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Function of non-visual arrestins in signaling and endocytosis of the gastrin-releasing peptide receptor (GRP receptor)

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ABSTRACT

Little is known about the role of arrestins in gastrointestinal hormone/neurotransmitter receptor endocytosis. With other G protein-coupled receptors, arrestins induce G protein-uncoupling and receptor endocytosis. In this study, we used arrestin wild-type and dominant-negative mutant constructs to analyze the arrestin dependence of endocytosis and desensitization of the gastrin-releasing peptide receptor (GRP-R). Co-expression of the GRP-R with wild-type arrestin2 and arrestin3 increased not only GRP-R endocytosis but also GRP-R desensitization in arrestin-overexpressing cells. Co-expression of the dominant-negative mutants V53D-arrestin2 or V54D-arrestin3 reduced GRP-R endocytosis. Notably, different trafficking routes for agonist-activated GRP-R-arrestin2 and GRP-R-arrestin3 complexes were found. Arrestin3 internalizes with GRP-R to intracellular vesicles, arrestin2 splits from the GRP-R and localizes to the cell membrane. Also, the recycling pathway of the GRP-R was different if co-expressed with arrestin2 or arrestin3. Using different GRP-R mutants, the C-terminus and the 2nd intracellular loop of the GRP-R were found to be important for the GRP-R-arrestin interaction and for the difference in GRP receptor trafficking with the two arrestin subtypes. Our results show that both non-visual arrestins play an important role in GRP-R internalization and desensitization.

1. Introduction

The signaling of G protein-coupled receptors (GPCR) is a highly regulated process. It is reported that seconds after agonist-induced activation the receptor desensitizes and is later internalized into an intracellular compartment [1]. The desensitization process is triggered by the phosphorylation of the GPCR by G protein-coupled receptor kinases (GRKs) and second-messenger kinases [2]. For many GPCRs arrestins are shown to be responsible for interrupting the interaction of the

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Abbreviations: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GRP receptor, gastrin-releasing peptide receptor; PLC, phospholipase C; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Bn, bombesin; DTT, dithiothreitol; GFP, green fluorescent protein; IP, inositol phosphate; JF-1, GRP receptor mutant lacking carboxyl terminal serines and threonines.

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phosphorylated GPCR with the G protein [3]. Furthermore, in some [4,5], but not all GPCRs [5,6], arrestins initiate the receptor endocytosis process by the desensitized receptor to clathrin and its cofactors [7]. Once internalized, GPCRs are either dephosphorylated and then recycled back to the cell membrane [8–10], or degraded in lysosomal compartments [11,12].

The gastrin-releasing peptide receptor (GRP receptor) is a GPCR that is expressed in the central nervous system and the gastrointestinal tract and accounts for a number of physiological effects, including the release of many gastrointestinal hormones, trophic effects, and the regulation of gallbladder and smooth muscle contractility as well as pathological effects like the stimulation of the growth of various human tumors including small cell lung cancer, prostate cancer and breast cancer [13,14]. After activation, the GRP receptor couples via $G\alpha_q$ to phospholipase C (PLC) [15] whose activation results in an increase in inositol phosphates (IP), mobilization of intracellular Ca^{2+} and activation of protein kinase C [16,17]. It is also known that activation of the GRP receptor triggers a biphasic receptor phosphorylation pattern – a rapid, GRK2-mediated and a slower, protein kinase C-mediated phosphorylation [18,19]. The rapid GRP receptor phosphorylation is believed to result in acute desensitization [19]. However, the mechanism that is responsible for the desensitization as well as the internalization of the phosphorylated GRP receptor is only partially understood. One study provides indirect evidence that arrestin function may not be needed for GRP receptor desensitization [19] and another study reports that arrestins participate in acute desensitization of the GRP receptor [20]. Similar to a number of other receptors for gastrointestinal (GI) hormones/neurotransmitters, the GRP receptor undergoes rapid internalization with agonist stimulation and this may participate in receptor desensitization. However, the mechanisms underlying the receptor endocytosis process in the GRP receptor as well as other GI hormone/neurotransmitter receptors are poorly understood. Although a number of structure–function studies in the GRP receptor have been done to reveal protein motifs important for activating processes involved in GRP receptor down-regulation, internalization and desensitization [21–23], no studies addressed the actual mechanisms and the proteins involved in this process.

In an attempt to answer these questions we investigated the effects of arrestin2 and arrestin3 on GRP receptor internalization and desensitization. We also focussed on arrestin subtype-specific differences in the interaction with the GRP receptor. We also report on the intracellular trafficking of the endocytosed GRP receptor relative to known markers of endocytic compartments like transferrin and the small GTPase rab5. Lastly, we assigned these findings on the interaction of arrestins with the GRP receptor to known GRP receptor motifs by using receptor mutants.

2. Materials and methods

2.1. Materials

pcDNA3, oligonucleotides were from Invitrogen (Carlsbad, CA). pEGFPN1 was from BD Biosciences/Clontech (Palo Alto, CA). All site-directed mutagenesis kits were from Stratagene (La Jolla, CA). Restriction endonucleases were from New England BioLabs (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, 100×, trypsin–EDTA, and Dulbecco's phosphate-buffered saline (PBS) were from Biofluids (Rockville, MD). Swiss 3T3, HEK 293 and Cos-7 cells were from American Type Culture Collection (Rockville, MD). Complete Mini protease inhibitor cocktail tablets and Fugene 6 Transfection Reagent were from Roche Diagnostics (Indianapolis, IN). Bombesin (Bn) was from Bachem Biosciences (King of Prussia, PA). $Na^{125}I$ (2200 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). 1,3,4,6-Tetrachloro-3α,6α-diphenylglucouril (IODO-GEN™), dithiothreitol, and enhanced chemiluminescence detection reagents were from Pierce Chemical (Rockford, IL). Bovine serum albumin fraction V was from ICN Pharmaceutical Inc. (Aurora, OH). Nystatin was from Calbiochem (San Diego, CA). Myo-[2-^{3}H(N)]inositol was from PerkinElmer Life Sciences (Boston, MA). Dowex AG1-X8 anion-exchange resin (100–200 mesh formate form) and the Bradford Protein Assay were from BioRad (Richmond, CA). Hydro-Fluor scintillation fluid was from National Diagnostics (Atlanta, GA). Rhodamine-transferrin was from Molecular Probes (Eugene, OR). BioMax MR film was from Eastman Kodak Co. (Rochester, NY). GraphPad Prism3 software was from GraphPad Software Inc. (San Diego, CA). If not labeled otherwise, all other substances were from Sigma (St. Louis, MO).

2.2. Plasmids

The GRP receptor mutants (R^{139}G[GRP receptor], A^{263}E[GRP receptor], JF-1[GRP receptor]) were a generous gift from Dr. Glenn Kroog. The arrestin-pcDNA3 constructs were a generous gift from Dr. Benovic (Thomas Jefferson University, Philadelphia, PA). The rab5-pEGFN1 construct was a generous gift from Dr. Brian Howell. For immunostaining of the GRP receptor, a HA-tagged GRP receptor was constructed. For this, the DNA sequence encoding the HA epitope (YPYDVPDYA) was inserted between the first (Met) and second (Ala) amino acid residue of the GRP receptor mutant using the ExSite PCR-based site-directed mutagenesis kit. Mutations in the arrestin constructs were made using the QuikChange site-directed mutagenesis kit. With both mutagenesis kits the manufacturer's instructions were followed, except for the DpnI treatment that was increased to 2 h. To construct the arrestin2 and arrestin3-GFP plasmids a PCR subcloning strategy was chosen using the parental vectors arrestin2-pcDNA3 and arrestin3-pcDNA3 as template DNA. PCR primers included in sense direction HindIII (arrestin2 and arrestin3), in antisense direction SmaI (arrestin2) and ApaI (arrestin3) restriction enzyme sites. PCR products were cut with the mentioned restriction enzymes, purified and ligated into pEGFPN1 (Clontech, Palo Alto, CA) using the Rapid Ligation Kit (MBI Fermentas, Hanover, MD). The DNA sequence of all constructs was confirmed using an automated DNA sequencer (ABI PRISM™ 377 DNA sequencer, Applied Biosystems Inc., Foster City, CA).

2.3. Cell culture and transfection

Swiss 3T3 cells, Cos-7 and HEK 293 cells were grown in standard medium (DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin). For transient transfection studies Cos-7 and HEK 293 cells were seeded

one day prior to transfection on 6 wells or 10 cm dishes at a density of 0.6×10^6 cells/well or 1×10^6 cells/dish, respectively. The Cos-7 and HEK cells were used for the transfection studies because Swiss 3T3 cells which possess native GRP-R receptors, only expressed low or non-detectable levels of arrestins after transient transfection. Cells were maintained at 37 °C with a 5% CO_2 atmosphere. The following morning, cells were transfected according to the manufacturer's protocol using 18 μl of Fugene 6 Transfection Reagent and either 0.5 μg (6 wells) or 1 μg (10 cm dishes) of GRP receptor-pcDNA3 DNA and 5 μg of pcDNA3, arrestin2-pcDNA3, or arrestin3-pcDNA3. The following day, the transfection medium was aspirated and cells were split on 24 wells (approximately 40,000 cells/well).

2.4. *Preparation of cell lysates from transiently transfected cells*

Three days after transfection, cells were scraped from the cell culture flasks and centrifuged (1500 rpm, 10 min, 4 °C). The pellet was washed, resuspended in 750 μl of ice-cold cell lysis buffer [50 mM Tris/HCl, pH 7.4, 25 mM β-glycerophosphate, 3.5 mM DTT, 5 mM EDTA, 1 mM sodium pyrophosphate, 25 mM NaF, 1% (v/v) Nonidet P40, 0.5% (w/v) deoxycholic acid, 1 Complete Mini EDTA-free protease inhibitor cocktail tablet per 10 ml lysis solution] and immediately sonicated for 5 s. After centrifugation (14,000 rpm, 15 min, 4 °C) the supernatant was saved. Protein concentration was measured with the Bio-Rad protein assay reagent.

