Available online at www.sciencedirect.com
ScienceDirect
Cellular Signalling 19 (2007) 1928–1938
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

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.

Received 14 November 2006; received in revised form 3 May 2007; accepted 9 May 2007
Available online 18 May 2007

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).

0898-6568/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2007.05.002

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.

2.9. Co-immunoprecipitation studies

Cells were harvested with or without doxycycline induction and resuspended in RIPA buffer (50 mM HEPES, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM NaF, 5 mM EDTA, 0.1 mM $NaPO_4$, 5% ethylene glycol). The cell pellet was disrupted and placed on a rotating wheel for 1 h at 4 °C. Samples were then centrifuged for 1 h at 100,000 ×*g* at 4 °C, and the supernatant transferred to a fresh tube containing 200 μl of 1×RIPA and 50 μl of protein G beads (Sigma) to pre-clear the samples. Following incubation on a rotating wheel for 1 h at 4 °C, samples were re-centrifuged at 20,817 ×*g* at 4 °C for 1 min, and the protein concentration of the supernatant determined. Samples containing equal protein amounts were incubated overnight with 40 μl of protein G beads, 1 μg of anti-VSV-G antibody (Roche) at 4 °C on a rotating wheel, and fractions were reserved to monitor protein expression in the cell lysates. Samples were centrifuged at 20,817 ×*g* for 1 min at 4 °C, and the protein G beads washed three times with RIPA buffer. Following addition of Laemmli buffer and heating to 85 °C for 4 min, both immunoprecipitated samples and cell lysate controls were revolved by SDS-PAGE using pre-cast 4–12% acrylamide Novex Bistris gel (Invitrogen). Proteins were transferred onto nitrocellulose. These membranes were incubated in 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution at room temperature on a rotating shaker for 2 h. A biotin-labelled secondary antibody was incubated overnight in 5% (w/v) low fat milk, 0.1% Tween 20/PBS (v/v) solution at 4 °C. Following further washes, the membrane was subsequently developed using ECL solution (Pierce).

3. Results

The human β_2-AR was modified to introduce the VSV-G epitope tag sequence at the N-terminus to generate VSV-G-β_2-AR. Following expression of VSV-G-β_2-AR in HEK293 cells isoprenaline produced a robust, concentration-dependent increase in cyclic AMP production with $EC_{50}=1.3\times10^{-7}$ M (Fig. 1a). When equivalent experiments were performed following expression of the point mutant VSV-G-Asp^{113} Serβ_2-AR, the potency of isoprenaline to stimulate adenylyl cyclase activity was reduced by almost 1000 fold ($EC_{50}=9.4\times10^{-5}$ M) (Fig. 1a). As anticipated from previous work [15], 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (also known as L-158,870) displayed no significant agonist activity at VSV-G-β_2-AR at concentrations below 1×10^{-3} M (Fig. 1b) but was a full agonist for adenylyl cyclase activation at VSV-G-Asp^{113}Serβ_2-AR with $EC_{50}=2.7\times10^{-5}$ M (Fig. 1b). Binding of the β_2-AR antagonist [^{3}H]dihydroalprenolol to VSV-G-β_2-AR was saturable, high affinity ($K_d=0.2$ nM) and was competed for by increasing concentrations of isoprenaline with estimated $K_i=1.4\times10^{-7}$ M (Fig. 1c). As anticipated from the adenylyl cyclase studies 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone had no significant affinity to compete with [^{3}H]dihydroalprenolol for binding to VSV-G-β_2-AR (Fig. 1c), whilst the estimated K_i for the antagonist propranolol was 3×10^{-10} M (Fig. 1c). Equivalent ligand binding studies could not be performed for VSV-G-Asp^{113}Serβ_2-AR because [^{3}H]dihydroalprenolol, like other catecholamine-based β_2-AR ligands, binds to this mutant with only low affinity.

Flp-In T-REx HEK293 cells express protein from DNA cloned into the single defined Flp-In locus in a tetracycline/doxycycline-dependent manner [16,17]. Parental Flp-In T-REx HEK293 cells, either with or without treatment with doxycycline (0.5 μg/ml, 24 h), did not display staining with an anti-

