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Modulation of α_{2C} adrenergic receptor temperature-sensitive trafficking by HSP90

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ABSTRACT

Decreasing the temperature to 30 °C is accompanied by significant enhancement of α_{2C}-AR plasma membrane levels in several cell lines with fibroblast phenotype, as demonstrated by radioligand binding in intact cells. No changes were observed on the effects of low-temperature after blocking receptor internalization in α_{2C}-AR transfected HEK293T cells. In contrast, two pharmacological chaperones, dimethyl sulfoxide and glycerol, increased the cell surface receptor levels at 37 °C, but not at 30 °C. Further, at 37 °C α_{2C}-AR is co-localized with endoplasmic reticulum markers, but not with the lysosomal markers. Treatment with three distinct HSP90 inhibitors, radicicol, macbecin and 17-DMAG significantly enhanced α_{2C}-AR cell surface levels at 37 °C, but these inhibitors had no effect at 30 °C. Similar results were obtained after decreasing the HSP90 cellular levels using specific siRNA. Co-immunoprecipitation experiments demonstrated that α_{2C}-AR interacts with HSP90 and this interaction is decreased at 30 °C. The contractile response to endogenous α_{2C}-AR stimulation in rat tail artery was also enhanced at reduced temperature. Similar to HEK293T cells, HSP90 inhibition increased the α_{2C}-AR contractile effects only at 37 °C. Moreover, exposure to low-temperature of vascular smooth muscle cells from rat tail artery decreased the cellular levels of HSP90, but did not change HSP70 levels. These data demonstrate that exposure to low-temperature augments the α_{2C}-AR transport to the plasma membrane by releasing the inhibitory activity of HSP90 on the receptor traffic, findings which may have clinical relevance for the diagnostic and treatment of Raynaud Phenomenon.

1. Introduction

The effects of circulating catecholamines are mediated by specific plasma membrane proteins, named adrenergic receptors. Adrenergic receptors are members of the G protein coupled receptors superfamily (GPCR) and are divided into β, α_1 and α_2-AR [1]. Three distinct genes have been identified that encode for separate subtypes of α_2-AR [2,3]. Lacking specific ligands, the progress in understanding α_2-AR pathophysiology was based on genetic models individually targeting each subtype [4,5]. These studies demonstrated distinct tissue distribution and functional roles for each α_2-AR subtype. Specifically, α_{2C}-AR is expressed in brain, atria, kidney, and hepatic cells, and in vascular smooth muscle cells (VSMC) from the peripheral vasculature [2–5]. Like other α_2-AR subtypes, the cellular effects of α_{2C}-AR are mediated by coupling to $G_{\alpha i}$ leading to inhibition of adenylate cyclase, inhibition of voltage Ca^{2+} channels, stimulation of phospholipase C, A_2 and D and activation of MAP kinases [2–5]. A functional coupling to $G_{\alpha s}$ has also been reported for α_2-AR, but it is apparent only at high agonist concentration or after inhibition of $G_{\alpha i}$ and its physiological significance remains unknown [6,7]. In the heterologous systems, α_{2C}-AR is poorly transported to the plasma membrane [8,9]. In contrast, in the neuroendocrine cell lines the receptor is efficiently targeted to the plasma membrane, suggesting a cell specific α_{2C}-AR intracellular trafficking [8,9]. Overall, α_{2C}-AR remains the least characterized α_2-AR subtype, and the mechanisms regulating the receptor intracellular trafficking are not fully understood.

However, a role of α_{2C}-AR in the pathology of Raynaud Phenomenon has been suggested. This disease is characterized by enhanced vasoconstriction in response to cold, emotional stress or exposure to vibrations [10,11]. The involvement of an unknown α_2-AR subtype was suggested by early publications of Flavahan and Freedman groups, based on the observation that the α_2-AR stimulation modulates the vasoconstriction at reduced temperature, whereas α_1-AR has no effect [12,13]. Subsequently, elegant work from Flavahan's group demonstrated that the vascular tone at low-temperature is specifically modulated by the α_{2C}-AR subtype, which is silent at 37 °C but it is functional at lower temperatures [14,15].

