Visualization of Agonist-induced Association and Trafficking of Green Fluorescent Protein-tagged Forms of Both β-Arrestin-1 and the Thyrotropin-releasing Hormone Receptor-1*

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A fusion protein (β-arrestin-1-green fluorescent protein (GFP)) was constructed between β-arrestin-1 and a modified form of the green fluorescent protein from Aequorea victoria. Expression in HEK293 cells allowed immunological detection of an 82-kDa cytosolic polypeptide with antisera to both β-arrestin-1 and GFP. Transient expression of this construct in HEK293 cells stably transfected to express the rat thyrotropin-releasing hormone receptor-1 (TRHR-1) followed by confocal microscopy allowed its visualization evenly distributed throughout the cytoplasm. Addition of thyrotropin-releasing hormone (TRH) caused a profound and rapid redistribution of β-arrestin-1-GFP to the plasma membrane followed by internalization of β-arrestin-1-GFP into distinct, punctate, intracellular vesicles. TRH did not alter the cellular distribution of GFP transiently transfected into these cells nor the distribution of β-arrestin-1-GFP following expression in HEK293 cells lacking the receptor. To detect potential co-localization of the receptor and β-arrestin-1 in response to agonist treatment, β-arrestin-1-GFP was expressed stably in HEK293 cells. A vesicular stomatitis virus (VSV)-tagged TRHR-1 was then introduced transiently. Initially, the two proteins were fully resolved. Short term exposure to TRH resulted in their plasma membrane co-localization, and sustained exposure to TRH resulted in their co-localization in punctate, intracellular vesicles. In contrast, β-arrestin-1-GFP did not relocate or adopt a punctate appearance in cells that did not express VSV-TRHR-1. Reciprocal experiments were performed, with equivalent results, following transient expression of β-arrestin-1 into cells stably expressing VSV-TRHR-1-GFP.

These results demonstrate the capacity of β-arrestin-1-GFP to interact with the rat TRHR-1 and directly visualizes their recruitment from cytoplasm and plasma membrane respectively into overlapping, intracellular vesicles in an agonist-dependent manner.

Many members of the seven transmembrane element, G protein-coupled receptor (GPCR) superfamily undergo rapid desensitization following exposure to agonist ligands (1, 2). In many circumstances, a key contribution to such desensitization is produced by phosphorylation of serine and threonine residues in the carboxyl-terminal tail of the GPCR mediated by members of the family of G protein-coupled receptor kinases (GRKs) (3, 4). Such phosphorylation, although integral to the process, is not the defining aspect that results in interference in the efficiency of interactions between GPCRs and G proteins. This is provided by the binding of members of the arrestin family to the phosphorylated GPCRs (5, 6). This process may also be integral to the internalization of GPCRs via clathrin-coated pits as the nonviral arrestins interact with clathrin (7). Although this process has been widely examined for rhodopsin, the GPCR of the visual system, and the β2-adrenoreceptor, in the majority of other studies, agonist-dependent GPCR phosphorylation following either addition of a recombinant GRK or transient expression of an appropriate cDNA is taken as an end point, and the direct contribution of an arrestin is hypothesized rather than being demonstrated directly.

Thyrotropin-releasing hormone (TRH) is a hypothalamic tripeptide intimately involved in controlling the production of thyrotropin and prolactin from the anterior pituitary (8, 9). At least in the rat, TRH functions via binding to a small group of GPCRs, the first cloned of which (10) is now known as TRH receptor-1 (TRHR-1). By selectively interacting with G13 and G11, this GPCR causes activation of phospholipase Cβ1 and the hydrolysis of phosphatidylinositol 4,5-bisphosphate (11–14). As with many other GPCRs, there has been great interest in mechanisms of regulation of the TRH receptors (15–18). However, although rapid desensitization of responses via TRHR-1 has been shown (15, 16), the contribution of GRKs or arrestins to this process have not been examined in detail.

Barak et al. (19) have recently provided evidence for the interaction of β-arrestin-2 with the β2-adrenoreceptor and the dopamine1A receptor by examining the movement of a β-arrestin-2-GFP fusion construct to the plasma membrane in response to agonist in cells transiently co-transfected with β-arrestin-2-GFP and the appropriate GPCR.