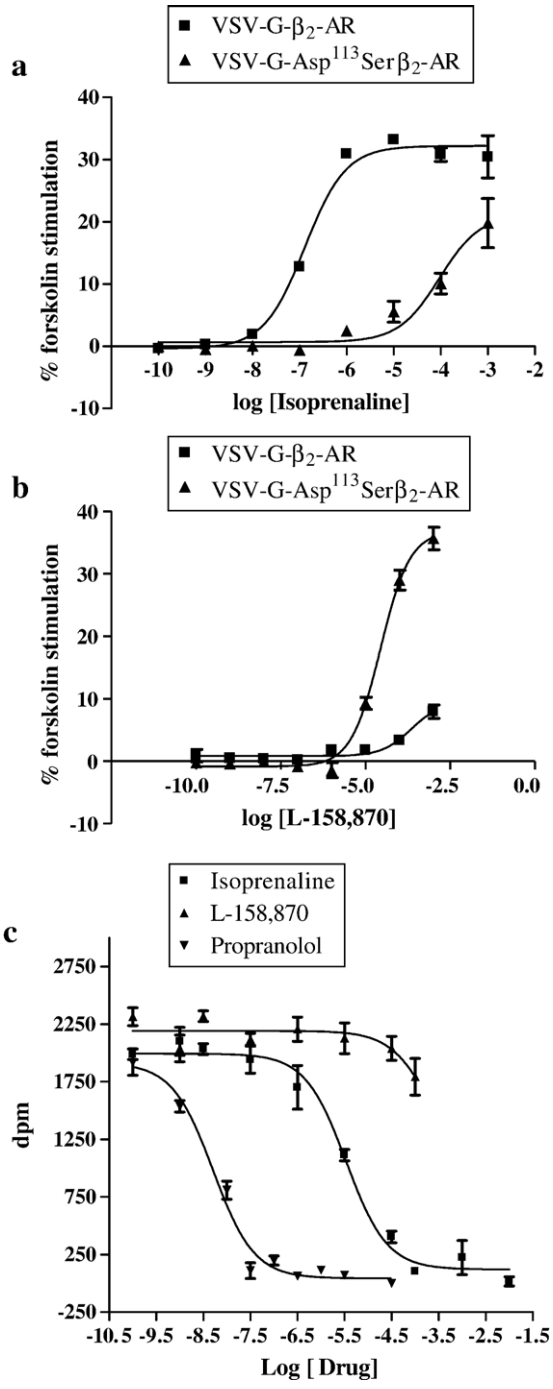


Fig. 1. Differences in potency and affinity of isoprenaline and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone for the wild type-and Asp^{113}Ser-β_2-adrenoceptor. N-terminally VSV-G tagged forms of the wild type-(squares) and Asp^{113}Ser-(triangles) β_2-AR were expressed in HEK293 cells. The capacity of varying concentrations of isoprenaline (a) and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (L-158,870) (b) to activate adenylyl cyclase activity and hence generate cyclic AMP was assessed. Data are presented relative to the stimulation produced by 10 μM forskolin. (c) The ability of varying concentrations of propranolol (inverted triangles), isoprenaline (squares) and L-158,870 (triangles) to compete with 1 nM [^{3}H]dihydroalprenolol for binding to VSV-G-β_2-AR was assessed in cell membranes.

VSV-G antiserum (Fig. 2a). By contrast, following cloning of VSV-G-β_2-AR into the Flp-In locus, treatment with doxycycline resulted in cell surface staining with the anti-VSV-G antiserum that was not detected in cells that had not been exposed to doxycycline (Fig. 2a). Cell surface ELISA studies using the anti-VSV-G antiserum confirmed the appearance of anti-VSV-G immunoreactivity in response to doxycycline in cells harbouring VSV-G-β_2-AR at the Flp-In locus (Fig. 2b). However, as anticipated from the foregoing, doxycycline treatment of parental Flp-In T-REx HEK293 cells did not result in the appearance of such immunoreactivity (Fig. 2b). Equivalent clones of Flp-In T-REx HEK293 cells were established to express either VSV-G-Asp^{113}Serβ_2-AR or VSV-G-Asp^{113}Asnβ_2-AR in a doxycycline-dependent manner. As with VSV-G-β_2-AR, expression of both VSV-G-Asp^{113}Serβ_2-AR and VSV-G-Asp^{113}Asnβ_2-AR was only detected following doxycycline treatment in both immunocytochemistry and ELISA studies (Fig. 2a and b). The extent of binding of the anti-VSV-G antiserum in the intact cell ELISA studies indicated that these two point mutant forms of the β_2-AR were expressed to similar levels as VSV-G-β_2-AR at the cell surface (Fig. 2b). Immunoblotting of lysates from doxycyline-treated cells habouring the various forms of the β_2-AR also detected the appearance of VSV-G-immunoreactive polypeptides (Fig. 2b) that were not present in lysates of doxycyline-treated, parental Flp-In T-REx HEK293 cells (Fig. 2b).

Following doxycycline-induction of VSV-G-β_2-AR expression, treatment with isoprenaline (10 μM, 30 min) caused substantial internalization of the receptor (Fig. 3). This effect was not produced by 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (100 μM, 30 min) (Fig. 3). By contrast, 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (100 μM, 30 min), but not isoprenaline (10 μM, 30 min), caused internalization of doxycycline-induced VSV-G-Asp^{113}Serβ_2-AR (Fig. 3), whilst at these concentrations neither isoprenaline nor 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone altered the plasma membrane distribution of VSV-G-Asp^{113}Asnβ_2-AR (Fig. 3).

We next generated cell lines able to express both the wild type β_2-AR and one of the mutants. For these, an N-terminally HA-tagged form of the wild type β_2-AR (HA-β_2-AR) was generated

