Real-time monitoring of ubiquitination in living cells by BRET

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Ubiquitin has emerged as an important regulator of protein stability and function in organisms ranging from yeast to mammals. The ability to detect *in situ* changes in protein ubiquitination without perturbing the physiological environment of cells would be a major step forward in understanding the ubiquitination process and its consequences. Here, we describe a new method to study this dynamic post-translational modification in intact human embryonic kidney cells. Using bioluminescence resonance energy transfer (BRET), we measured the ubiquitination of β-arrestin 2, a regulatory protein implicated in the modulation of G protein–coupled receptors. In addition to allowing the detection of basal and GPCR-regulated ubiquitination of β-arrestin 2 in living cells, real-time BRET measurements permitted the recording of distinct ubiquitination kinetics that are dictated by the identity of the activated receptor. The ubiquitination BRET assay should prove to be a useful tool for studying the dynamic ubiquitination of proteins and for understanding which cellular functions are regulated by this post-translational event.

Ubiquitination is a rapid and reversible post-translational modification that is involved in numerous aspects of biology. The 76-residue polypeptide ubiquitin fulfils essential functions in eukaryotes through its covalent attachment to other intracellular proteins[1,2]. Historically, the best-characterized role for this modification is the targeting of proteins for degradation by the 26S proteasome after the transfer of an ubiquitin chain of at least four units, referred to as polyubiquitination[3]. More recently, the addition of a single ubiquitin to one (monoubiquitination) or multiple (multiubiquitination) protein sites has been described[4]. In these cases, the post-translational modification serves as a reversible signal involved in a variety of key cellular functions including intracellular signaling, subcellular localization and protein-protein interactions.

Despite the growing interest in ubiquitination, inspired by the diversity of cellular functions that appear to be influenced by this process, the lack of tools for monitoring its dynamic regulation limits studies in this field. Western blot analysis using antibodies to ubiquitin allows the detection of protein ubiquitination. Although this assay can be used to determine whether a protein is ubiquitinated, it cannot be easily adapted to study the dynamic nature of the ubiquitination and deubiquitination cycles. To monitor the ubiquitination process in living cells, we took advantage of a bioluminescence resonance energy transfer (BRET) method, which allows real-time detection of protein-protein interactions *in vivo*. This technique is based on the nonradiative transfer of energy between a luminescent energy donor (here, *Renilla* luciferase, *R*luc) and a fluorescent energy acceptor (here, green fluorescent protein, GFP). This is a system of choice for monitoring both constitutive and regulated intermolecular interactions because of the strict dependence on molecular proximity (<100 Å) between the donor and acceptor molecules for energy transfer[5].

It was recently proposed that β-arrestin 2 (β-arrestin), a versatile protein involved in the regulation of G protein–coupled receptor (GPCR) signaling[6], is ubiquitinated in response to receptor activation[7]. Based on the stability of their interaction with β-arrestin, two broad classes of GPCRs are distinguishable[8,9]: GPCRs known as class A receptors (for example, the β_2-adrenergic receptor, β_2AR) interact only transiently with β-arrestin after their activation, whereas activated class B receptors (for example, the V_2-vasopressin receptor, V_2R) form stable complexes with β-arrestin. Recent findings[10] are suggestive of a link between the dynamics of β-arrestin ubiquitination and the nature of its interaction with the activated receptor. A stable interaction correlates with sustained β-arrestin ubiquitination, whereas a transient interaction results in short-lived ubiquitination. Using β-arrestin as a model ubiquitinated protein, we report here that BRET allows monitoring of the changes in the ubiquitination state of a protein in real time.

RESULTS

Ubiquitination monitored by BRET in living cells

As ubiquitination results from the covalent attachment of ubiquitin to specific protein substrates, BRET should be adaptable to monitoring this post-translational modification in living cells. The covalent attachment of a GFP-ubiquitin fusion protein to a substrate fused to *Renilla* luciferase (*R*luc) should bring the *R*luc and GFP moieties within a BRET-permissive distance, resulting in a detectable BRET signal. To determine whether we could use BRET

