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Agonist-induced internalization and desensitization of the apelin receptor

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Abstract

Apelin acts via the G protein-coupled apelin receptor (APJ) to mediate effects on cardiovascular and fluid homeostasis. G protein-coupled receptor (GPCR) trafficking has an important role in the regulation of receptor signalling pathways and cellular functions, however in the case of APJ the mechanisms and proteins involved in apelin-induced trafficking are not well understood. We generated a stable HEK-293 cell line expressing N-terminus HA-tagged mouse (m) APJ, and used a semi-automated imaging protocol to quantitate APJ trafficking and ERK1/2 activation following stimulation with [Pyr1]apelin-13. The mechanisms of [Pyr1]apelin-13-induced internalization and desensitization were explored using dominant-negative mutant (DNM) cDNA constructs of G protein-coupled receptor kinase 2 (GRK2), β-arrestin1, EPS15 and dynamin. The di-phosphorylated ERK1/2 (ppERK1/2) response to [Pyr1]apelin-13 desensitized during sustained stimulation, due to upstream APJ-specific adaptive changes. Furthermore, [Pyr1]apelin-13 stimulation caused internalization of mAPJ via clathrin coated vesicles (CCVs) and also caused a rapid reduction in cell surface and whole cell HA-mAPJ. Our data suggest that upon continuous agonist exposure GRK2-mediated phosphorylation targets APJ to CCVs that are internalized from the cell surface in a β-arrestin1-independent, EPS15- and dynamin-dependent manner. Internalization does not appear to contribute to the desensitization of APJ-mediated ppERK1/2 activation in these cells.

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1. Introduction

The apelin gene encodes a preproprotein of 77 amino acids that is processed into multiple shorter peptides including apelin-36, apelin-17, apelin-13 and apelin-12 (Tatemoto et al., 1998). Apelin-13 may undergo post-translational modification leading to the formation of a more stable and biologically active pyroglutamyl form, [Pyr1]apelin-13. Apelin acts via the single apelin receptor (APJ) subtype to mediate effects on the cardiovascular system (Reaux et al., 2001; Ishida et al., 2004), fluid homeostasis (O’Carroll and Lolait, 2003), glucose metabolism (Dray et al., 2008), and food intake (Taheri et al., 2002), influencing not only cAMP production but also PKC, PI3K, protein kinase B (Akt), S6 ribosomal protein kinase (p70S6K), ERK (Masri et al., 2002, 2004) and cytoplasmic Ca2+ concentration (Choe et al., 2000). APJ couples to G_i/o in assays measuring extracellular acidification rates (Hosoya et al., 2000) and phosphorylation of ERK and p70S6 kinase (Masri et al., 2002, 2004), and activates ERK1/2 and inhibits adenylate cyclase through G_q/11 and G_{q/11}-dependent pathways (Masri et al., 2006; Bai et al., 2008). However apelin activation of ERK1/2 is mediated via PKC in HEK293 cells expressing mouse APJ, indicative of coupling to either G_o or G_{i/o} (Masri et al., 2002). Additionally the beneficial inotropic effect of apelin in vivo is only partially abrogated by pertussis toxin (PTX) and by PKC inhibitors, indicating that some of the actions of APJ could be mediated by G_{q/11} and/or G_{i/o} coupling (Szokodi et al., 2002). Recently it has been shown that mechanical stretch signals via APJ to induce myocardial hypertrophy by a G protein-independent, β-arrestin-dependent pathway (Scimia et al., 2012). Interestingly APJ, when stably expressed in CHO cells, shows ligand


standing of the system of regulation that dynamically modulates ascertainment of the precise function of this receptor is under-
apelin-36, on inhibition of adenylyl cyclase and activation of ERK
mologous desensitization to effects of apelin-13, but not to those of
ported to occur for APJ-mediated effects on cytoplasmic Ca\textsuperscript{2+}
that have a brief interaction with
arrestin1 and then undergo rab-7-dependent traf
endosomes (Evans et al., 2001; Lee et al., 2010). Finally, truncation of the APJ C-terminus (in
apelin-36 stimulation the internalized APJ are co-localized with
cladthrin-mediated endocytosis (CME) (Wolfe and Trejo, 2007), and
process include epsin and EPS15, which act as adapter proteins for
vant for APJ as apelin causes clathrin-mediated APJ internalization
to form endosomes (Damke, 1996).

