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β -Arrestin biosensors reveal a rapid, receptor-dependent activation/deactivation cycle

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Abstract

(β -)Arrestins are important regulators of G-protein-coupled receptors (GPCRs)^{1–3}. They bind to active, phosphorylated GPCRs and thereby shut off ‘classical’ signalling to G proteins^{3,4}, trigger internalization of GPCRs via interaction with the clathrin machinery^{5–7} and mediate signalling via ‘non-classical’ pathways^{1,2}. In addition to two visual arrestins that bind to rod and cone photoreceptors (termed arrestin1 and arrestin4), there are only two (non-visual) β -arrestin proteins (β -arrestin1 and β -arrestin2, also termed arrestin2 and arrestin3), which regulate hundreds of different (non-visual) GPCRs. Binding of these proteins to GPCRs usually requires the active form of the receptors plus their phosphorylation by G-protein-coupled receptor kinases (GRKs)^{1,3,4}. The binding of receptors or their carboxy terminus as well as certain truncations induce active conformations of (β -)arrestins that have recently been solved by X-ray crystallography^{8–10}. Here we investigate both the interaction of β -arrestin with GPCRs, and the β -arrestin conformational changes in real time and in living human cells, using a series of fluorescence resonance energy transfer (FRET)-based β -arrestin2 biosensors. We observe receptor-specific patterns of

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conformational changes in β -arrestin2 that occur rapidly after the receptor- β -arrestin2 interaction. After agonist removal, these changes persist for longer than the direct receptor interaction. Our data indicate a rapid, receptor-type-specific, two-step binding and activation process between GPCRs and β -arrestins. They further indicate that β -arrestins remain active after dissociation from receptors, allowing them to remain at the cell surface and presumably signal independently. Thus, GPCRs trigger a rapid, receptor-specific activation/deactivation cycle of β -arrestins, which permits their active signalling.

Several lines of evidence suggest that GPCRs induce active conformations of (β -)arrestins, which facilitate interactions with effector proteins^{11–15}. X-ray crystallography of such active conformations revealed movements in the central loops that interact with GPCRs, plus a 20° twisting of the amino- versus carboxy-terminal domain^{8–10}. An intramolecular bioluminescence resonance energy transfer (BRET) sensor for β -arrestin2 activation showed activation over minutes, suggesting that it reports interactions with effectors rather than β -arrestin2 conformational changes¹⁶. Therefore, we set out to study the dynamics and the role and potential specificity of GPCRs in β -arrestin activation in living cells by FRET¹⁷. We generated eight different FRET-based β -arrestin2 biosensors by attaching an invariant cyan fluorescent protein (CFP) at the C terminus, and inserting a binding motif (CCPGCC) for the fluorescein arsenical hairpin (FIAsH) binder into different positions at the periphery of the N and C domains that were unlikely to be directly involved in receptor- β -arrestin interactions but might report conformational changes¹⁸ (Fig. 1a and Extended Data Table 1).

Confocal microscopy of transfected HEK293 cells showed that all β -arrestin2 sensors were expressed in the cytosol and labelled with FIAsH¹⁹ (Extended Data Fig. 1). With the exception of β -arrestin2-FIAsH8, all were rapidly recruited to the cell surface after stimulation of co-transfected parathyroid hormone type 1 receptors (PTH₁Rs; Extended Data Fig. 2), a receptor known to induce robust β -arrestin2 interactions²⁰.

For kinetic experiments, we used the β_2 -adrenergic receptor (β_2 AR), because its agonists have rapid on and off rates^{21,22}. The interaction was monitored by measuring FRET between the C-terminal CFP in the β -arrestin2 sensors and a C-terminal yellow fluorescent protein (YFP) in the co-transfected β_2 AR^{21,23}. Stimulation of the β_2 AR with 100 μ M isoproterenol promoted a β -arrestin-receptor interaction and increased FRET between CFP in the β -arrestin2 sensors and β_2 AR-YFP^{21,23} (Fig. 1b).

A phosphorylation-deficient β_2 AR-YFP construct²¹ failed to trigger such recruitment (Fig. 1c), indicating a high-affinity GRK-dependent β -arrestin-receptor interaction^{24,25}. Requirement for GRK-mediated phosphorylation made the interaction slower for the first than for subsequent stimuli (Fig. 1b), when receptors are already pre-phosphorylated²¹. Therefore, all further analyses refer to second stimuli, eliminating GRK-dependent phosphorylation as a potential issue.