During the last decade, significant progress was made in understanding the mechanisms controlling the intracellular protein traffic from the folding site represented by the endoplasmic reticulum to the functional destination [16,17]. It has been found that many newly synthesized proteins are transported along the biosynthetic pathway in an inefficient manner [17–19]. For example, within the GPCR class, only 50% of the newly synthesized δ-opioid receptors are transported to the plasma membrane [20]. The fate of the newly synthesized GPCR results from the interactions with several

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specialized proteins, generically named molecular chaperones [17–19]. These molecular chaperones are heterogeneous, with different subcellular localization (cytosol, Golgi, endoplasmic reticulum) and have different outcomes on the chaperoned protein, like improving the folding status and favoring the transport, or determining intracellular retention and proteasomal degradation. Thus, it is not surprising that interfering with the activity or expression of different molecular chaperones has been found to change the rate of intracellular transport for several proteins. Likewise, downregulation of the cellular levels of AHSA1, a HSP90 co-chaperone, enhanced the cell surface of CFTR Δ508 mutant [21]. In contrast, inhibition of HSP90 activity decreased the maturation rate of insulin receptor and nicotinic receptors [22,23]. Currently few specific pharmacological agents are available to modulate the activity of molecular chaperones. This deficit is partly compensated by several non-specific compounds, named pharmacological chaperones, which were shown to stabilize the misfolded proteins and allow their progression in the biosynthetic pathway [17,24,25]. The non-specific pharmacological chaperones are including osmolytes (dimethyl sulfoxide and glycerol), inhibitors of sarco(endoplasmic) reticulum Ca^{2+} ATP-ase and factors modifying the heat shock response. Interestingly, exposure to low-temperature has also been suggested to function in the same way as non-specific pharmacological chaperones, improving the subcellular transport of CFTR Δ508 mutant and potassium channels human ether-a-go-go-related gene channels [26–28].

Understanding the mechanisms regulating the intracellular trafficking of specific proteins can provide new therapeutic approaches to several diseases caused by accumulation of misfolded proteins. Therefore, in the present work we studied the subcellular localization of α_{2C}-AR at 37 °C and at low-temperature and we investigated the mechanisms underlying the particular receptor intracellular trafficking.

2. Materials and methods

2.1. Plasmids

Human α_{2C}-AR wild-type receptor in pcDNA3.1+ vector was a gift from Dr. D. Bylund (University of Nebraska Medical Center). Human HA-tagged-α_{2C}-AR was a gift from Drs. C. Hurt and T. Angelotti (Stanford University Medical School). Human $\alpha_{2C\text{-}322\text{–}325del}$-AR and not-tagged and 3×HA tagged α_{2B}-AR in pcDNA 3.1+ vector were bought from Missouri S&T cDNA Resource Center. HSP90AB and GRP94 in pCMV5 vector were from Origene. DsRed-Rab7 was from Addgene (plasmid 12661). Human α_{2C}-AR and α_{2B}-AR tagged with GFP at their C-termini were generated by PCR after the stop codon was mutated, and the sequences restricted with HindIII/SalI in frame with GFP were ligated into the pEGFP-N1 vector (Clonetech).

2.2. Antibodies and chemicals

The sources of the antibodies used in the present study were as follows: anti-GFP, anti-hemagglutinin (anti-HA), Na^+/K^+ ATP-ase and β-actin were from Santa Cruz Biotechnology; anti-HSP70 and anti-GM130 were from BD Biosciences and anti-HSP90 was from Enzo Life Sciences; rabbit polyclonal α_{2C}-AR antibody corresponding to the aminoacids 309–324 from the receptor third intracellular loop was from Abcam; fluorescently labeled secondary antibodies (Alexa Fluor 594-labeled anti-mouse and anti-rabbit), and 4,6-diamidino-2-phenylindole were obtained from Invitrogen. Macbecin and 17-DMAG were from Enzo Life Sciences and radicicol was from Sigma Aldrich. Lactacystin and MG132 were from Tocris.