2.5. *Western blotting*

Western blotting was performed as described previously [24] with minor modifications. Briefly, cell lysates were fractionated by SDS-PAGE using 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature using blotto [5% nonfat dried milk in a solution containing 50 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20] and were incubated for 60 min at room temperature with 1 μg/ml of a mouse monoclonal arrestin2-antibody (BD Biosciences Pharmingen, Palo Alto, CA) or 1 μg/ml of a mouse monoclonal arrestin3-antibody (BD Biosciences Pharmingen, Palo Alto, CA) in 10 ml of blotto. Membranes were washed twice for 10 min with blotto and were incubated for 1 h at room temperature with an anti-mouse or anti-goat IgG-horseradish peroxidase conjugate (1 μg/ml) in 10 ml of blotto. The membranes were finally washed twice for 5 min with blotto and twice for 5 min with washing buffer [50 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20], incubated with enhanced chemiluminescence detection reagents for 5 min and exposed to Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY).

2.6. *Internalization of GRP receptors*

Internalization was performed as described previously [25,26] with minor modifications. Cells were transfected three days prior to the experiment and split in 24 wells two days prior to the experiment. On the day of the experiment, cells were washed with PBS and pre-incubated for 15 min in binding buffer [24.5 M HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 2.2 mM KH_2PO_4, 5 mM theophylline, 2 mM glutamine, 11 mM glucose, 1% (v/v) amino acid mixture, 1% (v/v) essential vitamin mixture, and 0.2% (w/v) bovine serum albumin)] containing bacitracin (0.1%) and Sigma's protease inhibitor cocktail [1/1000 (v/v)]. Cells were incubated with 50 pM ^{125}I-[Tyr^4]Bn in binding buffer for various times at either 37 or 4 °C. After incubation, the 24

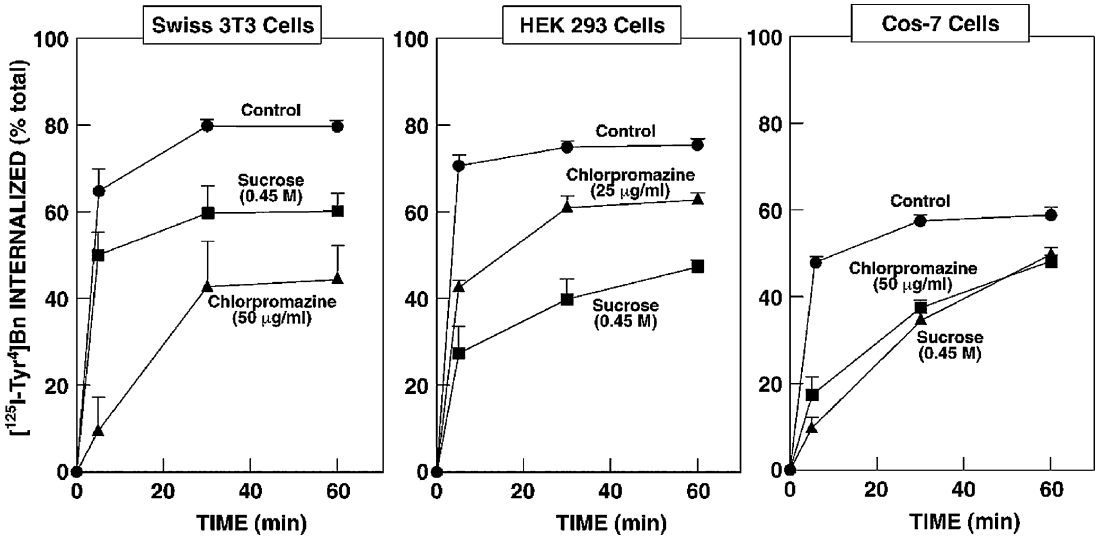

Fig. 1 – Effect of inhibitors of clathrin-mediated endocytosis on internalization of [^{125}I-Tyr^4]Bn. Native Swiss 3T3 cells, GRP receptor-transfected HEK 293, or Cos-7 cells (all at 50,000 cells/24 well) were pre-incubated with or without hyperosmolar sucrose (0.45 M) or chlorpromazine in the indicated concentrations at 37 °C for 30 min. After the pre-incubation, 50 pM [^{125}I-Tyr^4]Bn was added for the indicated times and the percent of total saturable binding internalized was determined using acid-stripping as outlined in Section 2. Data are expressed as the mean ± S.E. of the percentage of the total saturably bound [^{125}I-Tyr^4]Bn that was not removed by acid-stripping, from four separate experiments.

wells were put on ice. Cells were washed twice with PBS containing 1% BSA, then 1 ml of acid-stripping solution (0.2 M acetic acid, 0.5 M NaCl) was added for 10 min at 4 °C. The acid-stripped medium was collected into a first set of counting tubes. Cells in the 24 wells were trypsinized and collected in a second set of counting tubes. Both sets were counted using a Cobra II Autogamma Counter (Packard, Sterling, VA). In all cases parallel incubations with unlabeled bombesin to determine non-saturable binding were performed. Internalization results are expressed as percentage of saturable acid-resistant ^{125}I-[Tyr^4]Bn binding of the total saturable binding.

Analysis of internalization data was done as previously described [26] using the GraphPad Prism software and using the modification of the approach of Koenig and Edwardson [27] described previously [26]. Rearranging equation #2 from their review paper [27] yielded the relationship % internalized receptor = $100k_e\{1 - \exp[-(k_e + k_r)t]\}/(k_e + k_r)$, where k_e is the constant of endocytosis, k_r is the constant of recycling and t is the time of the incubation. Non-linear regression showed for the wild-type GRP receptor a k_e of 0.13 ± 0.019 min^{-1} and a k_r of 0.11 ± 0.013 min^{-1}, which is in the range of other G protein-coupled receptors [27]. The time needed for 80% of maximal internalization (i.e., t_{80}), expressed in minutes was then derived from the relationship $t_{80} = -\ln(0.2)/(k_e + k_r)$ [26].

2.7. *Measurement of [^{3}H]inositol phosphates ([^{3}H]IP)*

Changes in [^{3}H]IP were performed as described previously [28]. Briefly, cells were plated onto 24 well flasks at a density of 10^4 cells/well in standard medium. Twenty-four hours later, cells were loaded with 3 μCi/ml myo-[^{3}H(N)]inositol in DMEM containing 2% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h. After washing, cells were incubated with PBS and 20 mM LiCl for 15 min at 37 °C. Cells were incubated with varying concentrations of bombesin in IP buffer [135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 20 mM LiCl, 11.1 mM D-glucose, 0.5% bovine serum albumin] at 37 °C. After various incubation times, the reaction was stopped with 1% (v/v) HCl in methanol. Total [^{3}H]IP were isolated by anion-exchange chromatography as described previously [28]. Briefly, free [^{3}H]inositol was removed by washing with 3 volumes of water and then 5 mM disodium tetraborate in 60 mM sodium formate was used to remove [^{3}H]glycerophosphoryl inositol. Total [^{3}H]IP were eluted with 1 M ammonium formate in 100 mM formic acid. Eluates were assayed for their radioactivity after the addition of Hydro-Fluor scintillation fluid. Curve-fitting of [^{3}H]IP data was done using the sigmoidal dose–response function of the GraphPad Prism program.

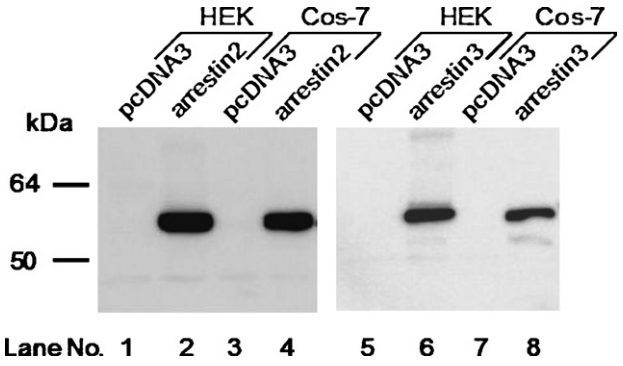

Fig. 2 – Expression of arrestins in transfected Cos-7 and HEK 293 cells. Cos-7 and HEK 293 cells (10^6 cells/10 cm dish) were transfected with 5 μg of wild-type arrestin2 or arrestin3 construct DNA (or Empty Vector DNA 'pcDNA3'). Three days after transfection, cells were lysed and the arrestin expression level was determined with Western blotting as described in Section 2. The Western blot on the left shows arrestin2 expression levels and on the right arrestin3 expression levels in Cos-7 cells and HEK cells. Results shown are representative of three independent experiments.

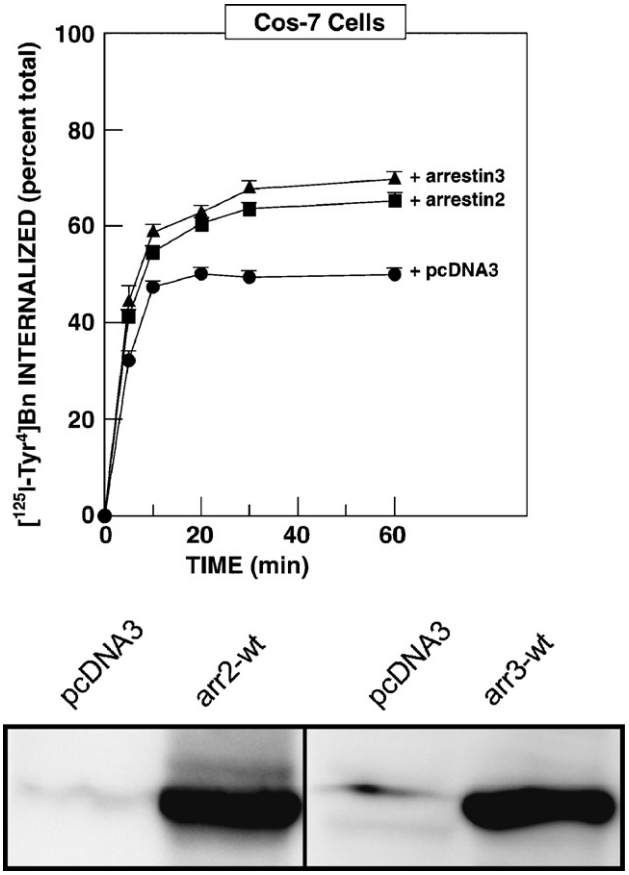

Fig. 3 – Effect of overexpression of arrestin constructs on [^{125}I-Tyr^4]Bn internalization in Cos-7 cells. Cos-7 cells (10^6 cells/10 cm dish) were co-transfected with 1 μg of GRP receptor-pcDNA3 and 5 μg of wild-type arrestin construct DNA [or Empty Vector DNA (pcDNA3)] and split to 24 wells the following day. Three days after transfection GRP receptor-mediated endocytosis using [^{125}I-Tyr^4]Bn was measured as described in Section 2. Data are expressed as the mean ± S.E. of the percentage of total saturably bound [^{125}I-Tyr^4]Bn that was not removed by acid-stripping, from four separate experiments. In the insert the expression levels of the two arrestin wild-types in Cos-7 cells are shown. Results shown are representative of three independent experiments.