Since the measurement of this interaction relied on the invariant CFP in all β -arrestin2 sensors, the receptor interactions of the different constructs can be directly compared (Fig. 1d). All β -arrestin2 sensors bearing FIAsH sequences in the N domain showed robust and quantitatively similar interactions with β_2 AR-YFP, whereas those with C-domain FIAsH

sequences, only the FIAsh1 construct showed a similar interaction. The interaction of the FIAsh6/7 constructs with PTH₁R but not β_2 AR indicates a distinct selectivity for β -arrestin2 between GPCRs. Receptor specificity was further suggested by an analogous M₂ muscarinic acetylcholine receptor (M₂AChR) construct stimulated with 100 μ M acetylcholine (Fig. 1e). Here, only β -arrestin2–FIAsh2 and β -arrestin2–FIAsh5 showed a similar receptor interaction, whereas all other constructs exhibited no detectable interaction.

Conformational changes within the β -arrestin2 sensors were investigated via intramolecular FRET between the CFP and FIAsh label (Fig. 1f–i), initially again using β -arrestin2–FIAsh2–CFP and the β_2 AR. Receptor stimulation caused a reversible reduction of intramolecular FRET in β -arrestin2–FIAsh2–CFP (Fig. 1f), which was, again, absent for the phosphorylation-deficient β_2 AR construct (Fig. 1g), indicating that agonist- and phosphorylation-dependent high-affinity receptor-binding was required for the conformational change. Only three β -arrestin2 sensors showed conformational changes after β_2 AR stimulation (Fig. 1h). FIAsh3 and FIAsh4, which showed ligand-dependent receptor interactions (Fig. 1d), revealed no β -arrestin2 conformational changes, and FIAsh1 showed only minor changes (Fig. 1h). This suggests that only distinct subdomains of β -arrestin2 move relative to its C terminus, and that the loop containing positions 2 and 5 (amino acids 154–158) is particularly sensitive. In line with their ability to report receptor-dependent activation, FIAsh2 and FIAsh5 increased isoproterenol-stimulated ERK phosphorylation in transfected HEK293 cells as much as wild-type β -arrestin2–CFP (Extended Data Fig. 3).

In analogous experiments with M₂AChR stimulated by 100 μ M acetylcholine (Fig. 1i), only β -arrestin2–FIAsh2–CFP showed an agonist-induced signal—that is, neither FIAsh5, which interacted with M₂AChR (Fig. 1e), nor FIAsh1, which changed conformation upon β_2 AR stimulation (Fig. 1h), responded to M₂AChR stimulation. Thus, both receptor interactions and conformational changes in β -arrestin2 occur in distinct receptor-specific manners.

Further evidence for receptor-specificity was illustrated by a FRET increase in β -arrestin2–FIAsh2 triggered by the free fatty acid receptor FFA4R (Extended Data Fig. 4), and by specific β -arrestin-mediated downstream signalling to kinases (Extended Data Fig. 5). In line with their different effects on β -arrestin2–FRET, M₂AChR caused significantly higher ERK1/2 activation than β_2 AR, and FFA4R was the only receptor causing no significant Src-kinase activation.

A comparison of the kinetics of the β_2 AR– β -arrestin2 interaction and of β -arrestin2 conformational changes in β -arrestin2–FIAsh2–CFP illustrates that the interaction step is faster (and thus precedes) the conformational change (Fig. 2a, b). While both processes began without an apparent lag period after agonist addition, the β_2 AR– β -arrestin2 interaction was clearly faster (rate constant $\tau = 1.3 \pm 0.17$ s (mean $\pm$ s.e.m.)) than the conformational change ($\tau = 2.2 \pm 0.22$ s; $P < 0.01$). Analysis with various isoproterenol concentrations revealed that these values represent maximal speeds (Extended Data Fig. 6).

When the superfusion system (which has a delay time of <10 ms) was switched from isoproterenol (100 μ M) to buffer alone, both the β_2 AR– β -arrestin2 interaction and the β -arrestin2 conformational changes reverted (Fig. 2c), albeit with significantly different

delays. This delay was 1.9 ± 0.51 s for the interaction, but 4.2 ± 0.85 s for the conformational change ($P < 0.05$; Fig. 2d). This difference indicates that β -arrestin2 remains in an active conformation after dissociation from the receptor. If β -arrestins continue to signal to effectors after their dissociation from receptors, this would allow signal amplification analogous to the well-established signal amplification occurring in G-protein-mediated signalling²⁶.

