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Melanocortin 5 receptor signaling and internalization: Role of MAPK/ERK pathway and β-arrestins 1/2

Adriana R. Rodrigues [a,b], Henrique Almeida [a,b], Alexandra M. Gouveia [a,b,c,*]

[a] Department of Experimental Biology, Faculty of Medicine, Universidade do Porto, Portugal
[b] IBMC- Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal
[c] Faculty of Nutrition and Food Sciences, Universidade do Porto, Portugal

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ABSTRACT

The Melanocortin 5 receptor (MC5R) is a G-protein coupled receptor (GPCR) that exhibits high affinity for α-MSH. Here we present evidence for MC5R-GFP internalization and subsequent recycling to cell surface, in α-MSH-stimulated HeLa cells. This melanocortin induces a biphasic activation of ERK1/2 with an early peak at 15 min, a G_i-protein driven, β-arrestins 1/2 independent process, and a late sustained activation that is regulated by β-arrestins 1/2. ERK1/2 lead to downstream phosphorylation of 90-kDa ribosomal S6 kinases (p90RSK) and mitogen- and stress-activated protein kinase 1 (MSK1). Only a small fraction (10%) of phosphorylated p90RSK and ERK1/2 translocates to the nucleus inducing c-Fos expression. α-MSH also activates CREB through cAMP/PKA pathway. In 3T3-L1 adipocytes, where MC5R is endogenously expressed, α-MSH also induces phosphorylation and cytosolic retention of the same signaling molecules. These findings provide new evidence on the signaling mechanisms underlying MC5R biological response to α-MSH.

1. Introduction

Melanocortins are neuropeptides derived from pro-opiomelanocortin (POMC), a polypeptide that upon processing originates the adrenocorticotropic hormone (ACTH), α, β and γ-melanocyte stimulating hormone (MSH) (Mountjoy, 2010), which are then recognized by a family of five melanocortin receptors (MCRs) termed MC1R to MC5R (Cooray and Clark, 2011). MC5R was the last melanocortin receptor to be cloned and although the precise functional role of this receptor is still unclear it seems to be related with the peripheral regulation of lipid metabolism. MC5R activation was directly implicated in the stimulation of fatty acid oxidation in skeletal muscle (An et al., 2007) and MC5R null mice show a down-regulation of sebaceous lipid secretion, leading to a defect in water repulsion and thermoregulation (Chen et al., 1997). Furthermore, in 3T3-L1 adipocytes, α-MSH and other melanocortins induce lipolysis (Cho et al., 2005; Moller et al., 2011). Since there is evidence that this cell line expresses only MC2R and MC5R (Norman et al., 2003; Boston and Cone, 1996), and MC2R has specificity for ACTH binding (Cooray and Clark, 2011), α-MSH effect on lipolysis should be mediated by MC5R.

MC5R belongs to the large family of GPCRs, plasma membrane receptors with seven transmembrane domains. After being activated by specific agonists, GPCRs trigger G proteins-dependent and independent signaling pathways (Woehler and Ponimaskin, 2009) and, subsequently, most of them are desensitized by internalization, primarily via clathrin-coated pits (Jean-Alphonse and Hanyaloglu, 2011). Usually this process involves the phosphorylation of the receptors by members of the GPCR kinase family and also the recruitment of cytoplasmic β-arrestins 1 and 2 (Jean-Alphonse and Hanyaloglu, 2011). Although the mechanism of GPCRs internalization is commonly linked to desensitization, some receptors continue to signal when integrated in endosomes mainly in complex with β-arrestins, which activate MAPK signaling molecules (Calebiro et al., 2010). Once internalized, GPCRs can be recycled back to the plasma membrane, targeted to lysosomes to be degraded or retained into endosomes. Thus, the final destination of GPCRs has also important functions in signal extinction or propagation.

