Selective recruitment of arrestin-3 to clathrin coated pits upon stimulation of G protein-coupled receptors

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SUMMARY

Non-visual arrestins (arrestin-2 and arrestin-3) play critical roles in the desensitization and internalization of many G protein-coupled receptors. In vitro experiments have shown that both non-visual arrestins bind with high and approximately comparable affinities to activated, phosphorylated forms of receptors. They also exhibit high affinity binding, again of comparable magnitude, to clathrin. Further, agonist-promoted internalization of many receptors has been found to be stimulated by exogenous over-expression of either arrestin-2 or arrestin-3. The existence of multiple arrestins raises the question whether stimulated receptors are selective for a specific endogenous arrestin under more physiological conditions. Here we address this question in RBL-2H3 cells, a cell line that expresses comparable levels of endogenous arrestin-2 and arrestin-3. When β2-adrenergic receptors are stably expressed in these cells the receptors internalize efficiently following agonist stimulation. However, by immunofluorescence microscopy we determine that only arrestin-3, but not arrestin-2, is rapidly recruited to clathrin coated pits upon receptor stimulation. Similarly, in RBL-2H3 cells that stably express physiological levels of m1AChR, the addition of carbachol selectively induces the localization of arrestin-3, but not arrestin-2, to coated pits. Thus, this work demonstrates coupling of G protein-coupled receptors to a specific non-visual arrestin in an in vivo setting.

Key words: Endocytosis, AP-2, Coated pit, GPCR

INTRODUCTION

Agonist stimulated G protein-coupled receptors (GPCRs) initiate cell responses by modulating the activity of effector molecules via activation of specific G proteins. Following this signaling event, activated GPCRs undergo phosphorylation by specific GPCR kinases (GRKs). This phosphorylation promotes the binding of arrestin molecules to the GPCR, which in turn uncouples the receptor from the G-protein leading to receptor desensitization, a temporary state during which the system becomes refractory to further stimulation (reviewed by Carman and Benovic, 1998; Lefkowitz, 1998). Recently, non-visual arrestins have been implicated in the process of receptor internalization which results in the removal of desensitized GPCRs from the plasma membrane and their sequestration in intracellular vesicular compartments (Ferguson et al., 1996). Many GPCRs are internalized by the cell via clathrin coated pits (Goodman et al., 1996; Zhang et al., 1996). For these GPCRs, non-visual arrestins have been shown to bind GPCR, and the coat proteins clathrin and AP-2 (plasma membrane restricted clathrin assembly protein), and are therefore thought to promote internalization by acting as adaptors to link the receptors with the coated pit (Goodman et al., 1997; Krupnick et al., 1997; Laporte et al., 1999). This internalization step is involved in receptor resensitization and down-regulation (Sterne-Marr and Benovic, 1995; Zhang et al., 1997).

Expression of visual arrestins (arrestin-1 and arrestin-4) are restricted to rod and cone cells, respectively. In contrast, non-visual arrestins (arrestin-2 (β-arrestin) and arrestin-3) are ubiquitously expressed in mammalian tissues, thus raising the question as to whether GPCRs selectively recruit either of the non-visual arrestins. In vitro assays have shown only minor differences in arrestin-2 and arrestin-3 binding affinity to various adrenergic and cholinergic receptors (Gurevich et al., 1995; Wu et al., 1997). Also, when transiently expressed, both arrestins appeared to be capable of mediating β2-adrenergic receptor internalization equally well (Ferguson et al., 1996). Because in vivo experiments aimed at addressing GPCR specificity for non-visual arrestins often require transfecting the cells with exogenous arrestins, the fine physiological regulation of the coupling mechanism may be masked (Barak et al., 1997), or the fate of the GPCR-arrestin complex altered altogether (Zhang et al., 1996).

To avoid the uncertainties of arrestin overexpression, we have surveyed cell lines to identify one that has comparable endogenous expression of both arrestin-2 and arrestin-3. RBL-2H3 cells, a rat mast cell line, fulfill this criterion. We have therefore investigated activation of several GPCRs in these
cells for their ability to induce localization of endogenous arrestins to clathrin coated pits.

