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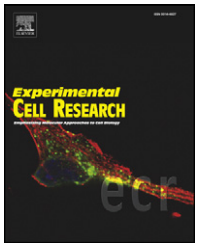

Research Article

Dual Mode of glucagon receptor internalization: Role of PKCα, GRKs and β-arrestins

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ABSTRACT

Glucagon levels are elevated in diabetes and some liver diseases. Increased glucagon secretion leads to abnormal stimulation of glucagon receptors (GRs) and consequent elevated glucose production in the liver. Blocking glucagon receptor signaling has been proposed as a potential treatment option for diabetes and other conditions associated with hyperglycemia. Elucidating mechanisms of GR desensitization and downregulation may help identify new drug targets besides GR itself. The present study explores the mechanisms of GR internalization and the role of PKCα, GPCR kinases (GRKs) and β-arrestins therein. We have reported previously that PKCα mediates GR phosphorylation and desensitization. While the PKC agonist, PMA, did not affect GR internalization when tested alone, it increased glucagon-mediated GR internalization by 25–40% in GR-expressing HEK-293 cells (HEK-GR cells). In both primary hepatocytes and HEK-GR cells, glucagon treatment recruited PKCα to the plasma membrane where it colocalized with GR. We also observed that overexpression of GRK2, GRK3, or GRK5 enhanced GR internalization. In addition, we found that GR utilizes both clathrin- and caveolin-mediated endocytosis in HEK-GR cells. Glucagon triggered translocation of both β-arrestin1 and β-arrestin2 from the cytosol to the perimembrane region, and overexpression of β-arrestin1 and β-arrestin2 increased GR internalization. Furthermore, both β-arrestin1 and β-arrestin2 colocalized with GR and with Cav-1, suggesting the possible involvement of these arrestins in GR internalization.

Introduction

Glucagon plays an important role in the regulation of blood glucose homeostasis. Dysregulation of glucagon metabolism is associated with several diseases, most importantly with diabetes, where the basal level of glucagon is elevated and the bihormonal insulin-to-glucagon relationship is altered [1–3]. Chronic hyperglucagonemia leads to increased hepatic production of glucose, aggravating hyperglycemia in diabetic patients. Suppression of glucagon secretion by somatostatin improves the condition of diabetic subjects. In recent

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years there has been an increased interest in the glucagon receptor (GR) as a therapeutic target in diabetes management [4]. However, application of GR antagonists in the chronic treatment of diabetes has had limited success thus far [5]. Therefore, gaining a better understanding of the mechanisms of GR desensitization is critical for evaluation of drug efficacy and identification of novel drug targets for diabetes.

The GR is a prototypical, Family B heptahelical G protein-coupled receptor (GPCR). Prolonged agonist stimulation attenuates signaling through GPCRs. This phenomenon, termed homologous desensitization, ensures precise spatiotemporal regulation of signal transduction by filtering input from multiple receptors and protecting against acute and chronic over-stimulation [6,7]. The first phase of desensitization involves phosphorylation of receptors by GPCR kinases (GRKs). GRK-mediated phosphorylation promotes binding of β-arrestins, which results in uncoupling of receptors from G proteins. In addition, it has been suggested that GRK2 and GRK3 can mediate GPCR desensitization through interaction with receptors in a phosphorylation-independent manner [8,9]. Other kinases, such as PKC and PKA, mediate desensitization and downregulation of some GPCRs, even in the absence of respective ligands (heterologous desensitization). Previous studies from other laboratories and ours have shown that glucagon-stimulated cAMP production is diminished upon treatment of cells with glucagon and other hormones, such as angiotensin and vasopressin, as well as with PKC activating agents, suggesting that GRs undergo both homologous and heterologous internalization [10,11].

Desensitized receptors are sequestered in the cytoplasm within minutes of agonist stimulation. Internalization of receptors is the primary mechanism of signal transduction attenuation and is also crucial for receptor resensitization. Whereas most GPCRs utilize clathrin-mediated endocytosis, utilization of clathrin-independent pathways, including caveolar endocytosis, has been reported [12].

We and others have previously shown that, upon 30 min of glucagon stimulation, GRs are internalized in vitro [13,14] and in vivo [15]. The receptor's carboxyl terminus is required for internalization of both the human [13] and rat GR [14]. Phosphorylation of certain Ser residues has been shown to be necessary for GR internalization [15]. However, the precise mechanisms of GR internalization are largely unknown. Therefore, the aim of the present study was to identify the key pathways of GR internalization. In this study, we have investigated (i) the role of PKC and GRK in GR internalization, (ii) utilization of different internalization pathways by GR, and (iii) ability of β-arrestin1 and β-arrestin2 to promote GR internalization.