The adaptive processes outlined above are thought to be rele-
vant for APJ as apelin causes clathrin-mediated APJ internalization
(Reaux et al., 2001; El Messari et al., 2004) and also translocation of
-arrin1 and -2 to the cell surface, indicating translocation to
phosphorylated APJ (Lee et al., 2010). Moreover, after agonist-
induced internalization, APJ can either be recycled to the cell sur-
faced or be degraded in lysosomes (Lee et al., 2010). Interestingly, APJ
trafficking displays ligand bias for both Class A and B -arrestin/
recycling behaviour as when internalization is stimulated by [Pyr\textsuperscript{1}]
apelin-13, internalized APJ is rapidly recycled to the plasma
membrane with none remaining in the cytoplasm at 60 min, whereas APJ
is retained within the cell for up to 120 min after
apel-36-stimulated internalization (Zhou et al., 2003). Similarly,
although apelin-13 causes -arrestin1 translocation to the plasma
membrane, the internalized receptors are not associated with
-arrin1 and are rapidly recycled to the cell surface via early
endosomes (Evans et al., 2001; Lee et al., 2010), whereas after
apel-36 stimulation the internalized APJ are co-localized with
-arrin1 and then undergo rab-7-dependent trafficking to lysos-
omes (Lee et al., 2010). Finally, truncation of the APJ C-terminus (in
order to delete potential GRK phosphorylation sites) prevents
homologous desensitization to effects of apelin-13, but not to those of
apel-36, on inhibition of adenyly cyclase and activation of ERK and
Akt (Masri et al., 2006; Lee et al., 2010).

Apelin/APJ has emerged as a major signalling pathway in
physiological homeostasis (O’Carroll et al., 2013) and central to
ascertaining the precise function of this receptor is an under-
standing of the system of regulation that dynamically modulates
APJ signalling. In peripheral tissues the apelinergic system appears
to be down-regulated in hypertensive disease – levels of apelin
immunoreactivity in plasma, and in ventricular and aortic tissues,
are lower in the spontaneously hypertensive rat, a genetic model of
hypertension, than in control Wistar-Kyoto normotensive rats
(Zhang et al., 2006a,b; Zhong et al., 2005). Additionally circulating
levels of apelin are decreased in patients with essential (Sonmez
et al., 2010) and pulmonary (Chandra et al., 2011) hypertension,
while there is a negative correlation between plasma apelin levels
and blood pressure (Zhu et al., 2013). This suggests a role for
decreased peripheral apelin signalling in the pathophysiology of
hypertension. Receptor trafficking is a key process for regulating
receptor signalling pathways and cellular functions, however in the
case of APJ the mechanisms and proteins involved in agonist-
induced trafficking are not well understood. To further under-
stand the signalling and regulation of APJ, and thus the efficacy of
ligands for potential therapeutic intervention, this study set out to
characterize the mechanisms underlying [Pyr\textsuperscript{1}]-apelin-13-induced
APJ desensitization and internalization, and to determine
whether agonist-induced APJ internalization contributes to its
functional desensitization. A stable HEK-293 cell line expressing N-
terminus HA-tagged mouse APJ (mAPJ) was generated, and a
semiautomated imaging protocol was used to quantitate ERLK1/2 activ-
ation and APJ trafficking in this cell line following agonist activa-
tion with [Pyr\textsuperscript{1}]-apelin-13. The mechanisms of [Pyr\textsuperscript{1}]-apelin-13-
induced internalization were further explored using dominant-
negative mutant (DNM) cDNA constructs of GRK2 (GRK\textsuperscript{DNM}),
-arrin1 (\textit{\textit{\alpha}}ARR\textsuperscript{DNM}), EPS15 (EPS\textsuperscript{DNM}) and dyanin (DYN\textsuperscript{DNM}),
known effectors of CME.