MATERIALS AND METHODS

Materials
Reagents were obtained from the following sources: p-nitrophenyl-N-acetyl-β-D-glucosaminide, bovine serum albumin (BSA), (-) isoproterenol (ISO), carbamylcholine (CBC), 5'-N-ethylcarboxamido-adenosine (NECA), and atropine from Sigma Chemical Company (St Louis, MO); antigen, DNP31-BSA, was from Calbiochem, La Jolla, CA; SlowFade from Molecular Probes (Eugene, OR); the Enhanced ChemiLuminescence (ECL) detection kit from Amersham Corp. (Arlington Heights, IL). Monoclonal anti-clathrin and anti-AP-2 antibodies were from American Type Cell Culture (Rockville, MD). Arrestin-2 and arrestin-3 were detected using affinity-purified rabbit antisera KEE (Santini and Keen, 1996), and 182-6 (Gaidarov et al., 1999), respectively. Total arrestins were detected using F4C1 monoclonal antibody (Dua et al., 1992). Fluorescein- or rhodamine-lissamine-conjugated affinity-purified donkey anti-mouse and Cy5- or fluorescein-conjugated affinity-purified donkey anti-rabbit polyclonal antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-conjugated affinity-purified goat anti-rabbit and anti-mouse polyclonal antibodies were purchased from Cappel.

Vectors, cell transfection and cell culture
Rat basophilic leukemia cell 2H3 subline (RBL-2H3) and RBL-2H3(m1) cells stably expressing the m1 muscarinic acetylcholine receptor were kindly supplied by Dr Michael A. Beaven (NIH, Bethesda, MD; Choi et al., 1993; Jones et al., 1991) and were cultured as described (Santini and Keen, 1996). The vector containing the cDNA coding for flag-β2 adrenergic receptor (flag-β2AR) has been described previously (Goodman et al., 1996). Transfection of RBL-2H3 cells was performed by electroporation (Wilson et al., 1995) and selection of transfected cells accomplished by using 1 mg/ml of G418 (Gibco-BRL, Gaithersburg, MD) in the culture medium.

Measurement of secretion
Release of hexosaminidase, a granule marker, was measured as described (Santini and Keen, 1996). As a control for positive A3AR (adenosine A3 receptor) stimulation, we measured NECA augmentation of antigen-induced secretory response (Ali et al., 1990).

Cell stimulation
Experiments were performed in buffer A: 5.6 mM glucose, 120 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 25 mM sodium PIPES (pH 7.2) that contained 0.1% BSA and 1 mM Ca2+. Antigen (DNP31-BSA) stimulation experiments were performed on cells that had been incubated overnight with 0.5 μg/ml of DNP-specific IgE, and then incubated with 20 ng/ml of DNP31-BSA for 3 minutes. β2AR, m1AChR (muscarinic acetylcholine receptor m1) and A3AR were stimulated at 37°C by the addition of either 10 μM ISO, 1 mM CBC or 100 μM NECA, respectively, for various periods of time before processing the cells for immunostaining.

Characterization of m1AChR and β2AR distribution by radioligand binding
Cells grown to ∼70% confluence on T75 tissue culture flasks were treated with serum-free medium containing either vehicle (control), 1 μM ISO, or 1 mM CBC for 0-30 minutes at 37°C. One minute prior to the termination of treatment, the medium was replaced with 2 ml of 0.05% trypsin/0.53 mM EDTA containing the PBS containing tryptic vehicle. Cells were harvested following 1 minute trypsinization, diluted into 20 ml of cold phosphate buffered saline (PBS) containing 0.5 mg/ml soybean trypsin inhibitor, washed 3 times in cold PBS, and resuspended in cold PBS. m1AChR binding was performed similar to that described previously for the m2AChR (Tsuga et al., 1994). For quantitation of cell surface m1AChR density, ∼250,000 cells were incubated in 500 μl PBS containing 1.5 nM [3H]-N-methylisococplamine (NMS) (84.5 Ci/mmol, NEN) ± 1 μM atropine for 4 hours at 4°C. [3H]NMS binding to cells was determined to be saturable and of high affinity (Kd = 0.4 pM) (data not shown). For assessment of total cellular m1AChR, cells were diluted into 1 ml of 10 mM Tris-HCl, pH 7.2, containing 2 mM MgCl2 and incubated with 1 nM [3H]-quinucilidyl benzilate (QNB) (52.3Ci/mmol, NEN) ± 1 μM atropine for 1 hour at 37°C. Cell surface β2AR density was determined as described previously (Goodman et al., 1996) by incubating 450,000 cells in 500 μl PBS containing 10 nM [3H] CGP-12177 (45 Ci/mmol, Amersham) ± 1 μM alprenolol for 4 hours at 4°C. All binding reactions were terminated by washing five times with 4 ml aliquots of ice-cold 10 mM Tris-HCl, pH 7.2, containing 2 mM MgCl2, and filtration through Whatman GF/C filters using a Brandel Cell Harvester.

