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β-Adrenergic Receptor Kinase (GRK2) Colocalizes with β-Adrenergic Receptors during Agonist-induced Receptor Internalization*

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Rapid regulation of G protein-coupled receptors appears to involve agonist-promoted receptor phosphorylation by G protein-coupled receptor kinases (GRKs). This is followed by binding of uncoupling proteins termed arrestins and transient receptor internalization. In this report we show that the β-adrenergic receptor kinase (βARK-1 or GRK2) follows a similar pattern of internalization upon agonist activation of β_2-adrenergic receptors (β_2AR) and that βARK expression levels modulate receptor sequestration. Stable cotransfected cells expressing an epitope-tagged β_2AR and βARK-1 show an increased rate and extent of β_2AR internalization compared with cells expressing receptor alone. Moreover, subcellular gradient fractionation studies suggest that βARK colocalizes with the internalized receptors. In fact, double immunofluorescence analysis using confocal microscopy shows extensive colocalization of β_2AR and βARK in intracellular vesicles upon receptor stimulation. Our results confirm a functional relationship between receptor phosphorylation and sequestration and indicate that βARK does not only translocates from the cytoplasm to the plasma membrane in response to receptor occupancy, but shares endocytic mechanisms with the β_2AR. These data suggest a direct role for βARK in the sequestration process and/or the involvement of receptor internalization in the intracellular trafficking of the kinase.

A general feature of G protein-coupled receptors (GPCR)[1] is the existence of complex regulatory mechanisms that modulate receptor responsiveness and which underlie important physiological phenomena such as signal integration, plasticity, and desensitization. The molecular mechanisms of desensitization have been investigated using the β_2-adrenergic receptor (β_2AR) as the main model system. Work from several laboratories has shown that rapid, short term β_2AR desensitization is due to functional uncoupling from G proteins as a consequence of receptor phosphorylation. Agonist occupancy triggers phosphorylation of the receptor by the β-adrenergic receptor kinase (βARK-1), a serine/threonine kinase that specifically phosphorylates the COOH-terminal cytoplasmic domain of the receptor. βARK1 is a member of a family of G protein-coupled receptor kinases (GRKs), which phosphorylate different GPCRs, and it is now also termed GRK2. The phosphorylated β_2AR interacts with additional regulatory proteins, the β-arrestins, which block signal transduction. The uncoupled receptors are subsequently removed from the plasma membrane in a process termed internalization or sequestration (1–4).

Despite the fact that agonist-promoted sequestration is a common feature of many GPCRs, the molecular mechanisms involved have remained elusive and controversial. However, recent data have shed new light into this field. It has been suggested that sequestration plays a key role in resensitizing uncoupled GPCRs by allowing the dephosphorylation and recycling of functional receptors back to the plasma membrane (5–8). On the other hand, the internalization compartments of β_2AR and other GPCRs have been identified as early endosomes (9–12). Finally, receptor phosphorylation and subsequent β-arrestin binding have been shown to facilitate the process of sequestration, leading to the suggestion that β-arrestin may play a direct role as an adaptor molecule for receptor trafficking (14–16).

It should be noted that agonist occupancy of GPCRs does not only promote changes in the subcellular distribution of the receptor. Upon receptor activation, βARK transiently translocates to the plasma membrane (17–20), in a process that seems to be facilitated by interactions of COOH-terminal regions of the kinase with G protein βγ subunits (21–23). On the other hand, we have shown recently that a significant amount of βARK is associated to internal, microsomal membranes (24–26). However, very little is known about the mechanisms governing such complex subcellular distribution. In particular, the way βARK is recycled after phosphorylating GPCR in the plasma membrane and its possible relationship with the subsequent receptor sequestration have not been investigated.

In this context, we have examined the effects of βARK overexpression on the internalization parameters of epitope-tagged β_2ARs and investigated the changes in the subcellular localization of βARK that take place during the sequestration process. Our results indicate a close relationship between the intracellular dynamics of β_2ARs and that of the kinase as a consequence of receptor activation.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All recombinant DNA procedures were carried out following standard protocols. A cDNA encoding bovine βARK-1 (donated by Dr. J. L. Benovic, Jefferson University, Philadelphia) was cloned into the mammalian expression vector pREP4 (Invitrogen). A cDNA encoding the human β_2AR modified to incorporate the Signal FLAG (SF) epitope at the amino terminus (gift of Dr. B. L. Kobilka, Stanford University, Ref. 27) was cloned into pREP4. After cleavage, the FLAG epitope can be specifically detected using the anti-FLAG M1 monoclonal antibody (IBI).