2. Methods and materials

2.1. Materials and cell culture

DMEM, FCS, penicillin (P), streptomycin (S), normal goat serum
(NGS), Alexa Fluor 488 goat anti-mouse IgG (H + L) and Alexa Fluor
546 goat anti-rabbit IgG (H + L) were purchased from Life Tech-
nologies (Paisley, UK). cDNA encoding DNMVs (provided by Professor
Eamonn Kelly, University of Bristol) included GRK\textsuperscript{DNM} (K220R),
\textit{\textit{\alpha}}ARR\textsuperscript{DNM} (319–418), EPS\textsuperscript{DNM} (EA95/295) and DYN\textsuperscript{DNM} (K44A).
Anti-HA antibody was from Cambridge Bioscience (Cambridge,
UK); rabbit anti-ERL1/2 antibody was from Cell Signalling Tech-
nology UK; Hercules II Fusion DNA polymerase was from Agilent
Technologies (Stockport, UK), Nanofectamin was purchased from
PAA Laboratories (Somerset, UK), and 4',6-diamidino-2-
phényldole (DAPI), adrenaline, EGF and mouse anti-ppERK1/2
antibody were from Sigma-Aldrich (Dorset, UK). [Pyr\textsuperscript{1}]-apelin-13
was purchased from Bachem (Bubendorf, Switzerland). Pertussis
toxin (PTX), bisindolylmaleimide I (BIM) and 1,4-diamino-2,3-
dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126) were
from Merck Chemicals (Nottingham, UK).

HEK293 cells, unless otherwise stated, were cultured in 10% FCS-
supplemented DMEM containing glutamine (4 mM) and P/S
(500 units/ml; 0.5 mg/ml). Cultures were maintained at 37 °C in 5% CO\textsubscript{2}. For imaging studies cells were seeded at 17,500 per well into
Costar black-walled 96-well plates (Corning, Arlington, UK).

2.2. Stable and transient transfection

Untagged and HA-tagged mouse (m)APJ cDNAs were generated by
PCR using 150 ng mouse 129/Sv genomic DNA (PCR conditions:
95 °C 2 min; 40 cycles of: 94 °C 45 s, 50 °C 1 min, 72 °C 1 min; and
final extension of 72 °C 10 min) using Hercules II Fusion DNA po-
lymerase. The integrity of the cDNA constructs was verified by DNA
sequencing. Primers for the untagged receptor were directed to 5'
and 3’-regions of mAPJ and corresponded to 8462–10,285 bp of the
mouse APJ gene (Genbank Accession number AC117228.2), generating a 1824 bp product. Primers for the tagged receptor were also directed to 5′ and 3′ regions of the receptor, but the 5′ primer contained an additional 27 bp, which coded for the Influenza HA epitope tag and generated a 1851 bp mouse product. The mAPJ gene, in the pcDNA3.1(+) vector (containing the neomycin resistance gene), was transfected into HEK293 cells by a calcium phosphate procedure (Chen and Okayama, 1988) and selected by G418. Stable cell lines highly expressing APJ were selected by the manufacturer’s protocol, with DNMM cDNAs (0.4 μg/well) was performed with Nanofectamin according to the manufacturer’s protocol, with DNMM cDNA-containing medium removed after 4 h and replaced with fresh DMEM (0.1% FCS). Alongside each transfection, transfection efficiency was estimated using a 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside (X-gal) staining assay. Control cells were transfected with a mammalian vector inserted with a LacZ gene (pSV-β-Galactosidase control vector; Promega, UK), and subsequent beta-galactosidase (β-gal) activity estimated from the percentage of blue cells. Approximately 40% transfection efficiency was observed with Nanofectamin with HEK293 cells, that did not deviate significantly between experiments.

2.4. ERK phosphorylation assay

Cell expression of total (terK) and di-phosphorylated ERK (ppERK) was visualized in stably transfected HEK293 cells with an immunocytochemistry protocol employing anti-terK and -ppERK antibodies. Quantification of ERK phosphorylation was performed by incubating mAPJ-HEK293 cells at 37 °C with [Pyr1]apelin-13 (100 nM) in DMEM (0.1% FCS) for 5 min. To explore homologous and heterologous desensitization mAPJ-HEK293 cells were pre-incubated for 2 h with medium in the presence or absence of [Pyr1]apelin-13. This time point has been used in previous studies on desensitization of APJ (Masri et al., 2006). Cells were then washed (×2) with PBS and exposed either to a second application of [Pyr1]apelin-13 (100 nM, 5 min) or to other ERK inducers, (adrenaline (1 μM), EGF (100 ng/ml), 5 min). Resensitization was monitored by varying the period between primary and secondary agonist incubation. For assays with DNMM cDNAs, mAPJ-HEK293 cells were transiently transfected with DNMM cDNAs before incubation with [Pyr1]apelin-13.