Immunofluorescence and confocal microscopy
Transfected RBL-2H3 cells grown overnight on coverslips were stimulated with ISO, CBC or vehicle for the specified period of time, and then fixed with 3.7% (w/v) formaldehyde in PBS and processed for immunostaining as previously described (Goodman et al., 1996; Santini and Keen, 2000). Clathrin was detected using X-22 (30 μg/ml), AP-2 with AP.6 (20 μg/ml), arrestin-3 with 182-6 (1:100), and arrestin-2 using KEE (1:100). Specificity of the arrestin antibody was confirmed by abolition of the signal upon preabsorption of each antibody with a GST-fusion protein containing the arrestin peptide sequence used as immunogen (data not shown). The primary
antibodies were detected with Cy5 anti-rabbit and fluorescein anti-mouse or fluorescein anti-rabbit and rhodamine-lissamine anti-mouse tagged second antibodies (1:100). Conventional fluorescence microscopy was performed on a Zeiss Axiolicht S100 TV microscope with Zeiss Plan-Neofluar ×100 1.40 NA oil immersion lens and images captured using a CCD camera (Quantix) containing a Kodak 1400 chip (Photometrics) and processed on a Macintosh running IP Lab Scientific Imaging software (Scanalytics, Fairfax, VA). Confocal analysis was performed on a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad, Eugene, CA) running CoMos™ 7.0a software and interfaced to a Zeiss Axiolicht 100 microscope with Zeiss Plan-Apo ×63 1.40 NA oil immersion objective. Only samples dual labeled with Cy5 and fluorescein were subject to confocal microscopy analysis. All dual-labeled samples were analyzed using sequential excitation at 488 nm and 647 nm and images collected using the photon counting mode. Fluorescein single label specimens were used as control for potential signal crossover; no significant crossover of the fluorescein signal into the Cy5 channel was detected using the data acquisition settings employed in our experiments. Images were processed using Adobe Photoshop.

**Gel electrophoresis and immunoblotting**

To study the level of expression of arrestin-2 and arrestin-3, cells were lysed in boiling Laemmli buffer and solubilized proteins were separated by electrophoresis on 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. To determine the distribution of arrestin-2 and arrestin-3 between Triton-soluble and Triton-insoluble pools, RBL-2H3(m1) cells were solubilized in 100 mM Na-Mes buffer, pH 6.5, containing 0.2% [v/v] Triton X-100, 0.5 mM magnesium chloride, 1 mM EGTA, 50 μM phenylarsine oxide and protease inhibitors (33 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and lightly homogenized in microcentrifuge tubes with a mini pestle. After 10 minutes on ice, cells were pelleted for 10 minutes at 75,000 rpm in a TLA 100.1 rotor using a Beckman Optima TLX ultracentrifuge at 4°C. Laemmli buffer was added to the supernatant and to the pellets. After boiling, each sample was fractionated by 10% SDS-PAGE and proteins were transferred onto nitrocellulose membranes. Total arrestin, arrestin-2 and arrestin-3 were detected using mouse monoclonal F4C1 (1:2000) and rabbit immunosera KEE (1:2000), and 182-6 (1:2000), respectively. Horseradish peroxidase-conjugated secondary antibodies were visualized with the ECL detection system. To generate the data presented in Table 1, bands were quantitated using a Personal Densitometer (Molecular Dynamics). The resultant intensities were then expressed relative to the intensity of endogenous arrestin-2 present in COS-1 cells, the latter set equal to 10.

**RESULTS AND DISCUSSION**

**Arrestin-2 and arrestin-3 are coexpressed in RBL-2H3 cells**

To evaluate the differential involvement of endogenous arrestin-2 and arrestin-3 in GPCR signaling, we sought a cell system with readily detectable levels of both proteins. In a series of initial experiments we analyzed lysates prepared from different cell lines by immunoblotting using a pan-arrestin antibody (F4C1). We focused our study on the rat RBL-2H3 cell line, a mast cell homolog, because it exhibited the highest expression of both forms of arrestins at comparable levels (Table 1).

