THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 40, pp. 29089–29100, October 5, 2007
© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Unraveling G Protein-coupled Receptor Endocytosis Pathways Using Real-time Monitoring of Agonist-promoted Interaction between β-Arrestins and AP-2*

Received for publication, January 19, 2007, and in revised form, July 3, 2007 Published, JBC Papers in Press, August 3, 2007, DOI 10.1074/jbc.M700577200

Fadi F. Hamdan[‡1,2,3], Moulay Driss Rochdi[‡1,3], Billy Breton[‡], Delphine Fessart[§], Douce E. Michaud[‡], Pascale G. Charest[‡4], Stéphane A. Laporte[§5], and Michel Bouvier[‡6]

From the [‡]Department of Biochemistry and Groupe de Recherche Universitaire sur le Médicament, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Québec H3C 3J7 and the [§]Department of Medicine, McGill University, Montréal, Québec H3A 1A1, Canada

The most widely studied pathway underlying agonist-promoted internalization of G protein-coupled receptors (GPCRs) involves β-arrestin and clathrin-coated pits. However, both β-arrestin- and clathrin-independent processes have also been reported. Classically, the endocytic routes are characterized using pharmacological inhibitors and various dominant negative mutants, resulting sometimes in conflicting results and interpretational difficulties. Here, taking advantage of the fact that β-arrestin binding to the β2 subunit of the clathrin adaptor AP-2 (β2-adaptin) is needed for the β-arrestin-mediated targeting of GPCRs to clathrin-coated pits, we developed a bioluminescence resonance energy transfer-based approach directly assessing the molecular steps involved in the endocytosis of GPCRs in living cells. For 10 of the 12 receptors tested, including some that were previously suggested to internalize via clathrin-independent pathways, agonist stimulation promoted β-arrestin 1 and 2 interaction with β2-adaptin, indicating a β-arrestin- and clathrin-dependent endocytic process. Detailed analyses of β-arrestin interactions with both the receptor and β2-adaptin also allowed us to demonstrate that recruitment of β-arrestins to the receptor and the ensuing conformational changes are the leading events preceding AP-2 engagement and subsequent clathrin-mediated endocytosis. Among the receptors tested, only the endothelin A and B receptors failed to promote interaction between β-arrestins and β2-adaptin. However, both receptors recruited β-arrestins upon agonist stimulation, suggesting a β-arrestin-dependent but clathrin-independent route of internalization for these two receptors. In addition to providing a new tool to dissect the molecular events involved in GPCR endocytosis, the bioluminescence resonance energy transfer-based β-arrestin/β2-adaptin interaction assay represents a novel biosensor to assess receptor activation.

G protein-coupled receptors (GPCRs)[7] are seven transmembrane domain receptors that constitute the largest family of cell surface proteins involved in signal transduction. In humans, it is estimated that GPCRs are encoded by ~800 distinct genes that control a variety of important physiological responses (1). Following agonist binding, GPCRs undergo conformational changes that regulate the activity of downstream effector systems to mediate various cellular responses. The extent and duration of GPCR signaling is tightly regulated by mechanisms that terminate the initial signaling and later re-establish the capacity of the receptors to respond to new agonist exposure. The removal of GPCRs from the cell surface, also known as internalization or endocytosis, plays an important role in these processes (2, 3). For most GPCRs, rapid feedback desensitization is initiated by G protein-coupled receptor kinases that phosphorylate agonist-occupied GPCRs to create high affinity binding sites for β-arrestins, which in turn uncouple the receptor from its cognate G protein (reviewed in Ref. 4). β-Arrestins also target receptors for endocytosis by linking them to the endocytic machinery, including clathrin and the clathrin adaptor AP-2 (5–11). By controlling receptor recycling following endocytosis, β-arrestins have also been shown to regulate the rate of receptor resensitization (12). Based on their interaction

* This work was supported in part by research grants from the Canadian Institute of Health Research (CIHR) (to M. B. and S. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Both authors contributed equally to this work.

[2] Current address: Saint-Justine Hospital Research Center, Dept. of Genetics, Université de Montréal.

[3] Recipients of CIHR postdoctoral fellowships.

[4] Current address: Biological Sciences Dept., University of California, San Diego.

[5] Holds a Canada research chair in molecular endocrinology.

[6] Michel Bouvier holds a Canada research chair in signal transduction/molecular pharmacology. To whom correspondence should be addressed: Institute for Research in Immunology and Cancer, Université de Montréal, C.P. 6128, succursale Center-Ville, Montréal, Québec H3C 3J7, Canada. Tel.: 514-343-6319; Fax: 514-343-6843; E-mail: michel.bouvier@umontreal.ca.

[7] The abbreviations used are: GPCR, G protein-coupled receptor; AVP, 8-arginine-vasopressin; AP-21967, synthetic heterodimerizer binding with high affinity to both cyclophilin FKBP and FRB fragments; AT1aR, angiotensin II receptor; β2AR, β2-adrenergic receptor; B2R, bradykinin B2 receptor; BRET, bioluminescence resonance energy transfer; C5a, complement component 5a; C5aR, the complement component C5a receptor; CCR5, CC-chemokine 5 receptor; DMEM, Dulbecco's modified Eagle's medium; dynI(K44A), dominant negative mutant of dynamin I; EP4R, prostaglandin EP4 receptor; ET1, endothelin; ETAR, endothelin A receptor; ETBR, endothelin B receptor; FKBP, cyclophilin fragments; FRB, cyclophilin fragment; G418, Geneticin; GFP, green fluorescent protein; HEK, human embryonic kidney cells; HRP, horseradish peroxidase; M2R, M2 muscarinic receptor; PEI, polyethyleneimine; *R*luc, *Renilla reniformis* luciferase; V1aR, V1a vasopressin receptor; V2R, vasopressin V2 receptor; VIP, vasoactive intestinal peptide 1; VIP1R, vasoactive intestinal peptide 1 receptor; EYFP, enhanced yellow fluorescent protein; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; siRNA, small interference RNA.

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

with β-arrestins, GPCRs are divided into two main classes. Class A, which includes receptors such as the β2-adrenergic (β2AR), endothelin A (ETAR), and V1a vasopressin (V1aR), interacts transiently with β-arrestins and can rapidly recycle back from the endosomes to the cell surface (13, 14). Class B receptors, such as vasopressin V2 (V2R), CC-chemokine 5 (CCR5), prostaglandin EP4 (EP4R), and vasoactive intestinal peptide 1 (VIP1R), interact more stably with β-arrestins, leading to a complex that resides for extended periods of time into endosomes. Receptors tightly associated with β-arrestins in endosomes are only poorly recycled to the cell surface and eventually targeted for lysosomal degradation (13–17). The two classes of receptors also differ by their binding preference toward β-arrestin1 and β-arrestin2. Indeed, whereas class A receptors binds with greater affinity to β-arrestin2, class B receptors do not show preference between the two β-arrestins (13). More recently, it was suggested that some receptors may not be easily classified in class A or B. For example, the bradykinin B2 receptor (B2R) was found to internalize with β-arrestins into endosomes but can then dissociate from β-arrestin and efficiently recycle to the plasma membrane after agonist removal (18). The authors suggested that receptors with such hybrid features could be referred to as class C.

Although the roles of β-arrestins and clathrin-coated vesicles in GPCR endocytosis have been well characterized, alternative pathways involving non-coated vesicles, such as caveolae, or other non-clathrin and non-caveolae mediated routes, have also been described for several receptors (reviewed in Refs. 2, 3, 19). Also, the requirement for β-arrestin does not seem to be universal, and endocytosis of some GPCRs through either clathrin-coated vesicles or caveolae was proposed to be β-arrestin-independent (20). To characterize the various endocytic routes used by different receptors, several pharmacological and biochemical tools have been used. These include blockers that do not discriminate between clathrin-dependent or caveolae-mediated endocytosis (*e.g.* hypertonic sucrose, low temperature, concanavalin-A, and dominant negative mutants of dynamin) as well as inhibitors that are believed to selectively inhibit clathrin-coated vesicle (*e.g.* monodansylcadaverine, chlorpromazine, as well as dominant negative mutants of β-arrestin and Eps-15) or caveolae-mediated endocytosis (*e.g.* filpin and nystatin) (3, 19, 21, 22). Although these approaches have been useful, the interpretation of their data was sometimes difficult and generated some controversies on the exact mechanism underlying the endocytosis of certain GPCRs. For example, inhibition of endocytosis by dominant negative mutants of β-arrestins (β-arrestin1(V53D), β-arrestin1-(319–418), and β-arrestin2(ΔLIELD/F391A) (7, 23, 24)) or a dominant negative of the AP-2 interacting protein Eps15 (25) has often been used to suggest the involvement of a clathrin-mediated process. However, in the case of the ETAR, endocytosis through caveolae was also inhibited by a dominant negative mutant of β-arrestin (20). Similarly, inhibiting Eps15 has been recently found to prevent caveolae-mediated endocytosis of the epidermal growth factor receptor (26). Off-target effects of dominant negative mutants can also complicate interpretation of the results. For instance, whereas it was initially believed that β-arrestins were specific regulators for GPCRs, growing evidences demonstrate its role in the regulation of non-GPCRs membrane proteins endocytosis (27). Although this may reflect direct interaction of β-arrestins with non-GPCR proteins (28), it could also result from the interference with components of the endocytic machinery that could be shared by clathrin- and caveolae-mediated routes.