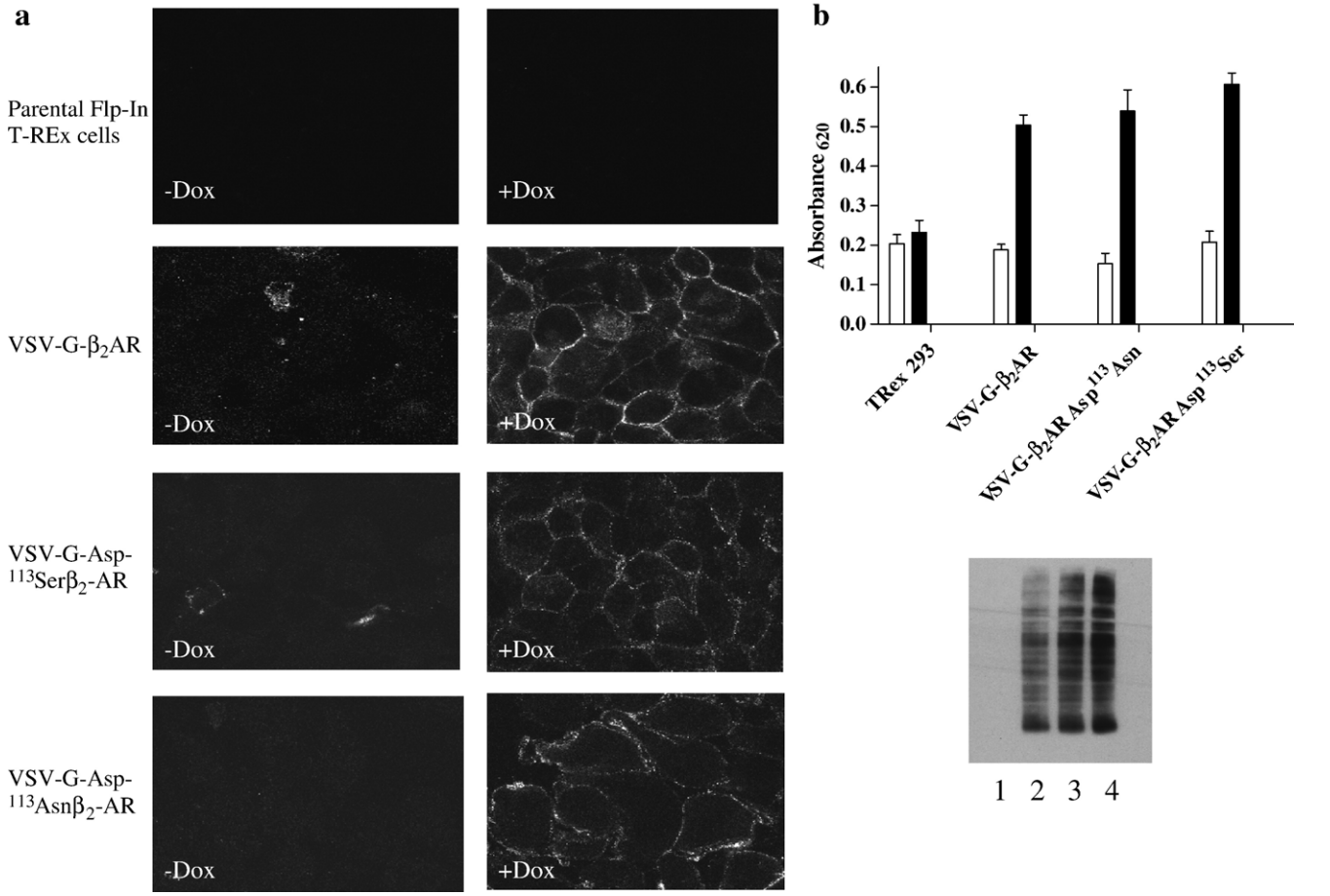


Fig. 2. Expression of VSV-G tagged forms of the β_2-adrenoceptor from the Flp-In locus of Flp-In T-REx HEK293 cells is dependent upon doxycycline treatment. (a) Parental, non-transfected Flp-In T-REx HEK293 cells and lines harbouring VSV-G-β_2-AR, VSV-G-Asp^{113}Serβ_2-AR or VSV-G-Asp^{113}Asnβ_2-AR at the Flp-In locus were untreated (left hand panels,-Dox) or treated with doxycycline (0.5 μg/ml, 24 h) (right hand panels,+Dox). Cells were fixed and stained for anti-VSV-G immunoreactivity. (b) Top panel: parental Flp-In T-REx HEK293 cells and Flp-In T-REx HEK293 cell lines harbouring VSV-G-β_2-AR, VSV-G-Asp^{113}Asnβ_2-AR or VSV-G-Asp^{113}Ser β_2-AR were untreated (open bars) or treated with doxycycline (filled bars) as in a. Intact cell ELISA assays employed an anti-VSV-G antiserum. Lower panel: lysates from the cell lines above (1) parental, (2) VSV-G-β_2-AR, (3) VSV-G-Asp^{113}Asnβ_2-AR and (4) VSV-G-Asp^{113}Ser β_2-AR that were treated with doxycycline were resolved by SDS-PAGE and immunoblotted with anti-VSV-G.

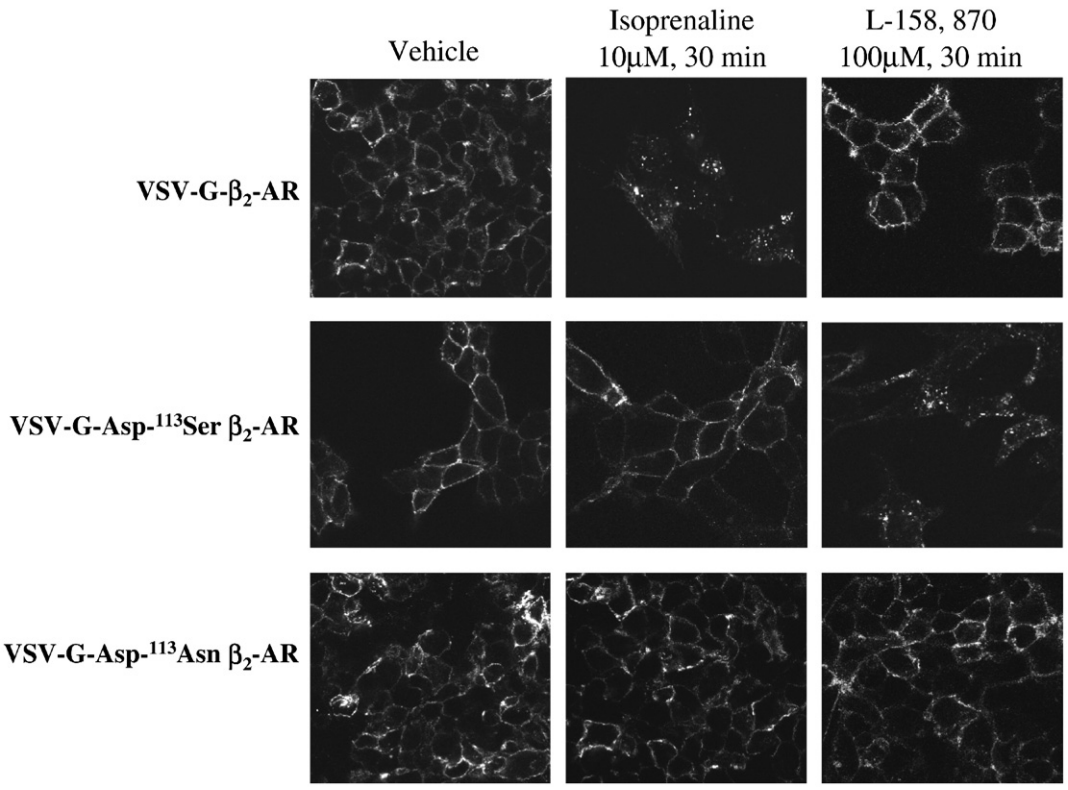


Fig. 3. Isoprenaline and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone cause selective internalization of the wild type-and $Asp^{113}Ser$-β_2-adrenoceptors. Flp-In T-REx HEK293 cells harbouring VSV-G-β_2-AR (top panels), VSV-G-$Asp^{113}Ser\beta_2$-AR (middle panels) or VSV-G-$Asp^{113}Asn\beta_2$-AR (lower panels) at the Flp-In locus were treated with doxycycline (0.5 μg/ml, 24 h) to induce expression. These cells were untreated (left hand panels) or treated with either isoprenaline (10 μM) (middle panels) or 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (L-158,870) (100 μM) (right hand panels) for 30 min prior to fixation and detection of the receptors with anti-VSV-G antiserum.