The MCR trafficking mechanisms have been intensively studied in last years. It was shown that the cell surface targeting of MC5R is negatively regulated by the MC2 receptor accessory protein (MRAP), which is required for MC2R expression at cell membrane

Abbreviations: CREB, cAMP-responsive element binding protein; ERK, extracellular-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HRP, horseradish peroxidase; MC5R, melanocortin 5 receptor; MCRs, melanocortin receptors; MSH, melanocyte-stimulating hormone; MSK1, mitogen- and stress-activated protein kinase 1; p90RSK, 90-kDa ribosomal S6 kinases; PKA, protein kinase A; PKC, protein kinase C.

* Corresponding author at: Department of Experimental Biology, Faculty of Medicine, Universidade do Porto, 4200-319 Porto, Portugal. Tel.: +351 22 551 36 54; fax: +351 22 551 36 55.
E-mail address: agouveia@med.up.pt (A.M. Gouveia).

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(Chan et al., 2009; Sebag and Hinkle, 2009). MC5R localizes at the plasma membrane in the absence of MRAP, but is trapped intracellularly when co-expressed with this accessory protein (Chan et al., 2009; Sebag and Hinkle, 2009). Regarding MC5R internalization mechanisms, there is only one report referring that the receptor activation leads to β-arrestins recruitment to plasma membrane and receptor internalization occurs by a clathrin-dependent endocytosis process (Cai et al., 2006).

The signaling mechanisms following MC5R activation are not completely understood but in skeletal muscle, PKA and AMP-activated protein kinase were implicated in the regulation of fatty acid oxidation (An et al., 2007). In lymphocytes, α-MSH binding to MC5R activates the Jak/Stat pathway (Buggy, 1998) and in HEK293 stably expressing the receptor it increases the production of cAMP and calcium (Hoogduijn et al., 2002; Mountjoy et al., 2001). We also have shown that in HEK293 cells stably expressing MC5R-GFP, the receptor uses a specific transduction pathway to activate ERK1/2 independently from adenylyl cyclase, PKA, PKC and Akt/PKB pathways but involving PI3K (Rodrigues et al., 2009). MC3R also induces a PI3K-dependent ERK1/2 phosphorylation independently from PKA and PKC but exhibits alterations on Akt phosphorylation (Nyan et al., 2008); MC4R promotes a PI3K-dependent ERK1/2 phosphorylation but requires PKC activation (Cai et al., 2007). MC1R triggers the ERK pathway by receptor tyrosine kinase transactivation, independently of cAMP and PKC (Herraiz et al., 2011). Different MCRs share the same agonist (α-MSH), so a distinct intracellular network seems to define the specificity and function of each MCR, in which ERK1/2 seems to play a pivotal role.

The present study is aiming to comprehensively investigate the signaling pathways promoted by α-MSH-activated MC5R and address the regulation of the receptor internalization/desensitization mechanisms.

2. Materials and methods

2.1. Materials

DMEM/Ham's F-12 and DMEM media, glutamine, insulin, fetal bovine and fetal calf serum (FBS and FCS, respectively) were purchased from Biochrom AG. 3-isobutyl-methyl-xantine (IBMX), α-MSH, dexamethasone, monensin sodium, pertussis toxin (PTX), cholera toxin (CTX), forskolin, phosphatase and protease inhibitor cocktails and siRNA oligonucleotides were obtained from Sigma–Aldrich. MEK1/2 inhibitors PD98059 and U0126 were purchased from Calbiochem and Cell Signaling, respectively. Rp-adenosine-3', 5'-cyclic mono-phosphorothioate triethylamine salt (Rp-cAMP) was from Santa Cruz. PKC inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide (GF109203X) was obtained from Calbiochem. Lipofectamine 2000 was from Invitrogen.

2.2. HeLa cells culture and transfection

The pMC5R–EGFP vector was obtained as previously described (Rodrigues et al., 2009). HeLa cells were cultured in DMEM/Ham's F-12 medium supplemented with 10% FBS and 2 mM glutamine until 70–80% confluence was reached. Then, cells were transfected with pMC5R–EGFP or pEGFP using Lipofectamine 2000, as recommended by the manufacturer, and selected for outgrowth of isolated colonies using 0.6 μg/μl Geneticin (GIBCO). Two colonies derived from independent transfection experiments were picked, after screening by inverted fluorescence microscopy. One clone was used in all the experiments presented in the paper. The characterization of the second clone involved the study of ERK1/2 activation by α-MSH in order to guarantee that observations were not clone specific.