Herein, we expand this strategy following construction of a β-arrestin-1-GFP fusion protein. Transient transfection of this construct into HEK293 cells stably expressing the long isoform of the rat TRHR-1 (20) results in an agonist and time-dependent translocation of β-arrestin-1-GFP to the plasma membrane. Visualization of this process by confocal microscopy further allowed subsequent detection of the internalization of β-arrestin-1-GFP into discrete, intracellular vesicles. To further explore this effect, we examined the movement of β-arrestin-1-GFP fusion constructs following exposure to agonist, and we confirmed these findings using a β-arrestin-1-GFP fusion construct in HEK293 cells transiently co-expressing the rat TRHR-1.
plore potential co-localization of the GPCR and β-arrestin-1 in response to agonist stimulation, HEK293 clones stably expressing either β-arrestin-1-GFP or TRHR-1-GFP were generated. Transient introduction of either β-arrestin-1 or an epitope-tagged form of TRHR-1 into appropriate cell lines allowed demonstration of TRH-induced co-localization of β-arrestin-1 and TRHR-1 in intracellular vesicles only in cells that had become dually transfected with a GPCR and an arrestin construct.

EXPERIMENTAL PROCEDURES

Materials—All materials for tissue culture were supplied by Life Technologies, Inc. Oligonucleotides were purchased from Cruachem Ltd. (Glasgow, United Kingdom).

Construction of the β-Arrestin-1-GFP Expression Construct—Production and subcloning of the β-arrestin-1-GFP fusion protein was performed in two separate steps. In the first step the coding sequence of a modified form of GFP (21) was altered by PCR amplification. Using the amino-terminal primer 5′-CCGTCGAGATAGAAAGGAGAAGAATTTTCAC-3′, an XhoI restriction site (underlined) was introduced to the sequence of codon 2 of GFP. The ATG initiator codon was removed. Using the carboxyl-terminal primer 5′-TGGCTAGATTTTGATGATGTTCATCCATGCC-3′, an XhoI restriction (underlined) and a partial Kozak site were introduced in front of the initiator Met of β-arrestin-1. Using the carboxyl-terminal primer 5′-AACCTGAGTCTTGTGGTGAAGGACACGAC-3′, an XhoI restriction site (underlined) was introduced just in front of the stop codon of β-arrestin-1. Finally, the GFP construct in pcDNA3 was digested with XhoI and HindIII and was ligated together with the PCR product of β-arrestin-1 amplification, which was digested with HindIII and XhoI. The open reading frame so produced represents the coding sequence of β-arrestin-1-GFP. This construct was fully sequenced prior to its expression and analysis. Functionality of the β-arrestin-1-GFP was characterized by binding experiments with rhodopsin (see below).

Transient and Stable Transfection of HEK293 Cells—HEK293 cells and clone E2M1 (14) cells were maintained in minimum essential medium (Sigma), supplemented with 0.292 g/liter L-glutamine, and 10% fetal bovine serum. Clones were seeded into 12-well plates (Nunc) at a density of 3 × 10⁵ cells per well 24 h before transfection. Transfection was performed using Lipo-2000 (Invitrogen) digested with XhoI (Invitrogen) (23) using bovine serum albumin as standard. Transfection was performed according to the method of Bensadoun and Weinstein (24). All protein samples were solubilized prior to gel loading by heating to 95 °C for 5 min in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 50 mM dithiothreitol, 10% (w/v) sucrose, 0.002% (w/v) bromphenol blue). Proteins were separated by electrophoresis on vertical slab gels (12 cm × 14 cm × 1.5 mm) containing a fixed percentage of polyacrylamide (10% (w/v)) using the buffer system of Laemmli (25). Electrophoresis was carried out at either 40 mA per gel constant current for 3 to 4 h, or 7.5 mA per gel constant current overnight in Tris-glycine buffer comprising 25 mM Tris-HCl, pH 8.9, 0.192 M glycine, and 0.1% (w/v) SDS. SDS-PAGE-separated polypeptides were electroblotted to polyvinylidene fluoride (NECNEN Science Products) according to the manufacturer’s instructions at 140 mA constant current for 90 min at 25 °C using an Amersham Pharmacia Biotech semidry electrophoretic transfer unit. Transferred proteins were visualized by staining the membrane in 0.1% (w/v) Ponceau S, 3% (w/v) trichloroacetic acid for 30 s to 3 min and destaining with deionized water. After removing the latter solutions, the blots were rinsed in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄) containing 0.3% (v/v) Tween-20 (PBS-T), and 5% (w/v) milk powder (Marvel) for 1 h in order to block binding sites on the membrane. Blots were incubated with the primary antibody (1:2000 to 1:10,000) for 1 h. Following incubation with primary antibody the blot was washed six times for 10 min each in PBS-T. The blots were subsequently incubated for 1 h with either horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (1:1,000 dilution) and then washed for 6 × 10 min with PBS-T. Cross-reacting polypeptides were detected by the enhanced chemiluminescent system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Confocal Laser Scanning Microscopy—Cells were observed using a laser scanning confocal microscope (Zeiss Axiovert 100) using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective, pinhole of 35, and electronic zoom 1 or 3. The GFP was exited using a 488 nm argon/krypton laser and detected with a 515–540 nm band pass filter. The images were manipulated with Zeiss LSM or MetaMorph software. Two different protocols for preparation of cells were used. When examining the time course of internalization, short time exposures to TRH were used. Cells were grown on glass coverslips and mounted on the imaging chamber. Cells were maintained in KRH buffer, and temperature was maintained at 37 °C. In other studies, fixed cells were used. Cells on glass coverslips were washed with PBS and fixed for 20 min at room temperature using 4% paraformaldehyde in PBS/5% sucrose, pH 7.2. After one wash with PBS, coverslips were mounted on microscope slides with 4% paraformaldehyde in PBS.