To assess whether these different delays might indeed be functionally relevant, we compared β_2 AR-induced translocation of β -arrestin2–YFP by confocal microscopy with the β_2 AR– β -arrestin2 interaction monitored under identical conditions by FRET (Fig. 3a–c). Because technical reasons prevented the use of the rapid perfusion system, these experiments were performed with direct application of isoproterenol to coverslips and subsequent washout with a peristaltic pump; thus, the two sets of experiments can be compared with one other, but not with the preceding experiments. After agonist washout, the translocation of β -arrestin2–YFP to the cell membrane lasted much longer than the direct β_2 AR– β -arrestin2 interaction (FRET). The latter was completely reversed 90 s after buffer addition (Fig. 3c), while the former clearly persisted at this time and took four times as long to reverse (Fig. 3b). This would be consistent with the hypothesis of an active, cell-surface-localized state of β -arrestin2–YFP lasting longer than the direct association with β_2 AR. Whether the cell surface retention is due to binding to lipids or to additional proteins, for example, clathrin^{5,7}, or is related to a biochemical modification remains to be determined.

Our data illustrate that GPCRs induce a complex, multi-step activation/deactivation cycle for β -arrestins that rapidly follows the well-known G-protein cycle. Provided sufficient GRK is present²¹, the rapid interaction between β -arrestins and receptors terminates G-protein activation long before downstream second messenger levels reach their new, receptor-triggered equilibrium²⁷.

Second, the distinct kinetics of receptor interaction versus conformational change (activation) of β -arrestin2 are consistent with the two-step activation model, which requires receptor phosphorylation and binding of the receptors' C terminus for β -arrestin activation by GPCRs^{12,15}. The time course of these steps reveals that GPCRs are as much β -arrestin-coupled receptors as they are G-protein-coupled receptors. Comparison of our β -arrestin2 sensors indicates that both steps occur in a receptor-specific manner. Thus, different activated forms of β -arrestins might exist that would allow signalling bias. The very recent determination of the X-ray structure of a rhodopsin–arrestin fusion protein¹⁰ is compatible with such a stepwise activation model as predicted from earlier structures of pre-activated (β -) arrestins^{8,9}. The stepwise activation would also allow active (β -) arrestins to dissociate from a GPCR.

Third, the persistence of active β -arrestin2 after dissociation from receptors has several important implications. It would allow signal amplification at this step, because a receptor becomes available for the activation of a subsequent β -arrestin while the first remains in an active state. It would allow β -arrestin coupling to its effectors at a site distinct from the cellular localization of the receptor, thus permitting distant β -arrestin-mediated signalling and thus making this process more versatile than previously thought.

While this work was in progress, we became aware of a complementary approach demonstrating β -arrestin conformational changes with BRET sensors and confirming the relevance of different active states of β -arrestin2 for downstream kinase signalling²⁸.

In summary, β -arrestins may orchestrate GPCR signalling by temporally and spatially segregating different, receptor-specific signalling ‘waves’^{29,30}, each lasting for a few seconds: first, G-protein-mediated signals from the cell surface, second, β -arrestin-mediated signals in its receptor-bound states, third, β -arrestin-mediated signals after dissociation from receptors, and finally signals from internal sites after β -arrestin-mediated receptor internalization. Thus, the interactions of GPCRs with β -arrestins are highly versatile, rapid and dynamic, and trigger an array of diverse, receptor-specific signals in space and in time.

METHODS

Reagents

Chemicals were purchased from the following sources: acetylcholine and isoproterenol from Sigma-Aldrich; penicillin (100 U ml⁻¹), streptomycin (100 μ g ml⁻¹), L-glutamine and G-418 from Invitrogen; Effectene transfection reagent from Qiagen. PCR primers were synthesized by MWG-Biotech, and sequencing reactions were done by Eurofins Medigenomix. DHA (*all-cis*-docosa-4,7,10,13,16,19-hexaenoic acid; 22:6(*n*-3)) was purchased from Sigma.