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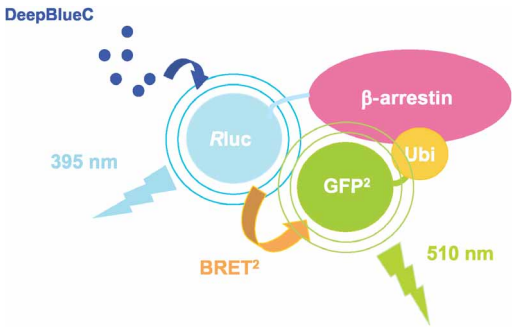

Figure 1 | Schematic representation of the β-arrestin ubiquitination BRET^2 assay. Upon degradation of its catalytic substrate (DeepBlueC coelenterazine), the *R*luc fused to the N terminus of β-arrestin emits blue light with an emission peak at 395 nm. If the β-arrestin is ubiquitinated, a nonradiative transfer of energy will occur between *R*luc and the GFP^2 fused to the N terminus of ubiquitin, resulting in re-emission of light with an emission peak at 510 nm.

to study ubiquitination of β-arrestin, we designed N-terminally tagged *R*luc–β-arrestin and GFP-ubiquitin (GFP^2-Ubi) fusion protein constructs. The close proximity of several GFP^2 moieties could cause quenching or interference phenomena that could lead to a decrease in the BRET signal, and indeed, we observed this (**Supplementary Fig. 1** online). To limit the formation of poly(GFP^2-Ubi) chains[11], we created a mutant GFP^2-Ubi protein by changing Lys48 and Lys63, the two residues through which linkages occur, to alanine residues.

To initiate the energy transfer between *R*luc and GFP^2 in cells coexpressing *R*luc–β-arrestin and GFP^2-Ubi, we used the luciferase substrate DeepBlueC coelenterazine, which readily crosses the plasma membrane. Hydrolysis of DeepBlueC coelenterazine leads to the emission of light with a spectrum overlapping the excitation spectrum of GFP^2, thus allowing BRET^2 (**Fig. 1**). Coexpression of a constant level of *R*luc–β-arrestin with increasing concentrations of GFP^2-Ubi led to an increase in the BRET^2 signal (**Fig. 2a**). The energy transfer increased hyperbolically as a function of the GFP^2-Ubi expression level, indicative of a specific interaction between the two proteins[12]. To confirm that the signal specifically reflects the ubiquitination process, we used either a GFP^2-tagged ubiquitin mutant that is unable to take part in the ubiquitination process as a result of the replacement of its last two glycines by alanine residues (GFP^2-Ubi$_{AA}$) or GFP^2 alone as negative controls. Coexpression of *R*luc–β-arrestin with GFP^2-Ubi$_{AA}$ or GFP^2 led to weaker signals that increased linearly rather than hyperbolically with the increase in the fluorescence/luminescence ratio, most likely reflecting random collision (bystander BRET) between *R*luc–β-arrestin and these two GFP^2 constructs. Western blot analysis of lysates from cells expressing GFP^2, GFP^2-Ubi$_{AA}$ or GFP^2-Ubi corroborated the lack of integration of GFP^2-Ubi$_{AA}$ into proteins (**Fig. 2b**). The smeared band detected above 63 kDa in lysates from cells expressing GFP^2-Ubi most likely reflects ubiquitination of multiple proteins, and the sharp bands observed in lysates from cells expressing GFP^2-Ubi$_{AA}$ probably correspond to monomeric and multimeric forms of the GFP^2-tagged ubiquitin. The BRET^2 signal detected between *R*luc–β-arrestin and GFP^2-Ubi did not result from the ubiquitination of the *R*luc moiety itself, as no signal was detected when only GFP^2-Ubi and *R*luc were coexpressed (**Fig. 2a**). To determine whether the GFP^2-Ubi protein can covalently modify β-arrestin, we immunoprecipitated Myc-tagged β-arrestin and detected GFP^2-Ubi–immunoreactive species, which probably correspond to a ubiquitinated β-arrestin population (**Fig. 2c**). Taken together, these results demonstrated that energy transfer between GFP^2-Ubi and *R*luc–β-arrestin reflects a nonrandom clustering of these two proteins and the covalent ubiquitination of β-arrestin *in vivo*.