Immunofluorescence Studies—After relevant TRH treatments, cells were prepared and fixed. If required, cell membranes were permeabilized with 0.4% (w/v) Triton X-100 in PBS for 3 min at room temperature and washed three times with PBS containing 0.1% goat serum and 0.2% gelatin (PBSGG) for 5 min at room temperature and then three times in PBS for 5 min at room temperature. Primary antibodies; anti-β-arrestin-1 (Transduction Laboratories) or anti-VSV (Roche Molecular Biochemicals) was diluted to a final concentration of 1:400 (1–4 µg/ml) in PBSGG and added to the coverslips for 1 h at room temperature. Coverslips were subsequently washed three times with PBSGG for 5 min and then three times in PBS for 5 min. An Alexa™ 594-labeled goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) diluted 1:200 was added to the coverslips for 1 h at room temperature. Coverslips were then washed with PBSGG and PBS as above and mounted onto microscope slides with 40% glycerol in PBS. Microscope slides were stored in the dark prior to confocal microscopic analysis. The Alexa™ 594 label was excited using a 543 nm argon/krypton laser and detected with a 590 nm long pass filter. Appropriate controls were routinely performed to exclude bleed-through from either red or green signals, potentially contributing to identified overlap of the signals.

β-Arrestin Binding to Rhodopsin—Bovine β-arrestin-1 and β-arrestin-1-GFP binding assays to either light exposed or dark adapted rhodopsin were performed according to the method described in Söhlemann et al. (26).

RESULTS

A PCR-based strategy was used to ligate together a cDNA encoding β-arrestin-1 and a humanized and thermostabilized form of the green fluorescent protein from Aequorea victoria with enhanced fluorescence characteristics (21) to generate β-arrestin-1-GFP (Fig. 1). Transient expression of wild-type β-arrestin-1-GFP or the segregated GFP was achieved in HEK293 cells following transfection with the appropriate cDNA species. Homogenization of the cells followed by a crude separation into particulate and supernatant fractions, resolution by SDS-PAGE, and immunoblotting with an antibody to GFP resulted in detection of the 27-kDa GFP as a cytosolic protein (Fig. 2). A polypeptide of some 82 kDa was detected in the supernatant...
fraction of cells transfected to express β-arrestin-1-GFP with the anti-GFP antibody. Such a protein was not detected in the cells transfected with the GFP cDNA. A polypeptide of the same size was also detected in the soluble fraction only of cells transiently transfected with β-arrestin-1-GFP by immunoblotting with an antibody to β-arrestin-1 (Fig. 2).

The functionality of β-arrestin-1-GFP compared with native β-arrestin-1 was tested by the capacity of both proteins to interact with rhodopsin. Both polypeptides interacted with rhodopsin in a manner that was entirely dependent upon light (Fig. 3).