Molecular biology

FRET-based biosensors for β -arrestin2 were designed such that they would be able to interact with receptors and at the same time pick up conformational changes occurring during β -arrestin2 activation, based on work by Gurevich and co-workers^{12,13,31,32}. To achieve this, cDNA encoding the six-amino-acid FAsH motif CCPGCC was inserted in different positions in the N or C domain of bovine β -arrestin2 cDNA, resulting in constructs β -arrestin2–FAsH1–FAsH8 (Fig. 1a and Extended Data Table 1). The enhanced variants of CFP or YFP, respectively (BD Bioscience Clontech) were fused to the C termini of the human M₂AChR, β_2 AR or the β -arrestin2–FAsH sensors by standard PCR extension overlap technique³³. In each case, the C-terminal stop codon of the receptor and the initial codon for methionine of the fluorescent protein were deleted. All resulting constructs were cloned into pcDNA3 (Invitrogen Life Technologies) and confirmed by sequencing.

Cell culture

HEK293 cells were cultured in DMEM supplemented with 4.5 g l⁻¹ glucose, 10% fetal calf serum (Biochrom AG), 100 U ml⁻¹ penicillin G, and 100 μ g ml⁻¹ streptomycin sulphate at 37 °C and 7% CO₂. Cells were passaged every 2–3 days.

HEK293 cells were routinely tested for mycoplasma contamination using a primer set specific for the highly conserved 16S rRNA coding region in the mycoplasma genome. A mycoplasma-positive sample shows a distinct band at 265–278 base pairs.

Transient expression of β -arrestin2 biosensors in HEK293 cells

For fluorescence measurements, HEK293 cells were seeded on poly-D-lysine-coated coverslips in 6-well-plates 4–6 h before transfection. The cells were transfected using Effectene according to the manufacturer's instructions. 0.3 μ g of each of the following cDNAs were used per well: β_2 AR or PTH₁R and one of the YFP-tagged β -arrestin2–FAsH sensors (β -arrestin2 translocation); M₂AChR–YFP or β_2 AR–YFP and one of the CFP-tagged β -arrestin2–FAsH sensors (receptor– β -arrestin2 interaction); M₂AChR, FFA4R or β_2 AR and one of the FAsH-labelled CFP-tagged β -arrestin2–FAsH constructs for measuring conformational changes. Cells were analysed 48 h after transfection.

FAsH labelling

The labelling was done as described^{19,34,35}. In brief, cells were washed twice, with Phenol red-free HBSS containing 1.8 g l⁻¹ glucose (Invitrogen) and incubated at 37 °C and 7% CO₂ for 1 h with 1 ml 250 nM FAsH in HBSS supplemented with 12.5 μ M 1,2-ethane dithiol (EDT). To reduce unspecific labelling, cells were washed again with HBSS, incubated for 10 min with 1 ml 250 μ M EDT in HBSS, and finally washed twice with HBSS before being used for fluorescence measurements.

Confocal microscopy

Confocal microscopy experiments were performed on Leica TCS SP5 or TCS SP8 systems. Coverslips with transfected HEK29 cells were mounted using an Attotfluor holder (Molecular Probes). Confocal images were taken with a 63 $\times$ oil objective (numerical aperture, 1.4). CFP was excited with a diode laser at 458 nm laser line according to the manufacturer's settings and fluorescence intensities were recorded from 470 to 550 nm. YFP and FAsH were excited with a 514 nm laser line according to the manufacturer's settings with the following modifications. Laser intensity was set to 2–4% and fluorescence intensities were recorded from 525 to 600 nm. Settings for recording were kept constant at 512 $\times$ 512 pixel format, line average 4, and 400 Hz. To avoid bleed through, parallel images of CFP and YFP/FAsH were taken in sequential scan mode using the settings described above.

Time series were recorded using the standard Leica software package (LAS AF 3). Images were taken at 30-s intervals (unless stated otherwise). For quantitative analysis of the β -arrestin2 translocation, regions of interest (ROIs) were defined on the membrane and fluorescence intensities were recorded over time. To correct the images in the ROIs for possible photobleaching, control regions including the whole cells were defined. To quantify β -arrestin2 translocation the resulting fluorescence intensities were then related to the initial values. Solely for display reasons, but not for quantitative analyses, individual images were corrected for autocontrast using Photoshop software (Adobe CS6).

