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Fluorescence correlation spectroscopy, combined with bimolecular fluorescence complementation, reveals the effects of β-arrestin complexes and endocytic targeting on the membrane mobility of neuropeptide Y receptors

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ABSTRACT

Fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) analysis are powerful ways to study mobility and stoichiometry of G protein coupled receptor complexes, within microdomains of single living cells. However, relating these properties to molecular mechanisms can be challenging. We investigated the influence of β-arrestin adaptors and endocytosis mechanisms on plasma membrane diffusion and particle brightness of GFP-tagged neuropeptide Y (NPY) receptors. A novel GFP-based bimolecular fluorescence complementation (BiFC) system also identified Y1 receptor-β-arrestin complexes. Diffusion co-efficients (D) for Y1 and Y2-GFP receptors in HEK293 cell plasma membranes were 2.22 and 2.15×10^{-9} $cm^2\ s^{-1}$ respectively. At a concentration which promoted only Y1 receptor endocytosis, NPY treatment reduced Y1-GFP motility (D 1.48×10^{-9} $cm^2\ s^{-1}$), but did not alter diffusion characteristics of the Y2-GFP receptor. Agonist induced changes in Y1 receptor motility were inhibited by mutations (6A) which prevented β-arrestin recruitment and internalisation; conversely they became apparent in a Y2 receptor mutant with increased β-arrestin affinity. NPY treatment also increased Y1 receptor-GFP particle brightness, changes which indicated receptor clustering, and which were abolished by the 6A mutation. The importance of β-arrestin recruitment for these effects was illustrated by reduced lateral mobility (D 1.20–1.33×10^{-9} $cm^2\ s^{-1}$) of Y1 receptor-β-arrestin BiFC complexes. Thus NPY-induced changes in Y receptor motility and brightness reflect early events surrounding arrestin dependent endocytosis at the plasma membrane, results supported by a novel combined BiFC/FCS approach to detect the underlying receptor-β-arrestin signalling complex.

1. Introduction

G protein coupled receptors (GPCRs) constitute a large and diverse array of cell surface receptors, which respond to signalling molecules ranging from metal ions to large polypeptide hormones. They are now known to activate a wide variety of signalling and regulatory pathways, beyond those mediated by classical heterotrimeric G proteins. Other effector proteins, most prominently the two non-visual isoforms of the β-arrestin family, can interact with the agonist bound GPCR to form alternative complexes [1,2]. β-Arrestins were originally described as simple terminators of G protein signalling, but their roles have rapidly expanded to those of multifunctional adaptors. Their association with clathrin, AP-2 and phosphotidylinositides drives internalisation of many GPCRs, and the stability of the internalised GPCR-β-arrestin complexes can dictate subsequent receptor trafficking to recycling or degradative pathways [2–8]. Moreover they recruit a range of other enzymes to the GPCR which can both regulate G protein dependent events [9,10] and initiate G protein independent signalling, for example through scaffolding of mitogen activated protein kinase cascades [1,2,11]. Structurally, several adaptor binding domains overlap on the β-arrestin surface, suggesting that some β-arrestin-based signalling complexes must form to the exclusion of others [2]. Understanding the spatiotemporal formation of different GPCR-β-arrestin scaffolds is thus an important element in defining how subsequent signalling is orchestrated. Potentially, this also influences the ability of some GPCR ligands to direct these events in a pathway specific manner [1]. Studying this organisation in part requires techniques to investigate such complexes within microdomains of single cells, rather than the overall population response.