We have previously established clone E2M11, which stably expresses the long isoform of the rat TRHR-1 and elevated levels of its cognate G protein G_s, by consecutive transfections of HEK293 cells with cDNAs encoding the receptor and murine G_s (14). Transient transfection of these cells with the GFP cDNA resulted in visualization of GFP in confocal microscopy as an evenly distributed cytoplasmic fluorescence. The distribution of GFP was not altered by addition of TRH (10 μM) to the cells for various time periods between 1 and 30 min (data not shown). Transient transfection of the β-arrestin-1-GFP cDNA into clone E2M11 cells also resulted in a uniform cytoplasmic distribution of fluorescence monitored in the confocal microscope (Fig. 4A). However, following addition of TRH (10 μM), real time visualization of β-arrestin-1-GFP allowed detection of the transfer of a substantial fraction of the cellular fluorescence to the plasma membrane within 10 min (Fig. 4B). In many cells, this bulk transfer of β-arrestin-1-GFP cDNA to the plasma membrane could be observed as rapidly (within 30 s) as measurement could be initiated (compare the cells in the center of the field of view in Fig. 5, a and b). At early time points, the plasma membrane distribution of the recruited β-arrestin-1-GFP was essentially homogenous, as we have previously noted for the distribution of a TRHR-1-GFP fusion protein stably expressed in HEK293 cells (27). Transfer of β-arrestin-1-GFP to the plasma membrane was more pronounced at time points between 0.5 and 2 min, and within this time frame, distinct, punctate foci of β-arrestin-1-GFP began to appear at sites around the plasma membrane (Fig. 5C) in many of the cells. At time points beyond 10 min, clear intracellular, punctate spots of β-arrestin-1-GFP were visible in many cells. This pattern was maintained over the longest time point monitored (30 min) (Fig. 5D).

Because we were unable to concurrently monitor the distribution of both β-arrestin-1-GFP and TRHR-1 in this system, β-arrestin-1-GFP was stably expressed in HEK293 cells, and a number of distinct clones were isolated. Expression of β-arrestin-1-GFP could be monitored in the confocal microscope in all of the cells as evenly distributed cytoplasmic fluorescence (Fig. 6A). These cells were transiently transfected with an amino-terminally VSV-epitope tagged form of TRHR-1, which we have previously constructed and characterized (27). Cell fixation with permeabilization and immunostaining with an anti-VSV antibody was followed by an Alexa™ 594-labeled goat, antimouse secondary antibody permitted detection of cells expressing VSV-TRHR-1 (Fig. 6F). As with many transient transfections of cDNA species encoding GPCRs, not all the expressed protein was effectively delivered to the plasma membrane. Clear evidence for appropriate plasma membrane delivery of a fraction of VSV-TRHR-1 in such transient transfections was obtained by fixing the cells without permeabilization followed by immunocytochemical detection with the anti-VSV antibody. Now only the plasma membrane located GPCR could be recorded (see later). Merging of the red and green signals in Fig. 6, A and B, indicated a lack of co-localization of VSV-TRHR-1 and β-arrestin-1-GFP (Fig. 6C). Following addition of TRH for 30 min, the cells expressing VSV-TRHR-1 showed distinct intracellular punctate relocalization of the GPCR (Fig. 6E). Punctate, intracellular bright patches of β-arrestin-1-GFP fluorescence could also be observed (Fig. 6D). These were only present in the cells that were immunopositive for the presence of VSV-TRHR-1 (Fig. 6E), and merging of the signals from the two proteins indicated a high degree of co-localization (Fig. 6F). To ascertain that such co-localization was seen routinely in response to TRH, the same experimental protocol was repeated with three distinct clones stably expressing β-arrestin-1-GFP. Equivalent results were obtained in all cases (data not shown).

We then assessed whether short term treatment with TRH of β-arrestin-1-GFP-expressing cells that had been transiently transfected with VSV-TRHR-1 would result in plasma membrane co-localization of the two proteins. Cells treated with or without TRH for between 20 s and 5 min were examined following cell fixation without permeabilization. With or without TRH treatment, over these time periods, transiently expressing VSV-TRHR-1 were detected by the presence of red plasma membrane defined immunoreactivity following immunocytochemistry with the anti-VSV antibody (Fig. 7, B and E). Without treatment with TRH, VSV-TRHR-1 and β-arrestin-1-GFP were clearly separated (Fig. 7, A and C). Following addition of TRH for 30 min, the cells expressing VSV-TRHR-1 showed distinct intracellular punctate relocalization of the GPCR (Fig. 7E). Punctate, intracellular bright patches of β-arrestin-1-GFP fluorescence could be observed (Fig. 7D). This occurred only in cells expressing VSV-TRHR-1 and merging of the red and green signals indicated obvious overlap of distribution of VSV-TRHR-1 and β-arrestin-1-GFP (Fig. 7F).