FRET

Dynamic FRET measurements in intact cells were performed as described previously^{34,36,37}. In brief, HEK293 cells transfected as described above were washed with HBSS and maintained in measuring buffer (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.3) at room temperature. FRET measurements using FAsH (after

labelling as described above) or YFP as acceptor and CFP as donor were done with an inverted microscope (Axiovert 135; Zeiss) equipped with an oil immersion 63× objective, a dual-emission photometric system (Till Photonics), and a polychrome IV light source (Till Photonics). Signals were converted with Digidata 1321A (Science Products GmbH) and stored with Clampex 8.1 software (Science Products GmbH). To minimize photobleaching, the fluorescence signals were recorded from entire cells with a frequency of 10 Hz with 20–40 ms illumination times. FRET was monitored from the emission ratio of YFP to CFP, F_{YFP}/F_{CFP} (emission intensities at 535 ± 15 nm and 480 ± 20 nm, beamsplitter DCLP 505 nm) upon excitation at 436 ± 10 nm (beamsplitter DCLP 460 nm). The adjusted FRET ratio was corrected for the direct YFP or FAsH excitation (YFP or FAsH emission at 436 nm excitation divided by YFP emission at 480 nm excitation was 0.065). The CFP bleed-through was recorded separately and subtracted from the YFP fluorescence to give a corrected normalized ratio F_{YFP}/F_{CFP} . To determine dynamic changes in FRET, cells were continuously superfused with measuring buffer, and agonist was applied using a computer-assisted solenoid valve-controlled rapid superfusion device ALAVM8 (solution exchange 5–10 ms; ALA Scientific Instruments).

FRET was recorded between receptors C-terminally labelled with YFP and β -arrestin2 labelled with CFP (or vice versa) to monitor receptor– β -arrestin2 interactions, or between FAsH bound to positions 1–8 in the various constructs and the C-terminal CFP in the β -arrestin2–FAsH sensors. For β_2 AR interaction experiments, the phosphorylation-deficient β_2 ARPD–YFP construct²¹ served as a control for GRK-mediated phosphorylation. Since β -arrestin2 binding to M2AChR has been reported to have a greater contribution of non-phosphate binding elements³², phosphorylation-deficient receptor mutants could not serve as negative controls as in the case of β_2 AR.

Ligand washout with peristaltic pumps

To compare the kinetics of the receptor– β -arrestin interaction versus β -arrestin translocation under identical conditions the ligand addition to the coverslip was performed with a pipette. The subsequent agonist washout was done with a peristaltic pump (Ismatec IPC, IDEX Health & Science GmbH) whereby the cells were continuously superfused with measuring buffer and the solution was simultaneously withdrawn by suction. The solution exchange occurred with a flow rate of 1.4 ml min^{-1} . Note that this results in a slower solution exchange than via the fast perfusion system used in Figs 1 and 2, but for technical reasons the latter system could not be fitted to the confocal system.

Immunoblot analysis for kinase activation

ERK1/2, Src and JNK phosphorylation were assessed in serum-starved (0.5% for 24 h) HEK293 cells expressing either the CFP-tagged wild-type β -arrestin2 or the β -arrestin2–FAsH2/5 sensor (Extended Data Fig. 3); these cells had been also co-transfected with M₂-muscarinic, β_2 -adrenergic or FFA4 receptors (Extended Data Fig. 5) at 80% confluence. Isoproterenol (100 μM), carbachol (100 μM) and DHA (10 μM) were added for the indicated times, and ERK1/2, Src or JNK phosphorylation was assessed by western blotting using phospho-specific antibodies (pERK, rabbit polyclonal anti-phospho-p44/42 MAPK (Thr-202/Tyr-204), Cell Signaling, 9101, 1:1,000; anti-phospho-Src (Tyr527), Cell

Signaling, 2105, 1:1,000; phospho-SAPK/JNK (Thr183/Tyr185), Cell Signaling, 9251, 1:1,000). Total ERK or G β was quantified as loading control using a rabbit polyclonal p44/42 MAPK antibody (Cell Signaling, 9102, 1:1,000) or G β antibody (Santa Cruz, sc-378, 1:5,000). Quantification was done by chemiluminescence using a horseradish-peroxidase-conjugated polyclonal goat anti-rabbit antibody (Dianova, 111-035-144, 1:10,000). pERK, pSrc and pJNK bands 5–10 min after agonist stimulation represent the β -arrestin2 dependent activation of the respective kinases³⁸.