Detection of agonist-induced ubiquitination

In a recent study, *ex vivo* western blot analysis suggested that GPCR activation modulates β-arrestin ubiquitination[7,10]. To assess whether BRET can detect receptor-regulated ubiquitination in living cells, we monitored the transfer of energy between *R*luc–β-arrestin and GFP^2-Ubi in the presence of coexpressed β_2AR and

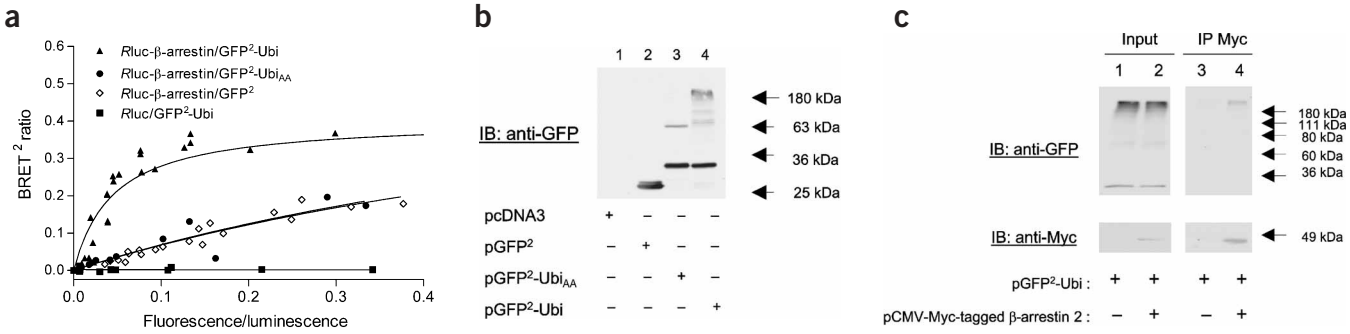

Figure 2 | Specific BRET^2 signal between *R*luc–β-arrestin and GFP^2-Ubi. (**a**) HEK 293 cells were cotransfected either with constant concentrations of *R*luc–β-arrestin, Myc-tagged V_2R and HA-tagged β_2AR DNA constructs and increasing concentrations of plasmids encoding GFP^2-Ubi (▲), GFP^2-Ubi$_{AA}$ (●) or GFP^2 (◇), or with a constant concentration of *R*luc and increasing concentrations of GFP^2-Ubi DNAs (■). The data shown represent pooled individual readings obtained from five to eight independent experiments. The curves were fitted using a nonlinear regression equation, assuming a single binding site (GraphPad Prism). (**b**) Lysates obtained from mock-transfected HEK 293 cells or cells transfected with pGFP^2, pGFP^2-Ubi$_{AA}$ or pGFP^2-Ubi were resolved by SDS-PAGE, and the attachment of ubiquitin to proteins was assessed by western blot analysis using an antibody to GFP. Numbers on the right indicate molecular weight of protein markers. The blot shown is representative of two independent experiments. (**c**) Immunoprecipitation with an antibody to Myc was performed on lysates prepared from cells expressing pGFP^2-Ubi or pGFP^2-Ubi and pCMV-Myc–tagged β-arrestin. GFP^2-Ubi protein associated with the immunoprecipitated β-arrestin was revealed as in **b** with an antibody to GFP, whereas the amount of β-arrestin immunoprecipitated was controlled with an antibody to Myc.