Western blot analysis of RBL lysate (Fig. 1A) showed that the F4C1 monoclonal antibody recognized two bands. The upper band was identified as arrestin-2 by virtue of being immunoreactive with a rabbit antiserum specific for a C-

**Table 1. Relative levels of arrestin-2 and arrestin-3 in various mammalian cell lines**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Arrestin-2</th>
<th>Arrestin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>BHK-21</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>CHO</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>CHRF</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>COS-1</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>DDT1-MF</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>H9C2</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>HEK293</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>HL 60</td>
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</tr>
<tr>
<td>Jurkat</td>
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<td>15</td>
</tr>
<tr>
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<tr>
<td>SaOS-2</td>
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<td>1</td>
</tr>
<tr>
<td>SHSY</td>
<td>10</td>
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</table>

Lysates prepared from different cell lines were analyzed by immunoblotting using F4C1, a pan-arrestin monoclonal antibody followed by densitometric analysis as described in Materials and Methods. Values are relative to the intensity of arrestin-2 levels in COS-1 cell, which was arbitrarily set equal to 10. ND, not detected.

**Fig. 2. Mobilization of endogenous arrestins induced by isoproterenol in RBL-2H3(β2) cells.** Comparison of the distribution of arrestin-3 in unstimulated (Unst.) and isoproterenol-treated (+ISO) cells. Agonist treatment induces substantial mobilization and coincidence of arrestin-3 with clathrin coated pits (AP-2). The distribution of arrestin-2, in contrast, is not substantially changed on stimulation. Bar, 10 μm.
terminal 16 amino acid peptide of arrestin-2 (Sterne-Marr et al., 1993). The lower band was identified as arrestin-3 by immunoreactivity with a polyclonal antibody specific for the C-terminal domain (aa 350-409) of arrestin-3 (Gaidarov et al., 1999). Using purified recombinant arrestin-2 as standard we estimate that RBL-2H3 cells contain approximately 0.12 \( \mu g \) of each form of arrestin per mg of total protein.

When cellular proteins were separated into Triton-soluble and -insoluble components and analyzed by immunoblotting, the distribution of arrestin-2 and arrestin-3 appeared to be quite different (Fig. 1B). While arrestin-2 was evenly distributed between the soluble and insoluble pool, arrestin-3 was predominantly associated with the latter. Interestingly, this distribution was not detectably changed in response to stimulation of m1AChRs or A3 ARs (data not shown). Detergent extractability is a criterion often used to differentiate between cytosolic proteins and those that are associated with the cytoskeleton and/or with glycosyl-phosphatidylinositol-rich membrane

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**Fig. 3.** Mobilization of endogenous arrestins induced by carbachol in RBL-2H3(m1) cells. Comparison of the distribution of arrestin-3 in cells unstimulated (Unst.) and in cells treated with carbachol for 3 minutes (+CBC). Agonist treatment induces substantial mobilization and coincidence of arrestin-3 with clathrin coated pits (Clathrin), as indicated by the appearance of yellow spots in the merged image. No substantial changes are observed in the distribution of arrestin-2 upon stimulation. Bar, 10 \( \mu m \).

**Fig. 4.** Arrestin-3 recruitment to clathrin coated pits induced by carbachol is rapid, consistent with m1AChR internalization. The relocalization of arrestin-3 to clathrin coated pits (AP-2) occurs rapidly upon m1AChR stimulation and proceeds with kinetics consistent with its role in mediating m1AChR internalization. Bar, 10 \( \mu m \).
domains which comprise caveolae (Draherova et al., 1996; Field et al., 1995). The different distribution of the two forms of arrestins may reflect their unique compartmentalization. Several proteins participating in the GPCR signaling cascade including certain GPCRs, GRKs and heterotrimeric G-proteins, have been reported to be compartmentalized in cells by their association with detergent-insoluble cellular compartments (Freeman et al., 1998; Okamoto et al., 1998; Pitcher et al., 1998).

**Agonist-induced β2AR stimulation mobilizes endogenous arrestin-3 to clathrin coated pits**

The β2AR is perhaps the best characterized non-visual GPCR. It is known that it couples primarily to Gs to induce activation of adenylyl cyclase in the cell. Recently our laboratory and others have reported that this receptor is internalized via clathrin coated pits (Ferguson et al., 1996; Goodman et al., 1996; Zhang et al., 1996). It is also known that, when transfected into COS cells, agonist-induced β2AR endocytosis is stimulated by the co-transfection of either arrestin-2 or arrestin-3 (Ferguson et al., 1996; Gaidarov et al., 1999; Goodman et al., 1996; Zhang et al., 1996) leaving open the question of whether selective coupling between β2AR and arrestin-2 and/or arrestin-3 exists in vivo.