The above discussion illustrates the importance of developing new tools that will allow the direct assessment of the molecular steps involved in the endocytosis of specific GPCRs. To generate one such tool, we took advantage of the fact that, for internalization, the β-arrestins binding to the β2-adaptin subunit of the clathrin adaptor AP-2 are needed for the β-arrestin-mediated targeting of GPCRs to clathrin-coated pits (7, 8, 10, 29). Specifically, we designed a bioluminescence resonance energy transfer (BRET)-based assay that allows real-time monitoring of the interaction between β-arrestins and β2-adaptin as a biosensor for β-arrestin-promoted clathrin-mediated endocytosis. BRET is a non-radiative energy transfer that occurs between the energy donor *Renilla* luciferase (*R*luc) and variants of the green fluorescent proteins (EYFP for BRET[1] and GFP, GFP[2], or Tsapphire for BRET[2]) as the energy acceptor only when the two proteins are within 100 Å from one another and situated in favorable orientation, making it an attractive assay for studying both inducible and constitutive protein-protein interactions (30, 31). Thus, in response to the activation of 12 different GPCRs, previously suggested to internalize via different endocytic routes, BRET[1] was assessed between β-arrestin-*R*luc and β2-adaptin-EYFP and used as an indicator of clathrin-mediated endocytosis involving β-arrestins. By combining the spectrally resolved BRET[1] and BRET[2] technologies, we could simultaneously assess the recruitment of β-arrestin to GPCR and β-arrestin/AP-2 interaction, which allowed us to monitor the kinetics of the two events. The data obtained herein show that some of the GPCRs previously suggested to internalize via a clathrin-independent pathway were capable of promoting β-arrestin interaction with AP-2. Thus, contrary to what was deduced from indirect methods, these receptors internalize via a β-arrestin- and clathrin-dependent mechanism. Other receptors, such as ETAR and ETBR, which are capable of recruiting β-arrestins but were shown to internalize via caveolae, did not promote β-arrestin interaction with β2-adaptin, indicating a β-arrestin-dependent but clathrin-independent endocytic process. In addition to providing a new tool to dissect the molecular events involved in GPCR endocytosis, the BRET-based β-arrestin/β2-adaptin interaction assay can also be used to detect constitutive receptor internalization and quantify receptor activation in a pharmacologically relevant manner.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, Geneticin (G418), L-glutamine, and penicillin-streptomycin were purchased from Wisent. Fugene6 was obtained from Roche Diagnostics. Coelenterazine-*h* was from Prolume, and DeepBlueC was from Biotium. Recombinant human RANTES (regulated on activation normal T cell expressed and secreted) was from PeproTech. Poly-D-lysine, 8-arginine-vasopressin (AVP), isoproterenol, bradykinin, human

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

recombinant C5a, prostaglandin E2, vasoactive intestinal peptide, endothelin-1 (ET1), and carbachol were from Sigma-Aldrich Canada. Antibodies against β2-adaptin, α-adaptin, and clathrin were obtained from BD Biosciences. The anti-GFP was from Molecular Probes. White opaque and clear bottom 96-well plates were from Corning. Linear polyethyleneimine (25 kDa) was from Polysciences. Plate readers used to measure BRET in this study were the Mithras LB940 from Berthold for $BRET^1$ and a modified TopCount from Packard for $BRET^2$.

Expression Plasmids—The constructs presented herein were made using standard molecular biology techniques employing PCR and fragment replacement strategies. β-Arrestin2-*R*luc and β-arrestin1-*R*luc were generated by PCR amplification of the coding sequences of the rat β-arrestin2 (kindly provided by S. Marullo, Institute Cochin, Paris) and the rat β-arrestin1 (a gift from Kathryn DeFea, University of California, Riverside) without their stop codons that were ligated upstream of a humanized *Renilla reniformis* luciferase (*R*luc, originally PCR-amplified from ph*R*luc-N1 from PerkinElmer Life Sciences) in pcDAN3.1zeo+ (Invitrogen); both β-arrestin1 and β-arrestin2 were fused to *R*luc via the same 6-amino acid linker (GSGTAT). *R*luc-β-arrestin2-EYFP construct was made as described by Charest *et al.* (42). β-Arrestin2-(R393E,R395E)-*R*luc DNA was obtained by subcloning the mutant β-arrestin2 into pcDNA3.1. V2R-EYFP construct was generated by amplifying V2R coding sequences by PCR without its stop codon and cloned upstream of EYFP in pRK5. β2-Adaptin-EYFP was generated by subcloning the coding region, minus the stop codon, and coming from a human β2-adaptin plasmid template (10) into pEYFP-N1 (Clontech). To construct V2R-Tsapphire, the coding sequence of Turbo Sapphire (32) was PCR-amplified and fused, in-frame, downstream of the V2R coding sequence lacking its stop codon (which was removed from V2R-VENUS-pIRESpuro3 (33)) and ligated into pcDNA3.1/zeo(+). EP4R and the complement component C5a receptor (C5aR) in pcDNA3.1(+) were from UMR cDNA Resource Center. The angiotensin II receptor (HA-AT1aR-pRc) expression construct was a gift from Dr. Sylvain Meloche (Université de Montréal, Montréal, Canada). The V2R-FKBP and FRB-β-arrestin2-*R*luc (34), Myc-V2R(R137H) (35), HA-β2AR (36), dynI(K44A) (37), Myc-V2R, Myc-V1aR (38), M2 muscarinic receptor (HA-M2R) (21), vasoactive peptide 1 receptor (FLAG-VIP1R) (21), endothelin receptor subtypes A and B (HA-ETAR and FLAG-ETBR (39)), CCR5 (18), and bradykinin receptor subtype 2 (HA-B2R) (40) expression constructs were previously described. Except for CCR5, EP4R, and C5aR, all GPCRs used in this study contained an N-terminal epitope tag (*e.g.* Myc, HA, or FLAG) that was previously shown not to compromise the activity of these receptors. For the purpose of brevity, unless stated otherwise, GPCRs used here will be identified by their names only without indicating their N-terminal epitope tag. All GPCRs used herein were from human cDNAs, except for the VIP1R, which was from rat.

Cell Culture, Plasmid Transfections, and Stable Cell Line Production—Unless otherwise stated, human embryonic kidney (HEK) 293T cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin-streptomycin, and 2 mM L-glutamine at 37 °C in a humidified chamber at 95% air and 5% CO_2. For transient transfections in 6-well plates, 400,000 HEK293T cells were seeded and transfected the next day using Fugene6 according to manufacturer's recommendations. For transfections in 100-mm plates, cells were seeded at a density of 2.5 million cells/plate in DMEM containing 5% fetal bovine serum and transfected the next day using polyethyleneimine (PEI, 25-kDa molecular mass, linear form prepared at 1 mg/ml in sterile distilled H_2O) at a DNA:PEI ratio of 1:3. Plasmid DNA and PEI were diluted, each in separate tubes, with 500 μl of NaCl solution (150 mM). The PEI solution was then added onto the DNA solution, vortexed at maximum speed for 5 s, and incubated at room temperature for 20 min prior to addition to the cells. To generate cells stably expressing β2-adaptin-EYFP (HEK293T/β2-adaptin-EYFP), transfected cells were selected with 1 mg/ml G418. Clonal cells expressing β2-adaptin-EYFP were obtained by limited dilution and were tested for expression of β2-adaptin-EYFP by fluorescence measurements and Western blotting. HEK293T cells stably expressing MycV2R (HEK293T/V2R) were generated by selection with 0.45 mg/ml G418, and a clonal cell line (41) expressing ~8 pmol/mg of V2R was used for transient co-transfection of β2-adaptin-EYFP and β-arrestin2-*R*luc.

Immunoprecipitation and Western Blotting—Immunoprecipitation experiments were carried out as described previously (65). Immunoprecipitated proteins were separated on 6% SDS-PAGE, whereas proteins from total cell lysates were separated on 10% SDS-PAGE before transfer onto nitrocellulose membranes. Protein immunodetection on membranes was assessed using either anti-β2-adaptin (0.2 μg/ml), anti-α-adaptin (0.25 μg/ml), anti-clathrin (0.25 μg/ml), or anti-GFP (2 μg/ml) (65) antibodies.

Total Fluorescence Measurements—To measure total fluorescence, cells were washed once with PBS-Mg (PBS containing 0.5 mM $MgCl_2$), gently detached by pipetting in PBS-Mg, and seeded at a density of ~100,000 cells/well in 96-well plates (white wall with clear bottom plates). All throughout this study, cell number quantification was based on measuring the A_{600} of the cell suspension and correlating it to a pre-established standard curve (A_{600} *versus* cell number). The fluorescence level was measured using a FlexStation II (Molecular Devices). For measuring fluorescence of V2R-Tsapphire, transfected cells were excited at 400 nm, and the emission was recorded at 511 nm (cut-off at 490 nm), whereas measurements of β2-adaptin-EYFP fluorescence were done at an excitation wavelength of 470 nm and emission wavelength of 535 nm (cut-off at 500 nm).

Monitoring Protein-Protein Interaction Using $BRET^1$—Approximately 18 h after transfection, cells were detached by trypsinization and seeded (~50,000 cells/well) into 96-well (white wall, clear bottom) tissue culture plates previously treated with poly-D-lysine, and re-incubated at 37 °C for another 18 h. On the day of the experiment, the culture medium was replaced by PBS-Mg and incubated with or without various concentrations of the tested agonist for the specified time at room temperature. To measure the $BRET^1$ signal, the transparent bottom of the 96-well plate was covered with a white-backed tape adhesive (PerkinElmer Life Sciences), and the $BRET^1$ substrate for *R*luc, coelenterazine-*h*, was added to all wells (5 μM final concentration), followed by $BRET^1$ measurement on the Mithras LB940 plate reader, which allows the

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

sequential integration of signals detected in the 480 ± 20 nm and 530 ± 20 nm windows. The BRET[1] signal was calculated as a ratio of the light emitted by EYFP (530 ± 20 nm) over the light emitted by *R*luc (480 ± 20 nm). Unless otherwise stated, BRET[1] ratios were corrected by subtracting the background signal detected when β-arrestin-*R*luc constructs were expressed in the absence of the EYFP fusion protein or the tested GPCR (in the case of the stable β2-adaptin-EYFP cell line) to generate BRETnet values. Agonist-promoted BRET[1] was calculated by subtracting the BRET[1] ratio obtained in the absence of agonist addition from the one obtained in the presence of an agonist. In the case of kinetic measurements, coelenterazine-*h* was added simultaneously with the agonist, followed by BRET[1] measurements. Receptor agonists used herein were: AVP for V2R and V1aR, isoproterenol for β2AR, bradykinin for B2R, vasoactive intestinal peptide for VIP1R, ET1 for ETAR and ETBR, carbachol for M2R, RANTES for CCR5, recombinant C5a for C5aR, prostaglandin E2 for EP4R, and angiotensin II for AT1AR.

Heterodimerizer-promoted β-Arrestin2 Interaction with V2R—HEK293T/β2-adaptin-EYFP cells were transfected with V2R-FKBP and FRB-β-arrestin2-*R*luc (34) or with V2R and β-arrestin2-*R*luc. Heterodimerization of V2R-FKBP and FRB-β-arrestin2-*R*luc was initiated by cell treatment with the heterodimerizer AP-21967 (20 min at room temperature), a chemically modified derivative of rapamycin. AP-21967 is a cell-permeable organic molecule with two separate motifs binding with high affinity to FKBP and FRB fragments, respectively (for details see Ariad on the web).