Table 1 – Comparison of maximal internalization and kinetics of internalization of different GRP receptor mutants

GRP receptor	Co-transfected with	% Internalization at 60 min	t_{80} [min]
Wild-type GRP receptor	pcDNA3	50 ± 1	7.3 ± 0.9
	Arrestin2-pcDNA3	66 ± 2[a]	7.6 ± 0.9
	Arrestin3-pcDNA3	70 ± 2[b]	7.8 ± 1.3
$R^{139}G$[GRP receptor]	pcDNA3	11 ± 1	13.3 ± 1.5
	Arrestin2-pcDNA3	14 ± 1	12.5 ± 2.1
	Arrestin3-pcDNA3	21 ± 1[a]	25.4 ± 6.0
$A^{263}E$[GRP receptor]	pcDNA3	50 ± 1	31.9 ± 5.3
	Arrestin2-pcDNA3	55 ± 0[a]	2.5 ± 0.1[a]
	Arrestin3-pcDNA3	64 ± 1[b]	7.0 ± 1.3[a]
JF-1[GRP receptor]	pcDNA3	20 ± 3	25.3 ± 1.7
	Arrestin2-pcDNA3	29 ± 4[a]	31.3 ± 7.2
	Arrestin3-pcDNA3	39 ± 7[a]	15.5 ± 3.1

The time-course of internalization of [^{125}I-Tyr^4]Bn ligand in Cos-7 cells by the wild-type GRP receptor and by each GRP receptor mutant shown in Figs. 1 and 11 was analyzed by the method of Koenig and Edwardson [27] as described in Section 2. The percentage of maximal internalization seen at 60 min was expressed as the percentage of total saturable binding occurring at 60 min and was calculated as described in Section 2. t_{80} is the time in minutes that was needed for internalizing 80% of maximal calculated as described in Section 2. Data are expressed as the mean ± S.E. of at least three separate experiments.

[a] $p < 0.05$ compared to pcDNA3 only co-transfected cells.

[b] $p < 0.01$ compared to pcDNA3 only co-transfected cells.

2.8. *Deconvolution imaging microscopy*

HEK 293 cells were used for these studies because they have been extensively used in the literature for studies of arrestin imaging in cells, and in preliminary studies we obtained much better imaging results with HEK cells than the smaller Cos-7 cells. One day after GRP receptor or arrestin2 or arrestin3 transfection, HEK 293 cells were trypsinized and plated on polylysine-coated glass coverslips in 12 well plates at a density of 100,000 cells/well. Two days later, cells were washed with PBS and treated with or without bombesin (100 nM) in DMEM for various incubation times at 37 °C. For staining of the recycling compartment of the cells, rhodamine-transferrin was incubated 30 min prior to fixation for the duration of 10 min at 37 °C. Cells were then washed with PBS and incubated in DMEM for the rest of the incubation. After incubation, cells were fixed (paraformaldehyde 4%, 10 min, RT), permeabilized (Triton X-100 0.5%, 5 min, RT) and blocked (2% BSA, 2% donkey serum in PBS, 30 min, RT). The GRP receptor was stained at its HA epitope using a mouse anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1/100 dilution) as first and a TRITC-labeled donkey anti-mouse antibody as a secondary antibody. Untagged GRP receptor was stained using a rabbit GRP receptor antibody (1/100 dilution) [20] and a TRITC-labeled donkey anti-rabbit secondary antibody. All secondary antibodies were used at a 1/200 dilution and were from Jackson ImmunoResearch Laboratories, West Grove, PA. Coverslips were mounted using Vectorshield (Vector, Burlingame, CA) and fixed on glass slides with nail polish. Microscopy studies were carried out using the Deltavision deconvolution imaging system (Applied Precision, Issaquah, WA). Deconvolution of image stacks was performed using the Softworx software (Applied Precision, Issaquah, WA).

2.9. *Statistics*

In IP experiments, EC_{50} values (potency) and maximal fold induction (efficacy) were determined by performing curve-fitting with the program GraphPad Prism3 (San Diego, CA). All analyses were performed using the Mann–Whitney *U*-test. p values < 0.05 were considered significant.

3. Results

To determine whether clathrin-coated vesicles contribute to GRP receptor internalization, we pre-incubated Swiss 3T3 cells which natively possess GRP receptors [33,35], and GRP receptor-transfected HEK and Cos-7 cells with hyperosmolar sucrose or chlorpromazine, both well-established inhibitors of clathrin-dependent endocytosis [36–39], and compared the GRP receptor internalization with cells that were pre-incubated with buffer only (Fig. 1). In the native GRP receptor expressing Swiss 3T3 cells and in the GRP receptor-transfected HEK and Cos-7 cells, GRP receptor internalization was significantly reduced by pre-incubating the cells with both inhibitors.

HEK and Cos-7 cells transiently transfected with GRP receptor were used for further experiments to determine the role of arrestins in the endocytotic process. Immunoblots were made to demonstrate the expression of arrestin2 and arrestin3 in HEK and Cos-7 cells that had been transfected with arrestin2- and arrestin3-cDNA (Fig. 2, lanes 2, 4, 6, 8). Note, that in both cell lines native expression of arrestin2 and arrestin3 was below detection level (Fig. 2, lanes 1, 3, 5 and 7).

To further investigate the possible role of arrestins in GRP receptor internalization, the effect of their overexpression on GRP receptor internalization was investigated in Cos-7 cells that are known to express arrestins at very low level [40]. Internalization of wild-type GRP receptor co-expressed with an empty pcDNA3 vector reached a maximal of 50 ± 1% after 30 min (Fig. 3; Table 1). Overexpression of arrestin2 or arrestin3 was achieved as shown by Western blotting (Fig. 3, insert) and significantly increased the maximal portion of internalized receptors to 66 ± 2% and 70 ± 2%, respectively.

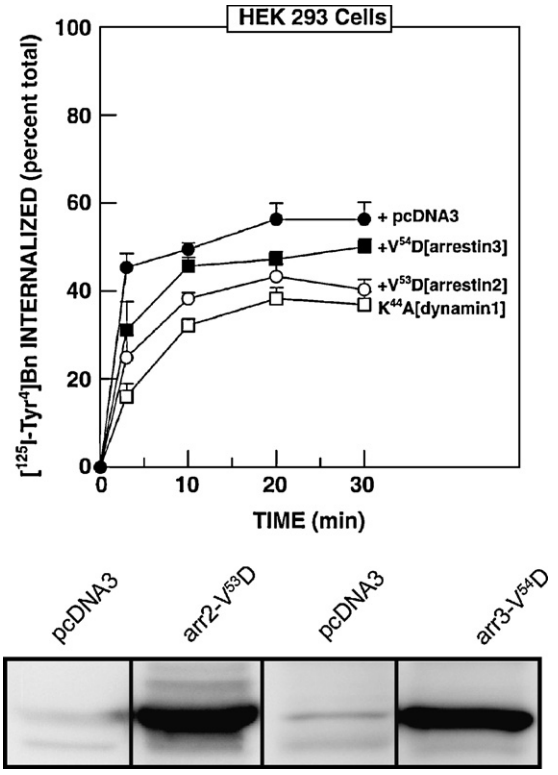

Fig. 4 – Effect of overexpression of mutant arrestin or dynamin constructs on [^{125}I-Tyr^4]Bn internalization in HEK 293 cells. HEK 293 cells (10^6 cells/10 cm dish) were co-transfected with 1 μg of GRP receptor-pcDNA3 and 5 μg of mutant arrestin or dynamin construct DNA [or Empty Vector DNA (pcDNA3)] and split to 24 wells the following day. Three days after transfection, GRP receptor-mediated endocytosis of [^{125}I-Tyr^4]Bn was measured as described in Section 2. Data are expressed as the mean ± S.E. of the percentage of total saturable bound [^{125}I-Tyr^4]Bn that was not removed by acid-stripping from four separate experiments. *Inset*: overexpression of arrestin mutants in HEK cells is shown with Western blotting.

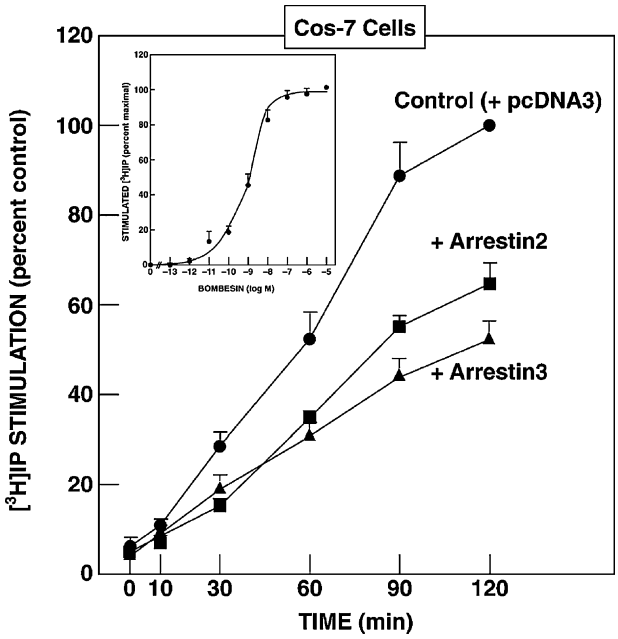

Fig. 5 – Effect of overexpression of arrestin on bombesin stimulation of phospholipase C activity in Cos-7 cells. Cos-7 cells were transfected with arrestin2 or arrestin3 and wild-type GRP receptor as described in the legend of Fig. 4 and split on 24 wells the following day. Two days after transfection, cells were loaded with 3 μCi/ml myo[2-^{3}H(N)]-inositol overnight. On the next day, cells were incubated with 0.3 nM bombesin for the indicated times at 37 °C, the reaction was stopped and cell lysates were processed as described in Section 2. Data are expressed as the percentage of the control value. The control value was 506 ± 80 dpm and the maximal stimulation seen at 120 min in the cells treated with empty vector (i.e., +pcDNA3) was 14,008 ± 3350 dpm. *Inset*: generation of inositol phosphates after incubation with different concentrations of bombesin for 1 h at 37 °C in Cos-7 cells transiently transfected with GRP receptor-pcDNA3. Results are expressed as the percentage of the maximal stimulation caused by 10 μM bombesin. The control and maximal values were 2128 ± 328 and 16,603 ± 3681 dpm, respectively. Data are expressed as the mean ± S.E. of three independent experiments.