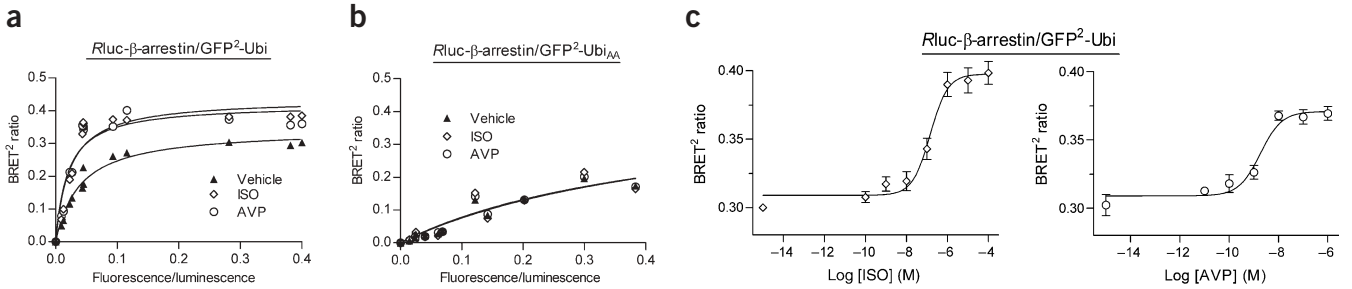

Figure 3 | Detection of GPCR-induced β-arrestin ubiquitination. (**a**,**b**) Cells cotransfected with a constant concentration of *R*luc–β-arrestin, Myc-tagged V_2R and HA-tagged β_2AR plasmids and increasing concentrations of GFP^2-Ubi (**a**) or GFP^2-Ubi_{AA} (**b**) constructs were unstimulated (▲) or stimulated with 10^{-5} M ISO (◇) or 10^{-6} M AVP (○) for 120 s before BRET measurement. DeepBlueC coelenterazine was added in the continuous presence of the agonists and the BRET measurements were taken 20 s after the addition of luciferase substrate. The data shown represent pooled individual readings obtained from three independent experiments. (**c**) Cells expressing the same construct as in **a** and displaying a GFP^2-Ubi/*R*luc–β-arrestin expression ratio of 0.1 ± 0.005 (assessed by measuring total fluorescence and luminescence signals) were stimulated with increasing concentration of ISO (left) or AVP (right) for 120 s; BRET measurements were then collected as in **a**. The curves shown represent the mean ± s.e.m. of three independent experiments.

V_2R. Activation of each receptor with its selective agonist, isoproterenol (ISO) for the β_2AR and arginine-vasopressin (AVP) for the V_2R, substantially increased the $BRET^2$ signal (**Fig. 3a**). In contrast, ligands did not have an effect on the bystander $BRET^2$ observed between GFP^2-Ubi_{AA} and *R*luc–β-arrestin (**Fig. 3b**), confirming the selectivity of the agonist-promoted ubiquitination observed with GFP^2-Ubi. This increase in $BRET^2$ signal was dose dependent, and the agonist concentrations yielding a half-maximal response (EC_{50}) were 140 and 1.8 nM for ISO and AVP, respectively, consistent with the affinity of these ligands for their receptors (**Fig. 3c**).

Concomitant detection of ubiquitination and recruitment

It has been suggested that receptor activation may regulate the ubiquitination of β-arrestin and its recruitment to GPCRs[10,13] in a coordinated fashion. To monitor these two events simultaneously in the same cells, we took advantage of the distinct spectral properties of two luciferase substrates that allow energy transfer with two different fluorescent proteins. Whereas the *R*luc-catalyzed oxidation of DeepBlueC coelenterazine leads to $BRET^2$ between *R*luc and GFP^2, the use of coelenterazine h leads to a distinct emission spectrum that promotes $BRET^1$ between *R*luc and a variant of GFP, the yellow fluorescent protein (YFP)[14–16]. We divided a culture of cells coexpressing *R*luc–β-arrestin, GFP^2-Ubi and V_2R-YFP into two samples. In one sample we monitored the β-arrestin ubiquitination by assessing the $BRET^2$ between *R*luc–β-arrestin and GFP^2-Ubi, and in the second sample we measured $BRET^1$ between *R*luc–β-arrestin and V_2R-YFP to evaluate the recruitment of β-arrestin to the receptor (**Fig. 4**). Stimulation with AVP resulted in an increase in both $BRET^2$ and $BRET^1$ signals, reflecting concomitant ubiquitination (**Fig. 4a**) and recruitment of β-arrestin (**Fig. 4b**). Each of the BRET signals detected was specific to the interaction monitored, as no substantial transfer of energy occurred between *R*luc and GFP^2 in the presence of coelenterazine h or between *R*luc and YFP in the presence of DeepBlueC coelenterazine.