Concomitant Monitoring of β-Arrestin2 Interactions with V2R and with AP-2 by Combining BRET[1] and BRET[2]—HEK293T/β2-adaptin-EYFP cells were seeded in 100-mm dishes at a density of 2.5 million cells per plate. The next day, cells were co-transfected with different concentrations of the V2R-Tsapphire plasmid (0.25, 0.5, and 1 μg) and 0.25 μg of β-arrestin2-*R*luc plasmid. Forty-eight hours post-transfection, cells were washed with PBS-Mg and then gently detached. Total fluorescence measurements were taken in 96-well plates containing ~100,000 cells/well, as described above. Cells were then dispensed into 96-well white opaque plates, and BRET[1] or BRET[2] kinetic measurements were done after the co-addition of 100 nM AVP and either 5 μM coelenterazine-*h* (*R*luc substrate for BRET[1]) or coelenterazine DeepBlueC (*R*luc substrate for BRET[2]). BRET[2] measurements were done using a modified TopCount plate reader that sequentially incorporates emission signals in the 370–450 nm and 500–530 nm ranges. The BRET[2] signal was determined by calculating the ratio of the light emitted by the V2R-Tsapphire (500–530 nm) over the light emitted by the β-arrestin2-*R*luc (370–450 nm). Agonist-promoted BRET[2] was calculated by subtracting the BRET[2] ratio obtained in the absence from the one obtained in the presence of agonist. Agonist-promoted BRET[1] was calculated as described above.

Measurement of Cell Surface Receptor Expression by ELISA—Agonist-promoted internalization was assessed as described previously (37). Briefly, the culture medium was removed and replaced with DMEM/0.5% bovine serum albumin/20 mM HEPES in the presence or absence of the specific agonist. After 30-min incubation at 37 °C, the medium was removed and cells were fixed with Tris-buffered saline/3.7% formaldehyde for 5 min at room temperature. Cell surface expression was measured using anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA, for ETAR), anti-Myc (for V1aR, V2R wt, and V2R(R137H)), or anti-FLAG M2 (Sigma, for ETBR) monoclonal antibodies. The percentage of agonist-promoted receptor internalization was determined as follows: (1 − stimulated/unstimulated) × 100.

β-Arrestin Knock-down—HEK293T cells were transfected with ETAR or ETBR plasmids along with minimal amount of β-arrestin1-*R*luc and β-arrestin2-*R*luc and either a specific siRNA targeting both β-arrestin1 and β-arrestin2 (AAAGCCTTCTGCGCGGAGAAT) or a control siRNA (Santa Cruz Biotechnology). 18 h post-transfection, cells were seeded in 24-well plates and incubated at 37 °C for an additional 18–24 h. siRNA efficacy for β-arrestins knock-down expression was assessed by measuring the luminescence obtained from the co-expressed β-arrestin1-*R*luc and β-arrestin2-*R*luc and expressed as the percentage of luminescence obtained in cells transfected with the control siRNA.

Confocal Microscopy—HEK293T cells were transfected with β-arrestin2-GFP and either ETAR or ETBR constructs and treated with ET1 (10 nM) for the indicated time. β-Arrestin2-GFP images were collected on a Zeiss LSM-510 Meta laser scanning microscope.

RESULTS

Generation and Expression of β2-Adaptin-EYFP—This study was designed to monitor agonist-induced interaction between β-arrestins and AP-2 in living cells, thus, providing a sensitive detection for the early events of β-arrestin-promoted clathrin-mediated endocytosis of GPCRs. For this, we used a β2-adaptin-EYFP fusion construct as a suitable BRET[1] partner (EYFP as energy acceptor) for β-arrestins fused to the energy donor *R*luc (β-arrestin2-*R*luc or β-arrestin1-*R*luc). Western blot analyses of HEK293T cells transfected with the β2-adaptin-EYFP construct confirmed that the protein was efficiently expressed. Indeed, a band migrating at ~130 kDa, corresponding to the expected molecular mass of the β2-adaptin-EYFP fusion protein was recognized by antibodies raised against either β2-adaptin (Fig. 1*A*, *lane 2*) or GFP (that recognizes EYFP; Fig. 1*A*, *lane 5*). A lower band of ~105 kDa, corresponding to the molecular weight of the endogenous β2-adaptin, was recognized only by the anti-β2-adaptin antibody (compare *lanes 1*, *2*, and *5* in Fig. 1*A*), in both transfected and untransfected cells. Transfection of wild-type β2-adaptin increased the immunoreactivity of the 105-kDa band (Fig. 1*A*, *lane 3*), confirming that this band corresponds to the native protein. To confirm the ability of β2-adaptin-EYFP to associate with its functional partners (43), co-immunoprecipitation experiments were carried out. As shown in Fig. 1*B*, immunoprecipitation of the fusion protein, using anti-GFP antibody, led to co-sedimentation of α-adaptin and clathrin, indicating that the fusion of β2-adaptin with EYFP did not prevent its association with the native AP-2/clathrin complex (Fig. 1*B*).

Monitoring Agonist-promoted Interaction between β2-Adaptin/EYFP and β-Arrestin-Rluc in Living Cells by BRET[1]—To determine whether GPCR-promoted interaction between

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

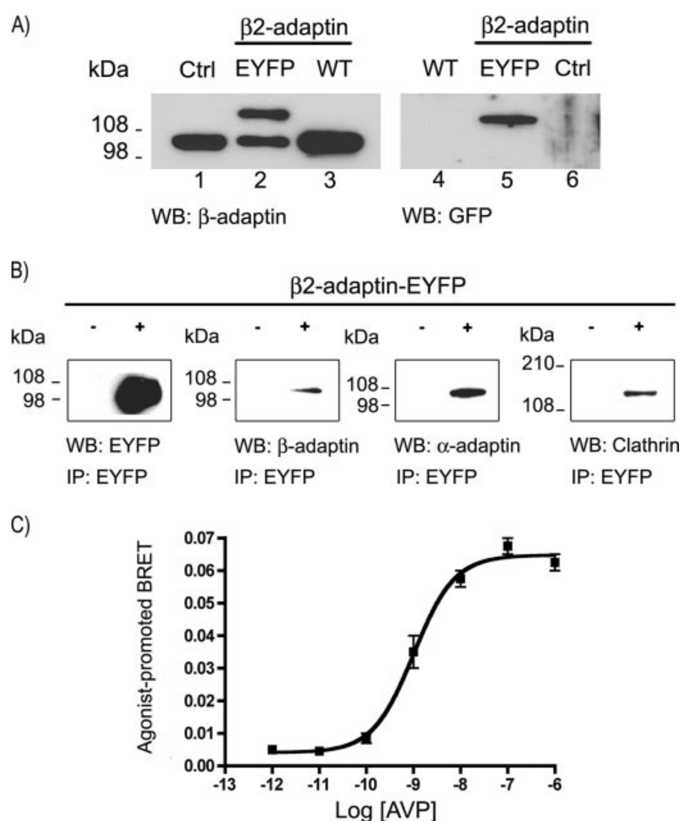

FIGURE 1. **Characterization of β2-adaptin-EYFP.** *A*, HEK293T cells were transiently transfected or not (Ctrl) with β2-adaptin wild-type (*WT*) or β2-adaptin-EYFP. Expression level of β2-adaptin in cell lysates was assessed by Western blot using anti-β2-adaptin (*left panel*) or anti-GFP (*right panel*) antibodies. *B*, cell lysates from HEK293T expressing (+) or not (−) β2-adaptin-EYFP were immunoprecipitated (*IP*) using an anti-GFP antibody, and the immunoprecipitates were analyzed by Western blot (*WB*) using either anti-GFP, anti-β2-adaptin, anti-α-adaptin, or anti-clathrin antibodies. *C*, HEK293T/V2R cells were co-transfected with β2-adaptin-EYFP and β-arrestin2-*R*luc, and $BRET^1$ measurements were done after 20-min incubation with the indicated concentration of the V2R agonist AVP. Data shown are representative of two to three experiments (*A* and *B*) or are the mean ± S.E. of three independent experiments (*C*).

β-arrestin and β2-adaptin could be detected by BRET in living cells, we initially measured $BRET^1$ between β-arrestin2-*R*luc and β2-adaptin-EYFP that were transiently co-transfected into HEK293T/V2R cells. The $BRET^1$ signal was measured in the absence and presence of increasing concentrations of the V2R agonist AVP. Fig. 1*C* shows that AVP promoted a concentration-dependent increase in $BRET^1$, indicative of an association between β-arrestin2-*R*luc and β2-adaptin-EYFP. The EC_{50} calculated for this agonist-promoted interaction was 1.2 nM, a value in good agreement with the published affinity of AVP for V2R (38). These data validate the use of the β2-adaptin-EYFP/β-arrestin-*R*luc pair as a biosensor to monitor the early event in the receptor-promoted formation of the endocytic complex involving β-arrestin and AP-2.

To develop a robust system that allows monitoring of β-arrestin/AP-2 interaction by distinct receptors, we generated a HEK293T/β2-adaptin-EYFP cells in which constant amounts of GPCR and β-arrestin-*R*luc constructs could then be co-transfected. Twelve GPCRs were selected for this study (Table 1). The GPCRs were chosen to include receptors belonging to classes A, B, and C, as defined by their β-arrestin interaction profile (13, 18). The list also included receptors that were previously proposed to undergo clathrin-dependent or -independent pathways. Agonist activation of VIP1R, V2R, V1aR, B2R, β2AR, CCR5, EP4, C5aR, AT1aR, and M2R resulted in an increase in agonist-promoted $BRET^1$, indicative of association between β-arrestin2-*R*luc and β2-adaptin-EYFP. This effect was selective, because agonist-promoted $BRET^1$ was detected only when GPCRs were incubated with their respective agonist and not with irrelevant agonists (data not shown). The time and the concentration dependence of the agonist-promoted association are illustrated in Fig. 2 (*A* and *B*) for representative receptors and the parameters ($t_{1/2}$, EC_{50}, and maximal $BRET^1$ values) of the curves obtained for all tested receptors are presented in Table 1. Although the $t_{1/2}$ varied from 1.7 to 8.9 min between individual receptors, in all cases the $BRET^1$ signal reached a plateau after 12 min of stimulation with saturating concentra-

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

TABLE 1

$BRET^1$ kinetics of association and comparison of generated EC_{50} with agonist affinity values obtained from other established assays

Receptor classification with respect to interaction with β-arrestins is indicated under "class" (Refs. 13–17, 51). The half-time ($t_{1/2}$) of the maximal association between β-arrestin2-*R*luc and β2-adaptin-EYFP in response to treatment with agonists (100 μM for M2R and 100 nM for all other receptors) was calculated from non-linear regression analysis of $BRET^1$ association kinetics curves. $BRET^1$max represents the agonist-promoted $BRET^1$ ratio obtained at maximum agonist stimulation. The $BRET^1EC_{50}$ values were determined from non-linear regression of dose-response $BRET^1$ curves. $BRET^1EC_{50}$ values were compared to previously published affinity values for the same agonists. Data are the mean ± S.E. of 3–5 independent experiments.