Whereas co-expression of arrestin2 and arrestin3 significantly increased the maximal amount of wild-type GRP receptor internalization, it had no effect on the kinetics of internalization; the time to 80% maximal internalization remained unchanged (t_{80} 7.3 to 7.8 min).

Because HEK cells are reported to express higher basal levels of arrestins than Cos-7 cells [40], HEK cells were used for GRP receptor endocytosis experiments with overexpression of dominant-negative arrestin mutants (Fig. 4, insert) [4]. In unstimulated cells, expression of V^{53}D-arrestin2 or V^{54}D-arrestin3 decreased maximal GRP receptor internalization by 29 ± 1% and 12 ± 0.4% (Fig. 4). Since dynamin, a large GTPase, is involved in clathrin-dependent as well as clathrin-independent internalization [41], the effect of overexpression of a dominant-negative dynamin mutant [K^{44}A (dynamin)] was investigated [42]. With overexpression of K^{44}A (dynamin), GRP-R internalization was decreased to a larger amount (33 ± 3%) than inhibition of either arrestin alone (Fig. 4).

To study the effects of arrestins on GRP receptor activation, we examined the ability of the GRP receptor to activate phospholipase C with and without overexpression of wild-type arrestin constructs (Fig. 5). GRP receptor transfected Cos-7 cells stimulated with bombesin showed a dose-dependent increase of total [^{3}H]inositol phosphates with an EC_{50} of 0.15 ± 0.03 nM (Fig. 5, inset), similar to the published EC_{50} in natively GRP receptor expressing Swiss 3T3 cells [28]. Maximal effective doses of bombesin caused a 7.8 ± 1.7-fold increase in [^{3}H]inositol phosphate production. A time-course study revealed that GRP receptor-transfected Cos-7 cells accumulated [^{3}H]inositol phosphates in a linear fashion after stimulation with 0.3 nM bombesin over a 120 min time-period (Fig. 5). After stimulation with 0.3 nM bombesin, cells co-

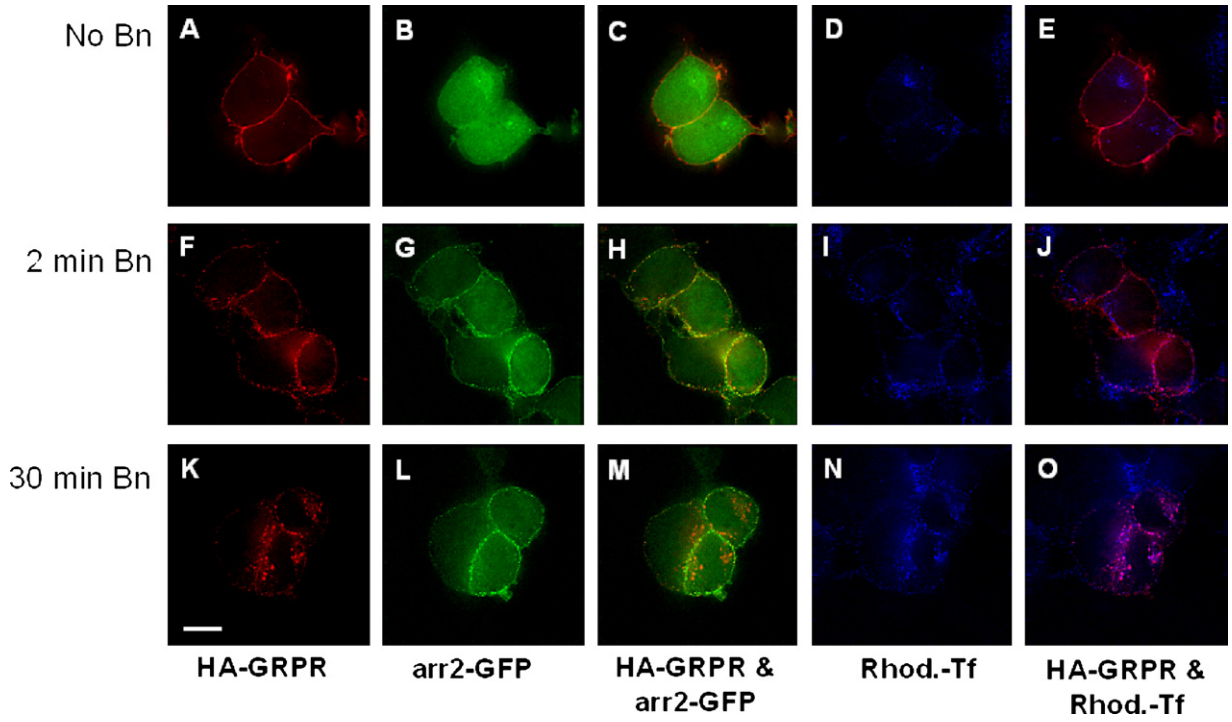

Fig. 6 – Effect of bombesin stimulation on cellular localization of arrestin2-GFP, relative to the HA-GRP receptor and to rhodamine-transferrin. HEK 293 cells were co-transfected with 1 μg of HA-GRP receptor-pcDNA3 and 1 μg of the arrestin2-pEGFPN1 construct DNA and split to a glass slide containing 12 wells the following day. On the third day after transfection, cells were incubated with or without 100 nM bombesin for the indicated periods of time. The same cells are shown in each row. Note that the secondary antibody to the HA antibody was Cy5 (infrared)-labeled and color-coded as red. At the same time, the rhodamine-labeled transferrin was computer color-coded to blue. Results shown are representative of three independent experiments. Scale bar, 10 μM.

transfected with GRP receptor and wild-type arrestins produced significantly less [^{3}H]inositol phosphates than cells co-transfected with GRP receptor and empty pcDNA3 vector. Specifically, overexpression of arrestin2 or arrestin3 caused a 35.6 ± 3.6% and 47.5 ± 4.7% decrease in bombesin-stimulated [3H]inositol phosphate generation at 120 min.

To study the intracellular trafficking of the GRP receptor relative to the intracellular movement of arrestins, deconvolution imaging microscopy was used (Figs. 6–9). HEK cells were transiently co-transfected with constructs expressing the HA-tagged GRP receptor and arrestin-GFP fusion proteins. Localization of receptor and arrestins was determined relative to the intracellular trafficking of rhodamine-transferrin, a well-established tracer of the recycling compartment of the cell [44]. Without stimulation of the cells, the GRP receptor was localized at the cell membrane (Fig. 6A). Arrestin2-GFP was homogeneously distributed in the cytosol and was also found in the nucleus as reported previously [45] (Fig. 6B). Two minutes after the addition of bombesin (100 nM), the GRP receptor changed to a more punctuate pattern, primarily on the cell surface (Fig. 6F). Arrestin2 translocated to the cell membrane (Fig. 6G), where it co-localized with the receptor (Fig. 6H). At this time point, only a minimal co-localization of the receptor with rhodamine-transferrin-filled vesicles existed (Fig. 6I and J). Later (30 min), the GRP receptor was internalized in intracellular vesicles (Fig. 6K) that also contained rhodamine-transferrin (Fig. 6N and O). Arrestin2-GFP primarily maintained at the cell membrane (Fig. 6L). Interestingly, the comparative cellular trafficking of arrestin3-GFP and GRP receptor was distinct from GRP receptor and arrestin2-GFP (compare Figs. 6 and 7). Without stimulation arrestin3-GFP also appeared homogeneously distributed in the cytosol (Fig. 7B). As reported previously [45], no nuclear staining of the cells with arrestin3-GFP was seen. Two minutes after addition of bombesin, arrestin3-GFP was translocated to the cell membrane and co-localized with the GRP receptor (Fig. 7F–H). A partial co-localization with rhodamine-transferrin-containing vesicles was seen (Fig. 7J). Thirty minutes after addition of bombesin, the GRP receptor was found in hollow vesicles (Fig. 7K) that were not found in arrestin2-GFP expressing cells (compare Figs. 6K and 7K). Contrary to the results with arrestin2-GFP, arrestin3-GFP followed the GRP receptor into the vesicles and co-localized with the GRP receptor (Fig. 7L and M). These vesicles also contained rhodamine-transferrin (Fig. 7N and O).

