can coexist. Fortunately, homo-FRET measurements can provide an estimate of higher-order oligomerization (13,14).

In this work, we explored the oligomerization state of the serotonin$_{1A}$ receptor by performing homo-FRET and fluorescence lifetime measurements on CHO cells stably expressing the EYFP-tagged serotonin$_{1A}$ receptor (5-HT$_{1A}$R-EYFP). Spatial resolution was achieved by using a microscope-based approach and homo-FRET was monitored by the increase in fluorescence anisotropy upon progressive photobleaching of the receptor. In anisotropy enhancement after photobleaching measurements, fluorescence depolarization due to energy transfer is prevented by photobleaching of FRET acceptors (15). We show here that analysis of the progressive enhancement in anisotropy due to increasing photobleaching provides evidence for the existence of constitutive serotonin$_{1A}$ receptor oligomers. We further report the effects of ligands, cholesterol depletion, and cytoskeletal disruption on receptor oligomerization.

MATERIALS AND METHODS

Materials

$MgCl_2$, *p*-MPPI, $CaCl_2$, penicillin, streptomycin, gentamicin sulfate, serotonin, MβCD, and CD were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F-12 (nutrient mixture F-12, Ham; 1:1)), fetal calf serum, and geneticin (G 418) were obtained from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cells and cell culture

CHO-K1 cells stably expressing the serotonin$_{1A}$ receptor tagged to EYFP (referred to as CHO-5-HT$_{1A}$R-EYFP) were used (~10^5 receptors/cell). Cells were grown on Lab-Tek (Nunc, Denmark) chambers in DMEM/F-12 (1:1) supplemented with 2.4 g/L of sodium bicarbonate, 10% fetal calf serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamicin sulfate in a humidified atmosphere with 5% CO_2 at 37°C. CHO-5-HT$_{1A}$R-EYFP cells were maintained in the above-mentioned conditions along with 300 μg/mL geneticin.

Treatment of cells

A stock solution of 2 mM CD was made in DMSO, and further concentrations were prepared upon dilution of the stock in buffer A (phosphate-buffered saline containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). The amount of DMSO was always <0.5% (v/v). Treatment of control cells with similar amounts of DMSO did not show any change in cellular morphology or receptor distribution. MβCD was directly dissolved in buffer A to make the desired concentration. Cells were treated with 5 μM CD and 10 mM MβCD unless otherwise mentioned. For treatment with ligands, 10 μM serotonin or *p*-MPPI was used. All experiments were performed at room temperature (~23°C).

FLIM experiments

FLIM measurements were carried out using a fluorescence lifetime imaging attachment (LIFA; Lambert Instruments, Leutingwolde, The Netherlands) mounted on an inverted microscope (TE2000U; Nikon, Japan). The tagged receptor in living cells were excited using epi-illumination with a sinusoidally modulated 470 nm LED at 40 MHz and observed with a 100x (NA 1.25) oil objective (Nikon Plan-Fluor; Nikon, Japan) through a filter set (Nikon FITC, DM505, EM 515–555 nm). The phase and modulation lifetimes were determined from a series of images taken at 12 phase settings using software provided by the manufacturer. A pseudo random recording order provided by the software was used to correct for any photobleaching (16). Rhodamine 6G in distilled water (lifetime 4.1 ns) was used as a reference (17). The mean and standard deviations for the phase and modulation lifetimes reported are from ~20 cells. We averaged the FLIM data on a cell-to-cell basis because we are interested in the statistics of a cell population.

Fluorescence anisotropy imaging microscopy

Anisotropy imaging was performed using the same setup as described for the FLIM experiments with the inclusion of a polarizer in the excitation path of the microscope and a dual-view polarizing beam splitter in the emission path before the LIFA intensifier-CCD camera. CHO-5-HT$_{1A}$R-EYFP cells were excited using epi-illumination with a 470 nm LED at constant illumination (unmodulated) and observed with a 100x (NA 1.25) oil objective, using a Nikon FITC filter, as described above. The parallel and perpendicular components of the emission were imaged synchronously on both halves of the CCD chip. Images were recorded at constant illumination as a function of time with the software provided by the manufacturer. The images were corrected for instrumental differences in the detection of parallel and perpendicular components of the emission by using Rhodamine 6G in distilled water as a reference and an anisotropy of 0.012, measured in a Varian Eclipse spectrofluorometer, as described previously (18). To correct for the depolarization caused by the high aperture objective used in this study, Rhodamine 6G was taken in solutions of varying glycerol content and anisotropies were simultaneously measured under the microscope and a Varian Eclipse spectrofluorometer. The calibration plot of the measured anisotropy of the same Rhodamine 6G solution under the microscope versus the spectrofluorometer is shown in the inset of Fig. 1. Correction for aperture depolarization in the microscope was made by multiplying the anisotropy determined under the microscope by a constant factor of 1.47, derived from the slope.

Individual cells were selected using region-of-interest tools in ImageJ (National Institutes of Health, Bethesda, MD), and the parallel ($I_{par}(t)$) and perpendicular ($I_{perp}(t)$) intensity values were analyzed as a function of illumination time. The total fluorescence as a function of time was given by

$$I(t) = I_{par}(t) + 2GI_{perp}(t),$$

where G is the correction factor for instrumental differences in detection in the parallel and perpendicular halves of the CCD. The mean anisotropy is given by

$$r(t) = \left[I_{par}(t) - GI_{perp}(t)\right]/I(t),$$

The fractional fluorescence remaining (after time zero) was calculated as

$$f(t) = [I_{t=0} - I_t]/I_{t=0}.$$

Plots of $r(t)$ versus $f(t)$ were constructed and interpreted using the theoretical framework of Yeow and Clayton (14) (see the Supporting