Statistical analysis

No statistical methods were used to predetermine sample size. All data shown in bar graphs are presented as mean $\pm$ s.e.m. of n independent experiments (biological replicates, that is only one cell per coverslip was analysed). Statistical analyses were performed with the OriginLab software (version 9.1). For kinetic analysis the data were fitted with a nonlinear mathematical function (exponential decay first order:

$$y(x)=A_0 \times (\exp(-(x-x_0)/\tau) - 1) \times H(x-x_0)+y_0, \text{ or}$$

$$y(x)=A_0 \times (1 - \exp(-(x-x_0)/\tau)) \times H(x-x_0)+y_0$$

where $H(x)$ is the Heaviside step function) using again the Origin Lab software.

To analyse statistically significant differences of >2 independent samples of equal or different sample sizes (with unknown distributions and/or unequal variances) the Kruskal–Wallis test was used. The Mann–Whitney U test was used as post-hoc test to identify where the stochastic dominance occurs or for how many pairs of groups stochastic dominance is obtained.

Extended Data

Extended Data Figure 1. Specific labelling of FRET-based β -arrestin2 biosensors in intact cells with FIASH

HEK293 cells were transfected with one of the CFP-tagged β -arrestin2 biosensors, labelled with FIASH and analysed by laser scanning microscopy. Confocal images show overlapping intracellular staining in both the CFP (blue) and the FIASH (yellow) channels.

Extended Data Figure 2. Translocation of the β -arrestin2 biosensors

HEK293 cells were transiently transfected with PTH₁R-CFP and either wild-type β -arrestin2-YFP or one of the eight β -arrestin2-FIAsH-YFP sensors. **a**, Increase in membrane fluorescence 10 min after stimulation with 1 μ M PTH 1–34 (N-terminal fragment) expressed as percentage increase of the initial fluorescence at $t = 0$ min. Data represent mean $\pm$ s.e.m. values of the indicated number of independent experiments. # $P < 0.01$ versus no effect (Kruskal–Wallis followed by Mann–Whitney U post-hoc analysis). **b**, Confocal images of the CFP-tagged PTH₁R (left) and wild-type β -arrestin2-YFP (top), or β -arrestin2-FIAsH2-YFP (bottom) before (middle) and 10 min after PTH stimulation (right).

Extended Data Figure 3. β -Arrestin-dependent ERK1/2 phosphorylation

HEK293 cells were transiently transfected with the indicated constructs or control vector (pcDNA3; Con) and treated without or with isoproterenol for 10 min (10 μ M) as indicated. Cell lysates were analysed for pERK1/2 and ERK1/2 by immunoblot analysis. Data represent mean $\pm$ s.e.m., $n = 6$ independent experiments. * $P < 0.05$ versus unstimulated samples; # $P < 0.05$ versus isoproterenol-stimulated control (Kruskal–Wallis followed by Mann–Whitney U post-hoc analysis).

Extended Data Figure 4. Conformational changes in the β -arrestin2–FlAsH2 biosensor after FFA4R stimulation

Representative traces of docosahexaenoic acid (DHA)-induced changes in the normalized FRET ratio ($F_{\text{FlAsH}}/F_{\text{CFP}}$) and the corresponding CFP (F_{CFP} , cyan) or FlAsH (F_{FlAsH} , yellow) emission recorded from one single HEK293 cell expressing the FFA4R and the FlAsH-labelled β -arrestin2–FlAsH2–CFP sensor. Application of 100 μM DHA is indicated. Representative trace of 10 experiments.

Extended Data Figure 5. β -Arrestin-mediated downstream signalling to kinases for M_2 -muscarinic, β_2 -adrenergic and FFA4 receptors

HEK293 cells were transfected with β_2 AR, M_2 -muscarinic or FFA4 receptors and stimulated with respective agonists at saturating concentrations (isoproterenol, 100 μ M; carbachol (CCH), 100 μ M; docosahexaenoic acid, 10 μ M) for 10 min. β -Arrestin downstream signalling was evaluated by phospho-specific antibodies for pSrc, pERK1/2 and pJNK. G β was used as loading control. Data represent mean $\pm$ s.e.m. of $n = 12$ independent experiments. * $P < 0.05$ versus unstimulated control; # $P < 0.05$ versus indicated column (Kruskal–Wallis followed by Mann–Whitney U post-hoc analysis).