Receptor	Agonist	Class	$BRET^1$ $t_{1/2}$	$BRET^1$max	$BRET^1EC_{50}$	Other EC_{50} or K_d	Ref.
			min			*nM*	
V1aR	AVP	A	1.7 ± 0.3	0.06 ± 0.002	1.6 ± 0.5	0.5 ± 0.2[a]	(38)
β2AR	Isoproterenol	A	3.5 ± 0.5	0.05 ± 0.002	10 ± 1.3	13.9 ± 3.9[b]	(66)
ETAR	Endothelin-1	A	ND[c]	ND	ND	0.2[a]	(67)
ETBR	Endothelin-1	?[d]	ND	ND	ND	0.3[a]	(67)
VIP1R	VIP	B	2.9 ± 0.6	0.13 ± 0.003	1.9 ± 0.2	4.0[e]	(17)
V2R	AVP	B	3.0 ± 0.1	0.13 ± 0.004	1.2 ± 0.2	0.7 ± 0.1[b]	(66)
AT1aR	Angiotensin II	B	1.8 ± 0.5	0.07 ± 0.002	2.8 ± 0.3	2.0[e]	(68)
EP4R	PGE2	B	5.3 ± 1.0	0.07 ± 0.004	1.3 ± 0.3	2.2 ± 0.6[b]	(16)
C5aR	C5a	B	4.2 ± 0.9	0.08 ± 0.003	9.5 ± 1.0	5.2 ± 2.5[e]	(69)
CCR5	RANTES	B	8.9 ± 0.5	0.09 ± 0.002	2.7 ± 0.7	1.0[a]	(70)
B2R	Bradykinin	C	3.8 ± 0.5	0.12 ± 0.009	0.5 ± 0.2	1.7 ± 0.1[e]	(18)
M2R	Carbachol	?[d]	3.3 ± 0.3	0.085 ± 0.005	800 ± 23	1100 ± 150[f]	(71)

[a] Phosphatidylinositol hydrolysis.
[b] cAMP accumulation.
[c] ND, signal not detected.
[d] ? = not classified.
[e] Radioligand binding.
[f] Inhibition of cAMP production.

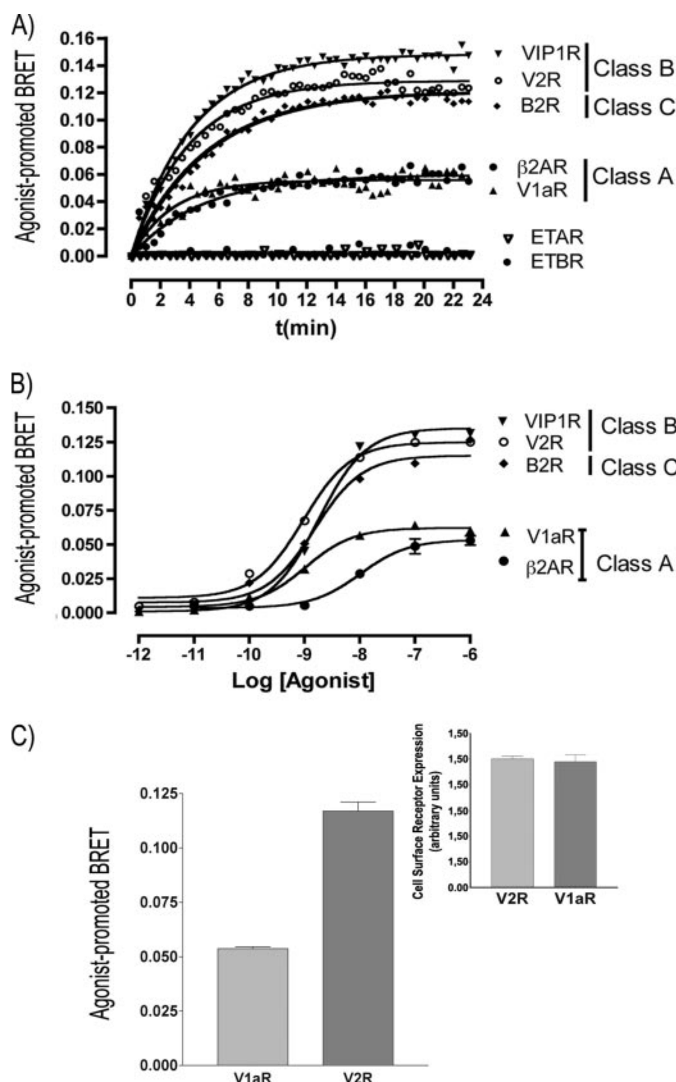

FIGURE 2. **Kinetics and dose dependence of agonist-promoted interaction between β2-adaptin-EYFP and β-arrestin-*R*luc.** HEK293T/β2-adaptin-EYFP cells were co-transfected with β-arrestin2-*R*luc and the indicated GPCRs. *A*, real-time BRET[1] measurements were taken at regular intervals for the indicated time period directly after the addition of coelenterazine-h and receptor specific agonists. *B*, cells were stimulated with the indicated concentrations of the receptor-specific agonists for 20 min at room temperature followed by BRET[1] measurements. For the agonist list, see Table 1. *C*, cells were stimulated with 100 nM AVP for 20 min followed by BRET[1] measurement. *Inset*, total cell surface expression of each receptor was assessed by cell surface ELISA assay using anti-Myc antibody.

tion of their respective agonists and remained constant for at least an additional 12 min. Therefore, for subsequent experiments, the BRET[1] signal was measured 20 min after agonist stimulation. For all GPCRs tested, the EC_{50} values obtained for the concentration-dependent increase in BRET[1] agreed well with the known affinities of the agonists used for each receptor (Fig. 2*B* and Table 1). The maximal BRET[1] signals (BRET[1]max) reached varied between 0.05 and 0.13 for the different receptors, with a tendency for class B receptors to promote higher BRET[1]max values. Although consistent with the greater propensity of Class B receptors to interact stably with β-arrestin2, it is difficult to directly interpret this difference, because the expression levels between receptors may have differed. However, in experiments where receptor expression levels were

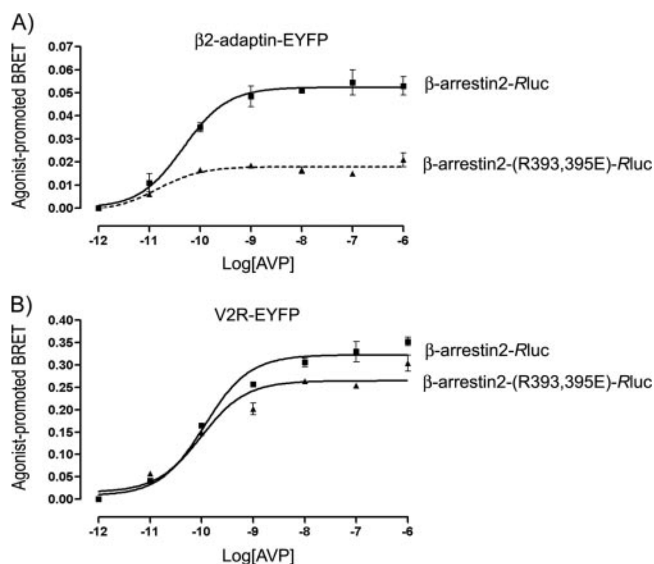

FIGURE 3. **Agonist-induced interactions of β-arrestin2-*R*luc and β-arrestin2-(R393–395E)-*R*luc with β2-adaptin-EYFP and V2R-EYFP.** *A*, HEK293T/β2-adaptin-EYFP cells were transiently co-transfected with V2R and either β-arrestin2-*R*luc or β-arrestin2-(R393–395E)-*R*luc. *B*, HEK293T cells were co-transfected with V2R-EYFP and either β-arrestin2-*R*luc or β-arrestin2-(R393–395E)-*R*luc. *A* and *B*, BRET[1] measurements were done after 20-min incubation with the indicated concentrations of AVP. Data are the mean ± S.E. of three to five independent experiments.

controlled by surface ELISA (using anti-Myc antibodies) for the class A (MycV2R) and class B (MycV1aR), significantly higher BRET[1]max signals were promoted by V2R as compared with V1aR. This indicates that the greater affinity of β-arrestin2 to interact with class B receptors can be reflected in the BRET-based β-arrestin/AP-2 interaction assay (Fig. 2*C* and *inset*).

To confirm that the BRET[1] signal observed truly reflects the association between β-arrestin and β2-adaptin, we took advantage of a mutant form of β-arrestin2, β-arrestin2-(R393E,R395E), which is unable to associate with β2-adaptin but can still undergo recruitment to the agonist-stimulated receptor (7, 44). For this purpose, HEK293T/β2-adaptin-EYFP cells were co-transfected with the V2R construct and either β-arrestin2-(R393E,R395E)-*R*luc or WT β-arrestin2-*R*luc. As can be seen in Fig. 3*A*, AVP promoted a robust increase in BRET[1] between β2-adaptin-EYFP and WT β-arrestin2-*R*luc but not β-arrestin2-(R393E,R395E)-*R*luc. The ability of β-arrestin2-(R393E,R395E)-*R*luc to be recruited to the receptor was confirmed in independent experiments monitoring the agonist-promoted BRET[1] between V2R-EYFP and both WT β-arrestin2-*R*luc and β-arrestin2-(R393E,R395E)-*R*luc (Fig. 3*B*). The detection of similar BRET[1] signals for the two β-arrestin2 constructs confirmed that the lack of signal between β2-adaptin-EYFP and β-arrestin2-(R393E,R395E)-*R*luc reflected the loss of association between these two proteins and not an impairment of β-arrestin translocation to the receptor, thus validating the β2-adaptin-EYFP/β-arrestin2-*R*luc pair as a specific biosensor for the receptor promoted β-arrestin/AP-2 interaction.