After experimental treatment cells were immunostained with primary antibody (mouse anti-ppERK1/2 (1:1600 dilution) or rabbit anti-ERK1/2 (1:800 dilution) in 1% NGS in PBS, 4 °C, overnight). Cells were then washed 3× and blocked with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L) or Alexa Fluor 546 goat anti-rabbit IgG (H+L) (1:500 dilution, 90 min)). Cells were washed (3×) with PBS, stained with DAPI and washed (2×) with PBS as above, before imaging. Responses for desensitization experiments are expressed as a percentage of maximal response, where the maximal response is defined as cells pre-treated with vehicle and then stimulated with [Pyr1]apelin-13.

2.5. Semi-automated image acquisition and analysis

Assays were quantified by semi-automated acquisition of digital fluorescence images using a high content imaging platform (In Cell Analyzer 1000, GE Healthcare UK) and validated algorithms for image segmentation and quantitation (In Cell Analyzer version 1.0 software) as described (Finch et al., 2008). Digital images were taken with a 10× objective (Plan Apochromat, numerical aperture 0.45), with excitation and emission filters for each channel as follows, blue (360 ± 40 nm; 460 ± 40 nm), green (475 ± 20 nm; 535 ± 50 nm), and red (535 ± 50 nm; 620 ± 60 nm) using a 61002 trichroic mirror. Four fields were acquired per well (each field capturing a 0.602 mm² area with a 10× objective), obtaining an average of 1000 cells per well.

For most experiments (cell surface or whole cell HA-mAP) measures, and whole cell ppERK measures) image analysis software (In Cell 1000 Multi-target Analysis) was used to define the perimeter of the nucleus (from the DAPI stain) and the perimeter of the cell (from the HA or ppERK stain). Average fluorescence intensity over the entire cell area was calculated for each cell and background values (obtained with no primary antibody) were also determined. The figures show background subtracted and population averaged data in arbitrary fluorescence units (AU). In most cases these are expressed as percentage of a vehicle control and for some experiments proportional cell surface expression was also calculated (PCSE; cell surface expression × whole cell expression) × 100). In receptor internalization assays, agonist exposure caused the internalized receptors to redistribute into punctate regions (presumably endosomes) in the cytoplasm and these “inclusions” were quantified using a Dual Area Analysis Algorithm (In Cell Analyzer version 1.0). The nuclear perimeter was determined from the DAPI stain and this was expanded with a 2 μm collar. The image analysis gave the number of inclusion over the
collar and nucleus for each cell and figures show population averaged inclusion counts. The agonist-induced appearance of the antibody in puncta is consistent with the wealth of data showing agonist-induced internalization of these and other GPCRs. We have previously used this methodology to investigate agonist-induced internalization of gonadotropin-releasing hormone receptors (Finch et al., 2009).

2.6. Statistical analysis

IN Cell Analyser 1000 experiments were performed in 3 replicate wells with triplicate fields within each well, and experiments were performed at least 3 times. Data are expressed in figures as mean ± SEM. Statistical analysis was with a one-way ANOVA and post hoc Dunnett’s test with GraphPad Prism software (version 4.0b) (as detailed in figure legends). p < 0.05 was considered as statistically significant.

3. Results

3.1. Imaging of HA-mAPJ in HEK293 cells and the ppERK response to [Pyr1]apelin-13

To facilitate functional characterization of APJ, a stable HA-mAPJ expressing cell line was generated. In the first experiments receptor expression was confirmed by immunohistochemical detection of the HA tags using automated image acquisition and analysis. As anticipated, essentially all cells expressed HA-mAPJ, that could be detected in permeabilized cells and also when the primary antibody was added to bind the exofacial HA-tag in intact cells (Fig. 1A).

![Fig. 1A](image_url)