To address this question by examining the behavior of endogenous arrestins, we stably transfected an epitope tagged β2AR in RBL-2H3 cells (RBL-2H3(β2)) and studied its mobilization upon isoproterenol stimulation. The clone used in this study expressed about 80,000 receptors/cell (data not shown), a number comparable to that of other GPCRs that are endogenously expressed in these cells (e.g. adenosine A3 receptors, see below). When RBL-2H3(β2) cells were challenged with 10 μM isoproterenol, the number of receptors on the cell surface rapidly decreased with t1/2 ≈ 3 minutes (data not shown), consistent with the kinetics of internalization of β2AR observed in COS cells (Goodman et al., 1996).

RBL-2H3(β2) cells were immunofluorescently stained to visualize arrestin-2 and arrestin-3 and to compare their localization with respect to clathrin coated pits. We observed that in unstimulated cells both arrestins were distributed throughout the interior of the cell in the cytoplasm in a predominantly punctate pattern (Fig. 2, Unst., and confocal microscopy analysis, data not shown). The staining patterns for arrestin-2 and arrestin-3 were very similar with both signals being concentrated in puncti. This pattern did not colocalize with the distribution of AP-2 (Fig. 2, AP-2), a clathrin adaptor protein that specifically associates with plasma membrane clathrin coated pits (Robinson, 1987). In addition to this punctate staining both arrestins also displayed a diffuse staining likely representing a soluble pool of the proteins (see above).

However, after 5 minutes of isoproterenol stimulation (Fig. 2, +ISO), a substantial amount of arrestin-3 was recruited to clathrin coated pits (Fig. 2, arrestin-3), as indicated by the coincidence of the arrestin-3 signal with that of AP-2. In contrast, stimulation by isoproterenol under the same conditions failed to induce a detectable mobilization of arrestin-2 to clathrin coated pits (Fig. 2, arrestin-2). Arrestin-2 immunostaining was never observed to colocalize with coated pits, even when cells were monitored for longer periods of time (up to 30 minutes).

**Agonist-induced m1AChR internalization**

The selective interaction of β2AR with arrestin-3 might be dictated by some downstream event initiated by the β2AR or specific to RBL-2H3 cells which preferentially regulates arrestin-3. Indeed, previous studies revealed that arrestin-2 was recruited to the plasma membrane and dephosphorylated in vivo following agonist-stimulation of β2AR and that only dephosphorylated arrestin-2 was associated with clathrin (Lin et al., 1997). However, as noted by the authors, the serine residue phosphorylated (Ser412) in arrestin-2 is lacking in arrestin-3, and is located in a region with very low homology to arrestin-3. Hence, a different mechanism might regulate arrestin-3 adaptor function.

To further investigate the arrestin specificity for GPCR interaction, we studied a subline of RBL that stably expresses the human m1 muscarinic acetylcholinergic receptors (m1AChR), RBL-2H3(m1) cells (Jones et al., 1991). The signaling cascade originating from m1AChR has been extensively characterized in this cell line. The receptor has previously been shown to be functionally coupled to phospholipase C and, in the presence of agonist, to induce IP3-dependent calcium release, protein kinase C activation, and

![Fig. 5. Stimulation of A3 AR does not induce recruitment of either arrestin-3 or arrestin-2 to clathrin coated pits. Following stimulation of A3 AR for 3 minutes, relocalization of arrestin-2 (arr2) or arrestin-3 (arr3) to clathrin coated pits (AP-2) is not observed. Bar, 10 μm.](image-url)
exocytic secretion of granules in these cells (Choi et al., 1993, 1996; Dippel et al., 1996; Jones et al., 1991). Indeed, when the cells were stimulated with carbachol, there was a rapid and robust release of hexosaminidase, a marker for secretory granule content (data not shown). Radioligand binding with $[^3]$H QNB revealed that RBL-2H3(m1) cells express $\approx 49,000$ m1ACh receptors/cell with a $K_d$ of $\approx 40$ pM (not shown). In response to 1 mM carbachol treatment, a time-dependent internalization of cell-surface m1AChR was revealed by the loss of binding sites for the hydrophilic ligand $[^3]$H NMS. The loss of $[^3]$H NMS binding was rapid ($t_{1/2} = 3$ minutes) and declined to $\approx 30\%$ of control (untreated) levels by 15 minutes. Conversely, no loss in the binding of the cell-permeant ligand $[^3]$H QNB occurred within 30 minutes of carbachol treatment. Together, these results suggest that the loss of $[^3]$H NMS binding during this time period reflects sequestration of m1AChR to a compartment(s) inaccessible to the hydrophilic ligand, and is not due to receptor degradation.

