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Molecular determinants and feedback circuits regulating type 2 CRH receptor signal integration

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ABSTRACT

In most target tissues, the adenylyl cyclase/cAMP/PKA, the extracellular signal regulated kinase and the protein kinase B/Akt are the main pathways employed by the type 2 corticotropin-releasing hormone receptor to mediate the biological actions of urocortins (Ucns) and CRH. To decipher the molecular determinants of CRH-R2 signaling, we studied the signaling pathways in HEK293 cells overexpressing recombinant human CRH-R2β receptors. Use of specific kinase inhibitors showed that the CRH-R2β cognate agonist, Ucn 2, activated extracellular signal regulated kinase in a phosphoinositide 3-kinase and cyclic adenosine monophosphate/PKA-dependent manner with contribution from Epac activation. Ucn 2 also induced PKA-dependent association between AKAP250 and CRH-R2β that appeared to be necessary for extracellular signal regulated kinase activation. PKB/Akt activation was also mediated via pertussis toxin-sensitive G-proteins and PI3-K activation but did not require cAMP/PKA, Epac or protein kinase C for optimal activation. Potential feedback mechanisms that target the CRH-R2β itself and modulate receptor trafficking and endocytosis were also investigated. Indeed, our results suggested that inhibition of either PKA or extracellular signal regulated kinase pathway accelerates CRH-R2β endocytosis. Furthermore, Ucn 2-activated extracellular signal regulated kinase appeared to target β-arrestin1 and modulate, through phosphorylation at Ser412, β-arrestin1 translocation to the plasma membrane and CRH-R2β internalization kinetics. Loss of this "negative feedback" mechanism through inhibition of the extracellular signal regulated kinase activity resulted in significant attenuation of Ucn 2-induced cAMP response, whereas Akt phosphorylation was not affected by altered receptor endocytosis. These findings reveal a complex interplay between the signaling molecules that allow "fine-tuning" of CRH-R2β functional responses and regulate signal integration.

1. Introduction

In mammals, the type 2 corticotropin-releasing hormone receptor (CRH-R2) plays critical roles in central and peripheral homeostatic mechanisms controlling energy balance [1–3] by controlling feeding behavior as well as glucose utilization and insulin sensitivity in skeletal muscle. In addition, CRH-R2 mediates important anxiolytic effects in the brain as well as cardioprotection, tissue angiogenesis and gastrointestinal regulatory effects at the periphery [4–7]. This heptahelical G-protein coupled receptor (GPCR) is activated by the family of CRH-related peptides, CRH and urocortins (Ucns), although it preferentially binds Ucn rather than CRH [8].

Similar to the closely related type 1 CRH-R (CRH-R1), the CRH-R2 can regulate diverse signaling pathways through activation of multiple G-proteins [9]. Second messenger studies suggest that the Gsα/adenylyl cyclase/cAMP, protein kinase B (PKB)/Akt and members of the family of mitogen-activated protein kinases (MAPK) are key signaling intermediates downstream of CRH-R2 activation important for physiological functions of urocortins and CRH in target cells [10–13]. The latter signaling pathway appears to involve multiple G-proteins (Gq-, Gi- and Go-) and a number of signaling molecules including, phosphatidylinositol-3 OH kinase (PI3-K), MAPK kinase 1, phospholipase C, Raf-1 kinase, tyrosine kinases, and intracellular Ca^{2+} [14–16]. Interestingly, recent studies suggest that CRH-R2–MAPK interactions appear to require exclusively G-protein dependent pathways but not β-arrestin dependent pathways [17]. This is in marked contrast to the CRH-R1, which utilizes both G-protein dependent and -independent pathways to activate the MAPK cascade [18]. Therefore, it appears that β-arrestin dependent CRH-R2 endocytosis is employed as a mechanism to terminate CRH-R2 receptor signaling rather than inducing a "second-wave" of intracellular signals.

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At present, the intracellular mechanisms employed by CRH-R2 to regulate signal integration are unknown. Studies on other GPCRs have demonstrated both synergistic and antagonistic actions of the cAMP pathway on MAPK activation [19]. The cAMP-dependent kinase PKA can modulate the functional activity of multiple intracellular proteins involved in extracellular signal regulated kinase (ERK1/2) signaling; for example in B-Raf negative cells, PKA-induced Rap1 activation leads to inhibition of Ras–Raf-1 interaction and ERK1/2 signaling. In contrast, in cells expressing B-Raf, PKA stimulates ERK1/2 phosphorylation via sequential activation of Rap-1 and B-Raf. This has important biological consequences and reflects the ability of cAMP to inhibit proliferation in many cell types but stimulate proliferation in others [19]. For some GPCRs, these cross-talk mechanisms involve feedback mechanisms targeting the receptor itself and regulating its signaling efficiency; for example, phosphorylation by PKA of the β2-adrenergic receptor switches its coupling from Gs to Gi leading to stimulation of the ERK1/2 signaling pathway [20], whereas a similar PKA action on CRH-R1 at Ser^{301} of the 3rd intracellular loop (IC3) attenuates receptor/Gq-protein coupling and ERK1/2 phosphorylation [21]. In addition to regulation of receptor-G-protein association, PKA can influence signaling potency of some GPCRs by either promoting or inhibiting receptor internalization [22,23] and thus modifying the amount of receptors expressed in the plasma membrane available for ligand binding and activation. PKA is not the only component of the cAMP/PKA pathway involved in the regulation of GPCRs signaling efficiency; A-kinase anchoring proteins (AKAPs) by association with specific GPCRs provide multivalent docking platforms for PKA, PKC, and other signaling regulators to influence spatial resolution and localization of downstream signaling events [24]. The presence of specific AKAP subtypes is crucial for propagation of ERK1/2 signal. Furthermore, MAPK, in particular ERK1/2, can inhibit GRK and β-arrestin-dependent receptor desensitization and internalization by phosphorylating β-arrestin1 at Ser-412 and GRK2 to a carboxyl-terminal serine residue (Ser-670) [25,26].

In the present study we used recombinant CRH-R2β stably expressed in HEK293 cells and specific kinase inhibitors to investigate signaling characteristics as well as potential interactions between the Ucn2 activated cAMP/PKA/AKAP and ERK1/2 cascades and the presence of feedback mechanisms regulating homologous CRH-R2β endocytosis.

2. Materials and methods

2.1. Materials

Ucn2 was purchased from Bachem UK Ltd (Helens, Merseyside, UK). Radiodinated rat [tyr°]Ucn1 was obtained from Amersham (GE Healthcare UK Ltd, Buckinghamshire England). Myristoylated PKA inhibitor, U0126 (MEK inhibitor) and forskolin were from Calbiochem/Merck Biosciences (Nottingham, UK). AKAP250 and CRH-R1/2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, and phospho-β-arrestin-1 (Ser 412) were from Cell Signaling (Chandlers Ford, Hampshire, UK); pan-arrestin and total β-arrestin-1 antibodies were from Abcam (Cambridge, UK). Secondary antibodies Alexa Fluor®405, Alexa Fluor®488, Alexa Fluor®594, Alexa Fluor®680 and gold slowfade mounting solution with DAPI were purchased from Invitorgen/Molecular Probes (Paisley, UK), and IRDye 800-conjugated goat antirabbit IgG was from Rockland Immunochemicals (Gilbertsville, PA, USA). VECTASHIELD Mounting Medium without DAPI was from Vector Laboratories, Inc. (Orton Southgate, Peterborough, UK). Cell culture media, gentamicin (G-418), Lipofectamine 2000, pcDNA3.1(+), restriction enzymes and *Pfu* polymerase were from Gibco/Invitrogen (Paisley, UK). dNTPs and DNA ladder were purchased from Bioline Ltd (London, UK). Primers were purchased from TANG (Gateshead, UK). All other chemicals were purchased from Sigma Aldrich Company Ltd (Gillingham, UK).

2.2. Transfection of CRH-R2β to HEK293 cells

Human CRH-R2β cDNAs subcloned in pcDNA3.1(+) (10 μg) were transfected in HEK293 cells using Lipofectamine 2000 reagent, according to the manufacturer's protocol. After 3 days of non-selective growth in normal growth media, followed by 15 days of growth in media containing 500 μg/ml gentamicin (G418, Gibco), clones were selected by serial dilution of surviving foci and maintained in 250 μg/ml gentamicin. The optimal concentration of gentamicin and the length of cell growth was determined by growing non-transfected HEK293 cells in media containing different concentrations of the antibiotic (50–1000 μg/ml) for 21 days and performing a cell viability MTT (thioazolyl blue) assay. After 2 months the expression of CRH-R2β was verified by RT–PCR, immunoblotting, confocal microscopy analysis, and functional assays (including cAMP production and ERK activation). At least two clones were selected and used for further studies. HEK293 cells stably expressing recombinant CRH-R2β (293-R2β) were maintained in high-glucose DMEM with Glutamax containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and 250 μg/ml gentamicin.

2.3. Transfection of β-arrestin1 siRNA to 293-R2β

Beta-arrestin 1 siRNA was purchased from QIAGEN (West Sussex, UK). AllStars negative siRNA with a 3′-AlexaFluor-488 modification (QIAGEN) was used as a control. One day before transfection 293-R2β were plated in 6-well plates in normal growth media without any antibiotics. Lipofectamine 2000 was used for the delivery of 1 nmol siRNA into cells according to manufacturer's instructions. Subsequent experiments were performed 48 h after transfections as previously described [17].

2.4. Receptor second messenger studies and Western blotting

Cells were grown in 12-well plates and when reached 70–80% confluency, media was aspirated and the cells were washed with plain DMEM. Following pre-treatment with inhibitor (U0126 10 μM) for 1 h, stimulation buffer (DMEM containing 10 mM $MgCl_2$ and 0.1 mg/ml 3-isobutyl-1-methylxanthine-IBMX) was added on the cells for 20 min at 37 °C and then cells were washed with plain DMEM and stimulated with 1–100 nM Ucn2, CRH or 10 μM forskolin for 15 min. The reaction was stopped by adding 10 μl of concentrated HCl for 15–20 min and cells were transferred in 1.5 ml tubes. After a brief spin, the cells and media were stored at −20 °C. The cAMP levels were determined by commercially available ELISA Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs Ins., Ann Arbor, MI, USA).

For MAPK phosphorylation assays, appropriate inhibitors were added for 30 min (1 μM myr-PKAi, 10 μM SQ22356, 10 μM st-Ht-31, 50 μM LY-294002, 100 μM brefeldin A) followed by stimulation of cells with 100 nM Ucn2 for various time periods (2–60 min). At the end of the stimulation period, media was removed and cells were briefly washed with ice-cold PBS and then were lysed by addition of 150 μl of 2× SDS–PAGE sample buffer (Sigma Aldrich Ltd). Solubilized material was collected, sonicated for 20 sec and boiled for further 5 min. After a brief centrifugation step (2000*g* for 30 s), protein lysates were stored at −20 °C until used. Before electrophoresis, protein extracts were centrifuged at 3000*g* for 5 min to remove insoluble material and 15 μl of the supernatant were loaded on 10% SDS–PAGE gels. For β-arrestin1 phosphorylation studies, cells were lysed in 100 μl of RIPA buffer containing protease and phosphatase inhibitors cocktail (Santa Cruz, CA, USA). Protein concentration was determined with BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA),

and 5 or 10 μg of proteins were loaded on 10% SDS–PAGE gels that run at 200 V for approximately 1 h. Proteins were electrophoretically transferred to PVDF membrane at 100 V for 1 h. After completion of transfer, the PVDF membrane was incubated with 8–10 ml Licor blocking buffer (LI-COR Biosciences, Cambridge, UK) for 1 h at room temperature with gentle agitation. To determine ERK1/2 phosphorylation the membrane was incubated simultaneously with the phospho-ERK1/2 (Thr202/Tyr204) antibody and total ERK2 antibody (dilutions 1:1500 and 1:2000, respectively) in 10 ml of Licor blocking buffer with gentle agitation overnight at room temperature. For β-arrestin1 phosphorylation, membranes were incubated simultaneously with phospho-β-arrestin1 (Ser412) and total β-arrestin1 antibodies (dilutions 1:500 and 1:1000, respectively) in 10 ml of Licor blocking buffer with gentle agitation overnight at 4 °C. After 18 h, the antiserum was removed and the membrane was washed 3 × 10 min with 15 ml TBS-T (TBS-0.1% Tween), before addition of secondary antibody conjugated to a fluorescent entity: IRDye™800-conjugated goat antirabbit IgG and/or Alexa Fluor®680-conjugated goat antimouse IgG (dilution 1:6000) in 10 ml of Licor blocking buffer with gentle agitation for 1 h at room temperature. At the end of the incubation period, membranes were washed twice with 15 ml of TBS-T and once with 15 ml of TBS. The membrane was dried, visualized and analyzed on the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK).

In some experiments PVDF membranes were treated with an alkaline phosphatase containing solution. The membrane was incubated at 37 °C overnight in 10 ml of alkaline phosphatase buffer and 1000 U of alkaline phosphatase. The following morning membrane was washed with TBS-T and incubated with the phospho-β-arrestin1 (Ser412) and total β-arrestin1 antibodies as described above. To monitor Akt phosphorylation, membranes were incubated with the phospho-Akt antibody (Ser473) (1:1000 in Licor buffer) with gentle agitation overnight at 4 °C. Following 3 washes with TBS-T, goat antimouse Alexa®Fluor 680 conjugated IgG (dilution 1:6000 in 10 ml of blocking buffer) was added for 1 h at room temperature, before visualization and analysis by the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK). Membranes were then stripped with 25 mM glycine buffer pH 2 containing 2% SDS and re-probed with total Akt antibody (dilution 1:500 in Licor blocking buffer) with gentle agitation overnight at room temperature.

2.5. Plasma membrane fractionation and β-arrestin immunoblotting

For plasma membrane fractionation, 293-R2β were grown in 10 cm Petri dishes, and when reached 80% confluency, cells were deprived from FCS for 1 h before treatment with 100 nM Ucn2 or CRH for the indicated time intervals. Membrane proteins were prepared using ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem/ Merck Biosciences, Nottingham, UK). Protein concentration was determined using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Protein fractions (5 μg) were analyzed by Western blot analysis. Following SDS–PAGE and electrophoretic transfer, membranes were incubated overnight at 4 °C with pan-arrestin antibody (recognizes both arrestins β-arrestin1 of 49 kDa and β-arrestin2 of 47 kDa) (dilution 1:500), after a 1 h incubation step in TBS-T containing 5% BSA at room temperature. Following incubation with the secondary horseradish peroxidase-conjugated antirabbit IgG (DAKO UK Ltd, Ely, UK) (1:2000 dilution in TBS-T for 1 h at room temperature), proteins were visualized using ECL reagent from Amersham/Pharmacia Biotech (GE Healthcare UK Ltd, Little Chalfont, UK).

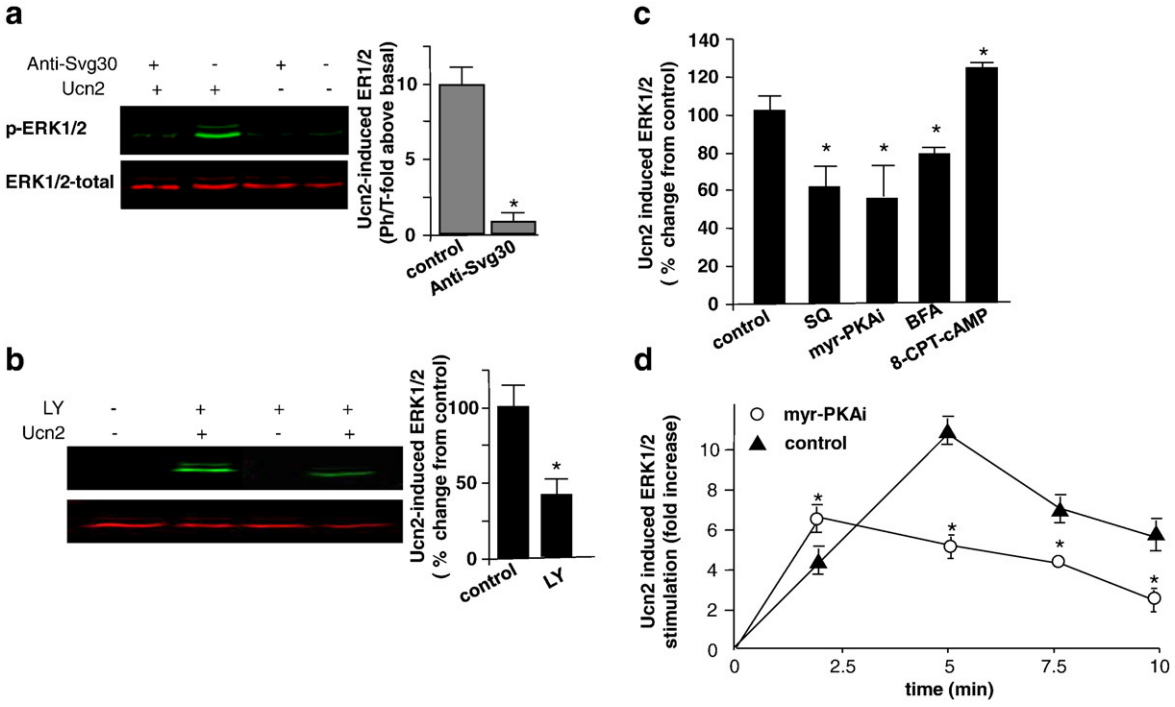

Fig. 1. Effect of CRH-R2 specific antagonists (a) and signaling molecules inhibition on ERK1/2 activation by Ucn2 in 293-R2β cells. Stimulation with Ucn2 (100 nM) for 5 min was carried out in the presence or absence of anti-sauvagine 30 (1 mM) (a) or after pre-treatment of cells with or without LY-294002 (50 μM), myr-PKAi (1 μM), SQ22356 (10 μM) or brefeldin A (BFA-100 μM) for 30 min (b–c). In some experiments (c) cells with treated with Ucn2 (100 nM) and 8-CPT-2′-O-Me-cAMP (10 μM) for 5 min. Alternatively (d) cells pre-treated with or without myr-PKAi were stimulated with Ucn2 (100 nM) for various time points (0–10 min) to determine the temporal profile of Ucn2 induced ERK1/2 activation. Following cell lysis and centrifugation, supernatants were subjected to SDS–PAGE and immunoblotted with primary antibodies for phospho-ERK1/2 (Thr204/Tyr202) and total-ERK2 to determine the phosphorylated/activated ERK1/2 and secondary antibodies conjugated to IRDye™ 800 (goat antirabbit IgG) and Alexa Fluor® 680 (goat antimouse IgG) [near-infrared (IR) fluorophore dyes] as described in Materials and methods. Top panels are representative Western blots. Data represent the mean ± SEM of 3 estimations from three independent experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between the treatment groups and controls (i.e., no antagonist or inhibitor pre-treatment; $^*P<0.05$).

2.6. Co-immunoprecipitation studies

Plasma membrane-rich fractions were prepared from 293-R2β as described above. Stimulated cells were then rinsed twice with ice-cold PBS and lysates were then incubated overnight at 4 °C with antibodies specific for CRH-R1/2 (Santa Cruz Biotechnology, Santa Cruz, California, USA) and protein A/G agarose (Pierce, Rockford, IL USA). The immunoprecipitates were collected, washed four times PBS before being resuspended in Laemmli sample buffer. After heating at 95 °C for 5 min, the samples were centrifuged briefly and the supernatants analyzed by SDS–PAGE on 10% gels. Following electrophoretic transfer, membranes were incubated overnight at 4 °C with pan-arrestin antibody (dilution 1:500) or AKAP250 (dilution 1:500). Membrane fractions were also incubated with pan-cadherin (dilution 1:500 in TBS-T) or GAPDH (1:60,000 in TBS-T) after a 1 h incubation step in TBS-T containing 5% BSA at room temperature. Following incubation with the secondary horseradish peroxidase-conjugated antirabbit IgG (DAKO UK Ltd, Ely, UK) (1:2000 dilution in TBS-T for 1 h at room temperature), proteins were visualized using ECL reagent from Amersham/Pharmacia Biotech (GE Healthcare UK Ltd, Little Chalfont, UK).

2.7. Immunofluorescent confocal microscopy and internalization studies

Cells were seeded on coverslips coated with 100 μg/ml poly-D-lysine in PBS. When reached 70–80% confluency, the cells were treated with various concentration of agonists and fixed with 4% PFA. Non-specific binding was blocked with 1% BSA in PBS-Triton X-100 (0.01%) for 1 h at room temperature. Following a 5 min wash with PBS, a PBS solution containing 1:50 monoclonal mouse antibody against AKAP250 was added for overnight incubation at 4 °C. Alternatively, slides were incubated with polyclonal goat antibody against the C-terminus of CRH-R1/2 (1:100 in PBS) for 2 h at room temperature. At the end of the incubation period, the primary antibody was removed and following three PBS washes for 5 min each, donkey anti-rabbit AlexaFluor®488 or donkey anti-goat AlexaFluor®594 were added in 1:400 dilution each for 1 h at room temperature. After three final washes with PBS, slides were mounted with VECTASHIELD Mounting Medium with DAPI after three final washes with PBS. The slides were examined under an oil immersion objective (×63) using a Leica model DMRE laser scanning confocal microscope (Leica Microsystems, UK, Buckinghamshire, Milton, Keynes, UK) with TCS SP2 scan head. Laser 543 nm at 50% of power and emission filter set at 555–620 nm were used to examine AlexaFluor®594 staining, and Laser 488 nm at 30% of power and emission filter set at 500–535 nm was used to examine AlexaFluor®488 staining. DAPI or AlexaFluor®405 staining was examined with Laser 405 at 10% of power and emission filter set at 410–450 nm. The scan speed was set at 400 Hz, and the format was 1024×1024 pixels. Optical sections (0.5 μm) were taken, and representative sections corresponding to the middle of the cells are presented. After indirect immunofluorescent staining, no specific fluorescence was observed in cells treated with secondary antibody only or when blocking peptide was incubated with the primary antibody prior to incubation on cells. For each treatment, between 20 and 30 individual cells in five random fields of view were randomly selected and examined. Fluorescence intensity profiles were generated along multiple line axes, analyzed, and quantified using ImageJ software developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/). Relative quantification of intracellular (internalized) receptor was carried out by measuring

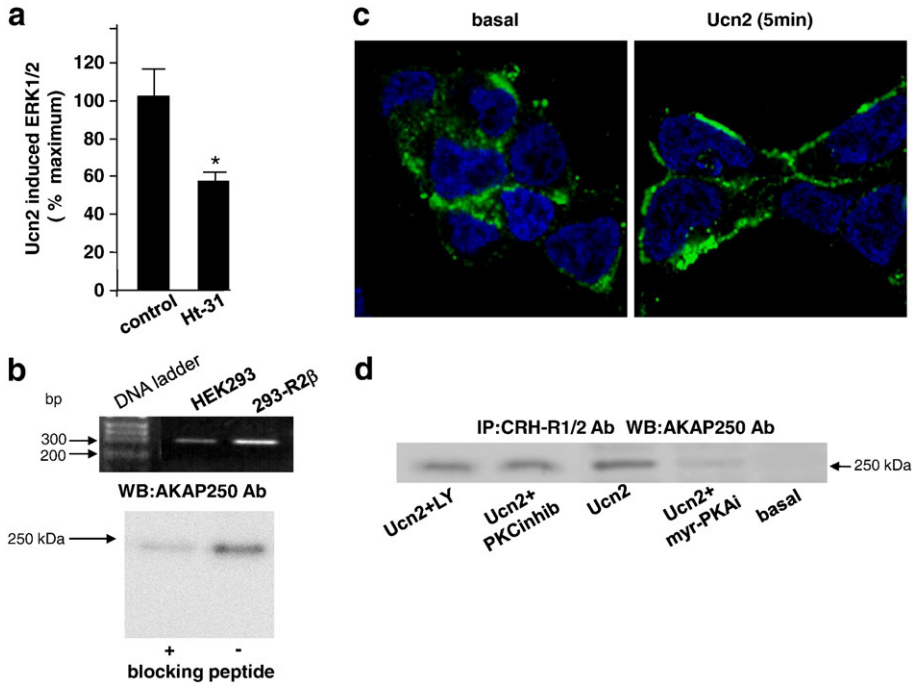

Fig. 2. AKAPs and CRH-R2β signaling. (a) Cells were pretreated with or without Ht-31 (10 μM) prior to stimulation with Ucn2 (100 nM) for 5 min. Activation of ERK1/2 was determined as described in Materials and methods. Data represent the mean ± SEM of 3 estimations from three independent experiments. *$P<0.05$ compared with cells without inhibitor pretreatment. (b) Top panel: RT–PCR of AKAP250 (gravin) mRNA present in untransfected HEK293 or 293-R2β cells using specific oligonucleotide primers. The size of the amplified DNA fragment was 300 bp. Bottom panel: Cell lysates from 293-R2β cells were fractionated on SDS–PAGE and subjected to immunoblotting with a specific AKAP250 antibody in the presence or absence of blocking peptide as described in Materials and methods. Identical results were obtained from three independent experiments. (c) The effect of Ucn2 on AKAP250 subcellular distribution was monitored by indirect fluorescence confocal microscopy. Cells were stimulated with Ucn2 (100 nM) for 5 min and AKAP250 distribution was monitored by using specific primary antibodies for AKAP250 and Alexa-Fluor® 488 secondary antibody (green). Identical results were obtained from 4 independent experiments. In addition (d), 293-R2β cells were pretreated with or without myr-PKAi, LY or bisindolylmaleimide II, for 30 min prior to stimulation with Ucn2 (100 nM) for 5 min. Subsequently, membrane-rich fractions were prepared, solubilized and immunoprecipitated with specific CRH-R1/2 antibodies. Proteins were resolved on SDS–PAGE gels, followed by immunoblotting with AKAP250 antibodies to identify potential complex formation. Representative immunoblots are presented. Identical results were obtained from 4 independent experiments.

the amount of total fluorescence along the longitudinal axis corresponding to the intracellular space (average 4–18 μm).

2.8. Statistics

The results obtained are presented as the mean ± SEM of each measurement. Statistical analysis was carried out using SPSS 18 software. Data were tested for homogeneity, and comparison between group means was performed by one-way analysis of variance and Dunnett's test. Probability values of $P<0.05$ were considered to be significant.

3. Results

3.1. Role of cAMP/PKA/AKAPs pathway and PI3-K on Ucn2-induced ERK1/2 activation

Our previous studies [14,17] demonstrated that treatment of HEK293 cells stably expressing CRH-R2β receptor (293-R2β) with either Ucn1 or Ucn2 (100 nM) significantly increased intracellular cAMP levels as well as ERK1/2. These findings were confirmed in the present study; we also showed that the specific CRH-R2 receptor antagonist anti-sauvagine 30 (1 μM) was able to block the stimulatory action of 100 nM Ucn2 on ERK1/2 signaling, demonstrating the specificity of Ucn2 actions (Fig. 1a).

The role of phosphatidylinositol-3 OH kinase (PI3-K), a signaling molecule previously shown to be involved in CRH-R1α and CRH-R2β ERK1/2 interactions [15,18], on CRH-R2β mediated ERK1/2 phosphorylation, was also investigated, by using the specific PI3-K inhibitor LY-294002. Pre-treatment of 293-R2β cells with 50 mM LY-294002 for 30 min, significantly impaired by 60 ± 5% Ucn2 (100 nM)-induced ERK1/2 (Fig. 1b). Moreover, a potential role for cAMP/PKA pathway in Ucn2 induced ERK1/2 activation was also investigated. For this purpose, 293-R2β cells were treated with adenylyl cyclase and PKA inhibitors (SQ22356 and myr-PKAi, respectively) and ERK1/2 phosphorylation after stimulation with 100 nM Ucn2 for 5 min was determined. Results demonstrated that both inhibitors significantly impaired (by 40% and 50%, respectively) Ucn2 induced ERK1/2 phosphorylation (Fig. 1c). We also examined the potential involvement of PKA-independent pathways downstream of cAMP mediating Ucn2-induced ERK1/2 activation, and specifically the role of Epac, a guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2. Epac signaling in the pituitary, locus ceruleus and heart has been implicated in CRH and Ucn1 activation of ERK1/2 [27–29]. In the 293-R2β cells, 8-CPT-2′-O-Me-cAMP (a selective activator of Epac) did not alter basal conditions; however, it increased Ucn2 (100 nM)-activated ERK1/2 phosphorylation by 30% (Fig. 1c). Pre-incubation of cells with brefeldin A, an Epac antagonist, exerted a significant inhibitory effect by 25% on Ucn2-induced maximal ERK1/2 activation at 5 min (Fig. 1c). Previous studies on Ucn2 activated ERK1/2 phosphorylation identified a transient response, which was maximal within 5 min of treatment and returned to basal levels after 30 min of agonist stimulation [17]. Interestingly, pre-treatment of 293-R2β cells with myr-PKAi attenuated by 60% Ucn2 (100 nM) induced ERK1/2 activation at 5–10 min only, whereas ERK1/2 early activation (within 2 min) was increased by 20–30% (Fig. 1d).

The involvement of signaling molecules downstream of cAMP/PKA such as AKAPs, on Ucn2-induced ERK1/2 activation was also investigated by using Ht-31 to interrupt PKA binding to AKAPs [30]. Results (Fig. 2a) showed that pre-treatment of 293-R2β cells with Ht-31 (10 μM) for 30 min markedly reduced (44 ± 6%) ERK1/2 phosphorylation induced by 100 nM Ucn2. Several types of AKAPs are expressed in mammalian cells [31]; of particular interest in GPCR signaling is AKAP250 (gravin), an adaptor protein important for agonist-induced GPCR internalization and recycling. Using RT–PCR and immunoblotting of cell lysates using specific antibodies, we showed that gravin mRNA and protein is expressed in 293-R2β cells (Fig. 2b) in agreement with previous reports [32]. Detection of gravin subcellular localization by indirect immunofluorescence confocal

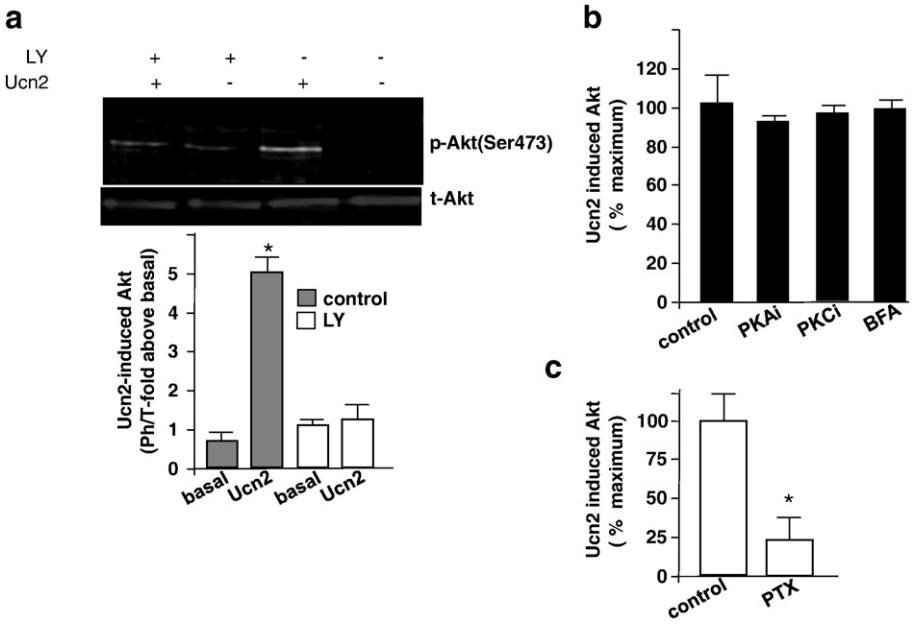

Fig. 3. Signaling characteristics of Akt activation by Ucn2 in 293-R2β cells. (a) Cells pretreated with or without LY-294002 (50 μM for 30 min) were stimulated with Ucn2 (100 nM) for 5 min. Following cell lysis and centrifugation, supernatants were subjected to SDS–PAGE and immunoblotted with primary antibodies for phospho-Akt (Ser473) and total-Akt to determine the phosphorylated/activated Akt and secondary antibodies conjugated to IRDye™ 800 (goat antirabbit IgG) and Alexa Fluor® 680 (goat antimouse IgG) [near-infrared (IR) fluorophore dyes] as described in Materials and methods. *Top* panels are representative Western blots. Alternatively (b) cells were pre-treated with or without myr-PKAi, or bisindolylmaleimide II or brefeldin A before Ucn2 stimulation for 5 min and determination of phospho-Akt. In some experiments (c), cells were pretreated with or without pertussis toxin (PTX) (12 h, 100 ng/ml) before stimulation with Ucn2 (100 nM) for 5 min. Data represent the mean ± SEM of 3 estimations from three independent experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between the treatment groups and controls (i.e. cells without inhibitor pretreatment; $^*P<0.05$).

microscopy experiments, showed that Ucn2 binding to 293-R2β cells led to a rapid translocation (within 5 min) of gravin to the plasma membrane (Fig. 2c). Co-immunoprecipitation studies showed that recruited gravin to the plasma membrane in response to CRH-R2β activation was associated with CRH-R2β receptor (Fig. 2d). This effect was PKA-dependent since inhibition of PKA with myr-PKAi blocked Ucn2-induced CRH-R2β-gravin binary complex formation. In contrast, inhibition of PKC or PI3-K had no effect on CRH-R2β-gravin association.

3.2. Signaling molecules involved in Ucn2-induced Akt activation

In addition to cAMP and ERK1/2, treatment of 293-R2β cells with Ucn2 (100 nM) for 5 min significantly induced by 5 fold Akt phosphorylation at Ser473 (Fig. 3a). Pre-treatment of cells with LY-294002 significantly impaired Ucn2 effect by 80%, identifying PI3-K as the major signaling molecule upstream of Akt. The potential role of cAMP/PKA and Epac pathways in Ucn2 induced Akt activation was also investigated; treatment of 293-R2β cells with myr-PKAi or brefeldin A had no effect on Ucn2-induced Akt phosphorylation (Fig. 3b). Similar results were obtained with bisindolylmaleimide II (100 nM), thus excluding the involvement of PKC in Akt activation (data not shown). By pre-treating cells with pertussis toxin (PTX) (12 h, final concentration, 100 ng/ml), we evaluated the possible involvement of Gi/Go proteins, downstream of CRH-R2β receptor in the activation of the PI3-K–Akt pathway. Results showed that PTX treatment abolished Ucn2-induced Akt activation, (Fig. 3c) confirming that Gi/Go proteins are crucial for Akt phosphorylation at Ser473.

3.3. Regulation of CRH-R2β internalization by the cAMP/PKA and ERK1/2 pathways

The demonstration of functional interactions between the cAMP and ERK1/2 signaling cascades led us to investigate whether these signals target the CRH-R2β receptor itself and modulate Ucn2 signaling potency through alteration in receptor internalization kinetics. Our previous studies characterizing the temporal properties of CRH-R2β internalization showed that substantial receptor internalization were evident after 30 min of Ucn2 treatment although some receptor trafficking in the cytoplasm was observed within 15 min of treatment [17]. Moreover, a functional role for the amino acid cassette TAAV at the end of the receptor C terminus was identified since loss of a potential phospho-acceptor site in mutant receptors containing deletion or Ala substitution of the cassette TAAV resulted in reduced ERK1/2 activation and accelerated receptor internalization.

To investigate potential effects of PKA on CRH-R2β internalization 293-R2β cells were pre-treated with myr-PKAi and CRH-R2β endocytosis was monitored by confocal microscopy. Results showed that in myr-PKAi pre-treated cells Ucn2 (100 nM) for 15 min elicited

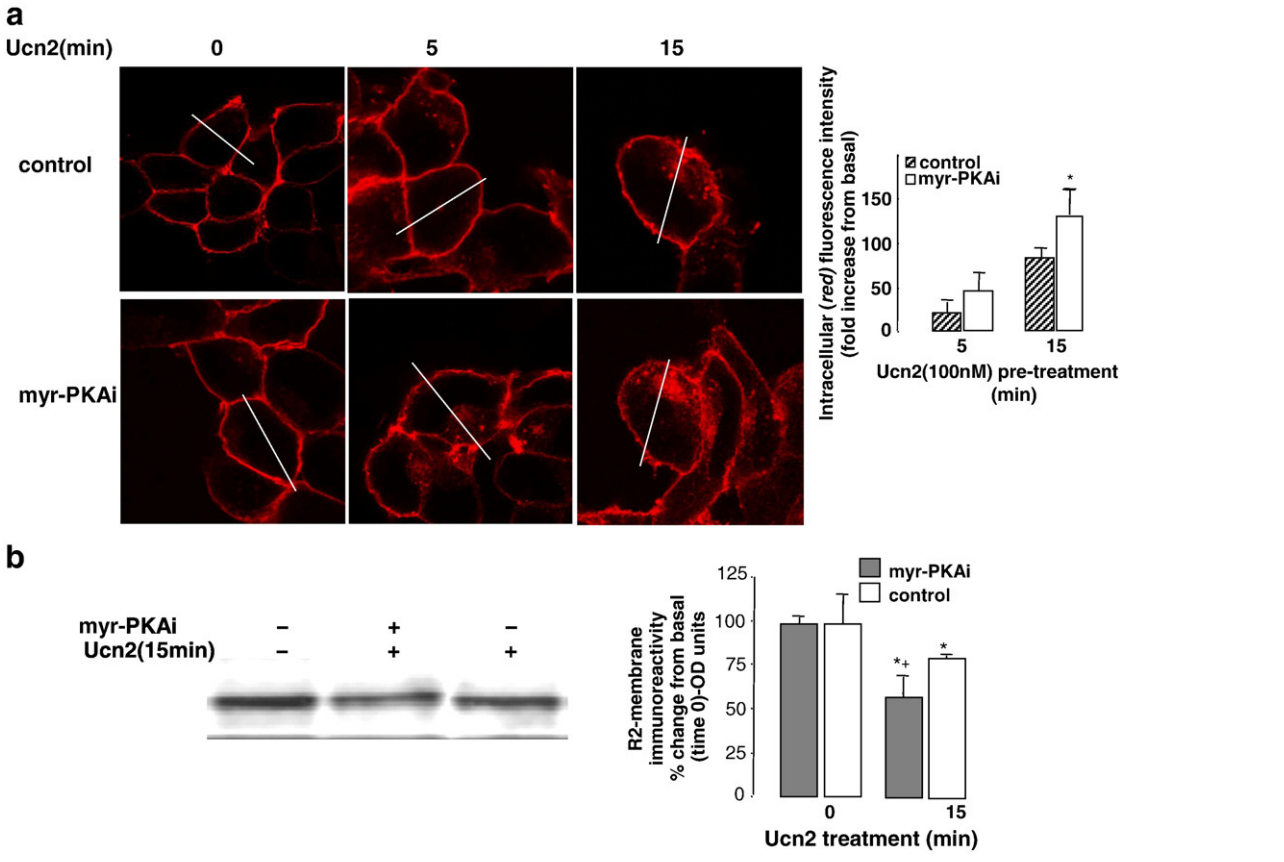

Fig. 4. Effect of PKA inhibition on Ucn2-stimulated CRH-R2β endocytosis. Cells were pre-treated with or without myr-PKAi for 30 min to inhibit PKA activity, before stimulation with Ucn2 (100 nM) for various time intervals (0–15 min). (a) Subsequent CRH-R2β internalization was monitored by indirect fluorescent confocal microscopy using specific primary antibodies and Alexa-Fluor 594 secondary antibody for CRH-R2 (red). For quantification of cytoplasmic CRH-R2β distribution, 20 individual cells in five random fields of view were examined, and profiles of fluorescence intensity, generated. The sum of fluorescence intensity of cytoplasmic (distance 4–18 μm) fluorescence along the lines was measured by using Image J software. Results are expressed as the mean ± SEM of three estimations from 20 individual cells. *$P<0.05$ compared with control (no inhibitor) values. (b) Alternatively, cell membrane-rich fractions were prepared and following SDS–PAGE, they were immunoblotted with antibodies against CRH-R1/2 C-terminus. Densitometry scanning was carried out to quantify agonist-induced loss of receptor expression from the cell membrane. Identical results were obtained from 4 independent experiments. Results are expressed as the mean ± SEM of three estimations from two individual experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between basal (time 0) vs Ucn2 stimulated (time 15 min) groups (*$P<0.05$) or between treatment groups and controls (i.e., cells without inhibitor pretreatment; +$P<0.05$).

considerable CRH-R2β internalization which was significantly increased compared to control cells (Fig. 4a). Quantification of red fluorescence in the intracellular space of 20 individual cells that were randomly selected showed 30–40% increased amount of red fluorescent signal throughout the intracellular space (4–18 mm) (indicative of receptor internalization), in myr-PKAi pre-treated cells compared to control (Fig. 4a), suggesting that PKA inhibition was associated with increased receptor internalization. Cell membrane-rich fractions were also prepared from cells pre-treated with or without myr-PKAi, before treatment with Ucn2 (100 nM) for 15 min. CRH-R2β proteins were detected as a single immunoreactive protein with an apparent molecular mass of approximately 60 kDa, by Western blotting using a specific antibody raised against the CRH-R1/2 C-terminus. Densitometric analysis revealed that PKA inhibition significantly increased by 24% Ucn2 induced (15 min) loss of receptors expressed in the cell membrane compared to untreated (control) cells (Fig. 4b), thus confirming the findings of the indirect fluorescence confocal microscopy. In the last set of experiments, the purity of the membrane-rich fractions and equal protein loading was confirmed by immunoblotting; results showed that all samples contained equal amounts of the plasma membrane protein cadherin whereas GAPDH, a cytosolic protein, was not detected in any of the samples (data not shown). Interestingly, disruption of PKA–AKAP interactions by incubation with Ht-31 also accelerated Ucn2-induced receptor internalization at 15 min was by 20–30%, highlighting the important role of AKAPs in agonist-induced CRH-R2β trafficking. In contrast, pretreatment of cells with BFA did not alter Ucn2-induced CRH-R2β internalization rate excluding Epac as a mediator of Ucn2-dependent CRH-R2β internalization and effects on receptor internalization machinery (data not shown).

Since PKA is involved in ERK1/2 activation, we next examined whether PKA modulates CRH-R2β trafficking indirectly through activation of ERK1/2. Similar to the studies described before, confocal microscopy was used to determine the effect of ERK1/2 in CRH-R2β internalization in 293-R2β cells where ERK1/2 activation was blocked by U0126. Preliminary experiments confirmed that Ucn2 ERK1/2 interaction is MEK dependent (Fig. 5, inset) since U0126 pretreatment blocked Ucn2 induced ERK1/2 activation. In U0126 pre-

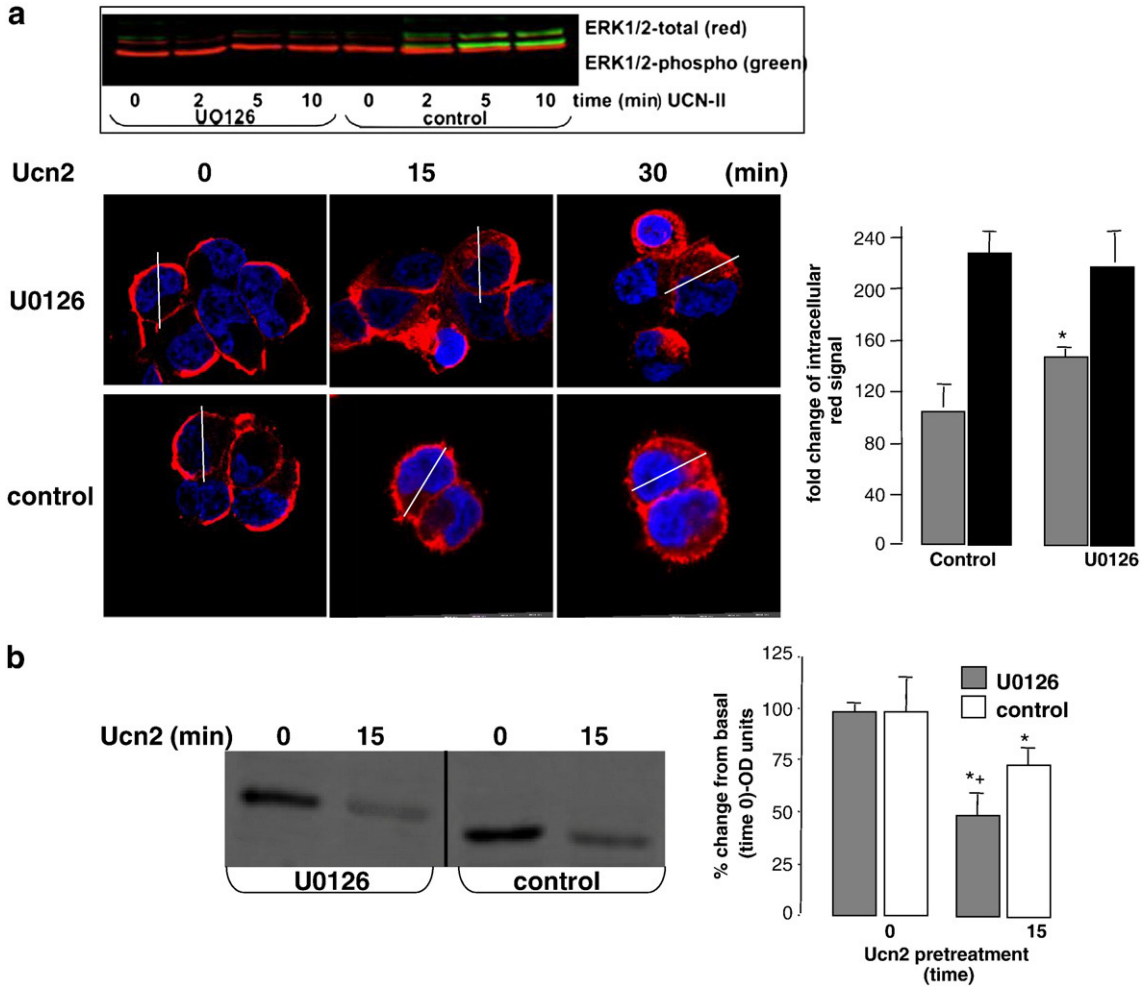

Fig. 5. Regulation of CRH-R2β internalization by ERK1/2 in 293-R2β cells. Cells were pre-treated with or without U0126 (10 μM) for 2 h to inhibit basal and Ucn2-dependent ERK1/2 activation (inset), before stimulation with Ucn2 (100 nM) for various time intervals (0–30 min). The effect of U0126 on Ucn2 induced CRH-R2β internalization was monitored by indirect fluorescent confocal microscopy using specific primary antibodies and Alexa-Fluor 594 secondary antibody for CRH-R2 (red). (a) For quantification of cytoplasmic CRH-R2β distribution following 15 min of agonist stimulation, 20 individual cells in five random fields of view were examined, and profiles of fluorescence intensity, generated. The sum of fluorescence intensity of cytoplasmic (distance 4–18 μm) fluorescence along the lines was measured by using Image J software. Results are expressed as the mean ± SEM of three estimations from 20 individual cells. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between the treatment groups and controls (i.e., cells without inhibitor pretreatment; $^{*}P<0.05$). Alternatively, (b) cell membrane-rich fractions were prepared and following SDS–PAGE, they were immunoblotted with antibodies against CRH-R1/2 C-terminus. Densitometry scanning was carried out to quantify agonist-induced loss of receptor expression from the cell membrane. Identical results were obtained from 4 independent experiments. Results are expressed as the mean ± SEM of three estimations from two individual experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between basal (time 0) vs Ucn2 stimulated (time 15 min) groups ($^{*}P<0.05$) or between treatment groups and controls (i.e., cells without inhibitor pretreatment; $^{+}P<0.05$).

treated cells, Ucn2 (100 nM) for 15 min increased by 30–50% the amount of red fluorescent signal throughout the intracellular space (4–18 mm) compared to control (Fig. 6a), suggesting that in conditions of ERK1/2 inhibition, receptor internalization is increased. No significant difference in intracellular fluorescent signal between control and U0126-treated cells was detected after 30 min of Ucn2 treatment. Furthermore immunoblotting experiments (Fig. 5b) showed that, in U0126-treated cells, Ucn2 was substantially more potent in attenuating cell membrane receptor expression, indicative of enhanced receptor internalization. Simultaneous inhibition of PKA and ERK1/2 (by myr-PKAi and U0126, respectively) did not exert an additional effect pointing towards the presence of a single pathway simultaneously involving PKA and ERK1/2 rather than two distinct independent cascades (data not shown).

3.4. Ucn2 induced ERK1/2 activation and phosphorylation of β-arrestin1 and feedback regulation of Ucn2-induced cAMP and Akt pathways

Previous studies have shown that ERK1/2 can potentially regulate GPCR endocytosis through a mechanism involving phosphorylation of β-arrestin1 at Ser412 [33]. Since our studies [17] implicated β-arrestin1 in CRH-R2β desensitization and internalization, we investigated whether β-arrestin1 is phosphorylated downstream of Ucn2-activated ERK1/2 and this signaling event is important for CRH-R2β internalization. The phosphorylation status of β-arrestin1 in 293-R2β cells was determined by immunoblotting using phospho-β-arrestin1 (Ser412) specific antibodies. The specificity of this antibody in recognizing phospho-β-arrestin1 was established in preliminary experiments (Fig. 6, inset): in total cell lysates prepared from resting 293-R2β cells, phospho-β-arrestin1 was detected as a single immunoreactive protein with an apparent molecular mass of approximately 50 kDa. When PVDF membranes were incubated overnight with a solution containing alkaline phosphatase, no immunoreactive band was detected although β-arrestin1 was detected by a specific β-arrestin1 antibody. Furthermore, phospho-β-arrestin1 immunoreactivity was absent in 293-R2β cells transfected with specific siRNA sequences that resulted in depletion of β-arrestin1 [17]. These results confirmed the specificity of the antibodies used to detect phospho-β-arrestin1.

Results showed that under basal (non-stimulated) conditions there were low levels of constitutively phosphorylated β-arrestin1 present in total cell lysates. Ucn2 treatment elicited a significant increase of phospho-β-arrestin1 immunoreactivity by 40% within 2 min (Fig. 6a); and after 10 min of Ucn2 treatment, phosphorylation of β-arrestin1 at Ser412 was reduced by 45–50% below basal levels. Ucn2 induced phosphorylation of β-arrestin1 appeared to be regulated by ERK1/2 since in U0126-pretreated cells, phospho-β-arrestin1 immunoreactivity was not increased in response to agonist; in contrast, Ucn2 treatment significantly reduced by 40% of basal phospho-β-arrestin1 immunoreactivity within 5 min (Fig. 6a).

We further investigated whether changes in β-arrestin1 phosphorylation status affected its recruitment to the plasma membrane and association with the receptor. Western blot analysis of 293-R2β cell membrane fractions using β-arrestin1 specific antibodies showed

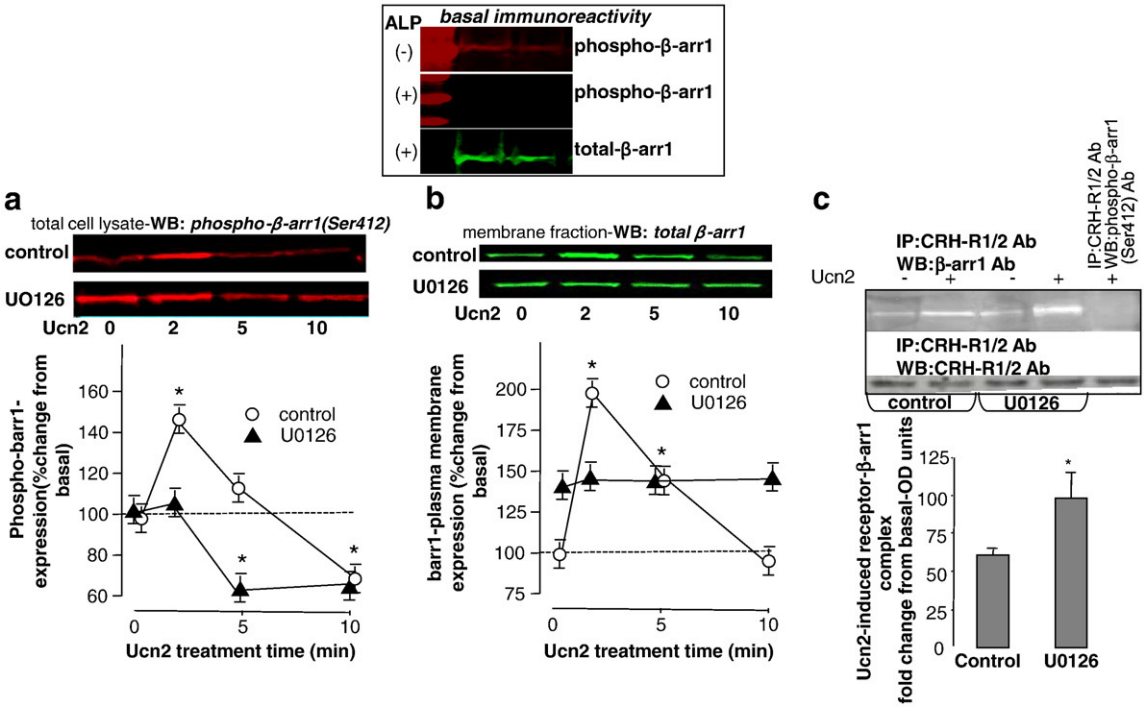

Fig. 6. Regulation of β-arrestin1 phosphorylation, membrane translocation and interaction with CRH-R2β by ERK1/2. Inset: Validation of phospho β-arr1 (Se412) antibody. Cell lysates were fractionated by SDS–PAGE and following protein transfer in PVDF membranes, phospho-β-arr1 immunoreactivity was detected by using specific antibodies and the Odyssey Infrared Imaging System. Membranes were also incubated in alkaline phosphatase (ALP)-containing solution prior to detection of phospho- or total β-arr1 immunoreactivity. (a–b) *β-arrestin1 phosphorylation, membrane translocation:* Cells were pre-treated with or without U0126 (10 μM) for 2 h before stimulation with Ucn2 (100 nM) for various time intervals (0–10 min). Following cell lysis in RIPA buffer, phospho-β-arrestin1 (Ser412) were determined by specific antibodies and Alexa Fluor®680-conjugated goat antimouse IgG using the Odyssey Infrared Imaging System (a). Alternatively (b), cell membrane-rich fractions were prepared and following SDS–PAGE, they were immunoblotted with antibodies against β-arrestin1 and IRDye™800-conjugated goat antirabbit IgG. (c) *Identification of β-arrestin and CRH-R2β complex formation by co-immunoprecipitation*: 293-R2β cells were pre-treated with or without U0126 (10 μM) for 2 h before stimulation with Ucn2 (100 nM) for 2 min before solubilization of membrane-rich fractions and immunoprecipitation with specific CRH-R1/2 antibodies. Proteins were resolved on SDS–PAGE gels, followed by immunoblotting with either β-arrestin1 (top panel), phospho-β-arr1 (Ser412) (top panel far right) or CRH-R1/2 (bottom panel) antibodies to identify potential complex formation. Representative immunoblots are presented. Identical results were obtained from 4 independent experiments. Data represent the mean ± SEM of two estimations from three independent experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between the treatment groups and controls (i.e., cells without inhibitor pretreatment; $^{*}P<0.05$).

that, within 2 min of Ucn2 treatment, β-arrestin1 recruitment to the plasma membrane was significantly increased by 70–90% above basal level, in agreement with previous studies [33]. This effect was transient and within 10 min the rise in β-arrestin1 membrane fraction was reduced by 50% (Fig. 6b). Both basal and Ucn2 induced trafficking of β-arrestin1 appeared to be regulated by ERK1/2 since inhibition of MEK and ERK1/2 by U0126 pretreatment significantly increased (by 50%) basal β-arrestin1 recruitment to the plasma membrane. In the absence of active ERK1/2, Ucn2 treatment of U0126-pretreated cells did not significantly alter β-arrestin1 recruitment to the plasma membrane and levels of membrane-bound β-arrestin1 remained unchanged for the period tested (up to 10 min) (Fig. 6b).

Potential changes in dynamics of CRH-R2β:β-arrestin1 binary complex formation and regulation by ERK1/2 were also evaluated. For this purpose, 293-R2β cells were pre-treated with U0126 or vehicle, before treatment with Ucn2 (100 nM) for 2 min. Plasma membrane fractions were prepared and solubilized before CRH-R2β receptor immunoprecipitation by a specific CRH-R1/2 antibody and β-arrestin co-purified with CRH-R2β receptors was detected by immunoblotting using β-arrestin1 specific antibodies. Results (Fig. 6c) showed that ERK1/2 inhibition significantly enhanced by 40–60% agonist-induced β-arrestin1 interaction with the CRH-R2β.

To confirm that the feedback mechanism involving ERK1/2-mediated phosphorylation β-arrestin1 could also modulate CRH-R2β signaling potency as a result of altered CRH-R2β internalization rate, we investigated whether the Ucn2-induced cAMP and Akt pathways are also sensitive to changes in ERK1/2 activity. Our results (Fig. 7a) showed that inhibition of ERK1/2 activity by U0126 significantly reduced (by 40%) Ucn2 (1–100 nM)-induced, but not basal, cAMP production. In contrast, agonist activated Akt phosphorylation was not affected by inhibition of intracellular Erk1/2 activity (Fig. 7b).

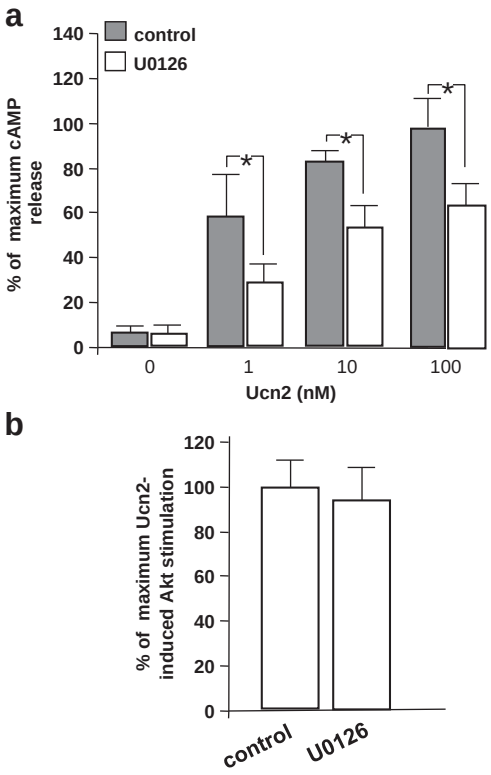

Fig. 7. Effect of ERK1/2 inhibition on Ucn2-stimulated cAMP production and Akt phosphorylation in 293-R2β cells. Cells were pretreated with U0126 (10 μM for 2 h) prior to stimulation with various concentrations of Ucn2 (0–100 nM) for 15 min (a) or Ucn2 (100 nM) for 5 min (b). Cyclic AMP production was determined by ELISA and Akt phosphorylation by immunoblotting using specific antibodies. Results are expressed as % of maximal response (obtained with 100 nM Ucn2) and data represent the mean ± SEM of two estimations from three independent experiments. Data analysis was carried out by using one-way ANOVA and Dunnett's test to compare mean values between the treatment groups and controls (i.e., cells without U0126 pretreatment; $^{*}P<0.05$).

4. Discussion

It now well-established that cAMP and MAPK pathways play prominent roles in mediating the physiological actions of CRH and CRH-related peptides in most target tissues [1]. There are numerous physiological paradigms where these two pathways interact to coordinate their activity and influence the final biological outcome. For example, CRH activation of the CRH-R1/cAMP/PKA pathway in the Ishikawa human endometrial adenocarcinoma and human breast cancer MCF7 cell lines inhibits cell growth and proliferation possibly by inhibiting MAPK activity [34,35]. Synergistic effects of the two pathways have also been described in human mast cells where CRH induces vascular endothelial growth factor release via the cAMP/PKA/p38MAPK pathway [36] and the hippocampus, where cAMP-dependent ERK1/2 activation is involved in the neuroprotective effects of CRH and Ucn1 against excitotoxicity [37]. In the latter paradigm, PKA-dependent ERK1/2 activation appears to be important for hippocampal CRH-R2 mediated regulation of stress and fear memory responses [13].

In this study, we provide novel insights into the mechanisms regulating CRH-R2β signaling involving intracellular "cross-talk" between cAMP and MAPK. Using a receptor over-expression system, we demonstrated presence of a complex signaling network required for maximal activation of ERK1/2 in response to Ucn2 activation that involves PI3-K as well as increases in cAMP levels and downstream PKA/AKAP interactions. In addition to PKA, Epac appears to play a role in cAMP-dependent ERK1/2 activation. Epac has been previously implicated in CRH and Ucn1 activation of ERK1/2 [27–29] and is also involved in other *Secretin* GPCRs such as the parathyroid hormone (PTH) and calcitonin receptors mediated ERK1/2 activation in bone and rat kidney cells, respectively [38,39]. The ability of 8-CPT-2′-O-Me-cAMP (a selective activator of Epac) to enhance Ucn2 effects without affecting basal ERK1/2 phosphorylation might suggest that Epac acts synergistically with another signaling pathway, possibly PKA, to augment ERK1/2 activation. Similar synergistic effects between Epac and PKA signaling pathways have been described in vascular smooth muscle cells and in the BON human endocrine cell line [40,41].

The effects of the cAMP cascade appears to be specific for ERK1/2 since Ucn2-induced PKB/Akt activation appears to depend on a distinct pathway activated downstream of PTX-sensitive G-proteins such as Gi/o and PI3-K activity which is independent of cAMP and PKA/AKAP or PKC activity. At present, the specific "cross-talk" points between cAMP/PKA and ERK1/2 signaling interactions are not known; it is possible that the CRH-R2β itself is a phosphorylation target of PKA. Similar PKA–GPCR interactions regulating signaling efficiency have been described before; for example, phosphorylation by PKA of the β2-adrenergic receptor switches its coupling from Gs to Gi to allow stimulation of the ERK1/2 signaling pathway [20]. Previous studies have shown that the CRH-R1, which is highly homologous to CRH-R2 especially in the intracellular loops (IC) and C-terminus, can also be targeted by PKA. A single serine residue (Ser^{301}) present in IC3 appears to be the main target of PKA phosphorylation; this signaling event leads to attenuation of receptor/Gq-protein coupling and ERK1/2 activation [21]. It is also possible that Ucn2-activated PKA directly modulates the activity of intracellular molecules downstream of PI3-K important for ERK1/2, but not Akt, activation. In FRTL5 and Caco-2 thyroid and adenocarcinoma cell lines, cAMP/PKA increases Ras and PI3-K association [42,43], possibly through phosphorylation of p85, which stabilizes the complex p110-p85. Interestingly, the early phase (2 min) of Ucn2-induced ERK1/2

activation appears to be independent of the cAMP/PKA effect. This observation might point towards the involvement of diverse signaling mechanisms determining the temporal characteristics of ERK1/2 activation. Alternatively, it is possible that PKA inhibition alters (accelerates) the transient kinetics of ERK1/2 activation.

One of the signaling events downstream of Ucn2-induced PKA activation is translocation of AKAP250 (gravin) to the plasma membrane that leads to association with the CRH-R2β via a mechanism that requires intact PKA activity, thus suggesting involvement of PKA-phosphorylation events. This scaffolding protein has been implicated in the mechanism of GPCR trafficking [24] and studies in other GPCRs suggest that AKAP250 maintains PKA in close proximity to the receptor and acts as scaffold for various kinases and phosphatases involved in receptor signaling modulation through trafficking. In line with these observations, our studies demonstrated the potential of PKA to regulate (delay) CRH-R2β internalization rate, a finding that might explain the diminished ERK1/2 activation in the absence of active PKA or disrupted PKA–AKAP interactions. Interestingly, not all CRH-R2β-driven signaling responses were susceptible to PKA effects and increased rate of receptor internalization since the Gi/o-dependent Akt response was not affected by inhibition of PKA activity. Given that CRH-R1 coupling efficiency to Gαs and $G\alpha q_{/11}$, but not Gαi, is susceptible to receptor desensitization and endocytosis [44], it is attractive to speculate that only CRH-R2β-mediated ERK1/2, but not Akt, activation is dependent on G-protein pathways sensitive to desensitization and receptor internalization. The physiological significance of this divergence in the regulation of signaling potency is not known; however, it is attractive to speculate that the dissociation of Akt regulation from the ERK1/2 and cAMP/PKA cascades might confer CRH-R2β the ability to selectively control independent biological outcomes. Although important actions of Ucns in the heart, such as the ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death, involve both Akt and ERK1/2 pathways [45] other urocortin actions are mediated by Akt but not cAMP or ERK1/2 activation; examples include the urocortin anti-inflammatory effects in cultured microglia that are PI3-K/Akt- but not cAMP pathway-dependent and the urocortin-induced inhibition of Beclin1 expression and autophagy as well as hypertrophy in cardiac myocytes mediated by activation of the PI3-K/Akt pathway but which does not involve activation of ERK1/2 [46–48].

Moreover, our data suggest that PKA becomes an important regulator of CRH-R2β internalization rate only in agonist-occupied receptor since inhibition of activation of PKA does not affect CRH-R2β trafficking at basal state. This might suggest the requirement for additional signaling molecules or mechanisms activated by Ucn2; ERK1/2 might be such a candidate molecule since our results showed that inhibition of ERK1/2 activity accelerates agonist-induced CRH-R2β endocytosis. Our studies suggest that β-arrestin1 might be one of ERK1/2 downstream targets involved in regulation of CRH-R2β endocytosis. It has been shown that β-arrestin1 phosphorylation and function are modulated by an ERK1/2-dependent mechanism that leads to attenuation of GPCRs internalization through negative feedback [25] since dephosphorylated β-arrestin1 at the plasma membrane is required for targeting agonist-occupied receptors to the clathrin-coated pits. Our studies suggest that Ucn2 dependent ERK1/2 activation, although β-arrestin independent [17], can potentially modulate β-arrestin1 phosphorylation and membrane translocation, binary complex formation with CRH-R2β and ultimately CRH-R2β endocytosis rate, in an autologous negative feedback mechanism. Experimental conditions that diminish ERK1/2 activity result in increased accumulation of β-arrestin1 to the plasma membrane, thus allowing increased association with CRH-R2β and rate of receptor endocytosis. The precise nature of the molecular determinants regulating β-arrestin1 dephoshorylation are unknown;

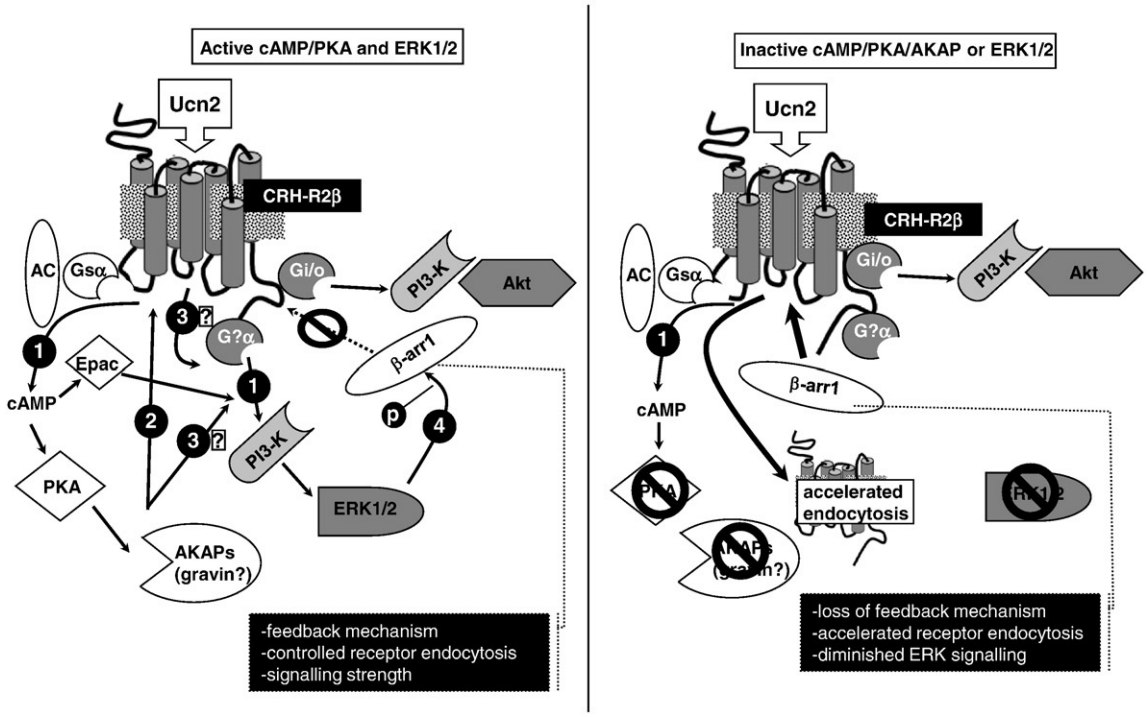

Fig. 8. Schematic representation of the proposed "cross-talk" mechanism regulating CRH-R2β signaling and endocytosis in HEK293 cells. According to this model (left panel), Ucn2 binding to its receptor leads to an increase in intracellular cAMP levels through activation of the Gs-adenylyl cyclase (AC) pathway and increased ERK1/2 phosphorylation through PKA and Epac-dependent pathways as well as involvement of PI3-K pathways (1). Activation of PI3-K by Gi/o pathways leads also to PKB/Akt phosphorylation. Downstream activation of PKA induces AKAP250 translocation to the plasma membrane and association with CRH-R2β (2). Moreover, PKA potentiates Ucn2-induced -ERK1/2, activation (3), through an AKAP-dependent mechanism potentially targeting the receptor and/or other signaling molecules. Activated ERK1/2 also regulates β-arrestin1 translocation, interaction with the receptor and ultimately the rate of receptor endocytosis (4). Inhibition of either PKA activity, PKA–AKAP interactions or ERK1/2 activation results in increased β-arrestin1 interaction with CRH-R2β, accelerated receptor endocytosis and impaired cAMP signaling (right panel). In contrast, Akt activation is insensitive to PKA or ERK1/2 inhibition.

previous studies have shown that protein phosphatase 2A (PP2A) is found in a molecular complex with β-arrestin1 and phosphatase inhibitors increase Ser-412 phosphorylation [33]. Thus it is possible that PP2A, through induction of β-arrestin1 dephosphorylation, directly modulates β-arrestin1 interaction with clathrin and ultimately receptor endocytosis. Evidence from other GPCR systems suggests that in this signaling assembly of GPCR/β-arrestin1, PP2A is capable of targeting multiple substrates including the receptor protein itself [49]. Interestingly, β-arrestin2 can also act as a signaling intermediate through an Akt/PP2A scaffold that can mediate inactivation of Akt in response to GPCR stimulation [50].

In conclusion, Ucn2 binding to CRH-R2β activates cAMP and ERK1/2 signaling cascades that interact through PKA-mediated phosphorylation events and AKAPs to regulate ERK1/2 signal intensity and rate of receptor endocytosis (Fig. 8). This mechanism induces AKAP250 translocation to the plasma membrane and interaction with the receptor. Furthermore, this interaction exhibits signaling selectivity since it does not appear to be important for activation of Akt, a mechanism that is mediated via Gi/o-dependent pathways involving PI3-K. ERK1/2 appears to regulate CRH-R2β endocytosis via direct phosphorylation of β-arrestin1 in an auto-regulatory mechanism that influences rate and extent of β-arrestin1 recruitment to the plasma membrane and interaction with CRH-R2β.

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