Real time monitoring of distinct ubiquitination kinetics

As described above, one can distinguish two GPCR classes based on their profile of interaction with β-arrestin. Because the ubiquitination state of β-arrestin seems intimately linked to its interaction profile with receptors[10], we followed the kinetics of agonist-promoted β-arrestin ubiquitination in real time in cells coexpressing β_2AR and V_2R. Both ISO and AVP induced rapid and similar increases in the BRET-detected ubiquitination of β-arrestin that reached a peak at 2 minutes after activation (**Fig. 5**). However, although it remained stable for at least 10 min after V_2R activation (**Fig. 5b**,**c**), the BRET signal returned progressively toward basal values after activation of the β_2AR (**Fig. 5a**,**c**). This reduction in BRET signal most likely reflects a deubiquitination process and not a degradation of β-arrestin. As BRET is a ratiometric measurement (GFP^2 emission divided by *R*luc emission), degradation of

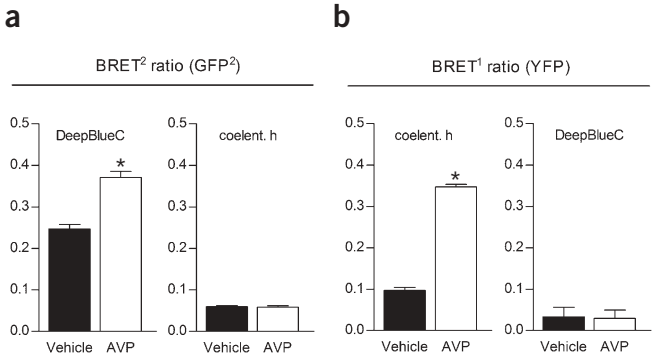

Figure 4 | Concomitant detection of β-arrestin ubiquitination and its recruitment to the activated receptor. (**a**,**b**) Cells cotransfected with *R*luc–β-arrestin, V_2R-YFP and GFP^2-Ubi were stimulated with 1 μM AVP or vehicle alone for 10 min. DeepBlueC coelenterazine ($BRET^2$, **a**) or coelenterazine h ($BRET^1$, **b**) were then added in the continuous presence of the agonist and BRET measurements collected 20 s after the addition of the luciferase substrate to monitor *R*luc–β-arrestin ubiquitination or its recruitment to V_2R-YFP, respectively. Bar graphs shown represent the mean ± s.e.m. of three independent experiments. The lack of cross-talk between $BRET^1$ and $BRET^2$ in the same samples was verified by measuring the BRET signal between *R*luc–β-arrestin and GFP^2-Ubi in the presence of coelanterazine h for $BRET^2$ and between *R*luc–β-arrestin and V_2R-YFP in the presence of DeepBlueC for $BRET^1$. Virtually no cross-talk signal was observed (right side of each panel). Statistical significance of the difference was assessed using paired Student's *t*-test. $^*P < 0.05$.

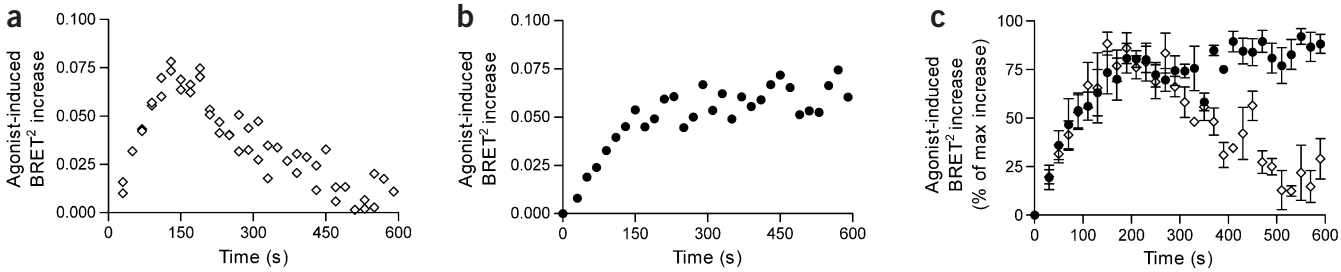

Figure 5 | Real-time assessment of receptor stimulated β-arrestin ubiquitination. Cells cotransfected with *R*luc–β-arrestin, Myc-tagged V_2R, HA-tagged β_2AR and GFP^2-Ubi (GFP^2-Ubi to *R*luc–β-arrestin ratio of 0.1 ± 0.005) constructs, were incubated in the presence of DeepBlueC coelenterazine immediately before stimulation with 10^{-5} M ISO (◇; **a**,**c**) or 10^{-6} M AVP (●; **b**,**c**). $BRET^2$ measurements were then collected immediately every 10 s in the continued presence of the agonists for 10 min. (**a**,**b**) The real-time agonist-promoted increases in BRET are represented as the difference in the absolute BRET values obtained in the presence and in the absences of ISO or AVP. (**c**) Compilation of three independent experiments carried out as in **a** and **b**. The data shown represent the mean ± s.e.m. and are expressed in percentage of the maximal $BRET^2$ signal obtained for each curve.