2.3. Cell culture and transient transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transient transfection of the HEK293T cells was carried out using LipofectAMINE 2000 reagent (Invitrogen), following the manufacturer instructions. In brief, HEK293T cells were cultured on 10 cm^2 dishes and transfected at ~80% confluency with 3 μg receptor construct in DMEM with no antibiotics and no FBS. Six hours later the cells were trypsinized and plated at a density of 10^6 cells/well in 6-well plates for western blot experiments, or 4×10^5 cells/well in 12-well plates for radioligand binding experiments and cAMP determination. For co-transfections experiments, the cells were cultured on 6-well plates and transfected with 0.5 μg α_{2C}-AR and 2.5 μg pcDNA3.1 or GRP94 per well. After six hours the cells were trypsinized and plated on 12-well plates as above. For siRNA studies, HEK293T cells in 10 cm^2 dishes were first transfected with α_{2C}-AR (3 μg/plate) and after 6 h were trypsinized and plated on 12-well plates together with siRNA complexes (3 nM/well) in Transfection Agent 1 following the manufacturer instructions (Applied Biosciences).

2.4. Ligand binding in intact cells

The cells in 12-well plates were serum starved for 24 h to prevent differential proliferation at different temperatures and we found no differences in cell number in these conditions. Eighteen hours before the experimental procedure, half of the plates were transferred to a similar incubator at 30 °C, whereas the other were incubated at 37 °C and served as control. Two days after transfection the medium was aspired and the cells were incubated in DMEM containing 20 nM [^{3}H]-RX821002 for four hours at 4 °C. The binding was terminated by aspiration of the radioactivity and the cells were washed three times with DMEM, digested with 1 M NaOH, and the bound radioactivity was determined in a β-scintillation counter. The non-specific binding determined in presence of non-radioactive rauwolscine (10 μM) represented less than 10% of the total radioactivity and it was subtracted from the presented results. In preliminary experiments we found that performing the binding procedure at low-temperature prevents [^{3}H]-RX821002 internalization. This was tested by washing the cells three times with 50 mM glycine (pH = 3) to remove plasma membrane bound radioactivity. Subsequently the cells were trypsinized and fractionated using Qproteome cell compartment kit (Qiagen) and the radioactivity was determined in each fraction. Most of the radioactivity (89 ± 3%, n = 3) was present in the initial acidic washouts, and the remaining was present in the membrane fraction (7 ± 2%) and in the cytosolic fraction (5 ± 3%, n = 3).

2.5. Flow cytometry

For measurement of total receptor expression, HEK293T cells were transiently transfected with 500 ng of GFP-tagged receptors for 48 h. The cells were collected, washed twice with PBS and resuspended at a density of 8×10^6 cells/mL. Total GFP fluorescence was then measured on a flow cytometer (BD Biosciences FASCalibur) as described previously [29,30].

2.6. Fluorescence microscopy

For fluorescence microscopic analysis of receptor subcellular localization, HEK293T cells were grown on coverslips pre-coated with poly-L-lysine in 6-well plates and transfected with 500 ng of GFP-tagged receptors. For colocalization of GFP-tagged receptors with the ER and lysosomal markers, HEK293T cells grown on coverslips were transfected with 500 ng of GFP-tagged receptors and 300 ng of pDsRed2-ER or pDsRed2-Rab7. The cells were fixed with 4% paraformaldehyde–4% sucrose mixture in PBS for 15 min and stained with 4, 6-diamidino-2-phenylindole for 5 min. For colocalization of GFP-tagged receptors with the *cis*-Golgi marker GM130 or with the plasma membrane marker Na^+/K^+ ATP-ase, HEK293T cells were