**Fig. 1.** (A) Thumbnail images from individual wells stained for HA-mAPJ cell surface and whole cell expression, after stimulation with [Pyr1]apelin-13. Representative regions of cell images are shown for DAPI (top panels A and C), and HA-mAPJ (bottom panels B and D) in HA-mAPJ-HEK293 cells with either non-permeabilized (left panels, cell surface) or permeabilized (right panels, whole cell) membranes, higher magnification inset. (B) Thumbnail images from individual wells stained for ppERK1/2 expression after stimulation with [Pyr1]apelin-13. Representative regions of cell images are shown for DAPI (top panels A and C), and ppERK1/2 (bottom panels B and D) in mAPJ-HEK293 cells stimulated with either vehicle control (left panels) or 100 nM [Pyr1]apelin-13 (right panels), higher magnification inset. Scale bars, 100 μm. mAPJ-HEK293 cells were pre-treated with or without (C) PTX (200 ng/ml, 16 h), (D) BIM (10 μM, 1 h) or (E) UO126 (10 μM, 30 min) and stimulated in the presence or absence of [Pyr1]apelin-13 (100 nM) for 5 min. (F) Tagging of mAPJ with the HA epitope did not interfere with receptor signalling. Non tagged mAPJ-HEK293 cells and HA-tagged mAPJ-HEK293 cells were stimulated with [Pyr1]apelin-13 (100 nM) for 5 min and compared with control cells treated with 1× PBS. For (C–F) cells were fixed, stained, and imaged for determination of whole-cell ppERK1/2 intensity using anti ppERK1/2 antibody. The value determined with no primary antibody present was designated as background and was subtracted from raw data to give arbitrary fluorescence units (AFU) and then normalized to a percentage of vehicle control. Data shown are mean ± SEM, of at least three separate experiments, each with triplicate wells and triplicate fields within wells. *p < 0.05, **p < 0.01, and ***p < 0.001 comparing stimulations to basal conditions, analysed by one-way ANOVA and Dunnett’s multiple comparison post hoc tests. ns – no statistical significant difference.
We used the pyroglutamyl form of apelin-13, [Pyr1]apelin-13, the most potent and abundant form in the brain (De Mota et al., 2004) and cardiovascular system (Maguire et al., 2009), to test for expression of functional receptors and found that 5 min stimulation with 100 nM [Pyr1]apelin-13 caused a marked increase in ppERK staining over the cytoplasm and nucleus of mAPJ-HEK293 cells (Fig. 1B). This effect was prevented by pre-treatment with PTX to prevent Gi activation; with BIM to prevent PKC activation; or with U0126 to inhibit MEK (Fig. 1C). The effects of [Pyr1]apelin-13 on ppERK levels in HEK-293 cells expressing non-tagged mAPJ and HA-tagged mAPJ were also compared and were found to be indistinguishable (Fig. 1F).

mAPJ-HEK293 cells were then treated for varied times with [Pyr1]apelin-13. The ppERK response was rapid (maximal at 5 min) and transient, reducing to near basal values by 10 min (Fig. 2A). We also varied [Pyr1]apelin-13 concentration and this revealed a concentration-dependent effect with an EC50 value of ~3 nM at 5 min (Fig. 2B). No significant variations were seen in ppERK levels in HEK-293 cells expressing non-tagged mAPJ and HA-tagged mAPJ were also compared and were found to be indistinguishable (Fig. 1F).

mAPJ-HEK293 cells were then treated for varied times with [Pyr1]apelin-13. The ppERK response was rapid (maximal at 5 min) and transient, reducing to near basal values by 10 min (Fig. 2A). We also varied [Pyr1]apelin-13 concentration and this revealed a concentration-dependent effect with an EC50 value of ~3 nM at 5 min (Fig. 2B). No significant variations were seen in

![Graph A](image)

![Graph B](image)

![Graph C](image)
total ERK1/2 levels in the mAPJ cell line stimulated with [Pyr1]apelin-13 in either time or dose response curves, consequently further experiments were conducted without measurement of total ERK levels.

3.2. Homologous and heterologous desensitization of the ppERK response to [Pyr1]apelin-13

The desensitization of the response to [Pyr1]apelin-13 was then investigated using a pretreatment protocol to test for homologous and heterologous desensitization. mAPJ-HEK293 cells were pre-treated for 2 h with 0 or 100 nM [Pyr1]apelin-13, washed and then immediately stimulated for 5 min with control medium or with medium containing 1 μM adrenaline, 100 ng/ml EGF or 100 nM [Pyr1]apelin-13. Adrenaline, EGF and apelin caused robust increases in ppERK in mAPJ-HEK293 control (PBS pre-incubated) cells (Fig. 3A, B and C). As shown (Fig. 3C), 2 h pre-incubation with [Pyr1]apelin-13 completely prevented the response to a subsequent 5 min stimulation with [Pyr1]apelin-13, but did not measurably alter the responses to adrenaline (Fig. 3A) or EGF (Fig. 3B).