and expressed stably, but this time constitutively, in Flp-In T-REx HEK293 cells that already harboured either VSV-G-$Asp^{113}Ser\beta_2$-AR or VSV-G-$Asp^{113}Asn\beta_2$-AR at the doxycycline-inducible locus. As neither VSV-G-$Asp^{113}Ser\beta_2$-AR nor VSV-G-$Asp^{113}Asn\beta_2$-AR bind [^{3}H]dihydroalprenolol with significant affinity and, as shown earlier (Fig. 2), these constructs are only expressed following treatment with doxycycline, preliminary screens for positive clones constitutively expressing HA-β_2-AR were performed by monitoring specific binding of a single (2 nM), near saturating concentration of [^{3}H]dihydroalprenolol (not shown). In clones that harboured VSV-G-$Asp^{113}Asn\beta_2$-AR and constitutively expressed HA-β_2-AR, then in the absence of doxycycline only HA-β_2-AR was present, and this was internalized by challenge with isoprenaline (Fig. 4a). Following treatment with doxycycline for 24 h both HA-β_2-AR and VSV-G-$Asp^{113}Asn\beta_2$-AR were expressed and displayed an overlapping distribution pattern at the cell surface (Fig. 4b). Treatment with isoprenaline caused internalization of HA-β_2-AR (Fig. 4b), but now also caused internalization of VSV-G-$Asp^{113}Asn\beta_2$-AR (Fig. 4b), whilst treatment of these cells with 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone had no detectable effect on the cellular distribution of either HA-β_2-AR or VSV-G-$Asp^{113}Asn\beta_2$-AR (Fig. 4c). As anticipated, in these HA-β_2-AR and VSV-G-$Asp^{113}Asn\beta_2$-AR co-expressing cells, isoprenaline was a potent and high efficacy activator of adenylyl cyclase, whilst 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone was again without effect at concentrations up to 100 μM (Fig. 4d).

In clones that harboured VSV-G-$Asp^{113}Ser\beta_2$-AR and constitutively expressed HA-β_2-AR, then, as expected, in the absence of doxycycline only HA-β_2-AR was present, and this again was internalized by challenge with isoprenaline (Fig. 5a). Following doxycyline-treatment of these cells, both isoprenaline and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone were able to stimulate adenylyl cyclase activity in a concentration-dependent manner (Fig. 5b) and with EC_{50} values akin to those observed with the expression of each receptor variant in isolation (Fig. 1). Combined anti-HA and anti-VSV-G immunostaining confirmed the co-expression of VSV-G-$Asp^{113}Ser\beta_2$-AR and HA-β_2-AR with an overlapping, cell surface distribution (Fig. 5c). Treatment of these doxycycline-exposed cells with isoprenaline resulted in internalization of both HA-β_2-AR and VSV-G-$Asp^{113}Ser\beta_2$-AR (Fig. 5c) and, in this case, treatment with 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone also resulted in the internalization of both VSV-G-$Asp^{113}Ser\beta_2$-AR and HA-β_2-AR (Fig. 5d). Pixel by pixel analysis of the distribution of the two receptor variants in single cells demonstrated a very high overlap correlation co-efficient (R^2=0.85) at the cell surface in the absence of adrenergic ligands (Fig. 5e) and following treatment of the cells with either