Cell Culture and Transfection—Human embryonic kidney cells (HEK-EBNA 293) were obtained from Invitrogen and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bo-

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[1] The abbreviations used are: GPCR, G protein-coupled receptors; βAR, β-adrenergic receptor; β_2AR, β_2-adrenergic receptor; βARK, β-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase.

vine serum and 0.2 mg/ml geneticin (Sigma). Cells were transfected with pREP4-βARK1 and/or pREP4-SF β_2AR by the calcium phosphate precipitation method. For selection of stable transfected cells, 0.25 mg/ml hygromycin (Calbiochem) was added to the culture medium following transfection. Colonies originating from single cells were subcloned into 96-well tissue culture plates and screened for βARK and β_2AR protein expression by immunofluorescence, immunoblot analysis, and [^{3}H]dyhydroalprenolol binding (see below). The level of β_2AR expression in the selected clonal cell lines was between 0.7 and 2 pmol/mg of whole cell protein. GRK2 levels were in the range of ~25 pmol/mg of protein (~20-fold higher than those of control cells). Experiments were performed in different clonal cell lines. In addition, similar experiments were carried out in HEK-293 cells transiently expressing both βARK-1 and β_2AR.

Sequestration—Receptor sequestration was quantitated by flow cytometry essentially as described previously (7). Following treatment in the presence of 0–10 μM isoproterenol (Sigma) for the indicated times at 37 °C in 96-well culture plates (50,000 cells/well), the cells were quickly chilled, washed by centrifugation, and labeled at 4 °C for 60 min with M1 FLAG antibody (1:2000 dilution) in phosphate-buffered saline supplemented with 2% fetal bovine serum. After washing by centrifugation in the same vehicle, the cells were subsequently labeled with a 1:100 dilution of fluorescein-labeled goat anti-mouse antibody (Amersham Corp.). Cells were washed and fixed in 3.6% formaldehyde and the fluorescence present at the cell surface analyzed within 1 h on a Coulter scientific flow cytometer. Base-line cell fluorescence intensity was determined with washed unlabeled cells and cells labeled only with the goat anti-mouse fluorescein-conjugated antibodies. The fraction of sequestered receptors was then calculated by comparing the signal obtained in the absence or presence of agonist.

Subcellular Fractionation—Aliquots of HEK-293 cells expressing both βARK-1 and β_2AR were incubated at 37 °C for 20 min in the presence or absence of 10 μM isoproterenol in the culture medium. The reaction was stopped by addition of ice-cold phosphate-buffered saline. The same number of control and treated cells (obtained from one 10-cm dish per assay) were harvested and resuspended at 2–3 × 10^6 cells/ml in homogenization buffer (0.25 M sucrose, 10 mM Hepes, pH 7.2, 1 mM EDTA, 1 mM benzamidine, 100 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor) and homogenized in ice in a Dounce homogenizer (40 strokes). Particulate fractions were obtained by centrifuging postnuclear supernatants (10 min, 750 × *g* at 4 °C) at 250,000 × *g* for 30 min at 4 °C. Gradient fractionation of membrane fractions was performed as described (28), with some modifications. Briefly, pellets were resuspended in the same volume of homogenization buffer and mixed with Percoll (from a stock solution containing 90% Percoll (Sigma) in 0.25 M sucrose) and bovine serum albumin to a final concentration of 27% (v/v) Percoll and 4 mg/ml bovine serum albumin in a final volume of 11.5 ml. The mixture was layered over a 1-ml cushion of 2.5 M sucrose and centrifuged at 29,000 × *g* for 90 min at 4 °C in a 50Ti rotor (Beckman). Fractions of 0.4 ml were collected from the bottom of the tube and tested for β_2AR binding activity by [^{3}H]dyhydroalprenolol binding as described (28) and for βARK protein by Western blot. Samples to be analyzed by Western blot were diluted with the same volume of SDS buffer, incubated for 30 min at 4 °C, and centrifuged at 250,000 × *g* for 45 min at 4 °C to sediment the Percoll. Fractions were resolved by 7.5% SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose filters, and probed with AB9, a polyclonal antibody raised against purified recombinant bovine βARK (generous gift of Dr. J. L. Benovic, Jefferson University) as described (25, 26), and developed using a chemiluminiscent method (ECL, Amersham). Densitometric analysis of the blots was performed using a Molecular Dynamics laser densitometer.