To further reveal the nature of the GRP receptor-containing vesicles, deconvolution imaging microscopy was performed on HEK cells co-expressing GRP receptor and rab5-GFP (Fig. 8), a small GTPase expressed primarily on early and recycling endosomes [46]. Without stimulation no co-localization of the GRP receptor and rab5-GFP was seen (Fig. 8A–C). After 5 min (Fig. 8D–F) and 30 min (Fig. 8G–I) of treatment with bombesin

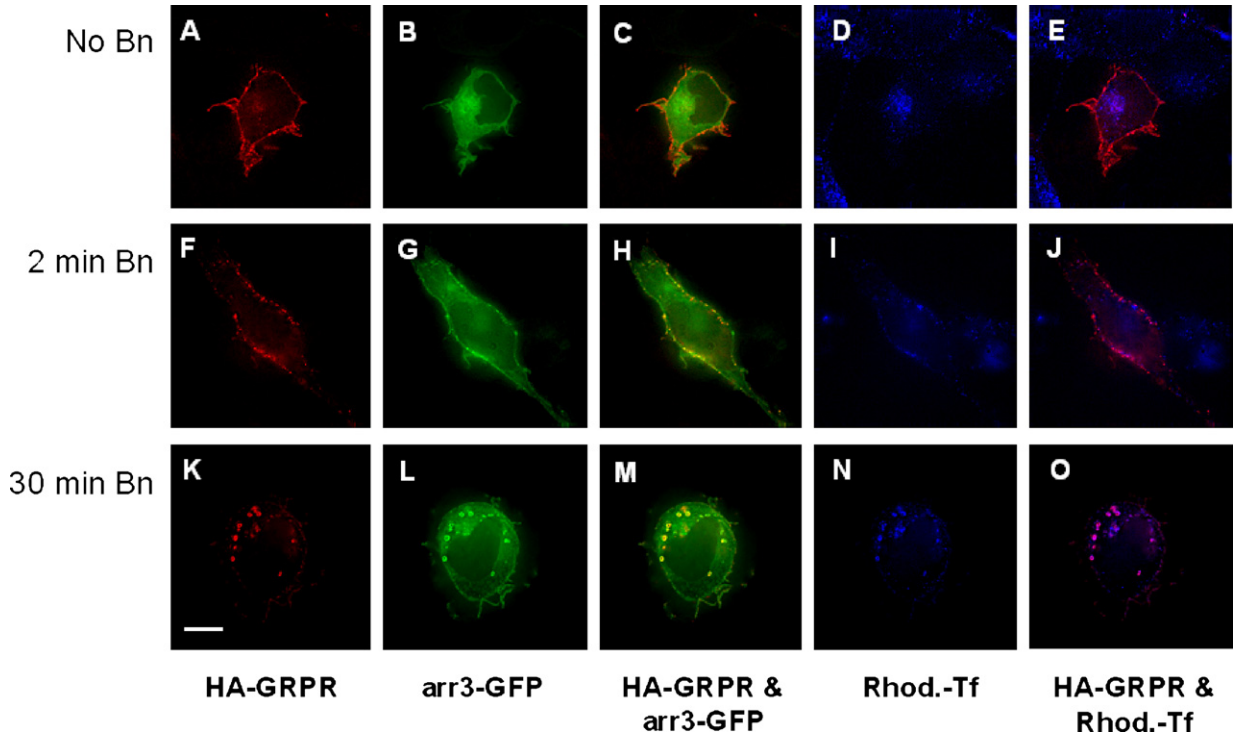

Fig. 7 – Effect of bombesin stimulation on cellular localization of arrestin3-GFP, relative to the HA-GRP receptor and to rhodamine-transferrin. HEK 293 cells were co-transfected with 1 μg of HA-GRP receptor-pcDNA3 and 1 μg of the arrestin3-pEGFPN1 construct DNA and split to a glass slide containing 12 wells the following day. On the third day after transfection, cells were incubated with or without 100 nM bombesin for the indicated periods of time. The same cells are shown in each row. The color-coding is as outlined in the legend of Fig. 6. Results shown are representative of three independent experiments. Scale bar, 10 μM.

(100 nM), co-localization of GRP receptor and rab5-GFP was seen in the same vesicles (Fig. 8F–I). To further investigate the intracellular trafficking of the GRP receptor, a receptor recycling study was performed (Fig. 9). After 30 min of stimulation with 100 nM bombesin, cells were washed and incubated with the bombesin receptor antagonist, [DPhe^{6}]Bn(6-13)methylester (ME-Bn, 1 μM) [47,48] for different periods of time. In HEK cells that co-expressed GRP receptor and arrestin2-GFP, receptor recycling to the cell membrane was observed after 60 min of incubation with the GRP receptor antagonist (Fig. 9A). However, full restoration of the cytosolic arrestin2-GFP pattern was not reached (Fig. 9B) and some of the arrestin2-GFP was still localized to the plasma membrane (Fig. 9C). Contrary to the findings with arrestin2-GFP, in cells expressing arrestin3-GFP GRP receptor was still localized to intracellular arrestin3-GFP-containing vesicles (Fig. 9D–F). Even 180 min after the addition of the GRP receptor antagonist, a substantial portion of GRP receptor was intracellular, although the extent of co-localization with arrestin3-GFP was decreased (Fig. 9G–I). Taken together, the microscopic results show that the interaction of the GRP receptor with arrestin2-GFP and its intracellular trafficking markedly differs from the interaction with arrestin3-GFP with the GRP receptor and its intracellular trafficking.

To explore the cellular mechanisms responsible for stimulation of receptor-arrestin interaction after GRP receptor activation as well as to provide insight into the amino acid sequences in the GRP receptor that might be involved in the interaction with the arrestins, various GRP receptor mutants were investigated. GRP receptor mutants of the second intracellular loop ($R^{139}G$) and the third intracellular loop ($A^{263}E$), and a GRP receptor mutant in which all C-terminal serines and threonines were substituted with alanines or glycines (JF-1) were analyzed.

We first analyzed the effect of the GRP receptor mutations on activation of phospholipase C and the production of total [^{3}H]inositol phosphates in Cos-7 cells transiently transfected with the GRP receptors (Table 2). Similar to previously reported findings in BALB 3T3 cells stably expressing the mutant GRP receptors [22,23], the potency of bombesin for $R^{139}G$ and $A^{263}E$ was slightly (2-fold) decreased. In contrast, the potency of the JF-1 mutant for bombesin was significantly increased. The ability of $R^{139}G$ and $A^{263}E$, but not JF-1, to maximally activate phospholipase C was impaired (Table 2). To assess whether decreased interaction with arrestins might contribute to the enhanced potency of bombesin in activating the JF-1 mutant, we examined whether overexpression of arrestin2 or arrestin3 would alter this mutant receptor's potency for bombesin activation (Fig. 10). The dose–response curve for bombesin to activate phospholipase C and increase [^{3}H]IP in Cos-7 cells that were co-transfected with the JF-1 GRP receptor mutant and arrestin3 was significantly shifted to the right relative to cells co-transfected with the JF-1 mutant and empty pcDNA3 vector. The shift was reflected by an increase in the potency

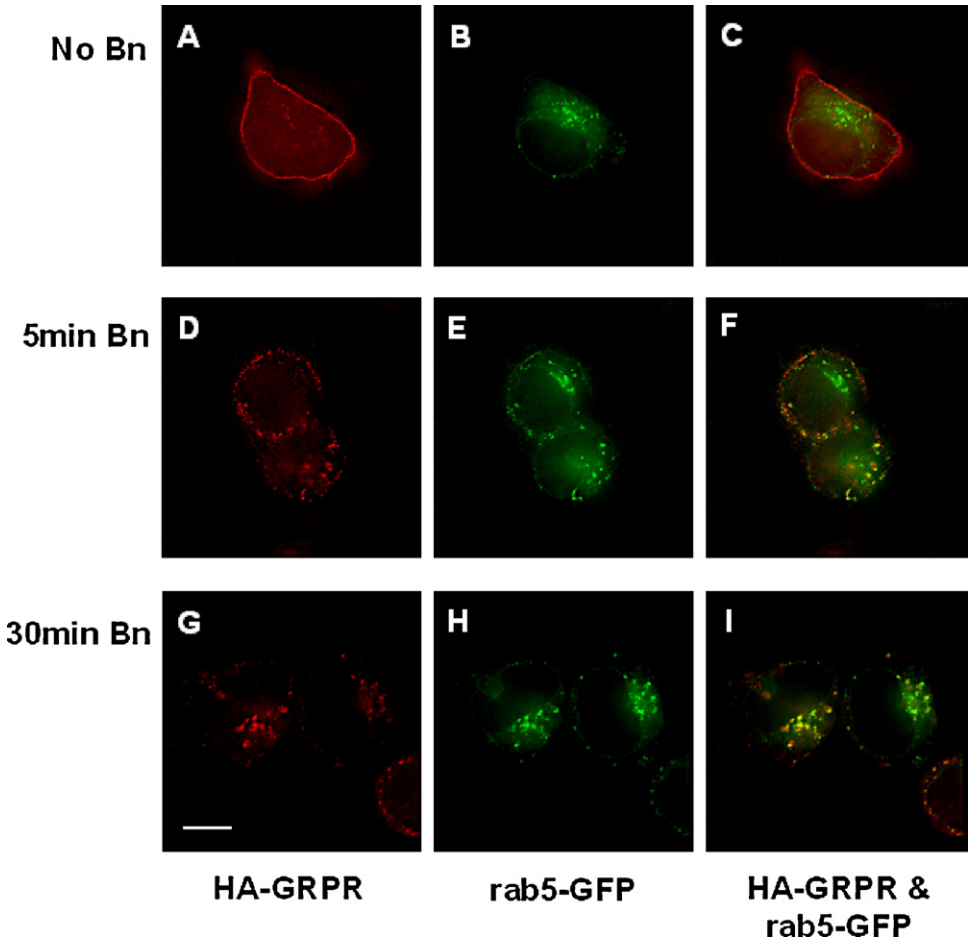

Fig. 8 – Effect of bombesin stimulation on cellular localization of the HA-GRP receptor and rab5-GFP. HEK 293 cells were co-transfected with 1 μg of HA-GRP receptor-pcDNA3 and 1 μg of rab5-pEGFPN1 construct DNA. The experiment was carried out as described in the legend of Fig. 6 without a transferrin chase. The same cells are shown in each row. Results shown are representative of three independent experiments. Scale bar, 10 μM.

of [^{3}H]IP induction (EC_{50} of JF-1 + pcDNA3: 6.9 ± 1.2 pM; EC_{50} of JF-1 + arrestin3: 15.2 ± 1.8 pM, $p = 0.004$). Overexpression of arrestin2 resulted in a right-shift relative to pcDNA3-expressing cells that did not reach statistical significance (EC_{50} of JF-1 + arrestin2: 9.4 ± 0.8 pM). The increase in EC_{50} for bombesin activation of phospholipase C induced by overexpression of arrestin3 in the JF-1 mutant was significant relative to the overexpression of arrestin2 ($p = 0.004$), suggesting a role for arrestin3 but not arrestin2 in determining receptor potency for phospholipase C activation.