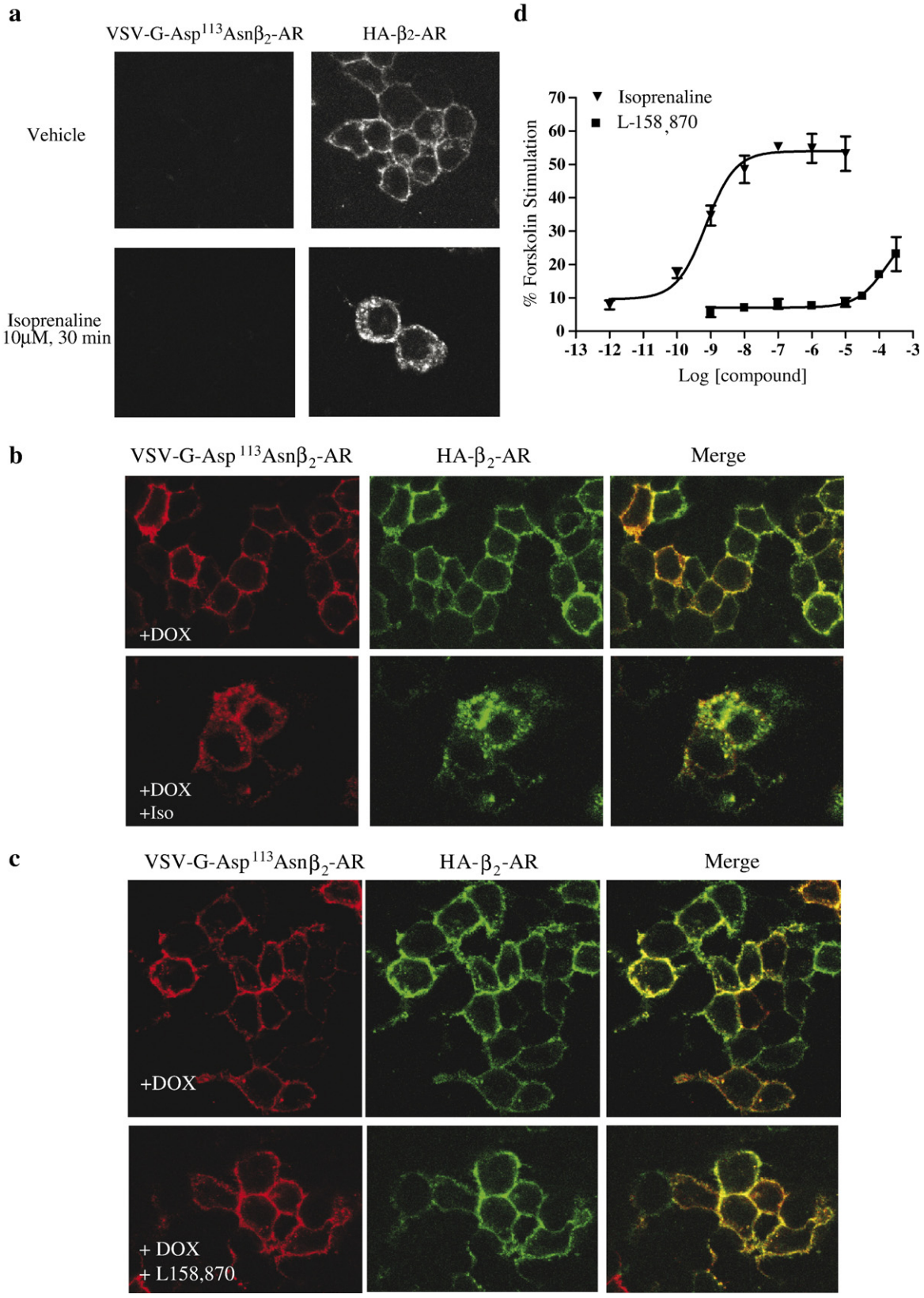
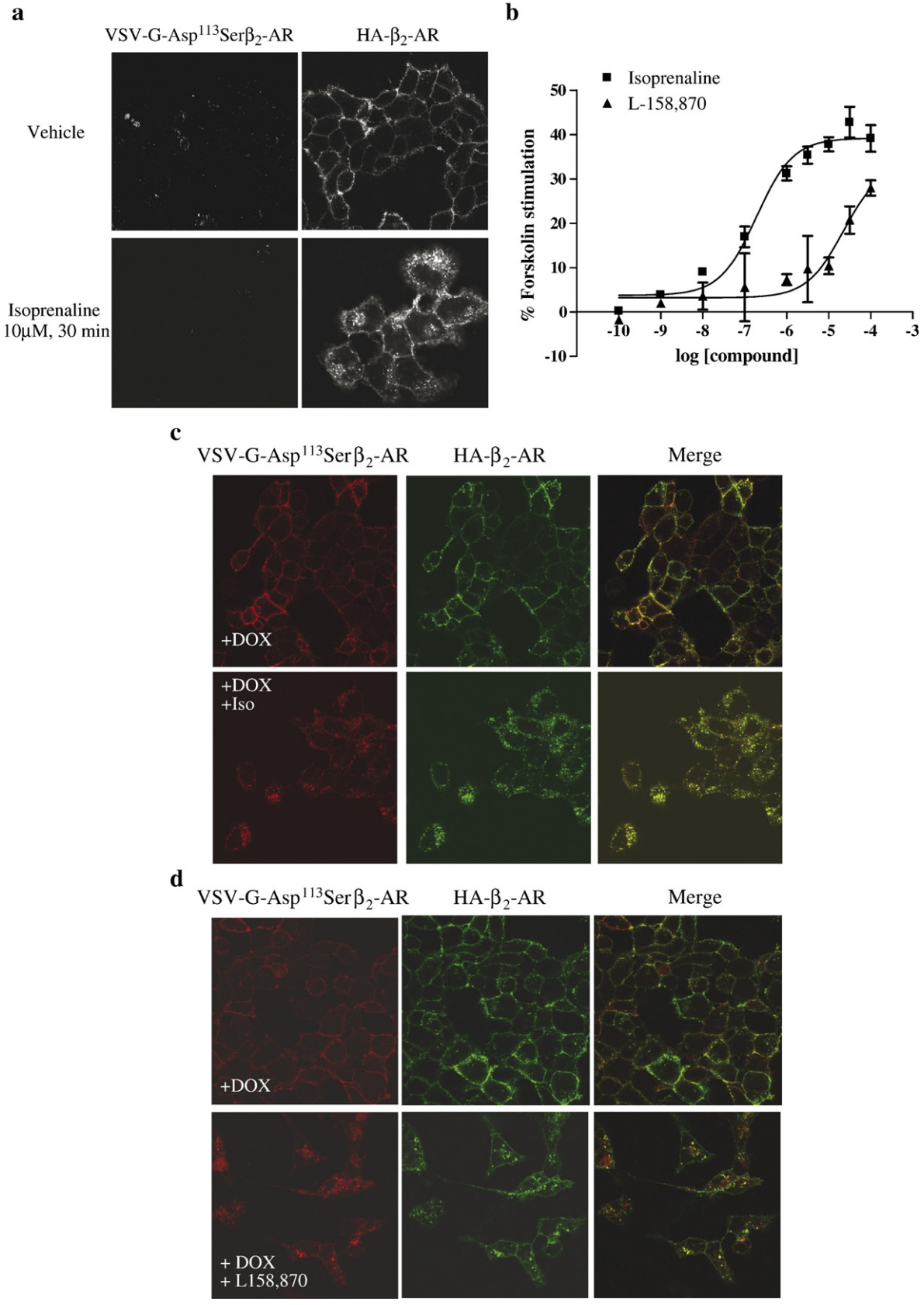


Fig. 4. Co-expression of wild type-and Asp[113]Asn-β_2-adrenoceptors results in their co-internalization in response to isoprenaline but not 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone. a, b, c, d, Flp-In T-REx HEK293 cells harbouring VSV-G-Asp[113]Asnβ_2-AR at the Flp-In locus and constitutively expressing HA-β_2-AR were not induced (a) or induced with doxycycline (0.5 μg/ml, 24 h) (b, c, d) and expression of the receptor isoforms measured via anti-HA or anti-VSV-G immunostaining. In cells induced to co-express HA-β_2-AR and VSV-G-Asp[113]Asnβ_2-AR merging of the signals (merge) indicated overlapping distribution patterns (yellow, b, c). Cells were then treated with vehicle or challenged with either isoprenaline (10 μM) or 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (L-158,870) (100 μM) as noted. In d stimulation of adenylyl cyclase activity was measured as in Fig. 1.

isoprenaline or 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone such pixel by pixel analysis indicated a very high degree of overlap of distribution in punctuate, intracellular vesicles (Fig. 5e). Although a range of previous studies have confirmed the ability of the β_2-AR to form a homo-dimer [6–8] we wished to confirm 'hetero-dimer' protein-protein interactions between co-