Extended Data Figure 6. Concentration dependency of the kinetics of the conformational changes in β -arrestin upon β_2 AR stimulation

HEK293 cells were co-transfected with the β_2 AR and β -arrestin2-FlAsH2-CFP and stimulated with different concentrations of isoproterenol. Kinetics of the agonist evoked intramolecular FRET changes were analysed by curve fitting according to Fig. 2. The bar graph shows the rate constants τ (s) for conformational changes detected with the β -arrestin2-FlAsH2 sensor upon stimulation with 1, 10, 30 or 100 μ M isoproterenol, respectively. Data represent mean $\pm$ s.e.m. of $n = 3$ independent experiments. The values are not significantly different ($P < 0.05$).

Extended Data Table 1

FRET β -arrestin2 sensor constructs used in this study

Abbreviation	Construct	FlAsH binding motif
FlAsH1	β -Arrestin2-FlAsH1	³³¹ CCPGCC ³³²
FlAsH2	β -Arrestin2-FlAsH2	¹⁵⁴ CCPGCC ¹⁵⁵
FlAsH3	β -Arrestin2-FlAsH3	⁴⁹ CCPGCC ⁵⁰

Abbreviation	Construct	FIAsH binding motif
FIAsH4	β -Arrestin2-FIAsH4	¹⁵⁰ CCPGCC ¹⁵¹
FIAsH5	β -Arrestin2-FIAsH5	¹⁵⁷ CCPGCC ¹⁵⁸
FIAsH6	β -Arrestin2-FIAsH6	³²⁶ CCPGCC ³²⁷
FIAsH7	β -Arrestin2-FIAsH7	³³⁵ CCPGCC ³³⁶
FIAsH8	β -Arrestin2-FIAsH8	¹⁹³ CCPGCC ¹⁹⁴

Insertion sites for the FIAsH-binding motif CCPGCC are denoted in single letter code with the amino acid positions in the β -arrestin2 sequence.

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Figure 1. FRET sensors for the β -arrestin2-receptor interaction and receptor-dependent conformational changes in β -arrestin2

a, FRET-based β -arrestin2 biosensors. Schematic representation of the β -arrestin2-FlAsH constructs used in this study derived from the crystal structure (Protein Data Bank (PDB) code 3P2D). CFP was attached C-terminally, and the FlAsH binding motif (CCPGCC) was inserted in different positions. Positions of the FlAsH binding motif are highlighted in the structure and specified in Extended Data Table 1. The sensors were termed β -arrestin2-FlAsH1–8-CFP (abbreviated: FlAsH1–8). **b–i**, Monitoring the β -arrestin2-receptor

interaction and receptor-dependent conformational changes in β -arrestin2 by FRET. **b, c, f, g**, Representative traces of isoproterenol-induced changes in CFP (F_{CFP} , cyan) and YFP or FAsH (F_{YFP} or F_{FAsH} , yellow) emissions and the corresponding normalized FRET ratio (F_{YFP} or $F_{\text{FAsH}}/F_{\text{CFP}}$) recorded from a single HEK293 cell expressing either YFP-fused $\beta_2\text{AR}$ (**b**) or $\beta_2\text{AR}$ (**f**) and the FAsH-labelled β -arrestin2–FAsH2–CFP. Isoproterenol application (100 μM) is indicated. Intermolecular (**c**) and intramolecular (**g**) FRET detected upon stimulation of a phosphorylation-deficient $\beta_2\text{AR}$ mutant ($\beta_2\text{ARPD}$) in HEK293 cells co-expressing β -arrestin2–FAsH2–CFP; these experiments were repeated more than ten times with similarly negative results. **d, e, h, i**, Quantification of agonist-evoked FRET changes. Shown are maximal FRET changes (percentage) for the interaction of $\beta_2\text{AR}$ –YFP (**d**) or M_2AChR –YFP (**e**) and the β -arrestin2–FAsH–CFP sensors, or by conformational changes detected with the β -arrestin2 sensors after stimulation of $\beta_2\text{AR}$ with 100 μM isoproterenol (**h**) or M_2AChR with 100 μM acetylcholine (**i**). Data represent mean $\pm$ s.e.m., for n independent experiments (biological replicates) as indicated. $\#P < 0.01$ (versus no effect); $*P < 0.05$ (versus FAsH2 and FAsH5).