3.3. Trafficking of HA-mAPJ

Following the lack of heterologous desensitization described above, that implies that the desensitization of the response to [Pyr1]apelin-13 may be due to upstream APJ-specific (rather than down-stream ERK-specific) adaptive mechanisms, we explored possible changes in the amount and compartmentalization of APJ by stimulating HA-mAPJ-HEK293 cells for varied periods (up to 6 h) with 0 or 100 nM [Pyr1]apelin-13 before determining cell surface and whole cell HA-mAPJ levels with the intact cell and permeabilized cell staining assays used for Fig. 1. As shown (Fig. 4A), [Pyr1]apelin-13 caused a reduction in cell surface HA-mAPJ, which reduced by >50% with a half-time of ~30 min. It also reduced whole cell HA-mAPJ (Fig. 4B) but the effect was less marked (reduction to ~60% of control) and slower (no measurable reduction until 1 h). We also used the cell surface and whole cell HA-mAPJ expression...
measures to calculate the proportional cell surface receptor expression (PCSE; cell surface expression ÷ whole cell expression) × 100) and found that in control cells ~76% of HA-mAPJ were at the cell surface and that this reduced to ~40% after 30 min stimulation with [Pyr1]apelin-13 before recovering to near control levels at 6 h (Fig. 4C).

3.4. HA-mAPJ internalization and desensitization of APJ-mediated ERK activation

To follow internalization more directly cell surface HA-mAPJ were preloaded with anti-HA antibody in the absence of agonist, and cells were then incubated for varied periods with 0 or 100 nM [Pyr1]apelin-13 before determining the number of anti-HA-containing inclusions (presumptive endosomes) by automated image analysis. As shown (Fig. 5A and B), [Pyr1]apelin-13 caused a rapid increase with the inclusion count being maximal after 30 min and remaining significantly elevated for 6 h.

This assay was also used to explore APJ internalization mechanisms using a 2 h [Pyr1]apelin-13 stimulation period. This revealed that pretreatment with hypertonic sucrose to block clathrin-mediated endocytosis completely blocked the [Pyr1]apelin-13 effect on inclusion counts (Fig. 6A). The dose-dependent effects of GRK, EPS, DYN and βARR DNMs on HA-mAPJ-HEK293 cells was then assessed. Co-transfection with the individual expression vectors for GRKDNM, EPSDNM and DYNDNM inhibited [Pyr1]apelin-13-induced HA-mAPJ internalization in a dose-dependent manner, with an optimal concentration of 0.4 μg/well, however the βARRDNM (Fig. 6B). The effects of GRKDNM, EPSDNM, DYNDNM and βARRDNM on [Pyr1]apelin-13-stimulated increase in inclusion count was not blocked by βARRDNM (Fig. 6B). The effects of GRKDNM, EPSDNM, DYNDNM and βARRDNM on [Pyr1]apelin-13-stimulated increase in inclusion count was not blocked by βARRDNM (Fig. 6B).
βARRDNM on [Pyr1]apelin-13-induced HA-mAPJ internalization are shown in Fig. 6C.

We also tested for effects of GRKDNM and DYNDNM cDNAs, both of which prevented HA-mAPJ internalization into inclusions (Fig. 6C), on the desensitization of APJ-mediated ERK activation. Acute (5 min) stimulation of mAPJ-HEK293 cells with [Pyr1]apelin-13 caused robust increases in ppERK, that did not alter in cells transfected with GRKDNM or DYNDNM cDNAs (Fig. 6D). Pre-treatment for 2 h with 100 nM [Pyr1]apelin-13 caused the expected reduction of subsequent responses to 5 min stimulation with 100 nM [Pyr1]apelin-13 in control cells (Fig. 6D, see also Fig. 3) and this reduction was also observed in cells transfected with GRKDNM or DYNDNM cDNAs (Fig. 6D).

3.5. Recovery of APJ levels after agonist removal

To explore recovery of APJ expression levels following pre-treatment with agonist, HA-mAPJ-HEK293 cells were treated for 2 h with 0 or 100 nM [Pyr1]apelin-13, washed and allowed to recover for varied periods (0–6 h) before quantification of cell surface HA-mAPJ and whole cell HA-mAPJ levels. As expected, the [Pyr1]apelin-13 pre-treatment reduced cell surface and whole cell HA-mAPJ levels by 40–50% (Fig. 7A and B; see also Fig. 4). Cell surface HA-mAPJ levels recovered slowly returning to control levels at 4–6 h after the pre-treatment (Fig. 7A), whereas whole cell HA-mAPJ levels remained low and were essentially unaltered during the 0–6 h recovery period (Fig. 7B). These data were used to calculate PCSE and this was reduced (from an initial ~76% to ~60%) by [Pyr1]apelin-13.
apelin-13 pre-treatment and recovered to almost 100% at 2–6 h after pre-treatment (Fig. 7C). A similar protocol was used to assess recovery from the effect of [Pyr1]apelin-13 on HA-mAPJ inclusion count. As expected, pre-treatment for 2 h with [Pyr1]apelin-13 increased the number of inclusions by ~75% and this effect was rapidly reversed so that there was no measurable increase in inclusions after 30 min of recovery (Fig. 7D).