Immunofluorescence Microscopy—Cells overexpressing βARK-1 and epitope-tagged β_2AR were grown on glass coverslips and incubated in the presence or absence of the desired concentrations of isoproterenol or other modulators for various times at 37 °C. After treatment, cells were fixed in 4% formaldehyde in phosphate-buffered saline and permeabilized with 0.2% Nonidet P-40, 5% dry non-fat milk, and 50 mM Tris-HCl, pH 7.4. Polyclonal βARK antibody AbFP1 (raised against a fusion protein containing amino acids 50–145 of bovine βARK1) (1:500, see Ref. 25) and M1 monoclonal anti-FLAG antibody to detect the tagged β_2AR were then applied to the specimen in the same blocking medium. After 60 min, the samples were extensively washed, and bound antibodies were detected using species-specific antibodies labeled with different fluorochromes (fluorescein-labeled goat anti-rabbit and Texas Red-labeled goat anti-mouse (Amersham) at a dilution of 1:100 for 30–45 min. In some experiments, colocalization of internalized β_2AR with transferrin receptors was established by serial double labeling

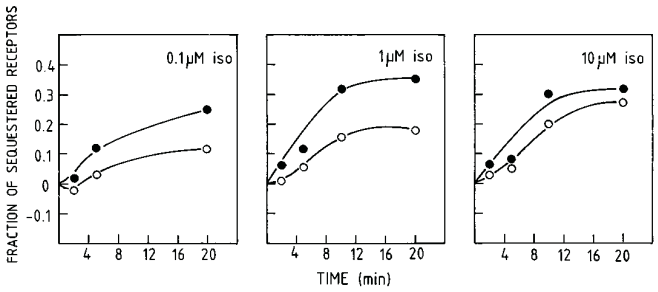

FIG. 1. **Effect of βARK1 overexpression on the agonist-promoted internalization of β_2-adrenergic receptors.** HEK-293 cells stably transfected with epitope-tagged wild-type human β_2AR alone (○, 1.6 pmol of β_2AR/mg of whole cell protein) or in combination with bovine βARK1 (●, 1.69 pmol of β_2AR/mg of whole cell protein) were incubated with 0.1, 1, or 10 μM isoproterenol for the indicated times. Surface receptors were then assessed by flow cytometry using M1 monoclonal antibody directed against the FLAG epitope engineered at the NH_2 terminus of β_2AR (see "Experimental Procedures" for details). Results are means of three independent experiments performed in triplicate (S.E. = 5–10%). Similar data were obtained using other HEK-293 cells clones.

immunofluorescence using M1 anti-FLAG and anti-transferrin receptor monoclonal antibodies. Confocal microscopy was performed using a Zeiss LSM 320 confocal laser scan microscope and conventional immunofluorescence microscopy by using a Zeiss Axiovert 35 microscope with 63 × NA 1.3 and 100 × NA 1.3 oil-immersion lenses. Absence of signal crossover was established using single-labeled samples.