2.3. 3T3-L1 differentiation

3T3-L1 pre-adipocytes were grown to confluence in DMEM supplemented with 10% FCS and 2 mM glutamine. Two days later, adipogenesis was induced by incubation with DMEM containing 10% FBS, 10 μg/ml insulin, 250 nM dexamethasone and 0.5 mM IBMX. After 3 days, the medium was replaced to DMEM, FBS and insulin for further 2 days. Cells were then allowed to differentiate for 7 more days in medium supplemented only with FBS.

2.4. Decrease of β-arrestins 1/2 function

siRNA sequences targeting human β-arrestin 1 and 2 and GAPDH were already described (Shenoy et al., 2006; Gong et al., 2008). HeLa cells stably expressing MC5R-GFP (2.5×10^5 cells) were plated in 6-well dishes and 24 h later were transfected with 60 nM β-arrestin 2 and GAPDH siRNAs and 6 nM β-arrestin 1 siRNAs using Lipofectamine 2000, according to the manufacturer's protocol. Forty-eight hours later, cells were split into 24-well dishes and serum-deprived during 16 h. All assays were performed 72 h after siRNA transfection. Silencing levels were confirmed by β-arrestin 1/2 immunoblotting detection using a mouse anti-β-arrestin 1 antibody (BD Biosciences) that cross-reacts with β-arrestin 2 (Luo et al., 2008; Fernandez et al., 2008).

The interference on β-arrestin 1 and 2 function was also obtained by transfecting HeLa cells stably expressing MC5R-GFP with dominant negative β-arrestin 1 and 2 mutants (delta-LIELD-F391A and pcDNA3-β-arrestin 2 290–409, respectively) and with both mutants. Cells transfected with the pcDNA3.1 vector were used as control. All assays were performed 48 h after transfection. The dominant negative β-arrestin 1 and 2 mutants were kindly provided by Dr. Jeffrey Benovic (Department of Biochemistry and Molecular Biology, Thomas Jefferson University) and Dr. Jean-Luc Parent (Rheumatology Division of the Faculty of Medicine at University of Sherbrooke), respectively.

2.5. Cell treatment and preparation of cellular extracts

HeLa cells expressing MC5R-GFP and 3T3-L1 differentiated adipocytes were serum starved 16 h prior to stimulation with 1 μM α-MSH for different periods of time. The concentration of α-MSH used was selected after ERK1/2 activation analysis by western-blotting of a dose–response experiment (Supplementary Fig. 1). Thirty minutes before α-MSH stimuli, cells were treated with PD98059 (100 μM) and UO126 (10 μM) for ERK1/2 signaling inhibition, with PKC inhibitor GF109203X (5 μM) and with the PKA blocker Rp-cAMP (10–100 μM). Cells incubated with PdBU (1 μM) and Forskolin (10 μM), during 15 min, were used as GF109203X and Rp-cAMP positive controls, respectively. The specific inhibition of G protein isoforms $G\alpha_s$ and $G\alpha_i$ was carried out by incubation with CTX (100 ng/ml) and PTX (100 ng/ml), respectively, overnight before α-MSH stimulus. Although CTX is a cAMP-elevating agent when administered for a short period of time, CTX sustained treatment results in a Gs down-regulation and, consequently, in a decline of cAMP levels (Lin et al., 1993; Mizuno et al., 2002). The selection of the proper inhibitors concentrations was based on the product datasheets and on the literature (Nishihara et al., 2004; Fricke et al., 2004). Cells were then washed with ice-cold PBS and solubilized in lysis buffer (50 mM Tris–HCl pH 7.6, 10 mM NaCl, 5 mM EDTA, 1 mM β-glycerophosphate, phosphatase and protease inhibitor cocktails) containing 0.25% Triton X-100. Lysates were sonicated and protein concentrations were determined using the Bradford protein assay (BioRad).