To assess the effects of the GRP receptor mutations on receptor internalization and arrestin interaction, the effect of arrestin overexpression on GRP receptor internalization was studied in each mutant receptor (Fig. 11). Intracellular interaction of the arrestins and the receptor mutants was assessed using deconvolution imaging microscopy (Fig. 12). As published previously in Balb 3T3 cells stably transfected with the JF-1 mutant [21] the internalization of the JF-1 mutant was reduced by 76% after 30 min compared to wild-type GRP receptor, and maximal internalization decreased by 60% (Fig. 11, upper panels; Table 1). Removal of the GRP receptor carboxy-terminal serines and threonines in the JF-1 mutant also altered the kinetics of receptor internalization, resulting in a significant slowing of the internalization rate [t_{80} – 25.3 ± 1.7 min (JF-1) vs. 7.3 ± 0.9 min (wild-type)] ($p = 0.036$, Fig. 11, top panels; Table 1). Overexpression of arrestin3 in the JF-1 mutant significantly increased maximal internalization but did not reach wild-type levels (Fig. 11, upper right panel; Table 1). Although overexpression of arrestin2 also increased maximal internalization significantly, it was not as effective as arrestin3. Neither overexpression of arrestin2 nor arrestin3 reversed the slower rate of internalization seen with the JF-1 mutant. Maximal internalization of the $R^{139}G$ mutant was decreased to 1/5 of the wild-type level and the rate of internalization was also significantly decreased (t_{80} – 13.3 ± 1.5 min vs. 7.3 ± 0.9 min) ($p = 0.032$, Table 2 and Fig. 11, lower left panel). Overexpression of arrestin3 with the $R^{139}G$ mutant resulted in a small, but statistically significant increase in maximal internalization; however, it had no effect on the rate of internalization ($p = 0.057$) (Fig. 11, lower left panel; Table 1). Overexpression of arrestin2 was not effective in increasing the maximal $R^{139}G$ mutant internalization, nor did it significantly alter internalization kinetics (Fig. 11, lower left panel; Table 1). In the case of the $A^{263}E$ mutant, the kinetics

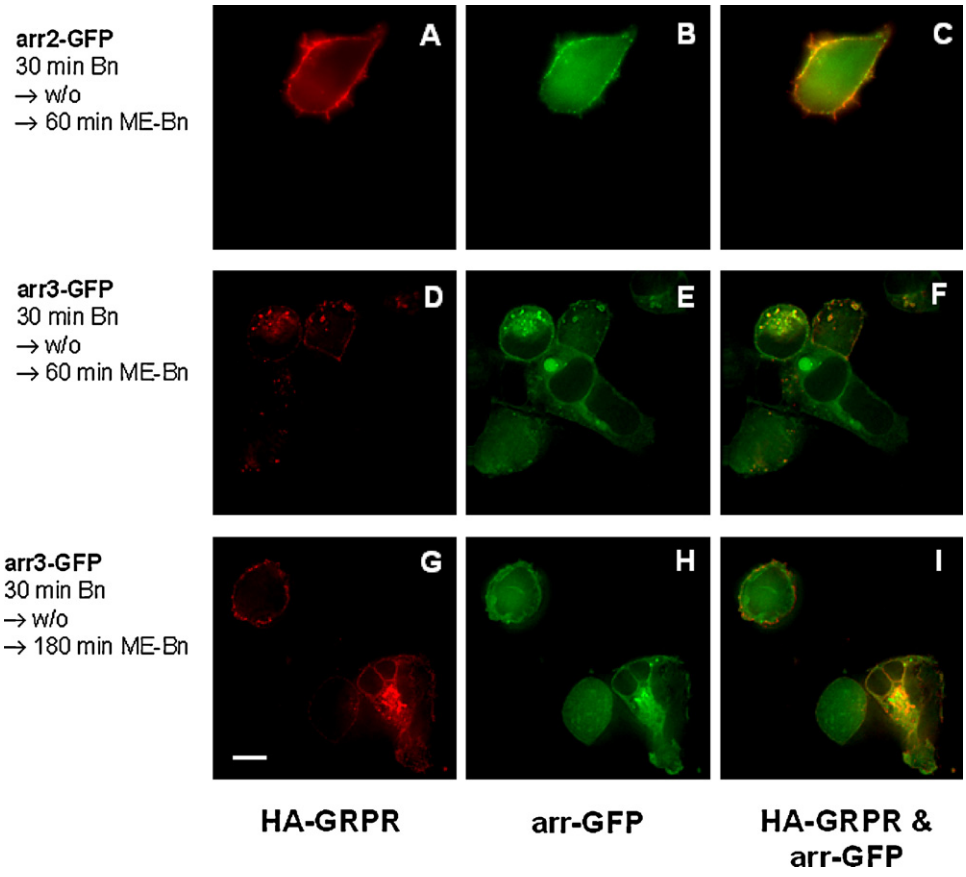

Fig. 9 – Recycling of the HA-GRP receptor to the cell membrane after GRP receptor activation. HEK 293 cells were transfected and split the following day as described in the legends of Figs. 6 and 7. On the third day after the transfection, cells were incubated with 100 nM bombesin for 30 min, washed twice with PBS (w/o) and then incubated with the bombesin receptor antagonist, [DPhe^6]Bn(6-13)methylester (ME-Bn) (1 μM) [47], for the indicated periods of time. The same cells are shown in each row. Results shown are representative of three independent experiments. Scale bar, 10 μM.

of internalization were markedly slowed compared to the wild-type GRP receptor (t_{80} − 31.9 ± 5.3 vs. 7.3 ± 0.9) ($p = 0.016$, Table 1), whereas the maximum internalization was not changed (Fig. 11, lower right panel; Table 1). With overexpression of arrestin3, the internalization rate of the A^{263}E mutant was restored to that seen with the wild-type GRP receptor (t_{80} = 7.0 ± 1.3 min) (Table 1). Arrestin2 overexpression also markedly increased the rate of internalization by the A^{263}E mutant and had a modest effect on the maximal amount internalized (Fig. 11, lower right panel; Table 1). Differences in the rescue effect of arrestin3 on internalization rate and differences in the effect on maximal internalization of A^{263}E compared to arrestin2 overexpression were significant ($p < 0.05$). The results of the pharmacological experiments on the different GRP receptor mutants suggest differences in the interaction of the GRP receptor with the two arrestin subtypes.

Table 2 – Potency and efficacy of bombesin for stimulating generation of [^{3}H]inositol phosphates in Cos-7 cells transfected with wild-type or mutant GRP receptors

	EC_{50} (nM Bn)	[^{3}H]IP (fold increase)
Wild-type GRP receptor	0.15 ± 0.03	7.71 ± 0.98
R^{139}G[GRP receptor]	0.40 ± 0.10[a]	1.99 ± 0.10[a]
A^{263}E[GRP receptor]	0.48 ± 0.10[a]	5.44 ± 1.05[a]
JF-1[GRP receptor]	0.03 ± 0.01[a]	6.13 ± 0.53

Stimulation of [^{3}H]IP was determined as described in the legend of Fig. 5. EC_{50} is the concentration necessary for half-maximal increases in total [^{3}H]IP calculated using the curve-fitting program GraphPad Prism. The efficacy of [^{3}H]IP is expressed as the maximal fold increase in total [^{3}H]IP. Data are expressed as the mean ± S.E. of three separate experiments.

[a] $p < 0.05$, compared to wild-type GRP receptor.

Since the R^{139}G, JF-1, and A^{263}E mutants were not HA epitope-tagged, a polyclonal anti-GRP receptor antibody was used for immunofluorescence detection [18] (Fig. 12). Deconvolution imaging microscopy demonstrated that without prior bombesin treatment both, the wild-type GRP-R and the GRP-R mutants were localized to the cell membrane (data not shown). In arrestin2-expressing cells that were stimulated for 30 min with 100 nM bombesin, the wild-type GRP receptor was primarily localized in small intracellular vesicles (Fig. 12A1) and arrestin2-GFP was translocated to the cell membrane (Fig. 12B1); no intracellular co-localization of GRP

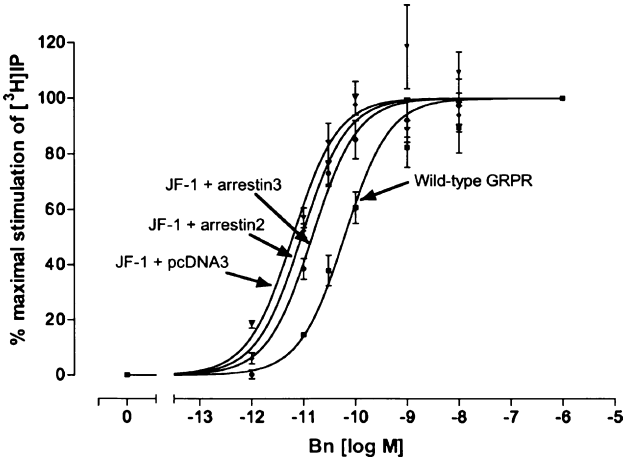

Fig. 10 – Ability of wild-type and mutant GRP receptor to activate phospholipase C and increase [^{3}H]IP alone or in the presence of high levels of wild-type arrestins. Cos-7 cells were co-transfected with wild-type or JF-1 receptor DNA (1 μg) and either empty pcDNA3, arrestin2 or arrestin3 DNA (5 μg) and loaded overnight with [^{3}H]myoinositol as described in the legend of Fig. 5. Cells were incubated with the indicated concentrations of bombesin for 1 h at 37 °C and [^{3}H]IP generation was measured as described in Section 2. Results are expressed as the percentage of the maximal stimulation seen with 1 μM bombesin with the wild-type GRP receptor or with the JF-1 mutant co-transfected with vector only. The control and maximal values for the wild-type GRP receptor with empty vector were 2182 ± 328 and 16,603 ± 3081 dpm, respectively (n = 3), and for the JF-1 mutant transfected with empty vector were 2002 ± 300 and 8220 ± 849 dpm, respectively. Data are expressed as the mean ± S.E. of three independent experiments. Curve-fitting was done using non-linear regression with GraphPad Prism3 software.