*R*luc–β-arrestin should induce a decrease in luminescence and lead to an equivalent reduction in the transfer of energy to the acceptor without affecting the signal. These results, therefore, confirm that in living cells, the nature of the activated receptor determines the dynamics of β-arrestin ubiquitination.

DISCUSSION

The aim of this study was to adapt an energy transfer assay to the detection of protein ubiquitination in living cells. Using β-arrestin as a model, we demonstrated that BRET can specifically detect both basal and dynamically regulated ubiquitination processes. As compared to western blot analysis, the use of BRET presents several advantages: (i) because the BRET assay is carried out in living cells, it avoids the possible signal alterations that could result from cell lysis, protein solubilization or any purification steps before western blot analysis; (ii) the ability to follow the evolution of the BRET signal in real time allows one to capture the dynamic nature of the ubiquitination process; (iii) one can monitor changes in ubiquitination levels resulting from specific treatments in one population of cells using BRET, whereas western blot experiments require distinct cellular pools; and (iv) because of the ratiometric nature of the BRET technique, a reduction in signal truly represents a decrease in ubiquitination and does not result from the degradation of the ubiquitinated protein target.

As the BRET method is a proximity-based assay that was previously used to detect noncovalent protein-protein interactions, it was important to validate that BRET detected between *R*luc–β-arrestin and GFP^2-Ubi truly reflected the covalent attachment of the ubiquitin moiety. This was achieved by comparing the BRET signals obtained between *R*luc–β-arrestin and either GFP^2-Ubi, GFP^2-Ubi_{AA} or the GFP^2 moiety alone. The BRET signal obtained between *R*luc–β-arrestin and GFP^2-Ubi increased hyperbolically with increasing concentration of GFP^2-Ubi before reaching a plateau once the covalent attachment of ubiquitin to *R*luc–β-arrestin attained the maximal steady state. In contrast, the smaller, linear BRET signal obtained between *R*luc–β-arrestin and GFP^2-Ubi_{AA} or GFP^2 most likely resulted from bystander BRET[12]. Because it cannot be incorporated into proteins, the concentration of free GFP^2-Ubi_{AA} available for random collision should be equivalent to that reached when the GFP^2 moiety alone is expressed, with both being much higher than that attained with GFP^2-Ubi for equivalent GFP^2 constructs. It follows that the bystander BRET component of the hyperbolic signal observed between *R*luc–β-arrestin and GFP^2-Ubi should then be much smaller than that observed with GFP^2-Ubi_{AA} or GFP^2. The lower signal detected between the *R*luc moiety alone and GFP^2-Ubi compared to that monitored between *R*luc–β-arrestin and GFP^2-Ubi_{AA} or GFP^2 confirms this prediction. This makes GFP^2-Ubi_{AA} a stringent control that allows one to distinguish between protein-protein interactions that are due to random collision and those due to covalent ubiquitination of a specific substrate.

Both BRET and immunoprecipitation approaches indicated that β-arrestin undergoes basal ubiquitination to a significant extent. These results are somewhat different from those of a published report[7,10] in which ubiquitination of β-arrestin was observed almost exclusively as a result of receptor activation. The specific reasons for this difference are not known, but it probably results from differences in the experimental conditions used, such as receptor expression level or the overexpression of exogenous ubiquitin protein.

