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Agonist occupancy of a single monomeric element is sufficient to cause internalization of the dimeric β_2-adrenoceptor

Nana Sartania, Shirley Appelbe, John D. Pediani, Graeme Milligan *

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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Abstract

A range of studies have indicated that many rhodopsin-like, family A G protein-coupled receptors, including the β_2-adrenoceptor, exist and probably function as dimers. It is less clear if receptors internalize as dimers and if agonist occupancy of only one element of a dimer is sufficient to cause internalization of a receptor dimer into the cell. We have used a chemogenomic approach to demonstrate that this is the case. Following expression of the wild type β_2-adrenoceptor, isoprenaline but not 1-(3′′4′-dihydroxyphenyl)-3-methyl-1-butanone, which does not have significant affinity for the wild type receptor, caused receptor internalization. By contrast, 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone, but not isoprenaline that does not have high affinity for the mutated receptor, caused internalization of $Asp^{113}Ser\beta_2$-adrenoceptor. Following co-expression of wild type and $Asp^{113}Ser\beta_2$-adrenoceptors each of isoprenaline and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone caused the co-internalization of both of these two forms of the receptor. Co-expressed wild type and $Asp^{113}Ser\beta_2$-adrenoceptors were able to be co-immunoprecipitated and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone produced internalization of the wild type receptor that was not prevented by the β-adrenoceptor antagonist propranolol that binds with high affinity only to the wild type receptor. These results demonstrate that agonist occupancy of either single binding site of the β_2-adrenoceptor dimer is sufficient to cause internalization of the dimer and that antagonist occupation of one of the two ligand binding sites is unable to prevent agonist-mediated internalization.

Keywords: Dimerisation; Internalization; Chemogenomics; Adrenoceptor

1. Introduction

In recent years it has become increasingly clear that G protein-coupled receptors (GPCRs)[1] can exist as dimers [1,2] and a growing body of evidence suggests that the dimer is probably the configuration able to interact with high affinity with a hetero-trimeric G protein [3,4]. As with many aspects of the mechanism of action and regulation of members of the GPCR superfamily the β_2-adrenoceptor (β_2-AR) has been a key model system [5]. Co-immunoprecipitation studies employing differentially epitope-tagged forms of this GPCR [6] were instrumental in providing compelling evidence of dimerization and the first application of bioluminescence resonance energy transfer to probe GPCR quaternary structure in intact cells utilized this GPCR [7]. In more recent studies the β_2-AR has been shown to dimerize during protein maturation and prior to plasma membrane delivery [8] and been used to indicate that transmembrane domain VI of this receptor contains sequences important for protein-protein dimer contacts [8].

Following agonist occupancy, the vast majority of GPCRs, including the β_2-AR [9] internalize into cells, and frequently then recycle back to the cell surface, as part of the complex series of process that are generically described as desensitization and resensitization [10]. Whether a class A GPCR homo-

Abbreviations: β_2-AR; β_2-adrenoceptor; GPCR; G protein-coupled receptor; HA-β_2-AR; haemagluttinin-epitope-tagged β_2-adrenoceptor; L-158,870; 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone; VSV-G-β_2-AR; VSV-G-epitope tagged β_2-adrenoceptor.

* Corresponding author. Tel.: +44 141 330 5557; fax: +44 141 330 4620.
E-mail address: g.milligan@bio.gla.ac.uk (G. Milligan).

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dimer can be activated and internalize in response to agonist-occupancy of only one of the two monomers is currently unclear and there is a highly variable literature on whether activated GPCRs internalize as dimers or dissociate into the corresponding monomers during this process [11–14].

The original demonstration that Asp^{113} in transmembrane domain III of the β_2-AR is the charge partner that allows high affinity interactions with catecholamine ligands [15] was the prototypic exemplar of the application of chemogenomics to GPCR function. Conversion of Asp^{113} to Ser results in substantial loss of affinity for catecholamines and related ligands but synthesis of 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (also known as L-158,870) [15], which has no significant affinity for the wild type β_2-AR but is capable of accepting hydrogen bonds from the β-hydroxymethyl side chain of Ser^{113}, demonstrated conclusively that the loss of affinity for catecholamines produced by this mutation was not simply a reflection of lack of expression or misfolding of the mutant receptor [15]. Herein, we take advantage of these observations to demonstrate that internalization of an $Asp^{113}\beta_2$-AR-$Ser^{113}\beta_2$-AR 'hetero-dimer' requires agonist occupancy of only one monomer within the dimer, that it does not matter which monomer is agonist-occupied, that agonist occupancy of one monomer is dominant when the other monomer is occupied by an antagonist and, as a consequence, that the β_2-AR is activated and internalizes as a dimeric complex.