To examine whether equivalent co-localization could be achieved by reversal of the above strategy, a clone stably expressing VSV-TRHR-1 with GFP linked to the carboxyl-terminal tail of the GPCR was established (27). VSV-TRHR-1-GFP was predominantly plasma membrane targeted in naïve cells (27) (Fig. 8A). This clone was transiently transfected with cDNA encoding β-arrestin-1 and, following fixation and cell permeabilization, stained with an anti-β-arrestin-1 antibody followed by an Alexa™ 594-labeled goat anti-mouse secondary antibody. Cells successfully transfected with β-arrestin-1 displayed an essentially homogenous cytoplasmic distribution of the protein (Fig. 8B) that was entirely distinct from the distribution of VSV-TRHR-1-GFP (Fig. 8C). Addition of TRH for 30 min resulted in the internalization of most of the VSV-TRHR-1-GFP (Fig. 8D), as we have reported previously (27), even in

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**Fig. 1. Construction of β-arrestin-1-GFP.** A cDNA species encoding β-arrestin-1 was amplified by PCR to incorporate a 5′ HindIII restriction site and a 3′ XhoI site with elimination of the stop codon. An equivalent PCR generated a form of GFP with a 5′ XhoI restriction site and a 3′ XhoI site. Ligation of these species produced β-arrestin-1-GFP, which was then ligated into the expression vector pcDNA3 digested with HindIII/XhoI.
cells that were not immunostained positive for β-arrestin-1. Although the bulk movement of the β-arrestin-1 population in immunopositive cells was not as profound and complete as in cells of clone E2M11, which express very high levels of TRHR-1 (14), intracellular, vesicular clustering of a proportion of the expressed β-arrestin-1 was observed (Fig. 8E). Merging of the signals again confirmed co-localization of VSV-TRHR-1-GFP in vesicles occupied by β-arrestin-1 immunoreactivity (Fig. 8F).

DISCUSSION

Modified forms of the GFP from A. victoria with enhanced fluorescence characteristics have become widely used to monitor gene expression (28–30) and in recent times have started to be applied to analysis of the dynamics and kinetics of regulation of the polypeptides of signal transduction cascades in single, intact cells (19, 29). For each of the β2-adrenoreceptor (31–32), the cholecystokinin type A receptor (33), and the TRHR-1 (27, 34), details of agonist-induced internalization have been elucidated following expression of these proteins with forms of GFP attached to their carboxyl terminus. Very recently, Barak et al. (19) have extended this idea to examine the cellular redistribution of a β-arrestin-2-GFP construct from cytosol to plasma membrane in an agonist-dependent manner following transient co-expression of β-arrestin-2-GFP and a range of GPCRs. As members of the arrestin family bind to GPCRs that have been phosphorylated by one or more members of the GRK family as part of the phenomenon of agonist-dependent homologous desensitization, the studies by
Barak et al. (19) presumably require both agonist occupancy of the GPCR and its subsequent phosphorylation. Support for this concept was derived from studies that demonstrated enhanced movement of the β-arrestin-2-GFP construct following co-expression with GRK2 (19).

In the current study, we extended this approach to using a GFP fusion protein with β-arrestin-1 and to introducing this construct into a cell stably expressing high levels of the long isoform of the rat TRHR-1 (14). Transient introduction of β-arrestin-1-GFP and monitoring of its intrinsic fluorescence in single cells and patches of cells grown on coverslips indicated the protein to be uniformly distributed within the cytoplasmic space, to be excluded from the nucleus, and to show no obvious plasma membrane concentration. (Fig. 4a). Simple partitioning of cells expressing the construct after homogenization into particulate and supernatant fractions confirmed the protein to be

**Fig. 6. Co-localization of stably expressed β-arrestin-1-GFP and transiently introduced VSV-TRHR-1 following exposure to TRH.** A clone of HEK293 cells stably expressing β-arrestin-1-GFP was monitored in the confocal microscope (green) (A). Following transient expression with VSV-TRHR-1 and GRK2 the cells were fixed with permeabilization. Distribution of the GPCR was monitored immunologically (red) (B). Merging of the signals indicated the two proteins to be differentially localized (C). Equivalent cell fields were visualized following 30 min exposure to TRH (10 μM) (D–F). Co-localization of the two proteins (yellow) (F) was observed in punctate intracellular vesicles. Vesicular concentration of β-arrestin-1-GFP in response to TRH (D) was only observed in cells expressing VSV-TRHR-1 (E).