receptor and arrestin2-GFP was seen (Fig. 12C1). In contrast, with arrestin3-GFP overexpression, both the GRP receptor and the arrestin3-GFP co-localized to hollow vesicles (Fig. 12C2). The JF-1 mutant showed a reduced fraction of intracellular receptor after addition of 100 nM bombesin after transfection with either arrestin2 (Fig. 12D1) or arrestin3 (Fig. 12D2). However, the level of activation of the JF-1 mutant was sufficient to translocate both arrestins to the cell membrane (Fig. 12E1 and E2). With arrestin3-GFP transfection, there was no co-localization of JF-1 and arrestin3-GFP in hollow vesicles (Fig. 12F2), suggesting a role for the C-terminus of the receptor in the stability of the GRP receptor arrestin3 interaction. In the $R^{139}G$ mutant, only a small fraction of the receptor was internalized after addition of 100 nM bombesin after transfection with either arrestin2 (Fig. 12G1) or arrestin3 (Fig. 12G2). In this GRP receptor mutant, translocation of both arrestins to the cell membrane was minimal (Fig. 12H1 and 12H2). The effect of arrestin co-expression on $A^{263}E$ trafficking was not investigated by deconvolution imaging microscopy because the anti-GRP receptor antibody binds to an epitope localized in the GRP receptor third intracellular loop sequence and no signal was obtained with the intracellular loop 3 mutant $A^{263}E$ [18] (data not shown).

4. Discussion

This study was done to explore the mechanisms that regulate the continued signaling of the GRP receptor, that is a member of the mammalian Bn receptor family [17]. Previous studies have shown that, after activation, the receptor preferentially couples to $G\alpha_q$, which activates phospholipase C and initiates signaling via the second-messengers 1,4,5-inositol-tris-phosphate and diacylglycerol [15,17]. These signaling events also activate the negative feedback loop that leads to the phosphorylation of the GRP receptor by GRK2 and protein kinase C to acutely desensitize the GRP receptor [19]. It is shown in other GPCRs that mechanistic correlates of acute desensitization include a reduction in affinity of the GPCR for the G protein by receptor phosphorylation, binding of arrestins to the phosphorylated receptor as well as the internalization of GPCRs from the cell membrane into intracellular compartments [3,49]. A recent publication by Ally et al. [20] provides support for a role of arrestins in acute desensitization of the GRP receptor. In the present study, we provide evidence for a role of arrestins in internalization and desensitization of the GRP receptor. In contrast to recent studies on other GPCRs, we found a distinct pattern of interaction for the two arrestin subtypes with the GRP receptor.

A number of our findings support the conclusion that the internalization of the GRP receptor is clathrin-, arrestin- and dynamin-dependent. First, classical inhibitors for clathrin-coated vesicle endocytosis decrease GRP receptor internalization in both native GRP receptor containing cells and HEK 293 or Cos-7 cells transfected with the GRP receptor; similar results were published by Grady et al. when GRP-R transfected KNRH cells were used [32]. Second, dominant-negative mutants of arrestins and dynamin partially inhibit GRP receptor internalization. Third, overexpression of arrestins increases internalization of the GRP receptor. Both, overexpression of dominant-negative mutants and wild-type constructs of arrestins and dynamin are well-established approaches to characterize a receptor's internalization mechanism as either arrestin- and dynamin-dependent [4,5,50,51] or independent [5,6,50]. Fourth, microscopic studies reveal a rapid translocation (<2 min) of both arrestin subtypes to the GRP receptor at the cell membrane. This is followed by the trafficking of the receptor to rab5-containing early endosomes, the intracellular compartment that was previously shown to receive endocytosed GPCRs by a dynamin-dependent mechanism [46,52].

Furthermore, the results of this study point to a role for arrestins in GRP receptor desensitization. First, overexpression of both arrestin-subtypes reduces the ability of GRP receptor activation to stimulate PLC activity, which is one of its principal intracellular mediators [17,28,53]. Second, overexpression of arrestin3 rescues the potency of agonists to stimulate PLC activity with the deregulated GRP receptor mutant JF-1, which lacks carboxyl serine and threonine and demonstrates markedly decreased internalization and association with arrestin3 intracellularly. Either an increase in the

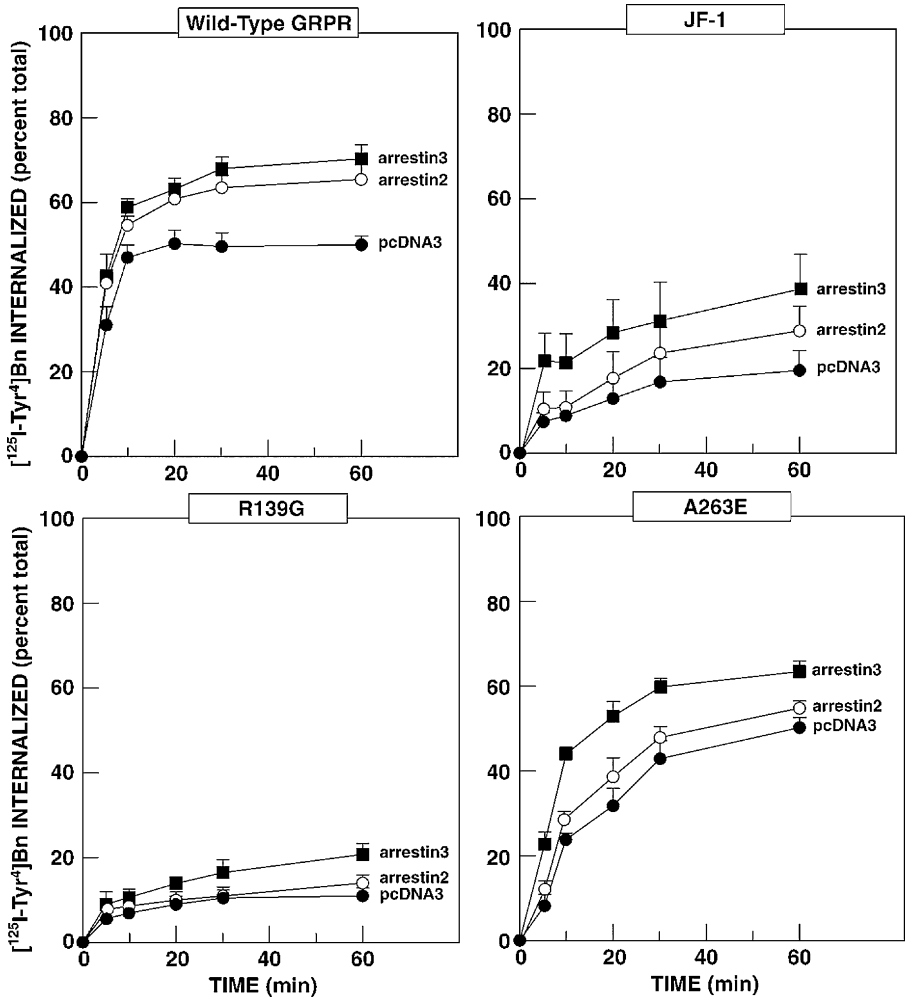

Fig. 11 – Effect of high levels of arrestins on the rate and magnitude of internalization of [^{125}I-Tyr^{4}]Bn by wild-type and mutant GRP receptors. Cos-7 cells were co-transfected with wild-type or three different mutant GRP receptor construct DNAs (1 μg) and either empty pcDNA3, arrestin2, or arrestin3 DNA (5 μg). Receptor internalization assays were carried out as described in the legend of Fig. 4. Data are expressed as the mean ± S.E. of the percentage of total saturable bound [^{125}I-Tyr^{4}]Bn that was not removed by acid-stripping from three separate experiments. Curve-fitting was done using non-linear regression with GraphPad Prism3 software.

extent of constitutively internalized receptors in arrestin-overexpressing cells or mechanisms different from receptor endocytosis (e.g. the physical prevention of the interaction between the GRP receptor and its cognate G protein by arrestins) could account for these effects [3,49]. These data agree with results on some GPCRs [2,54], where arrestins play a role in receptor desensitization. However, there are other GPCRs that are desensitized independently from arrestins [54].

Although this study was certainly not designed to find out subtle differences in the trafficking of arrestin2 and arrestin3 it has not escaped our notice that the two arrestins show different patterns of interaction with the GRP receptor. After GRP-induced translocation of both arrestins to the receptor at the cell membrane, arrestin3 but not arrestin2 further associates with the GRP receptor and follows the receptor to an endosomal compartment (Table 3). Also, the retardation of the recycling of the GRP receptor to the cell membrane by arrestin3 might hint at a higher ''stability'' of the arrestin3-GRP receptor complex. In previous extensive studies on a number of other GPCRs, Oakley et al. [55] showed that arrestin2 and arrestin3 have the same ''intracellular trafficking routes'' with the GPCR, although there are differences in intracellular arrestin traveling between different G protein-coupled receptors. Specifically, Oakley et al. divided G protein-coupled receptors into subgroups according to their intracellular trafficking with arrestins (Class A receptors that transiently interact with both arrestins at the cell membrane and Class B receptors that co-localize with both arrestin subtypes not only at the cell membrane but also later in endosomes, Table 3). However, it was also shown that arrestin3 translocates faster and to a higher extent than arrestin2 to Class A receptors, which was speculated to be secondary to differences in the affinity of the two arrestin subtypes for a GPCR [55]. This finding is in agreement with work done by Gurevich et al. who have shown *in vitro* that both the m2 muscarinic cholinergic receptor and the β2 adrenergic