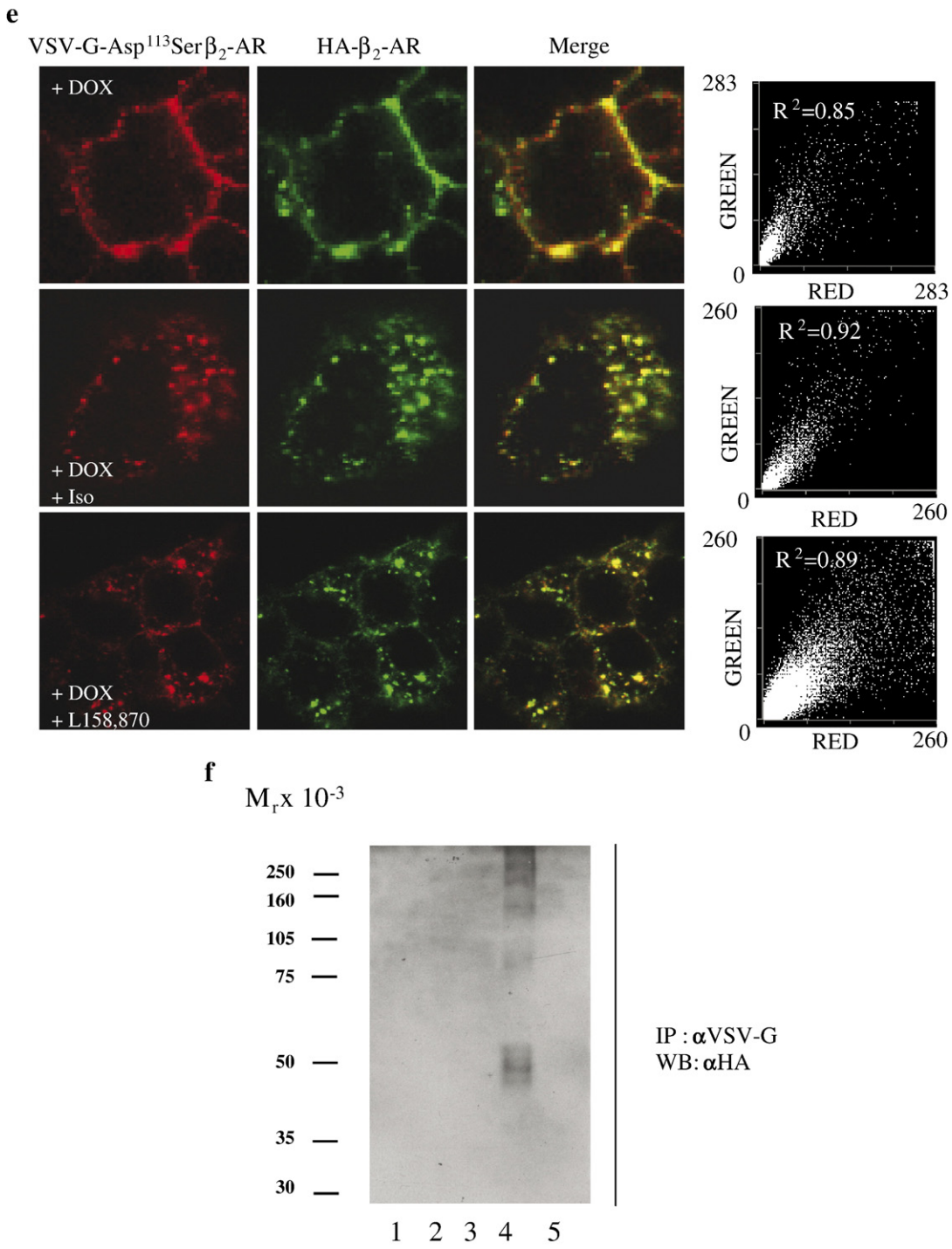


Fig. 5. Co-expression of wild type-and Asp^{113}Ser-β_2-adrenoceptors results in their dimerization and co-internalization in response to either isoprenaline or 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone. a, b, c, d, e, Flp-In T-REx HEK293 cells harbouring VSV-G-Asp^{113}Serβ_2-AR at the Flp-In locus and constitutively expressing HA-β_2-AR were not induced (a) or induced with doxycycline (b, c, d, e) as in Fig. 4. In b, stimulation of adenylyl cyclase activity was measured as in Fig. 1. a, c, d, e, cells were treated with vehicle or challenged with either isoprenaline (10 μM) or 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (L-158,870) (100 μM) as noted. Cells were subsequently fixed and stained with anti-HA antibody or anti-VSV-G antiserum and appropriate secondary antibodies as in Fig. 4. Red=VSV-G-Asp^{113}Serβ_2-AR, Green=HA-β_2-AR, Yellow=overlap of distribution. c, d, wide-field images, e, single cell, pixel by pixel analysis of the correlation of colour overlap. $R^2=1.0$ represents a perfect correlation, $R^2=-1.0$ represents no correlation. (f) Parental Flp-In T-REx HEK293 cells (1) or Flp-In T-REx HEK293 cells transfected to express HA-β_2-AR (2) VSV-G-Asp^{113}Serβ_2-AR (3) or both HA-β_2-AR and VSV-G-Asp^{113}Serβ_2-AR (4) were lysed and immunoprecipitation (IP) performed with anti-VSV-G. Samples were resolved by SDS-PAGE and subsequently immunoblotted (WB) to detect HA-β_2-AR. In (5) lysed cell samples individually expressing HA-β_2-AR and VSV-G-Asp^{113}Serβ_2-AR were mixed prior to immunoprecipitation.

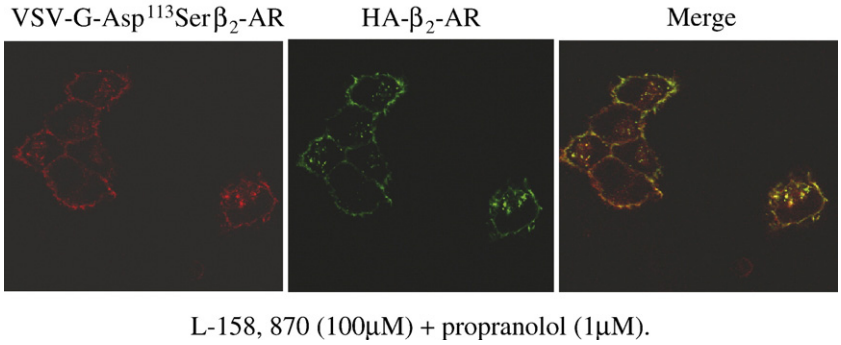


Fig. 6. Propranol does not block 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone-mediated internalization of HA-β_2-AR. Flp-In T-REx HEK293 cells harbouring VSV-G-Asp^{113}Serβ_2-AR (red) at the Flp-In locus and constitutively expressing HA-β_2-AR (green) were induced with doxycycline (0.5 μg/ml, 24 h). Both 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (100 μM) and propranolol (1 μM) were added for 30 min and the cellular distribution of the two receptor variants monitored as in Figs. 4 and 5. Yellow staining (merge) indicates overlapping distribution.

expressed VSV-G-Asp^{113}Serβ_2-AR and HA-β_2-AR. In cells co-expressing VSV-G-Asp^{113}Serβ_2-AR and HA-β_2-AR, immunoprecipitation with the anti-VSV-G antiserum resulted in the co-immunoprecipitation of anti-HA immunoreactivity and this only occurred with co-expression of the two receptor variants (Fig. 5 f).

