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Functional consequences of chemically-induced β-arrestin binding to chemokine receptors CXCR4 and CCR5 in the absence of ligand stimulation

Marcel Liebick*, Sarah Henze, Viola Vogt, Martin Oppermann

Department of Cellular and Molecular Immunology, University of Göttingen, Göttingen, Niedersachsen, Germany

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ABSTRACT

Chemokine receptor signaling is a tightly regulated process which was for a long time exclusively attributed to heterotrimeric G proteins. β-Arrestins constitute a separable signaling arm from classical heterotrimeric G proteins, in addition to their well-established roles in receptor desensitization and endocytosis. In order to clearly dissect β-arrestin- from G protein-dependent effects we forced the recruitment of β-arrestin to CXCR4 and CCR5 independently of agonist-promoted receptor activation through chemically-induced dimerization. Targeting β-arrestins to receptors at the plasma membrane prior to chemokine stimulation attenuated G protein-mediated calcium release. Association of β-arrestins to the receptors was sufficient to induce their internalization in the absence of ligand and this effect could be further enhanced by translocation of a constitutively active β-arrestin 1 variant. CXCR4 and CCR5 were targeted to different intracellular compartments upon chemical-induced dimerization with β-arrestins and reproduced the intracellular distribution of receptors after activation with their respective ligands. Our data further provide evidence for direct β-arrestin-mediated signaling via MAP kinases ERK 1/2. These results provide clear evidence that CXCR4- or CCR5-β-arrestin complexes induce receptor endocytosis and signaling in the absence of G protein coupling and ligand-induced conformational changes of the receptor.

1. Introduction

Chemokine receptors, members of the large family of G protein-coupled receptors (GPCR), are expressed on different cellular subsets including leukocytes and cancer cells [1,2]. In combination with a comprehensive arsenal of chemokines they orchestrate leukocyte trafficking and play fundamental roles either in physiological or pathological immune responses. The classical model of chemokine receptor signaling is based on heterotrimeric G proteins of the $G\alpha_i$ subset which are tightly regulated by arrestins [3–5]. Arrestins uncouple G proteins from the receptor but also fulfill important functions as scaffolding proteins for the assembly of signalosomes which are involved in downstream signaling pathways [6,7].

The 'arresting effect' of the two non-visual arrestins β-arrestin 1 and β-arrestin 2 was discovered in the context of β-adrenergic receptor signaling and is still regarded as the main mechanism underlying GPCR desensitization after ligand activation in non-retinal tissues [8]. Further studies revealed an important role for β-arrestins as adaptors to target receptors for internalization through its scaffolding of AP-2 and clathrin. This results in translocation of the activated receptor complex into clathrin coated pits from where they are internalized into early endosomes [9]. Following internalization receptors are sorted either for recycling or degradation where prolonged binding of β-arrestins favors receptor degradation rather than recycling since β-arrestins also interact with E3 ubiquitin ligases [10–12]. By shielding the phosphorylated C-terminus of the receptor from phosphatases β-arrestins also determine the level of dephosphorylation which goes along with receptor resensitization [13]. Besides termination of GPCR signaling β-arrestins are also involved in signal transduction to downstream effectors [14,15]. Interactions with proteins such as c-Src, ERK, JNK3 and p38 illustrate that β-arrestins actively link GPCRs to intracellular signaling pathways including MAPK cascades aside from classical G protein signaling [8,16].

Although β-arrestins are mandatory to maintain GPCR function a clear discrimination of G protein-mediated and β-arrestin-mediated effects is not easily achieved since recruitment of β-arrestin under physiological conditions requires ligand binding to the receptor which is accompanied by G protein activation [6,17]. A clear differentiation

Abbreviations: βArr, β-arrestin; AP2, adaptor protein 2; Dmr A/C, dimer A/C; FKBP12, 12 kDa FK506-binding protein; FRB, FKBP12-rapamycin-binding; GPCR, G protein-coupled receptor; MCF, mean channel fluorescence; mTOR, mechanistic target of rapamycin; p38, p38 mitogen-activated protein kinase; PFA, paraformaldehyde; PTX, pertussis toxin; Rec-DmrA, receptor-dimer A; s.d., standard deviation

* Corresponding author at: Department of Cellular and Molecular Immunology, Georg-August University, Humboldtallee 34, 37073 Göttingen, Germany.
E-mail address: marcel.liebick@med.uni-goettingen.de (M. Liebick).