Figure 2. Kinetics of the interaction of β -arrestin2 with β_2 AR and its conformational movements

a, Kinetics of the agonist (100 μ M isoproterenol)-induced interaction of β -arrestin2–FAsH2–CFP with β_2 AR (light grey) and its conformational changes (dark grey). Interaction and conformational changes (inverted to facilitate comparison) were quantified by FRET as in Fig. 1. FRET changes were plotted against time and analysed by exponential fitting to yield time constants (τ). Maximal values were set to 100%. Data represent mean $\pm$ s.e.m. of $n = 17$ (interaction) or 23 (conformational change) independent experiments (biological replicates). **b**, Bar graph of the time constants for the β_2 AR-agonist-induced interaction ($\tau = 1.3 \pm 0.17$ s) and the conformational changes detected with β -arrestin2–FAsH2–CFP ($\tau = 2.2 \pm 0.22$ s). Data represent mean $\pm$ s.e.m. of $n = 17$ (interaction) or 23 (conformational change) independent experiments (biological replicates). ** $P < 0.01$ (Mann–Whitney U test). **c**, Kinetics of FRET changes after agonist removal. FRET signals were recorded after

termination of exposure to the agonist (100 μ M isoproterenol; switch to buffer indicated by blue line). Traces are from a representative experiment showing distinct delays in the intermolecular FRET signal indicating β -arrestin2–receptor dissociation, and the intramolecular FRET signal indicating reversal of the active β -arrestin conformation. The rate constants of the two processes were not different ($\tau = 22 \pm 4.2$ s versus 23 ± 2.1 s). **d**, Bar graph of the delays for the agonist-induced β -arrestin–receptor dissociation (1.9 ± 0.51 s), and the reversal of the β -arrestin2 conformational changes from experiments as shown in **c** ($\tau = 4.2 \pm 0.85$ s). Data represent mean $\pm$ s.e.m. of $n = 12$ independent experiments (biological replicates). * $P < 0.05$ (Mann–Whitney U test).

Figure 3. Kinetics of β -arrestin2 translocation between cytosol and cell membrane after β_2 AR stimulation

a, Timeline of the experimental set up. HEK293 cells co-expressing β -arrestin2–YFP and β_2 AR were stimulated with isoproterenol (Iso 10 μ M) added to the coverslip, followed by washout with a peristaltic pump (flow rate 1.4 ml min⁻¹). Translocation of β -arrestin2–YFP to the cell membrane and subsequent dissociation upon washout were determined by confocal microscopy. Images were taken every 15 s. The experiment is representative of six independent experiments. **b**, Confocal images before and after ligand addition. I, β -

Arrestin2–YFP was diffusely cytosolic before agonist stimulation ($t = -90$ s). II, Maximal β -arrestin2 translocation to the cell membrane was observed 90 s after isoproterenol addition ($t = 0$ s; begin of washout). III, β -Arrestin2 movement back to the cytosol occurs after washout, but cell-surface localization is still clearly visible at 90 s after the beginning of washout ($t = 90$ s). IV, Translocation back to the cytosol is complete after 360 s of washout ($t = 360$ s). The images are representative of 4 independent experiments (biological replicates). **c**, Kinetics of the β -arrestin2– β_2 AR interaction after β_2 AR stimulation and ligand washout measured by FRET. HEK293 cells were transfected with β_2 AR–YFP and β -arrestin2–CFP. Isoproterenol (10 μ M) was added for 90 s and then washed out as above. Changes in CFP (F_{CFP} , cyan) and YFP (F_{YFP} , yellow) emissions and the corresponding normalized FRET ratio ($F_{\text{YFP}}/F_{\text{CFP}}$, red) were recorded from a single HEK293 cell. Note the artefact caused by switching on the pump for washout ($t = 0$ s). The traces are representative of 7 independent experiments (biological replicates). **d**, Model of an activation/deactivation and translocation cycle of β -arrestin2. After binding to an active phosphorylated receptor (1), β -arrestin2 adopts an activated conformation (2) that might facilitate fitting to the activated, phosphorylated receptor surface. Parts of C and/or N domain (labelled with FAsH in our studies) undergo further movements (3) to bring β -arrestin2 into a receptor-specific activated conformation. After agonist removal, β -arrestin2 dissociates from the receptor (4) and remains active for some time (5) before its active state is reversed (6). The major rearrangements in the loops associated with β -arrestin2 activation are schematically illustrated. Structural elements of β -arrestin2 are coloured as follows in each step: N and C domains, green; C-tail, red; FAsH2, cyan; FAsH5, blue; FAsH1, light green; finger, middle and gate loops, light grey.