Both of the cell systems expressing both the wild type and an Asp^{113} mutant β_2-AR produced an agonist-dependent redistribution pattern consistent with internalization of a β_2-AR dimer requiring agonist occupancy of only one element of the dimer. As anticipated from basic pharmacology, the antagonist propranolol (1 μM) prevented isoprenaline-induced internalization of HA-β_2-AR when this form of the wild type receptor was expressed alone (data not shown). To test whether occupancy of the second element of the dimer with an antagonist would exert a 'dominant negative' effect on agonist function, cells induced to co-express VSV-G-Ser$^{113}\beta_2$-AR along with HA-β_2-AR were concurrently treated with 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone (100 μM) and propranolol (1 μM). Propranolol did not prevent 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone-mediated internalization of HA-β_2-AR (Fig. 6).

4. Discussion

The β_2-AR is one of the most actively studied GPCRs [5] and key experiments that have contributed greatly to the now widely held view that members of the class A, rhodopsin-like family of GPCRs can exist as homo-dimers [1,2] have utilized this receptor as a model [6–8]. The β_2-AR has also been a key model system employed to understand processes and mechanisms involved in GPCR internalization and in agonist-mediated desensitization [5,10]. Two major questions in relation to the life history of family A, GPCR homo-dimers have been particularly difficult to address, however. These are whether a single molecule of agonist is sufficient to initiate signals and internalization and if so, whether the receptor is internalized as a dimer. The most obvious interpretation of the ability of the retina to respond to single photons of light is that the induced switch from 11-cis to all-trans retinal in a single monomer of a rhodopsin dimer is sufficient to generate signal within the highly organized paracrystalline arrays of rhodopsin dimers present in rod outer segments [4]. However, the rod outer segment is a very specialized structure with high levels of rhodopsin and it is unclear if these conclusions can be extrapolated to other receptors. The major limitation in addressing this question is, of course, that in a GPCR homo-dimer, the two monomers will bind the same ligand and with similar affinity. To overcome this limitation we have taken advantage of the classic chemogenomic demonstration that Asp^{113} in transmembrane domain III of the β_2-AR is the charge partner for the aminergic function of catecholamine ligands [15]. Although mutation of Asp^{113} to Ser resulted in loss of affinity and potency for aminergic ligands, potency was generated for 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone because this is able to form a hydrogen bond with the β-hydroxymethyl side chain of the introduced serine [15]. Initially we established cell lines able to express each of the wild type, Asp^{113}Ser and Asp^{113}Asn forms of the β_2-AR. Each of these was tagged with the VSV-G epitope to allow detection of expression of the Ser^{113} and Asn^{113} variants as these are not able to bind classic β-adrenoceptor antagonists with sufficient affinity to perform traditional ligand binding studies and the tag was placed at the N-terminus to allow intact cell ELISA measurement of trans-plasma membrane, cell surface delivery. Finally, each was expressed in a doxycycline-inducible manner in Flp-In T-REx HEK293 cells. Following induction, a similar level of each variant was detected at the cell surface and, as expected, isoprenaline treatment was only able to cause significant internalization of the wild type receptor. 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone was only able to cause internalization of the Asp^{113}Serβ_2-AR whereas neither ligand caused substantial internalization of the Asp^{113}Asnβ_2-AR variant. When cells harboring either Asp^{113}Serβ_2-AR or Asp^{113}Asnβ_2-AR at the inducible locus were further transfected to express HA-wild type β_2-AR in a constitutive manner, then without doxycycline-mediated induction, isoprenaline caused internalization of HA-β_2-AR, whilst following induction isoprenaline was now able to co-internalize both HA-β_2-AR and the VSV-G-β_2-AR variant. These results are entirely compatible with the generation of VSV-G-Asp^{113}Serβ_2-AR-HA-β_2-AR and VSV-G-Asp^{113}Asnβ_2-AR-HA-β_2-AR 'hetero-dimers' in the appropriate cells and with

isoprenaline occupancy of the wild type monomer resulting in co-internalization of the unoccupied dimer partner. We also showed direct interactions between co-expressed VSV-G-Asp^{113}Serβ_2-AR and HA-β_2-AR via co-immunoprecipitation studies. Equally because 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone was only able to cause internalization of the wild type β_2-AR when it was co-expressed with Asp^{113}Serβ_2-AR then these two examples of single agonist-induced co-internalization imply both that agonist occupancy of only one element of a β_2-adrenoceptor dimer is required to cause internalization, that it does not matter which monomer of the dimer is agonist-occupied and, as a corollary, that the receptor internalizes as a dimer.

It is important to recognize how and where GPCR dimers form. Strong evidence indicates that the β_2-AR dimerizes during protein synthesis and maturation and prior to plasma membrane delivery [8]. As such, in the 'double stable' cell lines used herein, prior to induction of expression of the VSV-G tagged β_2-AR mutants, the HA-β_2-ARs observed at the cell surface are likely to be true 'homo-dimers'. As there is no evidence to support the idea that agonists cause dissociation of the β_2-AR dimer [6,7], even if there is some evidence to suggest this for other GPCRs [2], then even upon induction of expression of the mutants, the HA-β_2-AR 'homo-dimers' will persist until the destruction of these proteins by normal cellular mechanisms. Induced expression of the mutants is produced in the face of ongoing constitutive expression of the HA-β_2-AR, thus a mixture of HA-β_2-AR-HA-β_2-AR 'homo-dimers', VSV-G mutant β_2-AR-VSV-G mutant β_2-AR 'homo-dimers' and HA-β_2-AR-VSV-G mutant β_2-AR 'hetero-dimers' should be generated. In cells co-expressing HA-β_2-AR and VSV-G-Asp^{113}Ser β_2-AR then isoprenaline treatment will cause internalization of HA-β_2-AR-HA-β_2-AR 'homo-dimers' as well as HA-β_2-AR-VSV-G-Asp^{113}Serβ_2-AR 'hetero-dimers'. VSV-G-Asp^{113}Ser β_2-AR-VSV-G-Asp^{113}Serβ_2-AR homo-dimers are not responsive to isoprenaline. Therefore, the merged HA and VSV-G immunofluorescent images identify the location of the internalized wild type+mutant 'hetero-dimers'. Similar logic defines interpretation of the internalization pattern in response to 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone. Of course, co-addition of isoprenaline and 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone resulted in very marked internalization of both HA-β_2-AR and VSV-G-Asp^{113}Serβ_2-AR in cells co-expressing the two variants (data not shown) and in such cells both ligands stimulated adenylyl cyclase activity with the expected potency. However, these internalization experiments cannot be usefully interpreted because of the different dimer forms present in the cells.