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between these two mechanisms becomes even more important in a refined model of classical GPCR signaling according to which 'biased' ligands stabilize receptor conformations which selectively activate distinct downstream signaling pathways either by G proteins or β-arrestin [18]. The recent discovery of G protein and β-arrestin pathway selective ligands has opened new prospects for the pharmaceutical development of such compounds which specifically promote beneficial effects while antagonizing detrimental ones [19,20]. In the light of β-arrestin-mediated effects and biased signaling a better understanding of downstream events which are conducted by β-arrestin is becoming more important.

By fusion of the rapamycin binding domain of mTOR (DmrC) to β-arrestin 1 or 2 and fusion of the rapamycin interacting protein FKBP12 (DmrA) to the CXCR4 or CCR5 carboxyl terminus we established an experimental system for the ligand-independent association of β-arrestin and the chemokine receptors CXCR4 and CCR5 in the absence of G protein activation [21–23]. In this study we provide data which expand the role of β-arrestin for receptor internalization and distribution by showing that β-arrestin alone can guide internalized chemokine receptors into distinct intracellular compartments. Furthermore, we demonstrate the capacity of β-arrestin to activate MAP kinases ERK1/2 in the absence of previous G protein activation.

2. Experimental procedures

2.1. Materials

Cell culture media and additives were from Biochrom, Thermo Fisher Scientific or Invitrogen. Cell culture consumables were from Greiner Bio-One. Chemicals, reagents western blot equipment and further consumables were obtained from Carl Roth, Sigma Aldrich, Sarstedt or Thermo Fisher. Primers were synthesized by Iba. Restriction enzymes, ligases and phosphatases were from NEB. DNA purification kits were from Machery & Nagel. Anti-receptor antibodies were from Biolegend (anti-CXCR4 clone 12G5) and RnD systems (anti-CCR5 clone T21/8). Anti-HA antibody was from Roche (clone 3F10). Anti-phospho ERK (D13.14.4E) and anti-pan ERK (137F5) antibodies were from Cell Signaling. Further used antibodies were self-established (anti-CCR5 clone R22; anti-β-arrestin 1/2 clone 21B1). Secondary antibodies and conjugates were from Jackson Immuno Research. Agonists and antagonists were obtained from Merck, Peprotech, Perkin Elmer or Sigma Aldrich. The Dimerization system was established using the iDimerize™ inducible heterodimer system from Clontech.

2.2. Eukaryotic expression constructs

Restriction sites were added to the cDNA of CXCR4, CCR5 and β-arrestin 1/2 or a phosphorylation state independent β-arrestin 1 varient [24] (provided by J.L. Benovic, Thomas Jefferson University, Philadelphia) by polymerase chain reaction. Receptor cDNA was ligated in frame into the eukaryotic expression vector pHet-Nuc1 (DmrA domain), β-arrestin 1/2 cDNA was ligated into the vector pHet-1 (DmrC domain). All cDNAs were integrated upstream of the coding sequence of the dimerizing domains to obtain C-terminally modified receptor or β-arrestin constructs. For cellular expression receptor-DmrA constructs were subcloned into the eukaryotic expression vector pEF1/Myc-His A. The integrity of all constructs was verified by Sanger sequencing. Before stable transfection all plasmids were linearized.

2.3. Cell culture and transfection

Human embryonic kidney 293 cells (HEK 293) were stably or transiently transfected by calcium phosphate precipitation. Stably transfected cells were selected with 1.2 mg geneticin per ml cell culture medium. Cells were cultivated in DME medium supplemented with 10% heat—inactivated fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin under an atmosphere of 5% CO_2 at 37 °C.