Although β-arrestin2 has been studied more extensively than other non-visual arrestins, β-arrestin1 has also been shown to interact with AP-2 upon activation of certain GPCRs (7, 10). Thus, we assessed the ability of the BRET-based assay to

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

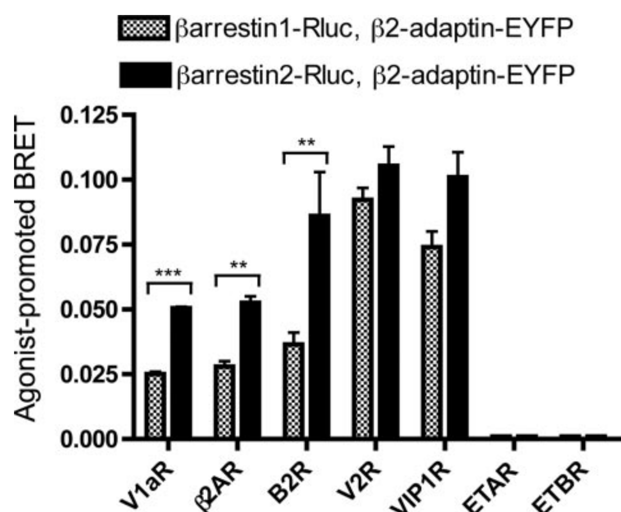

FIGURE 4. **Agonist-induced interactions of β-arrestin2-*R*luc and β-arrestin1-*R*luc with β2-adaptin-EYFP.** HEK293T/β2-adaptin-EYFP cells were co-transfected with either β-arrestin1-*R*luc or β-arrestin2-*R*luc and the indicated GPCRs. BRET[1] measurements were done after 20-min incubation with 100 nM receptor-specific agonists. Data are the mean ± S.E. of three to five independent experiments. Statistical significance of the difference was assessed using paired Student's *t* test. ***, $p < 0.0001$; **, $p < 0.005$.

detect this interaction on a representative subset of the GPCRs tested above. As shown in Fig. 4, agonist activation of the V1aR, β2AR, B2R, V2R, and VIP1R resulted in an increase in BRET[1] between β-arrestin1-*R*luc and β2-adaptin-EYFP, confirming that these receptors can promote the interaction between AP-2 and either β-arrestin1 or -2. Interestingly, the extent of signal generated by β-arrestin1 and β-arrestin2 was identical when promoted by the maximal activation of V2R and VIP1R, two prototypical class B receptors that display identical ability to interact with the two β-arrestins (13). In contrast, maximal activation of the class A receptors, V1aR and β2AR, led to weaker signals with β-arrestin1 than β-arrestin2, consistent with their reported lower propensity to associate stably with β-arrestin1 (13). For the B2R, although its propensity to co-localize in endosomes with β-arrestin2 (18) suggest a class B receptor, the weaker signal observed between β2-adaptin-EYFP and β-arrestin1-*R*luc as compared with β-arrestin2-*R*luc would be more consistent with a class A. This hybrid behavior is consistent with the observation by Simaan *et al.* (18) that, in contrast with prototypical class B receptors, the B2R once in the endosomes can dissociate from β-arrestin2 upon removal of the agonist, allowing the recycling of the receptor to the cell surface. The direct comparison of the interaction profile between β2-adaptin and the two β-arrestins promoted by different receptors is made possible by the fact that energy donor/acceptor ratio (*R*luc/EYFP) can be maintained constant independently of the receptor being expressed. In that respect, the present assay provides an advantage over the direct monitoring of β-arrestins to the receptor, because the identities of the BRET partners used are the same, independently of the receptor being studied. Thus, the β-arrestin-*R*luc/β2-adaptin-EYFP BRET assay provides a useful tool to classify GPCRs according to their profile of interaction with the β-arrestin/AP-2 complex.

Among the receptors tested, only two, ETAR and ETBR, failed to promote the interaction between either β-arrestin2-*R*luc or β-arrestin1-*R*luc and β2-adaptin-EYFP (Figs. 2*A* and 4). No BRET[1] signal could be detected even when agonist stimulation was maintained for 60 min (data not shown). To determine whether the lack of signal could result from the inability of these receptors to recruit β-arrestin, we used Double-Brilliance-β-arrestin (*R*luc-β-arrestin2-EYFP), a biosensor that allows monitoring of conformational changes of β-arrestin2 by intramolecular BRET[1] upon translocation to the receptor (42). As can be seen in Fig. 5*A*, agonist (ET1) activation of HEK293T cells co-transfected with ETAR or ETBR and Double-Brilliance-β-arrestin induced an increase in intramolecular BRET[1], indicating that these receptors recruited β-arrestin2 but failed to promote interaction between β-arrestin and AP-2. In addition, ET1-promoted translocation of β-arrestin2 to the plasma membrane was observed by fluorescence microscopy in HEK293T cells transfected with ETAR or ETBR (Fig. 5*B*). This is consistent with a previous report indicating that ETAR recruits β-arrestin2 following agonist activation (45). Also consistent with previous observations, ET1 stimulation led to the endocytosis of both ETAR and ETBR, as evidenced by the decrease in the receptor cell surface expression detected by ELISA following agonist treatment (Fig. 5*C*). Moreover, involvement of β-arrestins in ETAR and ETBR agonist-induced internalization could be demonstrated by interfering with β-arrestins expression by the use of siRNAs targeting β-arrestin1 and β-arrestin2. Indeed, transfection of these siRNAs, which led to a significant reduction in both β-arrestin1 and β-arrestin2 levels, significantly blocked ETAR and ETBR agonist-promoted internalization (Fig. 5*C*). Selective knock-down of β-arrestin2 led to a similar inhibition of the endocytosis (data not shown), indicating a predominant role for β-arrestin2. Taken together these data indicate that ETAR and ETBR can be endocytosed by a β-arrestin-dependent but AP-2-independent pathway, suggesting a non-clathrin-mediated endocytosis for these receptors.

Agonist-independent Association between β-Arrestin and β2-Adaptin Reveals Receptor Constitutive Internalization—Recently, mutations leading to constitutive endocytosis in the absence of ligand stimulation have been described for several GPCRs (46). Among those, substitution of arginine 137 by a histidine residue within the V2R has been found to promote the constitutive recruitment of β-arrestin to the receptor and the ensuing endocytosis of the complex (35, 47). To investigate whether constitutive internalization of GPCRs also involves β-arrestin/AP-2 interaction, we performed BRET[1] experiments on HEK293T/β2-adaptin-EYFP cells transiently co-transfected with β-arrestin2-*R*luc and either the wild-type or a constitutively internalizing V2R mutant, V2R(R137H). In the absence of agonist stimulation, V2R(R137H) promoted a slightly higher basal BRET[1] signal between β-arrestin2-*R*luc and β2-adaptin-EYFP than the wild-type V2R, and this, despite a lower cell surface expression, is consistent with its constitutive endocytosis (Fig. 6). Co-expression of the dominant negative mutant of dynamin I, dynI(K44A), that prevents the pinching off of endocytic vesicles from the plasma membrane further revealed the difference in BRET[1] signal promoted by V2R(R137H) *versus* WT (Fig. 6).

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

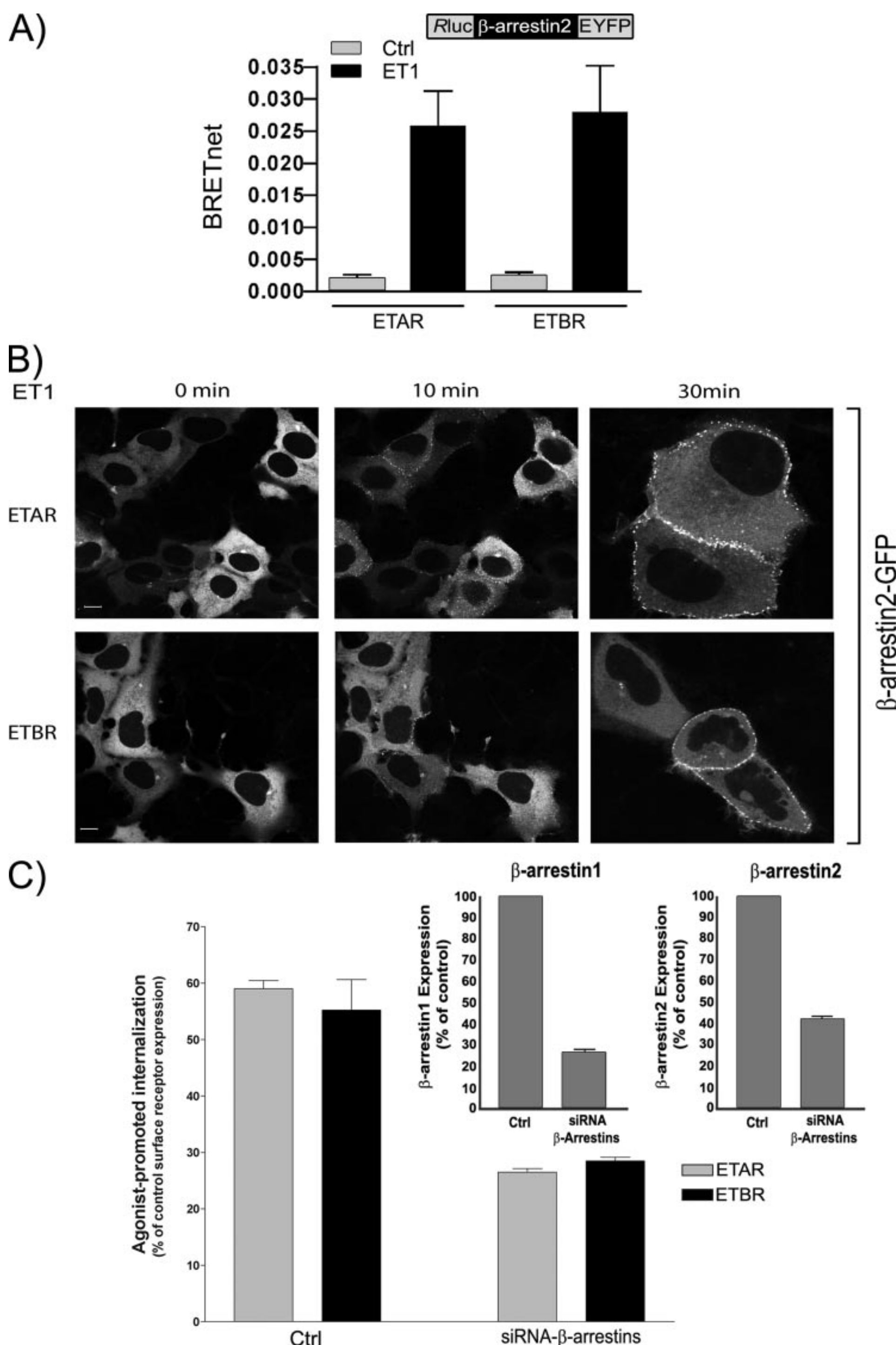

FIGURE 5. **Agonist-induced β-arrestin2 recruitment to ETAR and ETBR and receptor internalization.** *A*, HEK293T cells were co-transfected with ETAR or ETBR and *R*luc-β-arrestin2-EYFP (Double-Brilliance), and BRET[1] was measured after 20 min of agonist (+*ET1*, 10 nM) or vehicle treatment (*Ctrl*). *B*, HEK293T cells were transfected with β-arrestin2-GFP and either ETAR or ETBR and treated with 10 nM of ET1 for the indicated time period. β-Arrestin2-GFP translocation to the plasma membrane was observed using confocal fluorescence microscopy. *C*, HEK293T cells were transfected with HA-ETAR or FLAG-ETBR and either a control siRNA (*Ctrl*) or a siRNA targeting both β-arrestin1 and β-arrestin2 (*siRNA-β-arrestins*). Surface expression of each receptor was assessed by ELISA, in the absence or presence of 10 nM ET1, using anti-Ha (for ETAR) or anti-FLAG (for ETBR) antibodies, and the percentage of agonist-promoted receptor internalization was determined. The effect of siRNA on β-arrestin1 and β-arrestin2 expression levels was assessed by measuring the reduction of luminescence activity originating from low level of co-transfected β-arrestin1-*R*luc and β-arrestin2-*R*luc. Data are the mean ± S.E. (*A* and *C*) of four independent experiments or are representative (*B*) of two independent experiments.