Fluorescence correlation spectroscopy (FCS) is an imaging technique that is in theory capable of this resolution in living cells [12–14]. As fluorescent molecules pass in and out of a confocal detection volume fixed in position, they generate time-dependent fluctuations in intensity. Analysis of the fluctuations then provides information

Abbreviations: BiFC, bimolecular fluorescence complementation; BIBO3304, (*R*)-N_2-(diphenylacetyl)-*N*-[(4-(aminocarbonylaminomethyl-)phenyl)methyl]-argininamide; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; Gc, sfGFP fragment 155–238; Gn, sfGFP fragment 2–173; GPCR, G protein coupled receptor; HBSS, HEPES buffered saline solution; NPY, neuropeptide Y; PCH, photon counting histogram; (sf)GFP, (superfolder) green fluorescent protein; YFP, yellow fluorescent protein

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about the mobility and concentration of the fluorescent particles. Since the size of the confocal volume is small (~0.25 μm^3), FCS can investigate the properties of fluorescent species within a precise cellular region, for example containing ~0.1 μm^2 plasma membrane. Whilst not a true "single molecule" technique, FCS is highly sensitive and typically reflects the behaviour of 1–100 particles within the detection volume. The same fluctuation records can also undergo a separate statistical analysis. In this case the amplitude variation about the mean intensity is considered, using photon counting histogram (PCH) or fluorescence intensity distribution analysis [15,16]. PCH analysis provides complementary information to FCS, in particular by also estimating the particle brightness of individual fluorescent species. These techniques have been successfully applied in cells to study the plasma membrane diffusion and oligomeric state of GPCRs tagged with variants of green fluorescent protein (GFP) [17–21], and to monitor the binding of fluorescent GPCR ligands [17,22,23].

One of the inherent challenges for FCS and PCH analysis is to correlate changes in observed parameters, such as particle diffusion coefficients or brightness, with clearly defined signalling events and known molecular GPCR complexes. We and others have recently described one approach to this problem, in which bimolecular fluorescence complementation (BiFC) identifies the association between GPCRs and their partners [18,24–26]. BiFC involves the use of complementary fluorescent protein fragments as fusion tags for the interacting proteins of interest [25–27]. On protein–protein association these fragments (typically of yellow fluorescent protein, YFP) are brought together to allow refolding, chromophore maturation, and thus an indicative fluorescent signal. BiFC is irreversible, for example on association between GPCRs and β-arrestins [24], and thus unambiguously defines the associated complex for investigation. Moreover, the same range of fluorescence methods can be applied to wild type and complemented fluorescent proteins, since their underlying photophysical properties remain very similar [27]. For example, we have recently used FCS to study GPCR dimers identified by BiFC [18]. However a significant limitation of the combined technique has been that FCS measurements are more problematic using YFP tagged proteins. Such measurements are limited by the more complex photophysics and increased photobleaching inherent to these GFP variants [28].

Here we use a combination of FCS, PCH analysis, fluorescence recovery after photobleaching (FRAP) and novel BiFC approaches to study the plasma membrane mobilities and clustering of neuropeptide Y (NPY) Y1 and Y2 receptors. These are widely expressed G_i-coupled receptors with key roles in processes such as central control of food intake, cardiovascular regulation and bone metabolism [29]. Using GFP-tagged receptors and mutants that enhance or eliminate β-arrestin association and internalisation, we show how NPY-induced changes in receptor diffusion are clearly correlated with activated receptor targeting to arrestin dependent endocytosis mechanisms. We develop a new BiFC system based on a version of superfolder (sf)GFP [30], which for the first time allows study of molecularly defined Y1-β-arrestin complexes by FRAP, FCS and PCH analysis, and demonstrates their slow mobility, multimeric clustering and heterogeneity. Moreover brightness analysis supports a symmetric mode of recruitment of β-arrestins to individual Y1 receptor complexes.