RESULTS AND DISCUSSION

To investigate the relationship between agonist-dependent receptor phosphorylation by βARK and the internalization process, we used flow cytometry to measure the extent of epitope-tagged β_2AR remaining at the cell surface (7, 11) after treating with the β-agonist isoproterenol HEK-293 cells stably expressing similar levels of β_2AR alone or in combination with βARK1. Fig. 1 shows that the extent of receptor internalization attained at low concentrations of agonist (*left* and *middle panels*) is markedly increased in cells cotransfected with βARK. At a high concentration of isoproterenol (10 μM, *right panel*) there is only a slight effect of βARK cotransfection on the extent of internalization, although the rate of the process appears to be slightly enhanced with respect to control cells. An increased kinase/receptor ratio in the cotransfected cells would increase the proportion of phosphorylated receptors in response to a given concentration of agonist, thus leading to the observed increase in the extent of receptor internalization noted at low concentrations of agonists, as reported previously for m2 muscarinic acetylcholine receptors (14). The fact that previous reports (13, 29, 30) have failed to show an increased internalization of wild-type β_2AR as a consequence of βARK overexpression may be ascribed to the fact that only high agonist doses and long times of treatment (30 min) were investigated. Nevertheless, βARK overexpression has been shown to be able to rescue the sequestration of internalization-deficient β_2AR mutants (13, 15) and a dominant negative mutant of βARK decreased both wild-type and mutant β_2AR agonist-induced receptor internalization (13, 14). In line with these data, our results support a functional relationship between β_2AR phosphorylation by βARK and receptor sequestration.

We next investigated if changes in the subcellular distribution of the kinase can be observed during agonist-induced β_2AR endocytosis. Using subcellular gradient fractionation, a clear change in the pattern of β_2AR binding in particulate fractions can be detected in HEK-293 cells transfected with both β_2AR and βARK in the presence of 10 μM isoproterenol (compare *ISO versus CONTROL* in Fig. 2*A*). Interestingly, a relative increase in βARK protein is noted in the same fractions enriched in

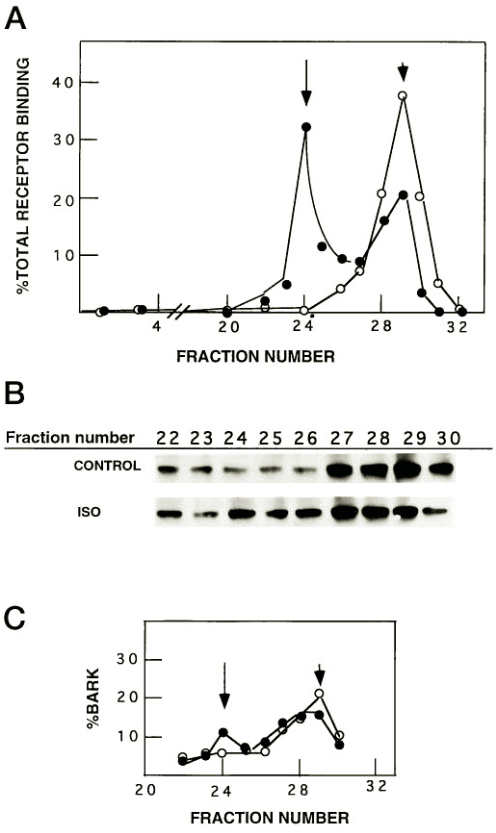

FIG. 2. **Effect of isoproterenol on the subcellular distribution of β_2AR and βARK as assessed by gradient fractionation studies.** HEK-293 cells stably expressing β_2AR and βARK1 were incubated for 20 min in the absence (○, control) or presence of 10 μM isoproterenol (●, iso). Membrane fractions were resolved by means of a Percoll density gradient and assayed for β_2AR binding and presence of βARK. The amount of β_2AR binding in a given fraction is expressed as a percentage of total specific binding in all the fractions (*A*). βARK protein across the receptor profile (*B*) was determined by Western blot analysis as detailed under "Experimental Procedures" and quantitated by laser densitometry. βARK levels in each fraction were expressed as a percentage of total kinase in the profile (*C*). The *short* and *long arrows* in *A* and *C* indicate the fractions containing the peak of plasma membrane and internalized receptors, respectively. Results are representative of four independent experiments.