The increase in the V2R(R137H)-promoted BRET[1] observed in the presence of dynI(K44A) most likely results from the accumulation of the receptor in the clathrin-coated pits at the cell surface (as illustrated by the increase in cell surface receptor detected by ELISA, Fig. 6, *inset*) where the constitutive formation of the V2R(R137H)/β-arrestin2/AP-2 complex can be detected. This is consistent with the role of dynamin that promotes severing of the clathrin-coated pits but is not involved in the assembly of the early endocytic machinery. The observation, that the BRET[1] signal promoted by V2R(R137H) in the presence of dynI(K44A) is of the same magnitude as that observed following agonist activation of the wild-type receptor by AVP, suggests that the R137H mutation can promote the formation of the receptor/β-arrestin/AP-2 complex as efficiently as the activated wild-type receptor. Taken together, these results confirm that constitutive endocytosis of V2R(R137H) and agonist-promoted internalization of the wild-type receptor involve the same initial steps of the clathrin-dependent pathway involving the interaction between β-arrestin and AP-2.

Receptor Activity-independent Recruitment of β-Arrestin2 Promotes Interaction between β-Arrestin2 and AP-2—Because it was previously shown that agonist stimulation of the α1A-adrenergic receptor (48) could directly promote the recruitment of AP-2 (through its μ subunit) to the receptor, one could envisage that the interaction between β-arrestin and AP-2 could follow their independent recruitment to the activated receptor. Alternatively, the interaction between β-arrestin and AP-2 may reflect a direct recruitment of AP-2 to β-arrestin following the translocation of β-arrestin to the receptor. To distinguish between these two possibilities, we assessed whether the recruitment of β-arrestin to the receptor, independently of agonist-promoted receptor activation, is sufficient to promote the interaction between β-arrestin and AP-2. For this purpose, we took advantage of a cyclophilin-based approach developed on the ability of a synthetic bivalent heterodimerizer compound,

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

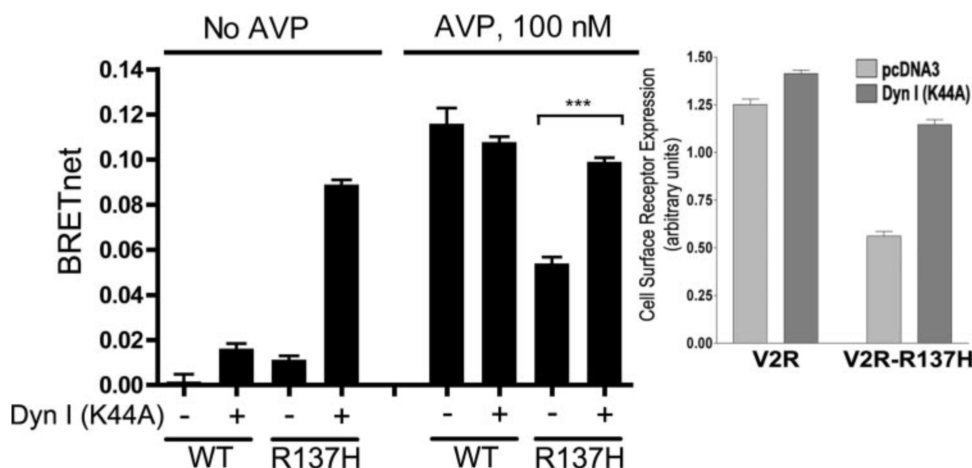

FIGURE 6. **V2R(R137H) promoted interaction between β-arrestin2-*R*luc and β2-adaptin-EYFP.** HEK293T/β2-adaptin-EYFP cells were co-transfected with wild type (*WT*) or mutant (*R137H*) V2R and β-arrestin2-*R*luc, with or without dynI(K44A). BRET[1] was measured in the absence or presence of 100 nM AVP (20-min treatment at room temperature). *Inset*: cell surface expressions of Myc-V2R and Myc-V2R(R137H) were assessed by cell surface ELISA assay using anti-Myc antibody. Data are the mean ± S.E. of three independent experiments. Statistical significance of the difference was assessed using paired Student's *t* test. ***, $p < 0.0001$.

AP-21967, to function as a high affinity adaptor to link the cyclophilin FRB and FKBP protein domains. The fusion of FRB to the N terminus of β-arrestin2-*R*luc and FKBP to the C terminus of V2R was previously shown to allow the recruitment of β-arrestin to the receptor upon AP-21967 treatment, in the absence of AVP. Such agonist-independent β-arrestin recruitment did not result in G protein activation but induced the internalization of the V2R-FKBP (34). Fusions with the cyclophilin domains were also shown not to affect the functionality of either β-arrestin2 or V2R (34). As shown in Fig. 7, treatment with AP-21967 induced a concentration-dependent BRET[1] between β2-adaptin-EYFP and FRB-β-arrestin2-*R*luc in cells co-expressing V2R-FKBP. No such BRET[1] was observed in cells expressing V2R and β-arrestin2-*R*luc not fused to FKBP and FRB, respectively. These data clearly indicate that recruitment of β-arrestin to the receptor, even in the absence of agonist activation, is sufficient to induce β-arrestin/AP-2 association. It has previously been suggested that the interaction between a phosphate sensor within β-arrestin and the phosphorylated tail of the receptor contributes to the conformational rearrangements leading to the engagement of AP-2 (7). Given that the agonist-independent recruitment of β-arrestin promoted by AP-21967 should not promote receptor phosphorylation, our data suggest that β-arrestin conformational rearrangements promoted by its interaction with the receptor, even in the absence of the phosphorylation, may be sufficient to trigger the interaction between β-arrestin and AP-2.

Association Kinetics between V2R/β-Arrestin2 versus β-Arrestin2/β2-Adaptin as Measured by Combining BRET[1] and BRET[2]—From the above discussion, one would predict that β-arrestin translocation to the receptor would precede β-arrestin/AP-2 interaction. To directly test this prediction, we took advantage of the availability of two different coelenterazine substrates whose oxidation by *R*luc results in resolved light emission spectra, allowing differential energy transfer to two different derivatives of GFP (49). Oxidation of DeepBlueC by *R*luc (used in BRET[2]) results in a maximal light emission at 400 nm, thus allowing efficient energy transfer to GFP variants such as GFP[2] or Tsapphire, whose maximum excitation wavelengths are ~400 nm, but permits only marginal energy transfer to EYFP (maximum excitation wavelength: ~511 nm). Conversely, *R*luc-catalyzed oxidation of coelenterazine-*h* (used in BRET[1]), which emits light with a peak emission at ~480 nm, allows energy transfer to EYFP but fails to excite Tsapphire. Therefore, it is possible to combine BRET[1] and BRET[2] in the same cells to follow two different protein-protein interaction events (49). For this, HEK293T/β2-adaptin-EYFP cells transiently co-transfected with V2R-Tsapphire and β-arrestin2-*R*luc were stimulated with AVP. BRET[1] and BRET[2] were then used to monitor kinetics of interaction for the β-arrestin2-*R*luc/β2-adaptin-EYFP and β-arrestin2-*R*luc/V2R-Tsapphire pairs, respectively. As shown in Fig. 8, the apparent association of β-arrestin2 to the receptor ($t_{1/2}$: 4.2 min) is faster than its interaction with AP-2 ($t_{1/2}$: 10 min). Because association kinetics may vary with different expression levels of the studied interactors, we further calculated the association halftimes ($t_{1/2}$) from BRET[1] and BRET[2] under conditions where we varied the expression levels of V2R-Tsapphire in the presence of fixed levels of β-arrestin2-*R*luc and β2-adaptin-EYFP (Table 2). Under all conditions, AVP stimulation resulted in β-arrestin2-*R*luc/V2R-Tsapphire association that was in average 2.5 ± 0.1 times faster than the interaction between β-arrestin2-*R*luc and β2-adaptin-EYFP. Taken together, these results are consistent with the notion that the formation of the receptor/β-arrestin/AP-2 complex is initiated by the recruitment of β-arrestin to the receptor, resulting in conformational changes leading to the association with AP-2.

DISCUSSION

This study was aimed at the development of a live cell approach that makes possible direct detection of the early events leading to β-arrestin- and clathrin-dependent internalization of GPCRs. For this purpose, we developed a BRET-based biosensor to monitor the agonist-induced interaction between β-arrestin-*R*luc and β2-adaptin-EYFP, a needed step for the β-arrestin-mediated targeting of GPCRs to clathrin-coated pits for internalization (7, 8, 10).

Out of the 12 different GPCRs tested, only the ETAR and ETBR were unable to promote β-arrestin association with AP-2 upon agonist-stimulated receptor internalization, indicating an alternative endocytic route for these two receptors. Among the receptors promoting β-arrestin/β2-adaptin interactions, those displaying lower and higher affinity for β-arrestins (class A and B, respectively) could be readily differentiated based on the strength of the BRET[1] signals detected between β2-adaptin-EYFP and both of β-arrestin2-*R*luc and β-arrestin1-*R*luc. The

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

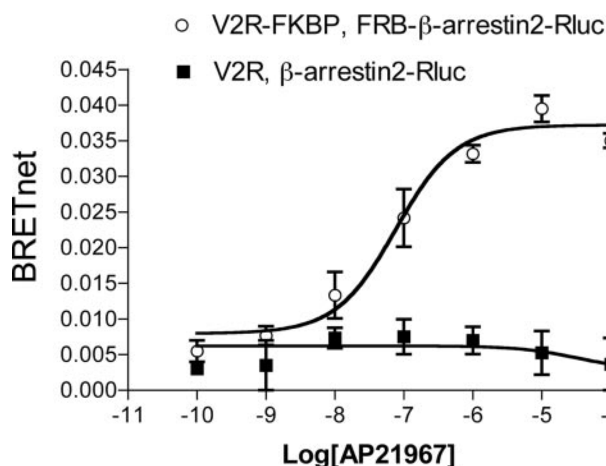

FIGURE 7. **AP-21967-induced interaction between β-arrestin2-*R*luc and β2-adaptin-EYFP in the absence of receptor activation.** HEK293T/β2-adaptin-EYFP cells were co-transfected with either FRB-β-arrestin2-*R*luc and V2R-FKBP or with β-arrestin2 and V2R. BRET[1] measurements were performed 20 min following the addition of increasing concentrations of AP-21967. Data are the mean ± S.E. of three independent experiments.