3.6. Resensitization of APJ-mediated ERK activation

We then followed recovery from desensitization (of [Pyr1]apelin-13-stimulated ERK activation) in control cells and in cells transfected with GRKDNM or DYNDNM cDNAs, both of which prevented HA-mAPJ internalization into inclusions (see Fig. 6), or βARRDNM cDNA. mAPJ-HEK293 cells initially exposed to vehicle control (1 × PBS; 2 h) showed significant activation of ERK1/2 after a 5 min exposure to [Pyr1]apelin-13. However 2 h pre-treatment of mAPJ-HEK293 cells with 100 nM [Pyr1]apelin-13 caused the expected reduction in response to a subsequent 5 min stimulation with 100 nM [Pyr1]apelin-13 (Fig. 8A). When cells were allowed to recover for varied periods (0–1 h) before the second stimulus, rapid recovery was observed, with maximal recovery and no measurable desensitization after just 15 min of recovery (Fig. 8A). Recovery was slower in the presence of GRKDNM (Fig. 8B) or DYNDNM cDNAs (Fig. 8C), as for both there was no measurable recovery at 15 min and recovery was near maximal at 1 h. The presence of βARRDNM did not alter the pattern of normal resensitization of [Pyr1]apelin-13-induced ERK1/2 activation.

4. Discussion

GPCR regulation in response to agonist stimulation is common to nearly all GPCRs and is essential in physiological systems to limit persistent signalling. In this study we have investigated the [Pyr1]apelin-13-induced trafficking and desensitization of mAPJ in mAPJ-HEK293 cells using a semi-automated imaging protocol and clearly
show that HA-mAPJ internalization is a GRK2-, dynamin- and EPS15-mediated event. A stable HA-mAPJ expressing cell line was generated and was used to quantify the proportion of APJ at the cell surface and within whole cells using semi-automated acquisition and analysis of digital fluorescence images. While the majority of epitope-tagged mAPJ was localized to the cell surface in these cells, a proportion of tagged APJ was distributed within the cell. This is in contrast to earlier studies that reported enhanced green fluorescent protein (eGFP)-APJ localization, under basal conditions, to be confined to the plasma membrane (El Messari et al., 2004). APJ acts primarily via G\(_i\) to inhibit adenylyl cyclase but has also been reported to activate other effectors including PKC, PI3K and ERK (Masri et al., 2002, 2004). As positioning of differing tags into the native receptor may have implications for receptor trafficking, we verified that the functional integrity of the receptor in our cell line remained intact and that these HA-mAPJ-HEK293 cells, like their non-tagged counterparts, mediate ERK activation. Significant and similar [Pyr\(^1\)]apelin-13-induced stimulation of ERK1/2 was seen in both HA-tagged and untagged mAPJ transfected HEK293 cells.

Many GPCRs show ligand bias (where different agonists bias signalling toward different effectors) and there is evidence that this may occur for APJ (Masri et al., 2006; Brame et al., 2015). It has been shown recently that the cyclic apelin analogue MM07 displays bias towards stimulation of a beneficial G-protein-dependent pathway, stimulating vasodilation and inotropic actions, over a more damaging G-protein-independent \(\beta\)-arrestin-dependent pathway that results in cardiac hypertrophy (Brame et al., 2015). In this regard, it is also of interest that APJ is most closely related to angiotensin 1 receptors (AT\(_1\)), for which ligand bias has been extensively explored. AT\(_{1}\) receptors are G\(_{qi}\) coupled GPCRs that also activate ERK. They undergo a process of rapid homologous receptor desensitization in which arrestins bind to the activated receptors preventing them from activating their cognate G proteins and targeting them for internalization via CCVs. The arrestins can also act as scaffolds for MAPK cascade components and mediate signalling to ERK. Activation of AT\(_{1}\) receptors can cause an initial phase of G protein-mediated ERK activation followed by a switch to a second phase of arrestin-mediated ERK activation and ligand bias is seen when angiotensin II activates both pathways whereas analogues (such as [Sar\(^1\),Ile\(^4\),Ile\(^8\)]AngII (SII)) engage only the latter signaling toward different effectors) and there is evidence that this may occur for APJ (Masri et al., 2006; Brame et al., 2015). We undertook therefore to monitor APJ compartmentalization, and found that incubation of HA-mAPJ-HEK293 cells with [Pyr\(^1\)]apelin-13 decreased both cell surface and whole cell expression of APJ in a time-dependent manner. These data are consistent with agonist-induced receptor internalization followed by degradation of a proportion of the internalized receptors, such that down-regulation follows the reduction in cell surface expression.