2. Materials and methods

2.1. Molecular biology

Standard molecular biology reagents were purchased from Fermentas (St. Leon-Rot, Germany), Promega (Southampton, U.K.) or Sigma-Aldrich (Poole, U.K.). Sequential site-directed Quikchange mutagenesis (Stratagene, La Jolla, CA, U.S.A.) generated our version of sfGFP, in which folding mutations M153T and V163A (amino acid numbering refers to wild type GFP), and the two most critical "superfolder" mutations S30R and Y39N [30] were introduced into an enhanced GFP template. PCR was then used to construct sfGFP fragments Gn (2–172) and Gc (155–238). SfGFP, Gn and Gc cDNAs (lacking start Met) were each placed between XhoI and XbaI sites in either pcDNA 4 TO or pcDNA3.1zeo+ (Invitrogen, Paisley, U.K.). Rat Y1 and human Y2 receptor cDNAs, with an N terminal FLAG epitope (DYKDDDDK), were inserted upstream in these vectors between KpnI and NotI sites. All receptor fusion proteins thus contained a consistent linker (LRPLE) between the C terminus of the receptor and fused GFP fragment. For transfection, Y1-Gc and Y2-Gc receptor cDNAs were transferred to the neomycin resistant pCMV FLAG vector (Stratagene). Full length human β-arrestin1, β-arrestin2, and the deletion mutant β-arrestin2 ΔLIEFD (amino acids 373–377) were all cloned in pcDNA3.1zeo+, removing the stop codons, to generate constructs fused to Gn at the C terminus (linker QRPLE). Construction of the Y1 6A and Y2 H155P mutants, and YFP BiFC cDNAs has been described previously [24]. A membrane tagged version of sfGFP was generated by the addition of the dual palmitoylated N terminal motif of GAP-43 (ML**CC**MRRTKQVEKNDEDQKILE…) [31] using synthetic oligonucleotide linkers (GAPsfGFP). All cDNAs were fully sequenced to confirm their identities, and primer sequences are available on request.

2.2. Cell culture and transfection

HEK293T and 293TR cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% foetal bovine serum, and passaged when confluent by trypsinisation (0.25% w/v in Versene). All transient and stable transfections were performed using Lipofectamine in Optimem (Invitrogen, standard protocol). Mixed population 293TR Y1sfGFP and Y2sfGFP cell lines were generated by transfection of receptor cDNAs in pcDNA4 TO and dual selection for the tetracycline repressor protein (blasticidin, 5 $\mu g\ ml^{-1}$) and receptor cDNA (zeocin, 200 $\mu g\ ml^{-1}$). Prior to experiments (18–21 h), receptor expression was induced by 1 $\mu g\ ml^{-1}$ tetracycline treatment. For BiFC experiments, stable HEK293T β-arrestin-Gn clonal cell lines were first generated by zeocin selection and subsequent dilution cloning. Y1 receptor-Gc (in pCMV FLAG) stable mixed populations were then established from selected clonal arrestin-Gn lines by dual G418 (0.8 $mg\ ml^{-1}$) and zeocin resistance. All stable transfected cell lines were routinely maintained in DMEM containing blasticidin (5 $\mu g\ ml^{-1}$), zeocin (50 $\mu g\ ml^{-1}$) and/or G418 (0.1 $mg\ ml^{-1}$) as appropriate.

2.3. [^{125}I]PYY competition binding studies

Membranes were freshly prepared from 293TR Y receptor-sfGFP cell lines (after tetracycline induction), or HEK293T Y receptor β-arrestin BiFC cells, as described previously [24]. Competition binding assays were performed for 90 min at 21 °C in buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1 $mg\ ml^{-1}$ bacitracin; pH7.4) and increasing concentrations of unlabelled ligands (0.1 pM–1 μM, duplicate). [^{125}I]PYY (Perkin Elmer, Seer Green, U.K.) was used as the radioligand, at 16 pM for Y1 receptors, and at 10 pM for the Y2 subtype. GTPγS displacements also included 30 $\mu g\ ml^{-1}$ saponin in the assay buffer. Membrane bound radioligand was separated by filtration through Whatman GF/B filters soaked in 0.3% polyethyleneimine on a Brandel cell harvester, and retained radioactivity was quantified using a gamma-counter (Packard Cobra II, Perkin Elmer, Waltham, MA, U.S.A.).

Non-specific binding in these experiments comprised less than 5% of total counts, and was subtracted from the data. IC_{50} values were calculated from displacement curves fitted using non-linear least squares regression in GraphPad Prism 5.01 (GraphPad software, San Diego CA, U.S.A.). Hill slopes for these curves ranged from −0.6 to −1.1. The Cheng–Prusoff equation converted IC_{50} measurements to pKi values, quoted as mean ± s.e.m. throughout. Homologous PYY