**Fig. 7. Short term exposure of cells co-expressing β-arrestin-1-GFP and VSV-TRHR-1 to TRH results in their co-localization at the plasma membrane.** A clone of HEK293 cells stably expressing β-arrestin-1-GFP was monitored in the confocal microscope (green) (A) as in Fig. 6. Following transient expression with VSV-TRHR-1 and GRK2, the cells were fixed without permeabilization. The expression of plasma membrane located GPCR was monitored immunologically in a fraction of cells in the field (red) (B). Merging of the signals indicated no overlap of distribution of the two proteins (C). Addition of TRH (10 μM) for 1 min resulted in the maintenance of plasma membrane VSV-TRHR-1 (E), a movement of significant levels of β-arrestin-1-GFP to the plasma membrane (D), and the overlap of distribution of the two at the plasma membrane (F).
Interactions of TRHR-1 and β-Arrestin 1

Fig. 8. Co-localization of stably expressed VSV-TRHR-1-GFP and transiently introduced β-arrestin-1 following exposure to TRH. A clone of HEK293 cells stably expressing VSV-TRHR-1-GFP was monitored in the confocal microscope (green) (A). Following transient expression with β-arrestin-1 and GRK2, the cells were fixed with permeabilization. Distribution of the arrestin as a cytoplasmic protein was monitored immunologically (red) (B). Merging of the signals indicated the two proteins to be differentially localized (C). Equivalent cell fields were visualized following 30 min of exposure to TRH (10 μM) (D–F). Co-localization of the two proteins (yellow) (F) was observed in punctate intracellular vesicles. Internalization of VSV-TRHR-1-GFP was observed in all cells in the field (D), but co-localization with β-arrestin-1 (yellow) (F) was only observed in cells that were doubly transfected. Not all of the expressed β-arrestin-1 was transferred to intracellular vesicles containing VSV-TRHR-1-GFP; some remained in the cytoplasm of cells (E and F). This is presumably a reflection of the relative levels of expression of VSV-TRHR-1-GFP and β-arrestin-1 in these cells.

Fig. 9. Model of agonist-induced subcellular trafficking of β-arrestin-1-GFP. In the absence of agonist, β-arrestin-1-GFP is evenly distributed throughout the cytoplasm. Following agonist occupation, hydroxyamino acids in the carboxyl-terminal tail of the receptor become phosphorylated by one or more members of the family of GRKs. This results in attraction of β-arrestin-1-GFP to the phosphorylated receptor and thus the translocation to the membrane observed in Figs. 4b and 7. As arrestins can interact with clathrin, redistribution of the receptor-β-arrestin-1-GFP complex occurs to punctate regions of the plasma membrane (Fig. 5), b and c) with subsequent internalization of the complex (Figs. 5d, 6, and 8).

cytosolic (Fig. 2). Addition of TRH produced a substantive redistribution of β-arrestin-1-GFP from the cytosol to the plasma membrane, which could be monitored in intact cells and in real time. Even without co-expression of excess GRK, this effect was dramatic (Fig. 4b). However, the co-expression of GRK2 appeared to shorten the kinetics of agonist-induced translocation of β-arrestin-1-GFP (data not shown).

The basis for the translocation of β-arrestin-1-GFP to the plasma membrane remains unclear, as in many cells (see Figs. 4 and 5) virtually the entire cellular content of β-arrestin-1-GFP was mobilized and redistributed. As noted also by Barak et al. (19) for β-arrestin-2-GFP, there did not appear to be a specific pool(s) of β-arrestin-1-GFP, closely apposed to the membrane, for example, which responded upon agonist addi-
tion. However, it is worth noting that the degree of effect varied between cells, even between those imaged in the same field during the same time period (see Fig. 5 for example). Two obvious possibilities may underlie such observations. Although clone E2M11 cells are a stable clone expressing TRHR-1, it would not be unusual for individual cells within the population to express the GPCR to differing levels. Secondly, to see the most effective bulk transfer of β-arrestin-1-GFP to the membrane in response to TRH, the cells transiently transfected with this construct must express it to sufficient levels to have high enough fluorescence for effective monitoring but not to such high levels that only a small fraction of the expressed protein is attracted/redistributed to the membrane. Each of these aspects can be observed in the field of cells visualized over the time course displayed in Fig. 5.