An important aspect of the BRET-based method is its ability to detect dynamic changes in the ubiquitination state of a protein and to monitor changes over time. The ubiquitination BRET assay allowed us to monitor distinct kinetics of β-arrestin ubiquitination resulting from the activation of different GPCRs. Activation of the β_2AR induced a fleeting β-arrestin ubiquitination, whereas a long-lasting ubiquitination of the regulatory protein was observed after activation of the V_2R. This is consistent with a previous study examining the ubiquitination status of β-arrestin at 1 and 15 min after receptor stimulation[10]. However, because the activated receptor recruits β-arrestin, the increase in ubiquitination BRET signal (**Fig. 2a**) could result from the ubiquitination of the receptor itself. This was not the case, as no BRET modulation occurred between β_2AR-*R*luc or V_2R-*R*luc and GFP^2-Ubi after brief agonist stimulation (**Supplementary Fig. 2** online). The data show that the ubiquitination BRET assay can faithfully reflect rapid and differential changes in the ubiquitination state of a specific substrate after specific stimuli.

The GFP^2-Ubi construct used for the BRET experiments was designed to lack the two major sites involved in polyubiquitination in mammals (Lys48 and Lys63) to prevent the formation of GFP chains that could interfere with the energy transfer process. Despite the constraint that such a construct could impose on the system, ubiquitination of β-arrestin, which is believed to be

polyubiquitinated[7,10], could be readily observed. This is not surprising given that the process of polyubiquitination permits the attachment of the GFP^2-Ubi through its terminal glycine either to β-arrestin targeted lysine(s) or to growing chains of ubiquitin. As the use of the GFP^2-Ubi construct, lacking the polyubiquitination sites, could interfere with the properties of ubiquitinated proteins, we compared the ubiquitination BRET signal obtained with this construct to the signal obtained in the presence of a wild-type GFP^2-Ubi protein (GFP^2-Ubi$_{WT}$). Although a specific hyperbolic BRET signal was detected between *R*luc–β-arrestin and GFP^2-Ubi$_{WT}$, it was lower than the one obtained with GFP^2-Ubi (**Supplementary Fig. 1** online). Despite the lower absolute signal, similar BRET modulations were observed with both constructs after agonist stimulation (**Supplementary Fig. 1** online). This indicates that GFP^2-Ubi expression, *per se*, does not influence the nature of the results obtained but facilitates the detection of BRET signal changes. The fact that GFP^2-Ubi$_{WT}$ could also be used in the ubiquitination BRET assay makes the method more broadly applicable.

Besides its usefulness in directly monitoring protein ubiquitination, the BRET assay offers the possibility of concomitant detection of additional protein interactions involving the ubiquitinated protein. Taking advantage of two spectrally distinct coelenterazines, we demonstrated the feasibility of such dual detection by monitoring both ubiquitination of β-arrestin and its recruitment to the V_2R in the same cells.

In addition to shedding new light on the dynamics of β-arrestin ubiquitination in living cells, our study represents proof of the principle that resonance energy transfer approaches allow the monitoring of ubiquitin attachment in real time. BRET and possibly fluorescence resonance energy transfer (FRET) could be used to study the ubiquitination of various proteins whose activity and fate are regulated by this post-translational modification. Studies of the growing number of ubiquitin-like proteins that regulate diverse cellular functions through their covalent attachment to specific protein substrates could also benefit from advances in ubiquitination BRET assays.

METHODS

Plasmids. To produce a wild type GFP^2-Ubi construct (GFP^2-Ubi$_{WT}$), we amplified the gene encoding human ubiquitin from a pQE32-Ubi plasmid and subcloned it into pGFP^2-C1 (Perkin Elmer BioSignal). We then mutated by PCR lysines 48 and 63 in the ubiquitin moiety of the GFP^2-Ubi$_{WT}$ construct into alanines (GFP^2-Ubi). Finally, we constructed the GFP^2-Ubi$_{AA}$[17,18] by mutating by PCR the two C-terminal glycines 75 and 76 into alanines. We previously described pcDNA3.1-*R*Luc–β-arrestin, pcDNA3.1-hemagglutinin (HA)–tagged β_2AR, pcDNA3.1-Myc–tagged V_2R and pRK5-V_2R-YFP[13,19–21]. pCMV-Myc–tagged β-arrestin was a kind gift from S. Marullo's laboratory.

Cell culture and transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, 2 mM L-glutamine (Wisent) and cultured at 37 °C in 5 % CO_2. We transfected the cells 24 h after seeding using the calcium phosphate precipitation method[22].