2.4. Agonist-/AP21967-induced internalization and flow cytometry

Receptor expression levels were determined by flow cytometry using anti-receptor antibodies. For the analysis of agonist-/AP21967-induced internalization cells were resuspended in binding medium (BM; DMEM, 0.2% BSA, 10 mM HEPES, pH 7.4) and treated with 125 nM agonist or 500 nM AP21967 (4 h, 37 °C). Samples were taken regularly and unbound ligand was removed by acid wash with EM medium (DMEM, 0.2% BSA, 10 mM MES, pH 2.5) at 4 °C. Receptors were stained with anti-receptor antibodies (1:50) and detected by flow cytometry. Receptor internalization was calculated as the percentage of expressed receptors on the cell surface at time 0 min. The initial rate of internalization was determined using the slope equation to calculate the slope between time points 0 min and 15 min.

2.5. Agonist-/AP21967-dependent intracellular calcium mobilization

For calcium monitoring cells were resuspended in DME medium (5% FCS) and loaded with Indo-1 AM dye (1 μM Indo-1 AM, 0.015% pluronic acid F127; 25 min, 30 °C). Subsequently, the solution was diluted with additional DMEM (10% FCS; 10 min, 37 °C). Loaded cells were washed and resuspended in Krebs-Ringer buffer. Receptor stimulation was done using agonist (50 nM) or AP21967 (500 nM) for 6 min. Changes in the ratio of Indo-$1_{405\ nm}$ and Indo-$1_{510\ nm}$ were monitored and further processed using FlowJo.

2.6. Membrane purification via ultracentrifugation

Cells were stimulated according to previous protocols and detached with membrane buffer (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2 * 6\ H_2O$). Cell lysates were loaded on discontinuous (20%; 35%; 50%) sucrose gradient and centrifuged (350,000 rpm, 2 h, 4 °C). Subsequently the interphase between 20 and 35% sucrose (membrane fraction) was collected and centrifuged again (50,000 rpm, 15 min, 4 °C). Dried pellets were resuspendend in lysis buffer (50 μM TRIS, 150 μM NaCl, 0.4 μM EDTA, 1% Igepal, 0.5% SDS) and were analyzed together with the cytosolic fraction by immunoblotting.

2.7. Immunoblotting

Cell lysates were resolved in SDS sample buffer containing 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.05% bromophenol blue and 10% β-mercaptoethanol and separated using electrophoresis on a 10% SDS polyacrylamide gel matrix. Samples were blotted on nitrocellulose membrane by semi dry blotting. β-Arrestin was detected using horseradish peroxidase (HRP) coupled anti-HA antibodies (100 ng/ml) in TRIS-buffered saline (TBS) supplemented with 0.1% Tween-20 and 1% bovine serum albumin (BSA) (60 min, rt). Proteins were detected by chemiluminescence.

2.8. Immunofluorescence

HEK 293 cells were grown on lysine-coated glass coverslips in 24-well plates (overnight, 37 °C). Cells were stimulated with agonist (125 nM) or AP21967 (500 nM) and fixed with 3% paraformaldehyde in PBS (20 min, 37 °C). Reactive aldehyde groups were saturated with 50 mM NH_4Cl in PBS (30 min, 37 °C). Cells were permeabilized with PBS supplemented with 0.1% saponin and 0.2% gelatin (15 min, 37 °C). Staining was done using biotinylated anti-HA antibodies (0.7 μg/ml) for β-arrestin and anti-receptor antibodies (5 μg/ml) (60 min, on ice). After washing with PBS-saponin FITC labeled goat anti mouse Ig and StAv-Alexa647 (2 μg/ml) was used for detection (60 min, on ice). After extensive washing, cells were mounted with Mowiol supplemented with 0.1% *p*-phenylenediamine. Cells were evaluated by confocal laser