Following stable expression of a TRHR-1-GFP construct in HEK293 cells, we have noted that in the absence of agonist, the plasma membrane distribution of this protein is entirely even around the plasma membrane (27). As such, it was not surprising that upon addition of TRH to clone E2M11, cells transiently transfected to express β-arrestin-1-GFP the protein moved to the plasma membrane with an equally homogeneous distribution, at least over short time spans (Figs. 4 and 5). Using the stably expressed TRHR-1-GFP construct we have previously examined the ability of TRH to cause internalization of the GPCR (27). We have noted a rapid agonist-induced internalization that proceeds via concentration into clathrin-coated vesicles. Following transfer of β-arrestin-1-GFP to the plasma membrane in these studies further examination of this process demonstrated the appearance of distinct plasma membrane-delineated, punctate spots of β-arrestin-1-GFP fluorescence (Fig. 5). Subsequently, internalized, punctate spots of β-arrestin-1-GFP were observed (Fig. 5). These could only be observed in cells in which virtually all of the expressed β-arrestin-1-GFP had previously been translocated to the plasma membrane, because in other cells, such internalization would be masked by the fluorescence from the remaining cytosolic construct.

Although the data of Fig. 5 represent the first clear visualization of a β-arrestin becoming internalized in response to agonist activation of a GPCR, transient introduction of β-ar-
restin-1-GFP into clone E2M11 cells did not allow direct monitoring of potential co-internalization of β-arrestin-1-GFP and TRHR-1. Schematic representations of the internalization of GPCRs via clathrin-coated vesicles usually indicate the co-internalization of the arrestin (Fig. 9), presumably owing to the known capacity of these proteins to interact with clathrin (6, 7, 35). To address this issue directly we generated clones of HEK293 cells stably expressing β-arrestin-1-GFP. A VSV-epitope tagged form of TRHR-1, which we have previously shown to bind TRH normally and to effectively cause activation of phosphoinositidase C in response to TRH (27), was subsequently transiently introduced. The green autofluorescence corresponding to β-arrestin-1-GFP was evenly distributed in the cytoplasm of all cells of the clone, and immunological detection of VSV-TRHR-1 allowed identification of individual cells in a field that were expressing the GPCR. The distribution of these proteins did not overlap in unstimulated cells (Fig. 6). However, following exposure to TRH (10 μM) for 30 min, both the GPCR and a significant amount of β-arrestin-1-GFP became internalized into punctate, intracellular vesicles. Merging of the signals indicated the location of these vesicles to overlap strongly. Interestingly, even without immunological detection of the GPCR, it was clear that intracellular punctate concentration of β-arrestin-1-GFP occurred in response to TRH only in a fraction of the cells (Fig. 6D). Immunolocalization of VSV-TRHR-1 (Fig. 6E) confirmed that these were the cells that were expressing the GPCR. Such studies provided the first direct evidence for co-internalization of a GPCR and β-arrestin-1-GFP.

To extend this analysis, we generated a clone of HEK293 cells stably expressing a carboxyl-terminally GFP-tagged form of VSV-TRHR-1 (27) and transiently introduced VSV-TRHR-1 (Fig. 6) into these cells. Now autofluorescent detection of the GPCR construct and immunological detection of β-arrestin-1-GFP into clone E2M11 cells did not allow direct monitoring of potential co-internalization of β-arrestin-1-GFP and TRHR-1. However, we have previously shown that β-arrestin-1-GFP does not overlap in unstimulated cells (Fig. 6). But, following exposure to TRH (10 μM) for 30 min, both the GPCR and a significant amount of β-arrestin-1-GFP became internalized into punctate, intracellular vesicles. Merging of the signals indicated the location of these vesicles to overlap strongly. Interestingly, even without immunological detection of the GPCR, it was clear that intracellular punctate concentration of β-arrestin-1-GFP occurred in response to TRH only in a fraction of the cells (Fig. 6D). Immunolocalization of VSV-TRHR-1 (Fig. 6E) confirmed that these were the cells that were expressing the GPCR. Such studies provided the first direct evidence for co-internalization of a GPCR and β-arrestin-1-GFP.