Interestingly, in cells co-expressing HA-β_2-AR and VSV-G-Asp^{113}Serβ_2-AR, propranolol did not prevent the co-internalization of HA-β_2-AR observed in response to 1-(3′4′-dihydroxyphenyl)-3-methyl-1-butanone. This indicates that when a β_2-AR dimer has one monomer occupied by agonist and the second by antagonist, the conformational changes associated with agonist function that result in interactions with β-arrestins and then internalization [10] are not prevented and, indeed, are dominant.

The conclusion that binding of a single agonist is sufficient to generate function in the β_2-AR dimer has parallels with the metabotropic glutamate-like, class C GPCRs including the γ-aminobutyric acid (GABA)b receptor. Although the GABAb receptor is a special case in that it is an obligate hetero-dimer [22], the GABAbR2 component is unable to bind GABA and thus activation must result from binding of a single agonist [22]. Despite this parallel, the current results are the first unequivocal demonstration that the homo-dimer of a mammalian class A GPCR requires binding of only a single agonist to induce internalization, and support the idea that class A GPCRs internalize as dimers. Previous studies using a combination of the wild type *Saccharomyces cerevisiae* Ste-2 pheromone receptor and a Ser^{184}Arg mutant of this receptor that does not bind α-factor have indicated that a receptor monomer lacking ligand can be co-internalized by the agonist-occupied wild type receptor [11]. However, these studies did not extend the analysis to utilize a ligand capable of binding only to the mutated receptor in a manner akin to the current studies. Equally, the ability of the β_2-AR to co-internalize with the delta opioid peptide opioid receptor when the two receptors are expressed at high levels [14,23] has been interpreted as evidence for GPCR hetero-dimer interactions. As others have concluded that the β_2-AR is generated as a dimer [8] and remains as a dimer upon ligand binding [7], the current results are supportive of the concept that family A GPCRs are constitutive dimers throughout their life history [2,24]. The basic chemogenomic approach applied herein should allow this concept to be tested for virtually any de-orphanized GPCR of choice and subsequent experiments will define if this is a generally applicable model.

Acknowledgements

These studies were supported by the Biotechnology and Biosciences Research Council.

References

[1] S. Angers, A. Salahpour, M. Bouvier, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 409.
[2] G. Milligan, Mol. Pharmacol. 66 (2004) 1.
[3] J.L. Baneres, J. Parello, J. Mol. Biol. 329 (2003) 815.
[4] D. Fotiadis, Y. Liang, S. Filipek, D.A. Saperstein, A. Engel, K. Palczewski, FEBS Lett. 564 (2004) 281.
[5] R.J. Lefkowitz, Trends Pharmacol. Sci. 25 (2004) 413.
[6] T.E. Herbert, S. Moffett, J.-P. Morello, T.P. Loisel, D.G. Bichet, C. Barret, M. Bouvier, J. Biol. Chem. 271 (1996) 16384.
[7] S. Angers, A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis, M. Bouvier, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3684.
[8] A. Salahpour, S. Angers, J.F. Mercier, M. Lagace, S. Marullo, M. Bouvier, J. Biol. Chem. 279 (2004) 33390.
[9] M. von Zastrow, B.K. Kobilka, J. Biol. Chem. 267 (1992) 3530.
[10] S.S. Ferguson, J. Zhang, L.S. Barak, M.G. Caron, Life Sci. 62 (1998) 1561.
[11] A. Yesilaltay, D.D. Jenness, Mol. Biol. Cell 11 (2000) 2873.
[12] R. Latif, R. Graves, T.F. Davies, J. Biol. Chem. 277 (2002) 45059.
[13] M.M. Berglund, D.A. Schober, M.A. Esterman, D.R. Gehlert, J. Pharmacol. Exp. Ther. 307 (2003) 1120.
[14] T.T. Cao, A. Brelot, M. von Zastrow, Mol. Pharmacol. 67 (2005) 288.
[15] C.D. Strader, T. Gaffney, E.E. Sugg, M.R. Candelore, R. Keys, A.A. Patchett, R.A. Dixon, J. Biol. Chem. 266 (1991) 5.
[16] M. Canals, L. Jenkins, E. Kellett, G. Milligan, J. Biol. Chem. 281 (2006) 16757.

[17] S. Milasta, J. Pediani, S. Wilson, S. Trim, M. Wyatt, P. Cox, M. Fidock, G. Milligan, Mol. Pharmacol. 69 (2006) 479.
[18] D. Ramsay, N. Bevan, S. Rees, G. Milligan, Br. J. Pharmacol. 133 (2001) 315.
[19] H.E. Moon, A. Cavalli, D.S. Bahia, M. Hoffmann, D. Massotte, G. Milligan, J. Neurochem. 76 (2001) 1805.
[20] T.T. Cao, H.W. Deacon, D. Reczek, A. Bretscher, M. von Zastrow, Nature 401 (1999) 286.
[21] S. Milasta, N.A. Evans, L. Ormiston, S. Wilson, R.J. Lefkowitz, G. Milligan, Biochem. J. 387 (2005) 573.
[22] J.H. White, A. Wise, M.J. Main, A. Green, N.J. Fraser, G.H. Disney, A.A. Barnes, P. Emson, S.M. Foord, F.H. Marshall, Nature 396 (1998) 679.
[23] B.A. Jordan, N. Trapaidze, I. Gomes, R. Nivarthi, L.A. Devi, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 343.
[24] G. Milligan, M. Bouvier, FEBS J. 272 (2005) 2914.