As the experimental procedure used could reflect agonist-stimulation of both anterograde and retrograde APJ trafficking, as has been described for the \(\delta\)-opioid peptide receptor (Zhang et al., 2006a,b), receptor internalization was more directly monitored by loading cell surface HA-mAPJ with anti-HA antibody before washing and incubation with agonist. We found a rapid increase in [Pyr\(^1\)]apelin-13-induced HA-mAPJ internalization that is consistent with previous confocal microscopy studies showing rapid agonist-induced internalization of eGFP-APJ from the plasma membrane (Lee et al., 2010; El Messari et al., 2004). [Pyr\(^1\)]apelin-13-induced HA-mAPJ internalization was inhibited in the presence of sucrose, which prevents formation of clathrin-coated pits (Heuser and Anderson, 1989), and by the expression of DNMs cDNAs of GRK2, dynamin and EPS15, known effectors of CME, but not by expression of \(\beta\)ARRDNM. We have used these DNMs successfully to assess GRK2-, \(\beta\)-arrestin1-, dynamin- and EPS-dependent internalization of several GPCRs (e.g. oxytocin receptor (Smith et al., 2006); gonadotropin-releasing hormone receptor (Hislop et al., 2001, 2005)). The GRK2\(^{DNM}\) construct (K220A) reduces agonist-INDuced phosphorylation (Mundell et al., 1997); \(\beta\)ARR\(^{DNM}\) (319–418) competes with wild-type arrestin for clathrin and AP2 binding, and impairs receptor-binding ability (Krupnick et al., 1997); EPS\(^{DNM}\) (E95/295) lacks domains recognising EPS15 itself and is required for coated pit formation; and DYNDNM (K44A) inhibits dynamin-mediated scission of CCVs from the plasma membrane (Danke et al., 1994). Together these data suggest that [Pyr\(^1\)]apelin-13 causes internalization of mAPJ via CCVs, and thereby reduces cell surface mAPJ levels. In accord with many other GPCRs, we suggest that GRK2-mediated phosphorylation targets the receptors to CCVs that are internalized from the cell surface in an EPS5- and dynamin-dependent manner. Interestingly, apelin-13-internalized APJ has been reported to dissociate from \(\beta\)-arrestin1 prior to receptor internalization (Lee et al., 2010), and we have shown that the [Pyr\(^1\)]apelin-13 effect on inclusion count was not prevented by transfection with the \(\beta\)ARR\(^{DNM}\) cDNA. Although not tested, it is
likely that both β-arrestin1- and β-arrestin2-mediated endocytosis would be inhibited by transfection of the βARRDNM construct (J.L. Benovic, personal communication). Further work such as using siRNAs specifically against each arrestin will determine whether either is necessary for APJ-mediated internalization. Therefore the means by which these receptors are targeted to CCVs for internalization remains unclear. A similar dynamin-dependent, β-arrestin independent internalization has been reported in the 5-hydroxytryptamine 2A (5-HT2A) receptor (Bhatnagar et al., 2001).

Desensitization of APJ has been shown not to occur in C-terminally truncated receptors that lack the majority of the serine and threonine residues that are liable to phosphorylation (Masri et al., 2006). Phosphorylation of GPCRs following ligand activation is the first step in receptor desensitization, occurring rapidly upon exposure to the agonist, and is conducted by second messengers (e.g. protein kinase C (Benovic et al., 1985)) and upon exposure to the agonist, and is conducted by second


Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

Author contributions

A-MOC and GRP conceived and coordinated the research. GRP, ST and A-MOC designed and performed the research. SJL designed the PCR primers and assisted with generation of the cell lines. GRP, ST, CMcA, SJL and A-MOC analysed and interpreted the data. All authors contributed to the drafting and revising of the manuscript, and approved the final version of the manuscript.

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