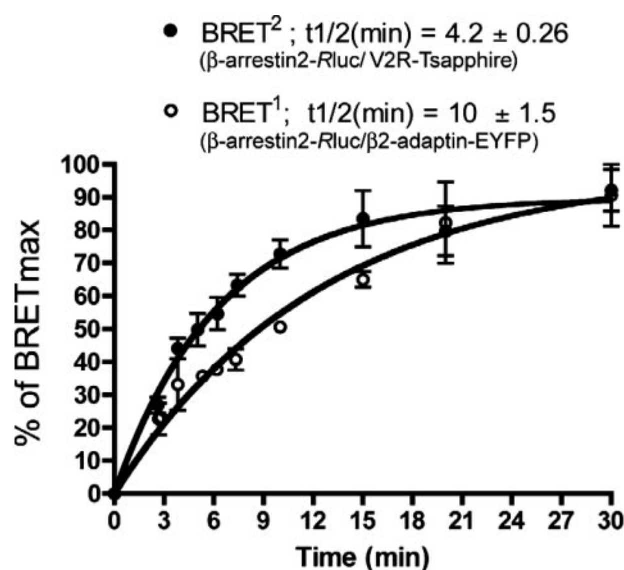

FIGURE 8. **Simultaneous detection of agonist-promoted interactions between β-arrestin2-*R*luc and both V2R-Tsaphire and β2-adaptin-EYFP by combining BRET[1] and BRET[2].** HEK293T/β2-adaptin-EYFP cells, co-transfected with β-arrestin2-*R*luc and V2R-Tsapphire, were stimulated with 100 nM AVP or vehicle alone in the presence of coelenterazine-*h* (BRET[1]) or DeepBlueC (BRET[2]), and the kinetics of the agonist-promoted BRET signals were measured. BRET values presented are the percentage of maximum response obtained from cells expressing the highest tested concentration of V2R-Tsapphire (2931 ± 81 fluorescence units). The BRET[1] ratio represents the interaction between β2-adaptin-EYFP and β-arrestin2-*R*luc, whereas the BRET[2] ratio corresponds to the recruitment of β-arrestin2-*R*luc to V2R-Tsapphire. Apparent $t_{1/2}$ (half-time) of maximal association is indicated. Data are the mean ± S.E. of three independent experiments.

comparison of the kinetics of association of β-arrestin with the receptor and AP-2, made possible by combining BRET[1] and BRET[2] in living cells, respectively, indicated that β-arrestin association with the activated receptors precedes and probably triggers the recruitment of AP-2 to the β-arrestin/receptor complex. Consistent with this notion, the recruitment of β-arrestin to a constitutively internalizing mutant of the V2R or forcing its binding to the wild-type receptor in the absence of agonist stimulation (using a cyclophilin-based approach) was sufficient for promoting the interaction of AP-2 with the complex.

The list of receptors tested in this study included GPCRs that were previously proposed to undergo clathrin-dependent or -independent pathways. Agonist activation of VIP1R, V2R, V1aR, B2R, β2AR, CCR5, EP4R, M2R, AT1aR, and C5aR resulted in an increase in agonist-promoted BRET[1] between β-arrestin and β2-adaptin, indicative that internalization of these receptors occurs via a β-arrestin- and clathrin-dependent pathway. Studies using pharmacological and biochemical inhibitors previously reported that VIP1R, V2R, V1aR, B2R, β2AR, CCR5, and EP4R undergo clathrin-dependent endocytosis (6, 17, 50–54). However, the internalization route previously proposed for M2R, AT1aR, and C5aR were more controversial. Indeed, it has been previously reported that the internalization of the M2R is atypical in being both non-clathrin and non-caveolae mediated, because it was not significantly affected by the co-expression of dominant negative mutants of β-arrestin1 (β-arrestin1(V53D)), dynamin (dynI(K44A)), or the heavy chain of clathrin (Hub) (55, 56). These results were surprising, considering that treatment with hypertonic sucrose, a classic inhibitor of clathrin-mediated endocytosis (57), was reported to strongly inhibit M2R internalization (55). Recently, by monitoring the trafficking of an M2R fused to GFP, it was reported that the M2R appeared to internalize initially via a clathrin-independent pathway but then quickly merged with the clathrin-endocytic pathway at the level of early endosomes (58). Our BRET[1] data clearly support the involvement of the clathrin pathway in M2R internalization and demonstrate that the M2R can promote the formation of the β-arrestin/AP-2 complex involved in the early steps of clathrin-mediated endocytosis. For the AT1aR and C5aR, dominant negative mutants of β-arrestin1 (β-arrestin1(V53D) or β-arrestin2-(319–418)) and dynamin (dynI(K44A)) were initially reported not to inhibit receptor internalization (59, 60). However, later studies showed that the internalization of both AT1aR and C5aR can be inhibited by dominant negative mutants of both dynamin and β-arrestins (51, 61, 62). The results obtained in the present study using the direct BRET[1]-based monitoring of the β-arrestin/AP-2 interaction are consistent with the latter studies, indicating that AT1aR and C5aR can undergo β-arrestin- and clathrin-mediated endocytosis.

Taken together, the conflicting results obtained in some studies illustrate the difficulties of interpretations associated with the exclusive use of dominant negative mutants to define the endocytic route used by specific receptors. The expression level of the mutant used and the endogenous levels of the proteins involved in the pathway are among the factors that can explain the contradictory results obtained. In this context, the BRET-based assays, directly monitoring the interactions between components of specific endocytic pathway, offer several advantages. For instance, the behavior of each receptor can be examined in an identical cellular background where the expression level of the components assayed can be selected and monitored in the course of the assay by measuring fluorescence and luminescence levels. Also, the biosensor system used to monitor the endocytic process is kept constant, independently

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

TABLE 2

Association kinetics determined by combining BRET[1] and BRET[2]

The different receptor expression levels were determined by fluorescence measurements (excitation 400 nm, emission 511 nm). BRET[1] ratio represents the interaction between β2-adaptin-EYFP and β-arrestin2-*R*luc, while BRET[2] ratio corresponds to the recruitment of β-arrestin2-*R*luc to V2R-Tsapphire. Apparent $t_{1/2}$ (half-time) of maximal association is indicated. Data are the means ± S.E. of three independent experiments.

Fluorescence (V2R-Tsapphire)	1244 ± 9	1851 ± 186	2931 ± 81
BRET[1], $t_{1/2}$ (min)	16.3 ± 5	16 ± 1.8	10.9 ± 1.5
BRET[2], $t_{1/2}$ (min)	7.25 ± 2.8	6.13 ± 0.3	4.17 ± 0.3
$t_{1/2}$ BRET[1]/$t_{1/2}$ BRET[2]	2.3	2.6	2

of the receptor studied, allowing direct comparison between receptors that are not modified. The assay is thus a direct readout of the ability of each receptor to engage specific components of the endocytic machinery and to evaluate the potential of the receptor to use specific internalization pathways in a given cell type.

As discussed above, the detection of receptor promoted BRET[1] between β-arrestin and β2-adaptin is a clear demonstration that this receptor can be internalized via clathrin-coated endosomes. However, a lack of BRET[1], although ruling out a β-arrestin-mediated clathrin-dependent process, does not exclude the possibility of a β-arrestin-independent/clathrin-dependent or a β-arrestin-mediated/clathrin-independent pathway. β-Arrestin-independent/clathrin-dependent endocytosis of GPCRs has been previously suggested for the HCMV-encoded chemokine US28 and 5-hydroxytryptamine 2A receptors (63, 64). Interestingly, direct interaction between a subunit of AP-2 (μ2-adaptin) and both the α1b-adrenergic receptor and the protease-activated receptor 1 (48, 72) has been documented. Whether such direct interactions between AP-2 and certain GPCRs could be responsible for the β-arrestin-independent clathrin-mediated endocytosis remains to be investigated.

For ETAR and ETBR, our data strongly suggest a β-arrestin-dependent but clathrin-independent endocytic pathway. This is consistent with previous data suggesting an internalization process involving both caveolae and β-arrestin for ETAR (20). In addition to providing additional support to the hypothesis that β-arrestin could be involved in non-clathrin-mediated endocytosis, these results reinforce the notion that inhibition of endocytosis by β-arrestin dominant negative mutants cannot be used as a marker of clathrin-dependent internalization.

When considering the β-arrestin/clathrin-dependent pathway, our data are consistent with the notion that the recruitment of β-arrestin to the receptor and the ensuing conformational changes are needed for the subsequent association with AP-2 leading to clathrin-mediated endocytosis. In particular, BRET[1] and BRET[2] clearly indicated that the β-arrestin/AP-2 complexes did not exist in the absence of receptor activation and that the recruitment of β-arrestin to the receptor precedes the association of β2-adaptin to the complex. Consistent with this notion, the recruitment of β-arrestin to the V2R using the cyclophilin-based recruitment system was sufficient to promote β-arrestin/AP-2 interaction and the consequent endocytosis, suggesting that GPCR/β-arrestin interaction may be the triggering signal for the recruitment of AP-2 to the complex. However, for ETAR and ETBR receptors, recruitment of β-arrestin was not sufficient for directing these receptors to the AP-2- and clathrin-dependent endocytic pathway. Thus, it is likely that AP-2 association with β-arrestin is determined by specific motifs or conformational rearrangements that are intrinsic to specific GPCR/β-arrestin complexes.

In addition to allowing detailed analysis of the molecular events involved in GPCR endocytosis, the BRET-based assay developed herein provides a robust tool to monitor the activation of receptors that internalize via the βarrestin- and clathrin-dependent process. Interestingly, the calculated EC_{50} values for the agonist-promoted interaction between β-arrestins and β2-adaptin correlated very well with the agonist affinities reported for GPCR functional assays. The β-arrestin/β2-adaptin BRET assay also presents the advantage of being independent of the G protein-coupling preference of the receptor ($G_{i/o}$-, G_s-, and $G_{q/11}$-coupled receptors) and does not require modification of the receptor, thus offering a universal functional assay for native GPCRs that internalize via the β-arrestin- and clathrin-mediated pathway. Finally, the present study opens the way to the development of similar BRET-based assays directly assessing the interaction between other partners of the endocytic machineries that will provide useful tools to dissect alternative pathways for GPCR endocytosis.

Acknowledgments—We are grateful to Drs. Audrey Claing and Stefano Marullo for providing some GPCR and β-arrestin2 constructs, respectively, and for their helpful discussions regarding the manuscript. We thank Drs. Joseph Sodroski, Oliver Griesbeck, Kathryn DeFea, and Sylvain Meloche for providing the CCR5, Tsapphire-GFP, β-arrestin1, and the AT1aR plasmids, respectively. We also thank Dr. Christian Le Gouill for his critical reading of the manuscript. We are grateful to Dr. Victor Rivera from Pharmaceuticals Ariad for the gift of the expression vectors pC4EN-F$_1$ (FKBP) and pC4-R$_H$E vectors (FRB) and for kindly providing the heterodimerizer AP-21967.