**Agonist-induced m1AChR stimulation mobilizes endogenous arrestin-3 to clathrin coated pits**

We next examined arrestin-2 and arrestin-3 distribution in RBL-2H3(m1) cells by immunofluorescence confocal microscopy. In untreated RBL-2H3(m1) cells both arrestins exhibited a predominantly punctate pattern (Fig. 3, Unst.), which did not colocalize with the distribution of clathrin (Fig. 3, merged panel). Within minutes of carbachol stimulation (Fig. 3, +CBC), arrestin-3 appeared to be recruited to clathrin coated pits (Fig. 3, arrestin-3), as indicated by the partial coincidence of arrestin-3 immunostaining with that of clathrin (note yellow complementary color in the merged panel of Fig. 3). Though the majority of arrestin-3 positive dots colocalize with clathrin, a significant proportion do not. Similarly, not all the clathrin-positive dots appear to contain arrestin-3, due at least in part to the fact that clathrin is also found in the trans-Golgi region (Keen et al., 1981) which, in these cells, extends well into the cell periphery (Santini and Keen, 1996), and on other endosomal structures (Stoorvogel et al., 1996). In contrast, stimulation by carbachol under the same conditions failed to induce a detectable mobilization of arrestin-2 to clathrin coated pits. Arrestin-2 immunostaining never colocalized with coated pits, even when cells were monitored for longer periods of time (up to 30 minutes; data not shown), when maximal agonist-induced receptor internalization from the plasma membrane had been attained.

Arrestin-3 mobilization induced by carbachol was further studied at various times after carbachol stimulation by double staining cells with arrestin-3-specific antibodies and anti-AP-2 antibody. As can be seen in Fig. 4, arrestin-3 localization to plasma membrane coated pits is very rapid, being detectable as early as 1 minute after agonist stimulation, consistent with the time course of receptor loss seen by radioligand binding. Five minutes after agonist stimulation arrestin-3 localization in clathrin coated pits is virtually complete, as indicated by the predominant yellow color in the merged panel. Also noticeable at this time is a decrease in the intensity of the arrestin-3 staining associated with the cytoplasm. After 10 minutes the two signals start to separate and more diffuse cytoplasmic staining for arrestin-3 again becomes visible, though m1AChR uptake appears to continue as judged by a plateau level of receptor loss from the cell surface.

Stimulation of the RBL cells’ endogenous IgE receptors by antigen induced cross-linking activates many of the same cellular responses induced by the muscarinic agonist carbachol (Hirasawa et al., 1995; Jones et al., 1991; Santini and Beaven, 1993). Additionally, the IgE receptor is known to be internalized efficiently through clathrin coated pits following activation (Mao et al., 1993; Pfeiffer et al., 1985; Robertson et al., 1986; Seagrave et al., 1991; Stump et al., 1989). Despite these similarities, IgE receptor activation by antigen did not elicit mobilization of either arrestin to clathrin coated pits (data not shown).

Recently, src kinases have been shown to be recruited to activated GPCRs by arrestins, and to be implicated in the assembly at the plasma membrane of a protein scaffold in which early signaling steps and interactions occur (Lefkowitz, 1998; Luttrel et al., 1999a,b). Because src kinases are found associated with coated membranes (Stenberg et al., 1997), and are known to phosphorylate clathrin (Martin-Perez et al., 1989; Mooibroek et al., 1992; Wilde et al., 1999), we tested the effect of the specific src kinase inhibitor PP1 (Hanke et al., 1996) on the recruitment of arrestin-3 to coated pits in RBL following m1AChR activation. While PP1 completely inhibits antigen-induced exocytosis, a response dependent on src-kinase activation (Eiseman and Bolen, 1992; Honda et al., 1997), we found that it had no effect on the ability of the m1ACHr to recruit arrestin-3 to coated pits (data not shown), suggesting independence of recruitment from src kinase activity.