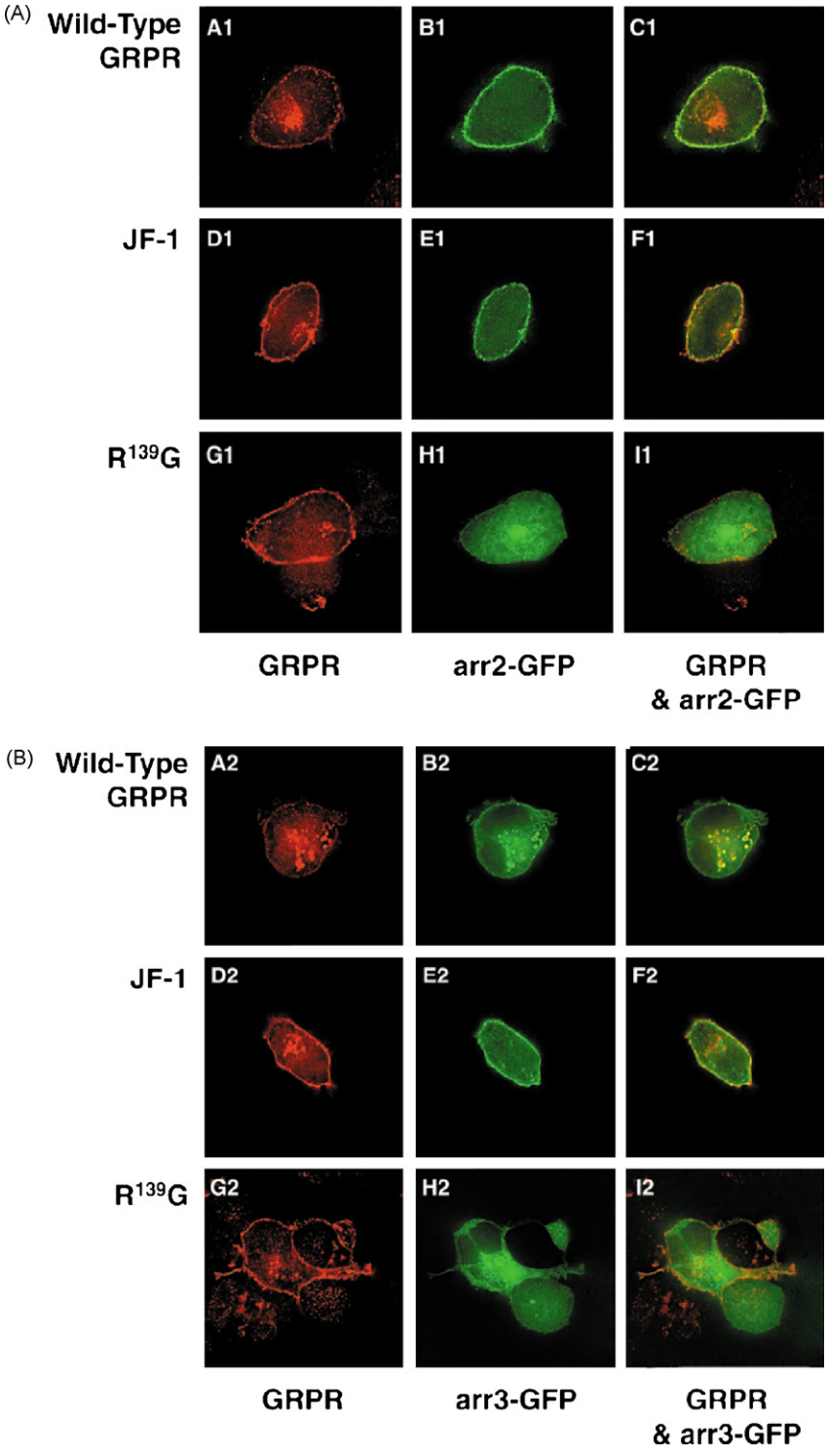

Fig. 12 – Localization of GRP receptor mutants and arrestin-GFP upon receptor stimulation. HEK 293 cells were co-transfected with 1 μg of wild-type or mutant GRP receptor DNA construct and 1 μg of either arrestin2-pEGFPN1 (panel A) or arrestin3-pEGFPN1 construct DNA (panel B) as described in the legends of Figs. 6 and 7. On the third day after transfection, cells were treated for 30 min with bombesin (100 nM) and immunostaining was performed using a native GRP receptor-antiserum as described in Section 2. The same cells are shown in each row. The top panels show cells expressing wild-type or mutant GRP receptor and arrestin2-GFP. The bottom panels show cells expressing wild-type or mutant GRP receptor and arrestin3-GFP. Results shown are representative of three independent experiments.

Table 3 – Comparison of microscopic findings in HEK cells co-expressing GRP receptor and arrestin-GFP constructs with the properties of Class A and Class B receptors delineated by Oakley et al. [55]

	Class A receptor	Class B receptor	GRP receptor and arrestin2	GRP receptor and arrestin3
Translocation to PM	Arr3 > Arr2	Arr3 = Arr2	Within 2 min	Within 2 min
Co-localization with GPCR	At PM	PM and C-vesicles	Only at PM	At PM and C
Arrestin localization	Stays at PM	Travels with GPCR	Arr2 stays at PM	Arr3 goes with GPCR
Recycling within	30 min	Hours	Within 1 h	≫1 h
Late association with arrestin	–	+	–	+

Class A and Class B receptor characteristics are from Oakley et al. [55]. GRP-R results with arrestin2 and arrestin3 were obtained from imaging studies of HEK cells transfected with HA-GRP receptor-pcDNA3 and arrestin2- or arrestin3-pEGFPN1 performed as described in the legends of Figs. 6, 7 and 9.
Abbreviations: PM, plasma membrane; GPCR, G protein-coupled receptor; Arr3, arrestin3; Arr2, arrestin2; C, cytosol.

receptor bind with a higher affinity to arrestin3 compared to arrestin2 [56].

Previous studies provide evidence that various G protein-coupled receptor domains may be particularly important for interaction with arrestins, especially the receptor carboxyl terminus [45,57–59]. To determine whether differential interaction of arrestins with the carboxyl terminus of the receptor or another receptor region might contribute to the differences seen with arrestin2 and arrestin3, studies were performed with mutated GRP receptors. Since it was previously shown that C-terminal serines and threonines of a GPCR are major determinants for Class A or Class B type interactions with arrestins [58], we examined the ability of the previously characterized GRP receptor mutant, JF-1, to interact with arrestin. The mutation of all C-terminal serines and threonines, which are putative phosphorylation sites for GRKs and PKC resulted in a markedly impaired receptor endocytosis [21]. Since arrestin3 overexpression substantially rescues the internalization of the JF-1 GRP receptor mutant, whereas overexpression of arrestin2 had only a minimal effect, the carboxyl terminus appears to be less essential for arrestin3 than arrestin2 GRP receptor interaction. Interestingly, microscopic studies showed that agonist stimulation of the JF-1 GRP mutant was able to stimulate translocation of arrestin3 and arrestin2 to the cell membrane. Apparently, the removal of all phosphorylation sites from the receptor carboxyl terminus does not completely abrogate the interaction with either arrestin. These results suggest that both arrestin2 and arrestin3 also interact with other GRP receptor domains. However, we did not find any intracellular (endosomal) co-localization of the JF-1 receptor mutant and arrestin3 with agonist stimulation, suggesting that C-terminal receptor phosphorylation sites are crucial for the late association of arrestin3 with the GRP receptor. The receptor C-terminus is known to be important for a number of non-GI hormone GPCRs (β_2-adrenergic, vasopressin V2, neurotensin-1, oxytocin, AT-1_A, endothelin A and B receptors [45,57–59]), but there is no evidence for a discriminating function of the receptor C-terminus for specific arrestin interaction. Studies on vasopressin V2 and V1A receptor chimeras have revealed a function for the V2 receptor C-terminus in the retardation of the receptor recycling involving both arrestins and the small GTPase rab11 [59]. No arrestin subtype-specific experiments were carried out in the vasopressin receptor study but our results lead one to speculate that arrestin3, rather than arrestin2, was responsible for this effect.

We explored the cellular mechanisms responsible for stimulation of receptor–arrestin interaction after GRP receptor activation. Also, we attempted to provide insight into the GRP-R amino acid sequences that might be involved in the interaction with the arrestins, by using various three GRP receptor mutants which have been extensively investigated in previous studies [20–24]. One GRP receptor mutant had a substitution in the 2nd intracellular loop ($R^{139}G$) and in previous studies [22,23] was shown to have decreased receptor affinity, decreased G protein coupling and impaired stimulation of phospholipase C; a third intracellular loop mutant ($A^{263}E$) which has decreased receptor affinity, full G protein coupling but impaired ability to activate phospholipase C [22,23] and finally a GRP receptor mutant in which all C-terminal serines and threonines were substituted with alanine or glycine (JF-1) which has full affinity, potency for phospholipase C activation but has impaired internalization [21,23]. In the present study, the JF-1 mutant had enhanced potency for phospholipase C activation whereas the other two had a decrease in potency likely due to difference in internalization, receptor affinity or G protein coupling. We found in the present study that the i3 loop Ala-263 residue – although important for GRP-R endocytosis in general – does not interact with arrestins, since the endocytosis deficit of the $A^{263}E$ mutant can be completely rescued by arrestin overexpression. The i2 loop Arg-139 residue might have a function in the immediate interaction of arrestins with the GRP-R since arrestin overexpression does not rescue the markedly reduced receptor endocytosis of this mutant. Alternatively, this effect is due to the decreased ability of this mutant to activate PLC [20,22,23]. However, it has already been shown that minimal levels of PLC activation can account for a substantial extent of internalization of other GRP receptor mutants [26], and this study shows some PLC activation for this receptor mutant. Furthermore, arginine residues in two other GPCRs that align with the Arg-139 of the GRP receptor are important for the interaction with arrestin [64,65]. Also, a CCR5 i2 loop peptide that includes this arginine residue has higher affinity to bind arrestin2 than the mutant CCR5 peptide lacking arginine [66].

In conclusion, we show that GRP receptor internalization and its regulation of PLC activity are arrestin-dependent. GRP receptor activation induces intracellular trafficking of both arrestin subtypes. The difference in the intracellular traffick-

ing routes of arrestin2 and arrestin3 after activation by the GRP receptor appears to be linked to C-terminal serines and threonines of the GRP-R. Other GRP receptor amino acid residues, that are important for receptor internalization, also had effects on receptor–arrestin interaction.

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