REFERENCES

1. Foord, S. M., Bonner, T. I., Neubig, R. R., Rosser, E. M., Pin, J. P., Davenport, A. P., Spedding, M., and Harmar, A. J. (2005) *Pharmacol. Rev.* **57,** 279–288
2. Claing, A., Laporte, S. A., Caron, M. G., and Lefkowitz, R. J. (2002) *Prog. Neurobiol.* **66,** 61–79
3. Marchese, A., Chen, C., Kim, Y. M., and Benovic, J. L. (2003) *Trends Biochem. Sci.* **28,** 369–376
4. Lefkowitz, R. J., and Shenoy, S. K. (2005) *Science* **308,** 512–517
5. Edeling, M. A., Mishra, S. K., Keyel, P. A., Steinhauser, A. L., Collins, B. M., Roth, R., Heuser, J. E., Owen, D. J., and Traub, L. M. (2006) *Dev Cell* **10,** 329–342
6. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383,** 447–450
7. Kim, Y. M., and Benovic, J. L. (2002) *J. Biol. Chem.* **277,** 30760–30768
8. Laporte, S. A., Miller, W. E., Kim, K. M., and Caron, M. G. (2002) *J. Biol. Chem.* **277,** 9247–9254
9. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J. Biol. Chem.* **275,** 23120–23126
10. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3712–3717
11. Santini, F., Gaidarov, I., and Keen, J. H. (2002) *J. Cell Biol.* **156,** 665–676
12. Prossnitz, E. R. (2004) *Life Sci.* **75,** 893–899

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

13. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) *J. Biol. Chem.* **275,** 17201–17210
14. Terrillon, S., Barberis, C., and Bouvier, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1548–1553
15. Fraile-Ramos, A., Kohout, T. A., Waldhoer, M., and Marsh, M. (2003) *Traffic* **4,** 243–253
16. Neuschafer-Rube, F., Hermosilla, R., Rehwald, M., Ronnstrand, L., Schulein, R., Wernstedt, C., and Puschel, G. P. (2004) *Biochem. J.* **379,** 573–585
17. Shetzline, M. A., Walker, J. K., Valenzano, K. J., and Premont, R. T. (2002) *J. Biol. Chem.* **277,** 25519–25526
18. Simaan, M., Bedard-Goulet, S., Fessart, D., Gratton, J. P., and Laporte, S. A. (2005) *Cell Signal.* **17,** 1074–1083
19. Chini, B., and Parenti, M. (2004) *J. Mol. Endocrinol.* **32,** 325–338
20. Okamoto, Y., Ninomiya, H., Miwa, S., and Masaki, T. (2000) *J. Biol. Chem.* **275,** 6439–6446
21. Claing, A., Perry, S. J., Achiriloaie, M., Walker, J. K., Albanesi, J. P., Lefkowitz, R. J., and Premont, R. T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1119–1124
22. Heuser, J. E., and Anderson, R. G. (1989) *J. Cell Biol.* **108,** 389–400
23. Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271,** 363–366
24. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* **272,** 32507–32512
25. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999) *J. Cell Sci.* **112,** 1303–1311
26. Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 2760–2765
27. Chen, W., Kirkbride, K. C., How, T., Nelson, C. D., Mo, J., Frederick, J. P., Wang, X. F., Lefkowitz, R. J., and Blobe, G. C. (2003) *Science* **301,** 1394–1397
28. Dalle, S., Ricketts, W., Imamura, T., Vollenweider, P., and Olefsky, J. M. (2001) *J. Biol. Chem.* **276,** 15688–15695
29. Orem, N. R., Xia, L., and Dolph, P. J. (2006) *J. Cell Sci.* **119,** 3141–3148
30. Hamdan, F. F., Percherancier, Y., Breton, B., and Bouvier, M. (2006) *Curr. Prot. Neurosci.* John Wiley & Sons, Inc., New York
31. Pfleger, K. D., and Eidne, K. A. (2004) *Biochem. J.* **85,** 625–637
32. Zapata-Hommer, O., and Griesbeck, O. (2003) *BMC Biotechnol.* **3,** 5
33. Hamdan, F. F., Audet, M., Garneau, P., Pelletier, J., and Bouvier, M. (2005) *J. Biomol. Screen.* **10,** 463–475
34. Terrillon, S., and Bouvier, M. (2004) *EMBO J.* **23,** 3950–3961
35. Barak, L. S., Oakley, R. H., Laporte, S. A., and Caron, M. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 93–98
36. Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) *J. Biol. Chem.* **271,** 16384–16392
37. Rochdi, M. D., and Parent, J. L. (2003) *J. Biol. Chem.* **278,** 17827–17837
38. Terrillon, S., Durroux, T., Mouillac, B., Breit, A., Ayoub, M. A., Taulan, M., Jockers, R., Barberis, C., and Bouvier, M. (2003) *Mol. Endocrinol.* **17,** 677–691
39. Freedman, N. J., Ament, A. S., Oppermann, M., Stoffel, R. H., Exum, S. T., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 17734–17743
40. Mirzabekov, T., Bannert, N., Farzan, M., Hofmann, W., Kolchinsky, P., Wu, L., Wyatt, R., and Sodroski, J. (1999) *J. Biol. Chem.* **274,** 28745–28750
41. Charest, P. G., Oligny-Longpre, G., Bonin, H., Azzi, M., and Bouvier, M. (2006) *Cell Signal.* **19,** 32–41
42. Charest, P. G., Terrillon, S., and Bouvier, M. (2005) *EMBO Rep.* **6,** 334–340
43. Collins, B. M., McCoy, A. J., Kent, H. M., Evans, P. R., and Owen, D. J. (2002) *Cell* **109,** 523–535
44. Milano, S. K., Pace, H. C., Kim, Y. M., Brenner, C., and Benovic, J. L. (2002) *Biochemistry* **41,** 3321–3328
45. Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G., and Ferguson, S. S. (1999) *J. Biol. Chem.* **274,** 10999–11006
46. Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P., and Clauser, E. (2002) *Trends Endocrinol. Metab.* **13,** 336–343
47. Bernier, V., Lagace, M., Lonergan, M., Arthus, M. F., Bichet, D. G., and Bouvier, M. (2004) *Mol. Endocrinol.* **18,** 2074–2084
48. Diviani, D., Lattion, A. L., Abuin, L., Staub, O., and Cotecchia, S. (2003) *J. Biol. Chem.* **278,** 19331–19340
49. Perroy, J., Pontier, S., Charest, P. G., Aubry, M., and Bouvier, M. (2004) *Nat. Methods* **1,** 203–208
50. Bowen-Pidgeon, D., Innamorati, G., Sadeghi, H. M., and Birnbaumer, M. (2001) *Mol. Pharmacol.* **59,** 1395–1401
51. Braun, L., Christophe, T., and Boulay, F. (2003) *J. Biol. Chem.* **278,** 4277–4285
52. Desai, S., and Ashby, B. (2001) *FEBS Lett.* **501,** 156–160
53. Mueller, A., Kelly, E., and Strange, P. G. (2002) *Blood* **99,** 785–791
54. Pizard, A., Blaukat, A., Muller-Esterl, W., Alhenc-Gelas, F., and Rajerison, R. M. (1999) *J. Biol. Chem.* **274,** 12738–12747
55. Roseberry, A. G., and Hosey, M. M. (2001) *J. Cell Sci.* **114**(Pt 4), 739–746
56. Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K. H., and van Koppen, C. J. (1999) *J. Biol. Chem.* **274,** 12333–12338
57. Hansen, S. H., Sandvig, K., and van Deurs, B. (1993) *J. Cell Biol.* **121,** 61–72
58. Delaney, K. A., Murph, M. M., Brown, L. M., and Radhakrishna, H. (2002) *J. Biol. Chem.* **277,** 33439–33446
59. Gilbert, T. L., Bennett, T. A., Maestas, D. C., Cimino, D. F., and Prossnitz, E. R. (2001) *Biochemistry* **40,** 3467–3475
60. Zhang, J., Ferguson, S. S., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J. Biol. Chem.* **271,** 18302–18305
61. Gaborik, Z., Szaszak, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J., and Hunyady, L. (2001) *Mol. Pharmacol.* **59,** 239–247
62. Suvorova, E. S., Gripentrog, J. M., and Miettinen, H. M. (2005) *Traffic* **6,** 100–115
63. Bhatnagar, A., Willins, D. L., Gray, J. A., Woods, J., Benovic, J. L., and Roth, B. L. (2001) *J. Biol. Chem.* **276,** 8269–8277
64. Droese, J., Mokros, T., Hermosilla, R., Schulein, R., Lipp, M., Hopken, U. E., and Rehm, A. (2004) *Biochem. Biophys. Res. Commun.* **322,** 42–49
65. Fessart, D., Simaan, M., Zimmerman, B., Comeau, J., Hamdan, F. F., Wiseman, P. W., Bouvier, M., and Laporte, S. A. (2007) *J. Cell Sci.* **120,** 1723–1732
66. Breit, A., Lagace, M., and Bouvier, M. (2004) *J. Biol. Chem.* **279,** 28756–28765
67. Williams, D. L., Jr., Jones, K. L., Alves, K., Chan, C. P., Hollis, G. F., and Tung, J. S. (1993) *Life Sci.* **53,** 407–414
68. Laporte, S. A., Servant, G., Richard, D. E., Escher, E., Guillemette, G., and Leduc, R. (1996) *Mol. Pharmacol.* **49,** 89–95
69. Crass, T., Ames, R. S., Sarau, H. M., Tornetta, M. A., Foley, J. J., Kohl, J., Klos, A., and Bautsch, W. (1999) *J. Biol. Chem.* **274,** 8367–8370
70. Raport, C. J., Gosling, J., Schweickart, V. L., Gray, P. W., and Charo, I. F. (1996) *J. Biol. Chem.* **271,** 17161–17166
71. van Koppen, C. J., and Nathanson, N. M. (1990) *J. Biol. Chem.* **265,** 20887–20892
72. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) *Mol. Cell. Biol.* **26,** 3231–3242

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016

Unraveling G Protein-coupled Receptor Endocytosis Pathways Using Real-time Monitoring of Agonist-promoted Interaction between β-Arrestins and AP-2

Fadi F. Hamdan, Moulay Driss Rochdi, Billy Breton, Delphine Fessart, Douce E. Michaud, Pascale G. Charest, Stéphane A. Laporte and Michel Bouvier

J. Biol. Chem. 2007, 282:29089-29100.
doi: 10.1074/jbc.M700577200 originally published online August 3, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M700577200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 47 of which can be accessed free at
http://www.jbc.org/content/282/40/29089.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at University of Arizona Library on April 24, 2016