2. Materials and methods

2.1. Materials

[^{3}H]dihydroalprenolol (94 Ci/mmol) was from GE Healthcare. 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (also known as L-158,870) was the kind gift of M. Candelore, Merck Research Laboratories (Rahway, NJ). Flp-In T-REx HEK293 cells were from Invitrogen (Paisley, U.K.). The anti-VSV-G antiserum was produced in house and the anti HA-antibody 12CA5 was from (Roche Molecular Biochemicals, Nutley NJ).

2.2. Molecular constructs

N-terminally HA-and VSV-G tagged forms of the human β_2-AR were generated using standard molecular biological procedures. Asp^{113}Ser and Asp^{113}Asn forms of VSV-G-β_2-AR were produced by site-directed mutagenesis. All constructs were fully sequenced prior to analysis.

2.3. Generation of cell lines

Flp-In T-REx HEK293 cell lines [16,17] harbouring N-terminally VSV-G tagged forms of each of wild type, Asp^{113}Ser and Asp^{113}Asn forms of the β_2-AR at the Flp-In locus were produced by following the manufacturers' instructions. Following confirmation of expression of these polypeptides in a doxycycline-dependent manner, cells harbouring either $Asp^{113}Ser\beta_2$-AR or $Asp^{113}Asn\beta_2$-AR were further transfected with cDNA encoding HA-β_2-AR in pcDNA3 and individual, hygromycin-resistant clones isolated. Constitutive expression of HA-β_2-AR was then monitored by the specific binding of [^{3}H] dihydroalprenolol.

2.4. [^{3}H]dihydroalprenolol binding studies

Were performed as described by Ramsay et al. [18]. When screening for clones constitutively expressing HA-β_2-AR total binding was assessed using 2nM [^{3}H]dihydroalprenolol whilst parallel samples also containing 10 μM propranolol defined non-specific binding of the radioligand.

2.5. cAMP production

The capacity of receptor ligands or forskolin to generate [^{3}H]cAMP was measured as described previously [19] in the various HEK293 cell lines following addition of [^{3}H]adenine to cells and its intracellular conversion to [^{3}H]ATP.

2.6. Cell surface ELISA

50,000 cells per well were seeded in poly-D-lysine coated 96 well plates with or without doxycycline. After 24 h the medium was replaced with 20 mM HEPES /DMEM (pH 7.4) containing anti-VSV-G antiserum (1:1000) and cells were incubated for 30 min. The cells were washed twice with 20 mM HEPES/Dulbeccos' modified Eagles' medium and then incubated with anti-rabbit horseradish peroxidase-conjugated IgG (GE Healthcare) at a 1:5000 dilution as secondary antibody and 1 μM Hoechst 33342 nuclear stain (Boehringer Mannheim GmbH, Germany) to determine cell number. Cells were washed twice in PBS, incubated with Sureblue TMB reagent (Insight Biotechnology) for 5 min at room temperature and the absorbance at 620 nm measured using a Victor^2 plate reader (Packard Bioscience). The absorbance was normalized to the cell number in the well.

2.7. Receptor internalization studies

Immunostaining was performed essentially according to the method of Cao et al. [20]. Cells were plated on to coverslips and induced with 0.5 μg/ml doxycycline. After 24 h, the medium was changed for 20 mM HEPES/Dulbeccos' modified Eagles' medium containing the appropriate antibody/antiserum diluted 1:100 and incubated for 40 min at 37 °C in 5% CO_2. Where required, 20 mM HEPES/Dulbeccos' modified Eagles' medium containing the desired concentration of agonist was added and incubated for 30 min at 37 °C in 5% CO_2. Coverslips were washed three times with phosphate buffered saline and then cells fixed with 4% paraformaldehyde in phosphate buffered saline/5% sucrose for 10 min at room temperature followed by three more phosphate buffered saline washes. Cells were then permeabilized in 0.15% Triton X-100/3% non-fat milk/phosphate buffered saline (TM buffer) for 10 min at room temperature. The coverslips were subsequently incubated with appropriate secondary antibodies (Molecular Probes, Eugene, OR) at a dilution of 1:400 (1–4 mg/ml), upside down on Nescofilm, for 1 h at room temperature, then washed twice in TM buffer and three times with phosphate buffered saline. Finally, coverslips were mounted on to microscope slides with 40% glycerol in phosphate buffered saline.

2.8. Confocal laser-scanning microscopy

Cells were observed using a confocal laser-scanning microscope (Zeiss LSM 5 Pascal) using a Zeiss Plan-Apo 63×1.40 NA oil immersion objective, pinhole of 20 and electronic zoom 1 or 2.5 [21]. Images were analysed with MetaMorph software. For the receptor internalization studies fixed cells were used.