**Stimulation of adenosine A3 receptors does not induce arrestin-2 or arrestin-3 mobilization**

RBL-2H3 cells have been shown to endogenously express high number of adenosine A3 receptors (A3Rs: 63,000 A3AR/cell; Olah et al., 1994). This GPCR couples to phospholipase C via a pertussis toxin-sensitive mechanism and, when stimulated by the agonist NECA, causes cellular responses similar to those observed with IgER and m1ACHr stimulation (Ali et al., 1990, 1994). Moreover, stimulated A3ARs are known to undergo rapid functional desensitization (Ramkumar et al., 1993). However, little is known regarding the internalization and trafficking of the A3AR.

When we treated RBL cells with NECA, despite the high number of A3ARs stimulated, we observed no recruitment of either arrestin-2 or arrestin-3 to coated pits, nor changes in their distribution (compare Fig. 5 with Fig. 2, Fig. 3 and Fig. 4, Unst.) induced by the agonist. In addition, no recruitment of either arrestin was observed in cells that had been preexposed to antigen and then stimulated with NECA (data not shown).

While our results could suggest that the fate of the stimulated A3AR is controlled by a different endocytic mechanism that does not involve arrestin-mediated clathrin coated pit internalization or that it is agonist-selective (Murray et al., 1998), we cannot exclude the possibility that arrestin recruitment below the limit of detectability occurred. Nonetheless, the results highlight the specificity of the observed preferential coupling of β2AR and m1ACHr to arrestin-3. They also indicate that when presented with the same complement of cellular arrestins (i.e. in the same cell) not all GPCRs are capable of inducing mobilization of endogenous arrestins.

The existence of two forms of non-visual arrestins, functionally different (e.g. in GPCR uncoupling) yet different in their primary sequence (Attramadal et al., 1992) and their...
regulation (Lin et al., 1997), implies that they participate in distinct pathways following receptor binding, perhaps by mediating specific protein-protein interactions. Indeed, recent studies involving functional deletion of non-visual arrestin genes in mice showed impaired desensitization of cardiac βARs (with arr2-ablation), and neuronal μ opioid receptors (with arr3-deletion) (Conner et al., 1997; Bohn et al., 1999). Additionally, it is known that receptor internalization is often determined not only by the presence of internalization motifs, but also by the cell type being examined (Koenig and Edwardsion, 1996). The m1AChR and the IL-8 receptors, for example, have been shown to undergo agonist-dependent internalization in some cells but not in others (Goldman et al., 1996; Barlic et al., 1999) leading to the conclusion that cellular factors are important to efficiently couple the stimulated phosphorylated receptor to coated pits. Noteworthy, it was previously observed that in different cell types, only the endogenous level of arrestin-3 and not that of arrestin-2 significantly correlates with GPCR sequestration (Menard et al., 1997), although in some cells both forms of endogenous arrestin have been seen to be mobilized in response to GPCR stimulation (McConalogue et al., 1999).

In the RBL-2H3 cells studied here, which express comparable levels of both non-visual arrestins, compartmentalization may also play a role. Partial segregation of arrestin-2 and arrestin-3 is indicated by the proteins’ finely punctate distribution observed by immunofluorescence (this paper and that of McConalogue et al., 1998). Our observation that a considerable larger proportion of arrestin-3 is associated with the membrane/cytoskeleton and resists detergent-extraction, suggests that its localization could contribute to its preferential recruitment to clathrin coated pits.

This study highlights the issue of specificity in the coupling of stimulated GPCRs to endogenous arrestin-2 and arrestin-3 in vivo. The results presented indicate the existence in cells of complex mechanisms regulating the interactions between internalizing receptors and a cell’s complement of non-visual arrestins. In vitro results have indicated similar capabilities of arrestin-2 and arrestin-3 in binding and uncoupling stimulated receptors from G proteins, but have also shown a 6- to 7-fold higher clathrin binding affinity for arrestin-3 compared to arrestin-2. Interestingly, co-transfection experiments have failed to reveal differences in the ability of arrestin-2 and arrestin-3 to support clathrin induced endocytosis.

At this time the molecular mechanisms responsible for β2AR- and m1AChR-specific coupling to arrestin-3 over arrestin-2 occurring in RBL cells are still not understood. However, the results presented indicate that in cells where both forms of non-visual arrestins are coexpressed, the two proteins do not perform redundant functions, but serve different, perhaps ancillary, purposes.

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