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RESEARCH PAPER

Physical and functional interaction between CB_1 cannabinoid receptors and β_2-adrenoceptors

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Background and purpose: The CB_1 cannabinoid receptor and the β_2-adrenoceptor are G protein-coupled receptors (GPCRs) co-expressed in many tissues. The present study examined physical and functional interactions between these receptors in a heterologous expression system and in primary human ocular cells.
Experimental approach: Physical interactions between CB_1 receptors and β_2-adrenoceptors were assessed using bioluminescence resonance energy transfer (BRET). Functional interactions between these receptors were evaluated by examining receptor trafficking, as well as extracellular signal-regulated kinase (ERK) and cyclic AMP response element binding protein (CREB) signalling.
Key results: Physical interactions between CB_1 receptors and β_2-adrenoceptors were demonstrated using BRET. In human embryonic kidney (HEK) 293H cells, co-expression of β_2-adrenoceptors tempered the constitutive activity and increased cell surface expression of CB_1 receptors. Co-expression altered the signalling properties of CB_1 receptors, resulting in increased $G\alpha_i$-dependent ERK phosphorylation, but decreased non-$G\alpha_i$-mediated CREB phosphorylation. The CB_1 receptor inverse agonist AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) attenuated β_2-adrenoceptor-pERK signalling in cells expressing both receptors, while the CB_1 receptor neutral antagonist O-2050 ((6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) did not. The actions of AM251 and O-2050 were further examined in primary human trabecular meshwork (HTM) cells, which are ocular cells endogenously co-expressing CB_1 receptors and β_2-adrenoceptors. In HTM cells, as in HEK 293H cells, AM251 but not O-2050, altered the β_2-adrenoceptor–pERK response.
Conclusion and implications: A complex interaction was demonstrated between CB_1 receptors and β_2-adrenoceptors in HEK 293H cells. As similar functional interactions were also observed in HTM cells, such interactions may affect the pharmacology of these receptors in tissues where they are endogenously co-expressed.
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Keywords: G protein coupled receptor; CB_1 receptor; β_2-adrenoceptor; bioluminescence resonance energy transfer; trabecular meshwork

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, N-arachidonoylethanolamine, anandamide; BRET, bioluminescence resonance energy transfer; $BRET_{Eff}$, BRET efficiency; CREB, cyclic AMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; Dox, doxycycline; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HERG, human *ether-a-go-go* related gene; HTM, human trabecular meshwork; IOP, intraocular pressure; mGluR6, metabotropic glutamate receptor 6; PTx, *Pertussis* toxin; Rluc, *Renilla* luciferase; WIN, WIN 55,212-2.

Introduction

The CB_1 cannabinoid receptor is a rhodopsin-like, family A, G protein-coupled receptor (GPCR) that is widely expressed both within the CNS and the periphery. The CB_1 receptor (nomenclature follows Alexander *et al.*, 2009) was originally

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described as a receptor for the primary psychotropic agent in the plant *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Matsuda *et al*., 1990), but has since been shown to also bind endogenous ligands including N-arachidonoylethanolamine or anandamide (AEA), and 2-arachidonoylglycerol (2-AG) (Devane *et al*., 1992; Mechoulam *et al*., 1995). CB_1 receptors are involved in a wide range of biological functions both in the CNS and the periphery. Within the CNS, they are present presynaptically and act to inhibit neurotransmitter release, while in the periphery CB_1 receptors are involved in the regulation of energy and metabolism, bone formation, embryo implantation, cardiovascular function and intraocular pressure (IOP) (Kunos *et al*., 2000; Wang *et al*., 2004; Szczesniak *et al*., 2006; Cota, 2007; Hashimotodani *et al*., 2007; Bab and Zimmer, 2008).

Functionally, CB_1 receptors have been reported to couple primarily to *Pertussis* toxin (PTx)-sensitive $G_{i/o}$ proteins to inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels, while activating mitogen-activated protein kinases (Demuth and Molleman, 2006). However, it has recently been shown that CB_1 receptors also couple to some degree with both G_s and $G_{q/11}$ proteins to activate adenylyl cyclase and increase intracellular Ca^{2+} respectively (Maneuf and Brotchie, 1997; Lauckner *et al*., 2005).

Like many other family A GPCRs, CB_1 receptors physically interact with other GPCRs to form both homodimers, as well as heterodimers with the D_2 dopamine receptor; the μ-, κ- and δ-opioid receptors; the orexin-1 receptor; and the A_{2A} adenosine receptor (Wager-Miller *et al*., 2002; Kearn *et al*., 2005; Mackie, 2005; Ellis *et al*., 2006; Rios *et al*., 2006; Carriba *et al*., 2007). These interactions have been shown to influence many aspects of CB_1 receptor function including ligand pharmacology, receptor trafficking and G protein coupling. Although, to date, these are the only GPCRs reported to physically interact with CB_1 receptors, given the biological significance and widespread distribution of this receptor it is likely that CB_1 receptors interact with additional GPCRs.

The β_2-adrenoceptor is also a rhodopsin-like, family A, GPCR that is widely expressed in several tissue and cell types. β_2-adrenoceptors are predominantly coupled with G_s, but also to a lesser extent with G_i (Xiao *et al*., 1995). Like CB_1 receptors, β_2-adrenoceptors have been shown to form homodimers and heterodimers with other family A GPCRs, including the β_1- and β_3-adrenoceptors, the prostaglandin EP_1 receptor and the μ-opioid receptor (Hebert *et al*., 1996; Angers *et al*., 2000; Mcvey *et al*., 2001; Lavoie *et al*., 2002; Breit *et al*., 2004; Mcgraw *et al*., 2006). The tissue distribution of β_2-adrenoceptors overlaps significantly with that of the CB_1 receptors, including parts of the cardiovascular system, reproductive tract, brain, eye and bone (Jampel *et al*., 1987a; Wanaka *et al*., 1989; Tsou *et al*., 1998; Stamer *et al*., 2001; Wang *et al*., 2004; Pacher and Hasko, 2008). Despite this overlapping distribution, possible direct physical and functional interactions between CB_1 receptors and β_2-adrenoceptors have not been examined. Instead, most studies on the interactions between the cannabinoid and adrenergic systems have focused on inhibition of noradrenergic neurotransmission by presynaptic CB_1 receptors (Schlicker *et al*., 1997; Schultheiss *et al*., 2005; Pakdeechote *et al*., 2007; Tam *et al*., 2008).

One organ where interactions between CB_1 receptors and β_2-adrenoceptors may be of particular interest is the eye. Agonists targeting CB_1 receptors and antagonists targeting β_2-adrenoceptors in the eye are known clinically to decrease IOP (Hepler and Frank, 1971; Borthne, 1976; Pate *et al*., 1998; Mccarty *et al*., 2008). In fact, β_2-adrenoceptor antagonists are a front-line treatment for glaucoma, a blinding eye disease for which the major risk factor is elevated IOP. In humans, IOP is maintained by the balance of aqueous humour production in the ciliary body epithelium and outflow through trabecular meshwork and uveoscleral pathways (Woodward and Gil, 2004). Interestingly, both ciliary epithelial cells and trabecular meshwork cells co-express CB_1 receptors and β_2-adrenoceptors, and these receptors have been implicated in the regulation of both aqueous humour production and outflow (Jampel *et al*., 1987b; Wax *et al*., 1989; Straiker *et al*., 1999; Stamer *et al*., 2001; Njie *et al*., 2006). Thus, these ocular cells provide an ideal model for studying endogenous interactions between these two receptors.

In the present study, novel physical and functional interactions between CB_1 receptors and β_2-adrenoceptors were identified in human embryonic kidney (HEK) 293H cells. These interactions were found to influence both signalling and trafficking of the two receptors. The functional consequences of this CB_1/β_2-adrenoceptors interaction were then further examined in primary human trabecular meshwork (HTM) cells. Together, our observations in HEK 293H and HTM cells suggest complex cell type-specific physical and functional interactions between CB_1 receptors and β_2-adrenoceptors that may be relevant to the cells that co-express these two receptors *in vivo*.

Methods

Constructs

Human CB_1 cannabinoid receptor (CB_1) carboxy-terminal GFP^2 and *Renilla* luciferase (Rluc) constructs were generated by PCR; the CB_1 sequence was amplified without its stop codon from the Rc/CMV-CB_1 plasmid (from Tom Bonner, NIH, Bethesda, MD, USA) using forward (CGACGAATTC-CAGCCTAATCAAAGACTGAGGTT) and reverse (TGACATG-GATCCCACAGAGCCTCGGCAGAC) primers. The PCR product was digested with EcoRI and BamHI and inserted into the $pGFP^2$-N3 and pRluc-N1 plasmids (PerkinElmer) to produce CB_1-GFP^2 and CB_1-Rluc respectively. Constructs of human β_2-adrenoceptors (β_2AR-GFP^2, or β_2AR-Rluc) and of the human *ether-a-go-go* related gene (HERG-GFP^2) were prepared as previously reported (Lavoie *et al*., 2002; Dupre *et al*., 2007). The human metabotropic glutamate receptor 6 RcCMV(m-GLuR6) and mGluR6-GFP^2 constructs were kind gifts from Dr Robert Duvoisin (Oregon Health, and Science University, Portland, OR, USA). The HA-tagged β_2-adrenoceptor in pcDNA3.1/Zeo(–) (Invitrogen Canada Inc., Burlingon, ON, Canada), HA-β_2AR(Zeo), was generated by inserting HA-β_2AR into the EcoRI and HindIII sites of pcDNA3.1/Zeo(–). The neomycin resistant HA-β_2AR(Neo) construct was generated by transferring the HA-β_2AR sequence from HA-β_2AR(Zeo) to pcDNA3.1(+) (Invitrogen Canada Inc.) using NheI and HindIII. To generate pTreHA-β_2AR, the HA-β_2AR sequence was

cut out of HA-β_2AR(Neo) using NotI and inserted into pTRE2hyg (Clontech Laboratories Inc., Mountain View, CA, USA). The pTet-ON plasmid was from Clontech.

Cell culture and transfection

Human embryonic kidney 293H cells (Invitrogen Canada Inc.) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were carried out using the Lipofectamine 2000 transfection reagent (Invitrogen Canada Inc.) according to the manufacturer's protocol. Stable cell lines for various expression constructs were generated by selection with the appropriate antibiotics. Primary HTM cells were obtained from ScienCell (Carlsbad, CA, USA) and maintained in DMEM with 10% FBS. Cell cultures were split every 3–4 days before reaching confluency and were passaged up to 10 times before their experimental use.

Generation of stable cell lines

CB_1-GFP^2 was transfected into HEK 293H cells and stably expressing clones were selected using the antibiotic Zeocin. These CB_1-GFP^2 cells were then transfected with the Tet-ON plasmid and selected using G418. Finally, cells expressing CB_1-GFP^2 and Tet-ON were transfected with pTreHA-β_2AR and selected using hygromycin B to generate a cell line stably expressing CB_1-GFP, Tet-ON and pTreHA-β_2AR (CB_1-GFP^2/TreHA-β_2AR cells). Using On-Cell Western, the addition of doxycycline (Dox) to CB_1-GFP^2/TreHA-β_2AR cells was shown to produce a dose-dependent induction of HA-β_2AR expression (data not shown). A cell line stably expressing both CB_1-GFP^2 and HA-β_2AR was generated by transfecting HA-β_2AR(Neo) into CB_1-GFP^2 cells and selecting using both Zeocin and G418. Cells only expressing HA-β_2AR were generated using the HA-β_2AR(Zeo) plasmid and selected using Zeocin.

Bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) experiments were carried out using a combination of the GFP^2 BRET acceptor, and the DeepBlueC coelenterazine Rluc substrate (PerkinElmer, Waltham, MA, USA), as part of the previously described $BRET^2$ technique (Ramsay *et al.*, 2002). Cells were transfected with the GFP^2 and Rluc constructs and cultured for 24–48 h before their use in BRET experiments. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) before being suspended in PBS supplemented with 1.0 $g{\cdot}L^{-1}$ glucose, 10 $mg{\cdot}L^{-1}$ benzamidine, 5 $mg{\cdot}L^{-1}$ leupeptin and 5 $mg{\cdot}L^{-1}$ soybean trypsin inhibitor (Roche Canada, Mississauga, ON, Canada). Cells were dispensed into a white 96-well plate and their GFP^2 emission was measured using a FLx800 fluorescence plate reader (BioTek Instruments Inc., Winooski, VT, USA) with excitation and emission filters of 485/20 and 528/20 nm respectively. BRET measurements were carried out using a Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA, USA) immediately following the addition of DeepBlueC coelenterazine substrate (PerkinElmer, Waltham, MA, USA) to a final concentration of 5 μM. All BRET measurements were taken by setting the plate reader to make dual luminescent emission measurements using 510 and 405 nm filters with the integration time set to 10 s and the photomultiplier tube voltage set to 1200. BRET measurements were then converted to BRET efficiencies ($BRET_{Eff}$) according to a previously described method (James *et al.*, 2006). Briefly, $BRET_{Eff}$ values were calculated by normalizing the ratio of luminescent emissions at 510/405 nm for each sample to the minimum and maximum 510/405 nm emission ratios obtained using empty Rluc and GFP^2-Rluc fusion constructs respectively.

Immunofluorescence and confocal microscopy

Cells expressing HA and/or GFP^2 constructs or HTM cells were plated onto glass coverslips and maintained for 24–48 h. Cells were then treated as indicated in FBS-free DMEM before being fixed for 5 min in ice cold 100% methanol. After washing with PBS, cells were permeabilized with 100 μM digitonin, washed with PBS and blocked with 1% BSA in PBS. Cells were incubated with primary antibodies: monoclonal mouse anti-HA (Covance, Emeryville, CA, USA), or polyclonal mouse anti-β_2AR (Abnova, Neihu District. Taipei City, Taiwan) and polyclonal rabbit anti-CB_1 (Caymen Chemical, Ann Arbor, MI, USA) overnight at 4°C. After washing with PBS, coverslips were incubated with Cy3-conjugated anti-mouse IgG and/or fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. Coverslips were then washed again with PBS before being mounted on slides using Fluorsave reagent (Calbiochem, San Diego, CA, USA), and imaged using a Nikon Eclipse E800 microscope fitted with the D-Eclipse C1 confocal system (Nikon Canada Inc., Mississauga, ON, Canada). GFP^2 and fluorescein isothiocyanate were imaged using a 488 nm air-cooled argon laser (Spectra-Physics Lasers Inc., Mountain View, CA, USA), while Cy3 was imaged with a 543 nm He-Ne laser (JDS Uniphase, Milpitas, CA, USA).

In-Cell Western blot analyses

Phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK) and cyclic AMP response element binding protein (CREB) was assessed using a modified In-Cell Western protocol, as previously reported (Mcintosh *et al.*, 2007). Briefly, HEK 293H cells expressing CB_1-GFP and/or HA-β_2AR constructs or HTM cells were plated in 96-well plates and cultured to confluency. Cell culture media was then replaced with FBS-free DMEM and cells were maintained for 24 h prior to experiments. Cells were then treated as indicated for either 5 or 10 min in ERK experiments, or 30 min in CREB experiments, before being fixed for 1 h with 4% paraformaldehyde. Rabbit anti-phospho ERK1/2 and goat anti-total ERK2 primary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used to assess ERK phosphorylation, while goat anti-phospho-CREB and rabbit anti-total CREB primary antibodies (Santa Cruz Biotechnology Inc.) were used to assess CREB phosphorylation. Secondary antibodies used were a IRDye800CW-conjugated donkey anti-rabbit IgG (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and an Alexa Fluor680-conjugated donkey anti-goat IgG (Invitrogen

Canada Inc.). Plates were scanned to measure their fluorescent emission from the IRDye800CW- and Alexa Fluor680-conjugated antibodies using the Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE, USA). In order to convert these data to relative pERK and pCREB values, background fluoresence was first determined and then subtracted using wells of the plate that received only the secondary antibodies. The ratio of the pERK/ERK2 or pCREB/total CREB signals (with background subtracted) were then determined for each well and normalized to the ratios obtained from the appropriate vehicle or untreated wells in order to obtain relative pERK or pCREB values. Experiments were in all cases repeated several times, and within each experiment, each condition was repeated in 2–8 wells. The total numbers of individual wells used are presented as the '*n*' number in each figure.

On-Cell Western blot analyses

Cell surface expression of CB_1-GFP^2 and HA-β_2AR was assessed using a modified In-Cell Western protocol utilizing non-permeablized cells (Miller, 2004). Cells were plated in 96-well plates and cultured for 24–48 h before experiments. Cells were treated as indicated in FBS-free DMEM and fixed in 4% paraformaldehyde. After washing with PBS, cells were blocked with 1% BSA in PBS at room temperature for 2 h. Primary antibodies directed at N-terminal epitopes of either the CB_1-GFP^2 or HA-β_2AR constructs were polyclonal rabbit anti-CB_1 (Cayman Chemical, Ann Arbor, MI, USA) or monoclonal anti-HA (Covance, Emeryville, CA, USA) antibodies respectively. Primary antibodies were diluted in 1% BSA in PBS and applied for 1 h at room temperature. After washing, cells were incubated in secondary antibodies: anti-rabbit IgG IRDye800CW and goat anti-mouse IgG Alexa Fluor680 (Invitrogen Canada Inc.), diluted in 1% BSA in PBS for 1 h at room temperature. Cells were washed with PBS then with distilled water before drying. Once dry, the plates were imaged using an Odyssey infrared imaging system (Li-Cor Biotechonology).

Statistical analysis and curve fitting

All data are presented as mean ± SEM. Statistical analysis and curve fitting of the data were performed using Graphpad Prism v.4 (GraphPad Software Inc., San Diego, CA, USA). To fit data to dose–response curves, vehicle treatments were plotted at a concentration equal to one and a half log units less than the lowest drug treatment concentration then data were fitted to a sigmoidal dose–response curve with variable slope. Statistical significance for curve fits was determined by the *F*-test comparing global fits, by *t*-test when comparing means of only two groups, or by one-way, two-way, or repeated measures analysis of variance (ANOVA), as appropriate, when comparing the means of multiple treatment groups. Tukey's *post hoc* analysis was used to determine differences among groups for one-way ANOVA, while Bonferroni's *post hoc* analysis was used for two-way ANOVA. $P < 0.05$ was considered statistically significant.

Materials

Pertussis toxin, hygromycin B and G418 sulphate were from Calbiochem. (R)-(+)-WIN 55,212-2 mesylate ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate), AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone), O-2050 ((6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), ICI 118,551 ((±)-erythro-(S*,S*)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl) oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride) and CGP 20712 (1-[2-((3-carbamoyl-4-hydroxy)phenoxy) ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propan ol dihydrochloride) were from Tocris Bioscience (Ellisville, MO, USA). Zeocin and Opti-MEM were obtained from Invitrogen Canada Inc. FBS was from PAA laboratories Inc. (Etobicoke, ON, Canada). Restriction enzymes, DNA polymerases and other enzymes were from ferments Canada Inc. (Burlington, ON, Canada). All other chemicals and reagents were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Results

Physical interactions between CB_1 receptors and β_2-adrenoceptors in HEK 293H cells

Bioluminescence resonance energy transfer^2 ($BRET^2$) was used to demonstrate an interaction between CB_1 receptors and the β_2-adrenoceptors in HEK 293H cells. $BRET_{Eff}$ was measured from cells co-transfected with either CB_1-Rluc or β_2AR-Rluc, and one of CB_1-GFP^2, β_2AR-GFP^2, HERG-GFP^2 or mGluR6-GFP^2 (Figure 1A). When co-expressed with CB_1-Rluc, CB_1-GFP^2 and β_2AR-GFP^2 produced significantly increased $BRET_{Eff}$ ($P < 0.001$) compared with either HERG-GFP^2 or mGluR6-GFP^2, two different membrane proteins not expected to interact with either CB_1 receptors or β_2-adrenoceptors. Similarly, when co-transfected with β_2AR-Rluc both CB_1-GFP^2 and β_2AR-GFP^2 produced significantly increased $BRET_{Eff}$ compared with the HERG-GFP^2 ($P < 0.001$) and mGluR6-GFP^2 ($P < 0.01$) controls. In all cases, GFP^2 expression levels were equal to or less than those of the HERG-GFP^2 negative control (data not shown). These data confirm previous reports of both CB_1 receptor and β_2-adrenoceptor homodimerization (Hebert *et al.*, 1996; Angers *et al.*, 2000; Wager-Miller *et al.*, 2002), but also suggest a novel physical interaction between CB_1 receptors and β_2-adrenoceptors.

Bioluminescence resonance energy transfer saturation experiments were performed according to a previously described protocol (Roy *et al.*, 2006). A fixed amount of the CB_1-Rluc construct was co-transfected with increasing amounts of β_2AR-GFP^2, CB_1-GFP^2, HERG-GFP^2 or mGluR6-GFP^2. $BRET_{Eff}$ values were plotted against the ratio of GFP^2 fluorescent emission obtained by directly exciting GFP^2 (measuring GFP^2 emission in the absence of the Rluc coelenterazine substrate) to the Rluc bioluminescent emission and fitted to rectangular hyperbola curves (Figure 1B). Significantly different $BRET_{50}$ values ($P < 0.05$) of 0.6 ± 0.1 and 0.19 ± 0.07, and $BRET_{Max}$ values ($P < 0.001$) of 0.53 ± 0.03 and 0.24 ± 0.03, were obtained from the saturation curves when β_2AR-GFP^2 and CB_1-GFP^2 were used as BRET acceptors respectively. These

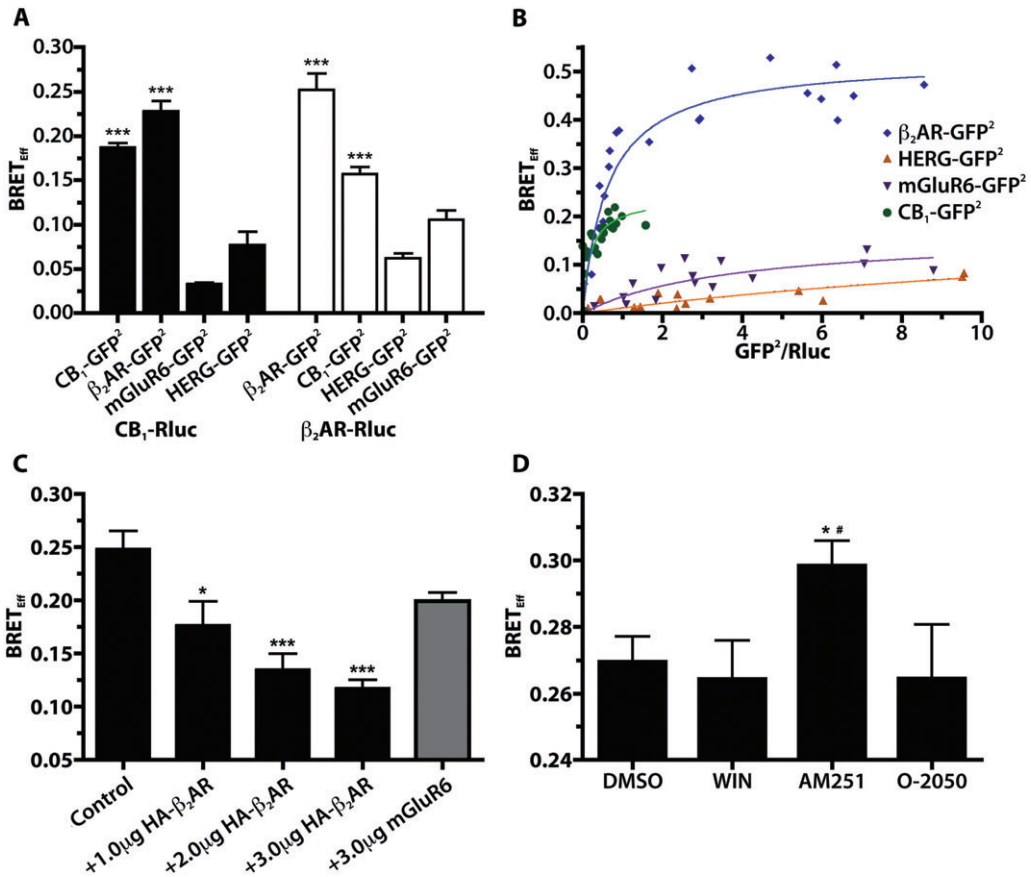

Figure 1 CB_1 cannabinoid receptors physically interact with β_2-adrenoceptors when expressed in 293H cells. (A) $BRET_{Eff}$ values obtained from 293H cells transiently transfected with either CB_1-Rluc or β_2AR-Rluc and CB_1-GFP^2, β_2AR-GFP^2, mGluR6-GFP^2 or HERG-GFP^2. ***$P < 0.001$ compared with HERG-GFP^2 controls; $n = 4$–9. (B) BRET saturation curves for CB_1-Rluc with β_2AR-GFP^2, CB_1-GFP^2, HERG-GFP^2 and mGluR6-GFP^2. $BRET_{Eff}$ is plotted against the ratio of GFP^2 fluorescence (obtained by directly exciting GFP^2) and Rluc emission and the data were fitted to a rectangular hyperbola. (C) $BRET_{Eff}$ values from 293H cells transfected with a fixed amount of CB_1-Rluc and β_2AR-GFP^2 (Control) and pcDNA or increasing amounts of HA-β_2AR or mGluR6. *$P < 0.05$ and ***$P < 0.001$ compared with control column; $n = 4$–10. (D) $BRET_{Eff}$ values obtained from cells transfected with CB_1-Rluc and β_2AR-GFP^2 and treated for 15 min at room temperature with DMSO (0.05%), WIN (10 μM), AM251 (10 μM) or O-2050 (10 μM) prior to measuring $BRET_{Eff}$. *$P < 0.05$ compared with WIN; $^{\#}P < 0.05$ compared with O-2050; $n = 9$. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; BRET, bioluminescence resonance energy transfer; $BRET_{Eff}$, BRET efficiency; DMSO, dimethylsulphoxide; HERG, human *ether-a-go-go* related gene; mGluR6, metabotropic glutamate receptor 6; O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; Rluc, *Renilla* luciferase; WIN, WIN 55,212-2.

saturation curves demonstrate that there is a specific interaction between CB_1 receptors and β_2-adrenoceptors that is observed at low levels of receptor expression.

A BRET competition experiment was performed to demonstrate that the BRET signal between CB_1-Rluc and β_2AR-GFP^2 could be reduced in a dose-dependent manner by co-transfection with HA-β_2AR (Figure 1C). CB_1-Rluc/β_2AR-GFP^2 $BRET_{Eff}$ was significantly reduced by co-transfection with 1 μg of HA-β_2AR cDNA ($P < 0.05$) and further reduced with 2 and 3 μg of HA-β_2AR plasmid ($P < 0.001$), but was not significantly affected by 3 μg of RcCMVmGluR6 ($P > 0.05$) plasmid, indicating the specificity of the CB_1/β_2-adrenoceptors interaction.

The effect of various CB_1 receptor ligands on the $BRET_{Eff}$ for the CB_1-Rluc/β_2AR-GFP^2 pair was then assessed (Figure 1D). The CB_1 receptor inverse agonist AM251 resulted in a $BRET_{Eff}$ that was significantly elevated compared with either the agonist WIN 55,212-2 (WIN) or the neutral antagonist O-2050 ($P < 0.05$). This finding suggests that AM251 modulates the CB_1/β_2-adrenoceptor heterodimer, either by altering the number of receptors interacting, or by altering the conformation of the dimer, such that it affects the orientation of the BRET donor and acceptor.

Functional interactions between CB_1 receptors and β_2-adrenoceptors in HEK 293H cells affect CB_1 receptor constitutive activity

When CB_1-GFP^2 was stably expressed in HEK 293H cells a punctate pattern of internalized CB_1 receptor distribution was observed in addition to a less intense pattern of receptors detected at the cell surface. Following addition of the CB_1 receptor inverse agonist AM251 (10 μM, 24 h), CB_1-GFP^2 expression was redistributed to the cell surface (Figure 2A). Using On-Cell Western blots, a quantitative measure of the effect of AM251 (10 μM, 24 h) was obtained, confirming a significant ($P < 0.001$, 360%) increase in CB_1-GFP^2 cell surface expression in response to treatment with the inverse agonist

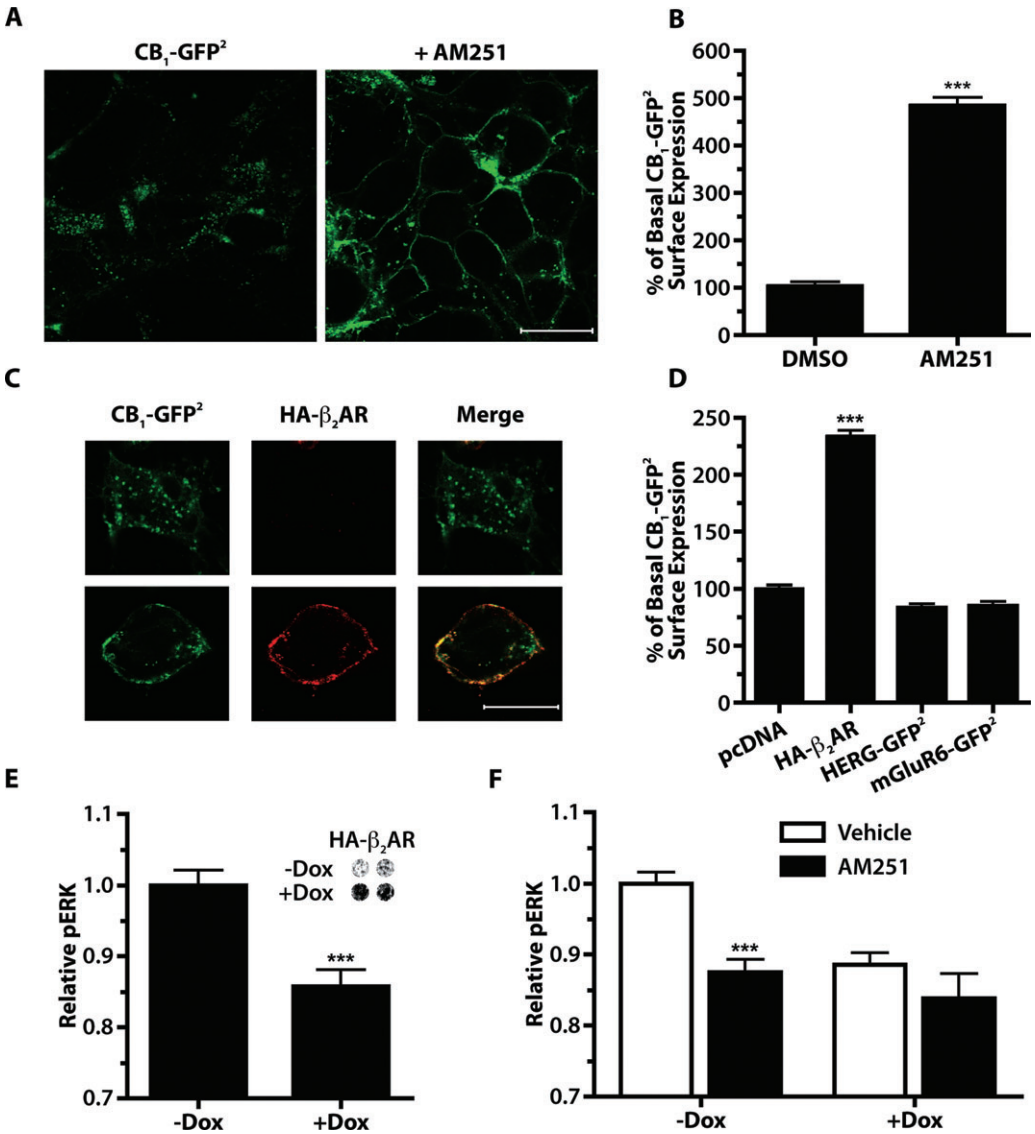

Figure 2 Co-expression of HA-β_2AR reduces the constitutive activity of CB_1-GFP^2 in 293H cells. (A) Confocal images of 293H cells stably expressing CB_1-GFP^2 treated for 24 h with 0.05% DMSO vehicle (left panel) or 10 μM AM251 (right panel). