Biochimica et Biophysica Acta 1813 (2011) 896–907

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

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

Danijela Markovic [1,2], Anu Punn [1], Hendrik Lehnert, Dimitris K. Grammatopoulos *

Division of Endocrinology and Metabolism, Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK

ARTICLE INFO

Article history:
Received 9 January 2011
Received in revised form 1 February 2011
Accepted 7 February 2011
Available online 19 February 2011

Keywords:
CRH receptor
PKA
Ucn2
β-arrestin1
ERK1/2
AKAP

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.

* Corresponding author. Tel.: +44 2476 524206; fax: +44 2476 574637.
E-mail address: d.grammatopoulos@warwick.ac.uk (D.K. Grammatopoulos).
[1] D.M. and A.P. should be considered equal first co-authors by virtue of their unique contributions to this work.
[2] Current address for D.M.: MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, UK.

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.02.005

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),