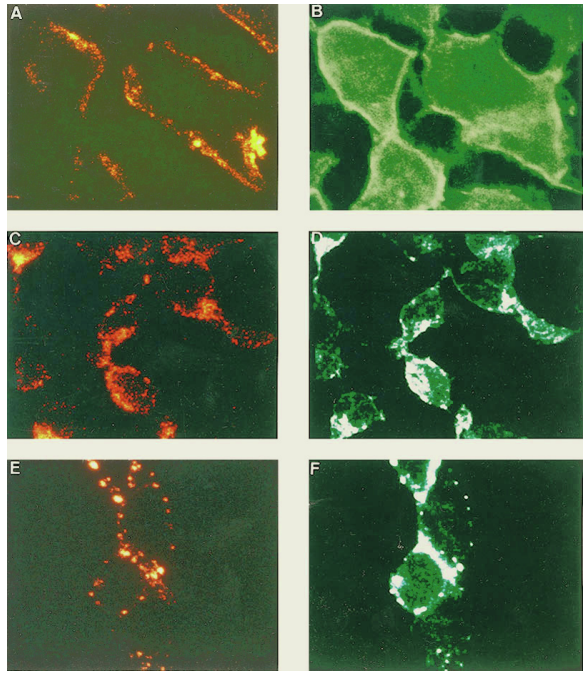

FIG. 3. **Colocalization of epitope-tagged β_2AR with βARK in internalization vesicles.** HEK-293 cells stably transfected with epitope-tagged β_2AR and βARK1 were incubated for 10 min at 37 °C in the absence of agonists (*A, B*) or challenged with different concentrations of isoproterenol (0.1 μM for 5 min in *C* and *D*, 10 μM for 10 min in *E* and *F*). After fixation and permeabilization, receptors were localized by immunoflourescence using M1 monoclonal anti-FLAG antibody detected with Texas Red-conjugated anti-mouse antibody (*A, C*, and *E*) and βARK distribution assessed with the specific polyclonal AbFP1 antibody visualized with fluorescein-conjugated anti-rabbit antibody (*B, D*, and *F*). Samples were analyzed using confocal microscopy as detailed under "Experimental Procedures." The cells shown are representative of large number of specimens from independent experiments examined at various planes of focus.

internalized β_2AR in the agonist-treated cells (Fig. 2, *B* and *C*). Fraction 24 (showing most of the internalized receptors) contains 1.88 ± 0.23-fold more βARK protein than the average of fractions 22–25 in the isoproterenol-treated cells, compared with 0.98 ± 0.07 in fraction 24 of control cells (average ± S.E. of three experiments, $p < 0.05$). Consistent with an agonist-dependent redistribution of βARK, a decrease in the proportion of kinase associated to plasma membrane fractions is noted (Fig. 2*C, small arrow*). These results suggested that βARK may colocalize with receptors during the internalization process. Both β_2AR and GRK2 levels in fraction 24 have been estimated to increase in treated cells in the range of 10–20 pmol (data not shown). Unfortunately, a more detailed quantitative analysis of the stoichiometry of β_2AR and GRK2 in internalized vesicles is not possible using this experimental approach.

To further study the changes in subcellular distribution taking place upon ligand binding, we performed double immunofluorescence confocal microscopy studies. Cells were transfected with epitope-tagged β_2AR and βARK, so the localization of both proteins can be analyzed in the same samples by using monoclonal antibodies that recognize the receptor tag and specific polyclonal antibodies raised against βARK (25) coupled to different chromophores. Fig. 3*A* shows that in control conditions the receptor is located in the plasma membrane whereas βARK (Fig. 3*B*) displays a diffuse cytoplasmic localization as well as a plasma membrane staining. The plasma membrane localization of βARK can be detected even in the absence of agonists in cells overexpressing β_2AR, probably as a consequence of the basal activity of receptors[2]; the same effect was observed for β-arrestin localization in similar experimental conditions (31). After agonist stimulation (0.1 or 10 μM isoproterenol for *C* and *D* and *E* and *F* in Fig. 3, respectively), the β_2AR distribution is markedly and gradually changed (Fig. 3, *C* and *E*). A similar punctate pattern, intracellular structures or vesicles, can be observed for βARK, with extensive colocalization with the receptor (Fig. 3, *D* and *F*). These data were further confirmed by image merging (not shown). Similar results were obtained in transiently transfected cells using either confocal or conventional double labeling immunoflourescence microscopy (not shown). It should be noted that sequestered receptors colocalize with transferrin receptors in endocytic vesicles (not shown), in agreement with previous reports investigating β_2AR internalization (10, 11). It is worth noting that immunoflourescence studies appear to show a more clear and extensive colocalization of internalized receptor and kinase than anticipated by the gradient fractionation data. This could be ascribed to a better preservation in the former experimental approach of GRK2 association to endosomal vesicles, which could be partially lost during cell lysis and fractionation procedures given the peripheral nature of kinase association to membranes (18, 25), as well as to the favored visualization of