Materials and methods

Materials

Cell culture media, Penicillin/Streptomycin, L-glutamine and amino acids were from Cellgro (Kansas City, MO). Nystatin, Filipin III, DABCO, Poly-L-lysine, phenylarsine oxide, Geneticin/G418, Tetracycline, Sodium-butyrate, bovine serum albumin (BSA), 1,4-diazobicyclo [2,2,2] octane (DABCO), paraformaldehyde, phenylmethylsulphonyl fluoride (PMSF), phosphate-buffered saline (PBS), Tris-buffered saline, and Di(N-succinimidyl) 3,3′-dithiodipropionate were from Sigma (St Louis, MO). Glucagon was purchased from Bachem (King of Prussia, PA). ^{125}I-glucagon was purchased from Linco (St. Charles, MO). Phorbol 12-myristate 13-acetate (PMA), Forskolin, Phorbol 12,13-Dibutyrate (PDBu), 4α-phorbol ester, and Mowiol were purchased from Calbiochem (San Diego, CA). The transfection reagents Lipofectamine 2000 and OptiMEM were from Invitrogen (Rockville, MD). Lysotracker and Texas Red – Phalloidin and Hoescht 33258, as well as the fluorescently labeled secondary antibodies, goat anti-rabbit Alexa fluor 568, goat anti-mouse Alexa fluor 350, goat anti-mouse Alexa fluor 568 and goat anti-mouse Alexa fluor 488 were from Molecular Probes (Carlsbad, CA). The mouse anti-clathrin heavy chain antibody and the rabbit anti-EEA1 antibody were from Affinity Bioreagents (Golden, CO). The GR antibody (ST-18) was generated in rabbit for an epitope in the last 18 amino acids as previously reported [16]. The rabbit anti-caveolin (Cav)-1 antibody and the mouse anti-FLAG antibody were from Sigma (St. Louis, MO). The rabbit anti-PKCα antibody used for Western blots was from Research Diagnostics (Concord, MA). The rabbit anti-PKCα antibody used for immunoprecipitation experiments was from Cell Signaling Technology (Danvers, MA). The rabbit anti-PKCδ antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-GAPDH antibody was from Trevigen (Gaithersburg, MD).

Plasmids

β-arrestin1-GFP and β-arrestin2-GFP plasmids were a generous gift of Dr. Cornelius Krasel (University of Würzburg, Würzburg, Germany). Dominant negative (DN) β-arrestin1 (V53D), β-arrestin2 DN (arr2 319–418), GRK2, GRK3 and GRK5 were a generous gift from Dr. Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA). PKCα and PKCα DN were kindly provided by Dr. Jae-Won Soh (Inha University, Incheon, South Korea). PKCα-YFP was kindly provided by Dr. R. Kubitz (Heinrich-Heine University, Dusseldorf, Germany). Plasmid pcDNA 3.1, used as an empty vector control, was purchased from Invitrogen (Rockville, MD).

Cell culture and transfection

Hepatocytes were isolated by the collagenase perfusion technique as previously described [17] from male Golden Syrian hamsters (Harlan Sprague Dawley, Indianapolis, IN, 100–130 g body wt) fed a rodent chow diet ad libitum. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine (200 μM), MEM non-essential and MEM essential amino acids, Penicillin (5000 IU/mL) and Streptomycin (5000 μg/mL). HEK-293 cells stably expressing the rat glucagon receptor (HEK-GR, [18] were maintained under the culture conditions described above. HEK-293 cells stably expressing a tetracycline-inducible FLAG-tagged GR (HEK-FLAG-GR) [16] were maintained in DMEM/F-12 (50:50) supplemented with 200 μg/mL Geneticin/G418, 10% fetal calf serum and Penicillin/Streptomycin. The GR expression was induced by incubation of cells in an induction medium, DMEM/F-12 supplemented with sodium butyrate, tetracycline, 10% serum and Penicillin (5000 IU/mL) and Streptomycin (5000 μg/mL) for 24–48 h. The cells were transfected with 4–8 μg plasmid DNA using Lipofectamine 2000, following the manufacturer's instructions. Control cells were transfected with the equivalent amount of vector plasmid (empty vector). Twenty-four hours after transfection, the cells were seeded onto poly-L-lysine coated coverslips and glass-bottom dishes (for microscopy) or onto