BRET measurement. Transfected cells were washed twice with PBS, detached in PBS with EDTA and resuspended in PBS with 0.1% glucose. We determined the cell number by measuring protein concentration using the Dc protein assay kit (Bio-Rad). We obtained BRET measurements using two different sets of energy transfer partners. Energy transfer known as BRET^2, between *R*luc and GFP^2, was determined after the addition of the *R*luc substrate, DeepBlueC coelenterazine[14] (Perkin Elmer), whereas BRET^1, between *R*luc and YFP, was assessed in the presence of another Rluc substrate, coelenterazine h[15,16].

BRET^2 monitoring of β-arrestin ubiquitination. We distributed cells coexpressing Myc-tagged V_2R, HA-tagged β_2AR, *R*luc–β-arrestin and GFP^2-Ubi constructs in white 96-well Microplates (Corning) at a density of 100,000 cells per well. We collected BRET^2 measurements 20 s after the addition of the luciferase substrate, DeepBlueC coelenterazine, at a final concentration of 5 μM. Readings were obtained using a modified TopCount NXT instrument (Packard Bioscience) that allows the sequential integration of the signals detected in the 370–450 and 500–530 nm ranges. The BRET^2 signal corresponds to the ratio of the light emitted by the GFP^2 (500–530 nm) over the light emitted by the *R*luc (370–450 nm). We corrected the values by subtracting the background ratio value detected when the *R*luc constructs were expressed alone. For titration experiments, the BRET signal was plotted as a function of the total fluorescence signal over the total luminescence signal (see below), a ratio used as an index of the concentration of GFP^2-Ubi (or GFP^2-Ubi$_{AA}$) expressed.

BRET^1 monitoring of β-arrestin recruitment to the V_2R receptor. After dividing the cells coexpressing *R*luc–β-arrestin, GFP$_2$-Ubi and V_2R-YFP into two samples, we initiated BRET^1 between *R*luc–β-arrestin and V_2R-YFP by the addition of 5 μM coelenterazine h (the recruitment of β-arrestin to the receptor). We collected the readings at the appropriate emission wavelengths after the addition of 1 μM AVP using a Mithras LB 940 instrument (Berthold Technologies). We analyzed results as described above by calculating the ratio of the light emitted by the YFP (510–550 nm) to the light emitted by the *R*luc (460–500 nm). We corrected the values by subtracting the background ratio value detected when the *R*luc constructs were expressed alone.

Total fluorescence and luminescence measurements. We dispersed cells in white 96 well plates with clear bottoms (Costar) at a density of 100,000 cells per well[12]. We measured total fluorescence of cells in a Fluorocount (Packard Bioscience) using an excitation filter at 400 or 485 nM and an emission filter at 510 or 530 nM in the case of GFP^2 or YFP, respectively. We then incubated cells for 10 min with coelanterazine h to measure the total luminescence using a LumiCount instrument (Packard Bioscience). In the case of fluorescence, we subtracted the background value detected in untransfected cells to give a specific fluorescence value. In the case of luminescence, where the background was negligible, we used absolute values.

Immunoblotting and immunoprecipitation. We solubilized cells expressing pcDNA3, GFP^2-Ubi$_{AA}$ or GFP^2-Ubi in lysis buffer A containing 50 mM HEPES (pH7.5), 0.5 % Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 5 mM *N*-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml

aprotinin and 100 μM benzamidine. After separation of protein on SDS-PAGE and transfer to nitrocellulose, we revealed the immunoreactive bands using an antibody to GFP (Clontech), a horseradish peroxidase–conjugated antibody (Amersham) and the Western Lightning chemiluminescence Reagent Plus (Perkin Elmer). For immunoprecipitation experiments, we used cells expressing or not expressing pCMV-Myc–tagged β-arrestin 2 and pGFP^2-Ubi and solubilized them in buffer A for 30 min with rocking at 4 °C. We then incubated soluble extracts with an antibody to Myc (9E10) and protein G–agarose beads overnight at 4 °C. Bound extracts were resolved by SDS-PAGE and transferred to nitrocellulose. We performed western blot analysis with an antibody to GFP, as described above, to reveal basal β-arrestin ubiquitination, and used A14 antibody to Myc (Santa Cruz) to detect the presence of Myc-tagged β-arrestin after its immunoprecipitation.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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