[2] A. Ruiz-Gómez and F. Mayor, Jr., manuscript in preparation.

structures displaying concentrated antigens (either β_2AR or βARK) by the indirect immunofluorescence technique. The marked change in βARK subcellular distribution does not appear to be a consequence of signaling pathways downstream receptor activation, since treatment of cells transfected only with the kinase with forskolin or aluminum fluoride does not promote any apparent changes in the βARK localization pattern (data not shown). The presence of β_2AR and βARK in intracellular vesicles is not detected when agonist treatment is performed at low temperature, in line with previous observations (Ref. 10 and data not shown). It is also worth noting that the colocalization with the β_2AR during the sequestration process is not extended to other proteins involved in signal transduction, since receptor-activated $G\alpha_s$ does not colocalize with β_2AR in endosomes (32).

Taken together, our results confirm that βARK expression levels can modulate the extent of receptor sequestration at certain agonist concentrations and, more importantly, show that βARK does not only translocates to the plasma membrane upon receptor activation, but colocalizes with β_2AR during receptor internalization. Previous experiments have indicated that β_2AR and other GPCRs are internalized via the clathrin-coated vesicle-mediated endocytic pathway. Sequestered receptors have been shown to colocalize with endosomal markers, such as transferrin, rab 5, or clathrin (10–12, 31), and dynamin is essential for β_2AR (but not angiotensin AT_{1A} receptor) internalization (16). Very recent reports have focused on the role of the uncoupling protein β-arrestin in this process. Overexpression of β-arrestin rescues the sequestration of internalization-defective β_2AR mutants, and dominant negative arrestins inhibit wild-type β_2AR sequestration (15, 16), thus suggesting that these uncoupling proteins would act as adaptor molecules by helping to target GPCRs to the endocytic machinery. In fact, during the writing process of this manuscript, a report showing an interaction of β-arrestin with clathrin "*in vitro*" and agonist-dependent colocalization of β_2AR, β-arrestin, and clathrin has been published (31). In this context, the fact that the expression of wild-type or dominant negative βARK facilitates or decreases internalization, respectively (this report and Refs. 13, 14, 30, and 33; see above), may be explained by a facilitation of the binding to the phosphorylated receptor of endogenous β-arrestin, which would then directly mediate the internalization process. However, the colocalization of βARK with β_2AR in endocytic vesicles that we report here may suggest that the kinase, in addition to β-arrestin, plays a direct role in receptor sequestration, either by contributing to a correct conformation of the various domains of the receptor involved in sequestration (34, 35) as a consequence of the kinase interaction with cytoplasmic receptor domains other than the phosphorylation sites (reviewed in Ref. 2) or by direct interaction of βARK with as yet unidentified proteins of the endocytic machinery.

Alternatively, or in addition, the presence of βARK in the internalization vesicles may indicate that the endocytic system plays a role in the recycling of the kinase that translocated to the plasma membrane upon receptor stimulation. Such rapid kinase sequestration would be in agreement with the transient nature of its agonist-induced association with the plasma membrane (17–20) and may contribute to the modulation of βARK subcellular distribution (24–26). It is also possible that βARK serves other unknown cellular functions in the internalization vesicles. The recently reported functional relationship between βARK and heterotrimeric G proteins in intracellular organelles (26) and the role of these G proteins in regulating intracellular trafficking is an intriguing possibility in this regard (36 and references therein).

The coexistence of β_2AR and the regulatory proteins βARK and β-arrestin in the same cellular structures during agonist-induced receptor internalization raises important questions to be addressed in future research. Whether βARK (and β-arrestin) are bound (simultaneously or not) to the β_2AR or to other components of the endocytic vesicles (G protein βγ subunits, lipids, etc.) should be investigated. On the other hand, and in line with the recent report by Benovic and colleagues (31), the identification of additional cellular proteins able to interact with βARK and β-arrestin (or receptor-kinase-arrestin complexes), and the characterization of its functional relevance, may help to better understand the internalization pathways of GPCRs and their physiological role in the modulation of cellular responses to messengers.

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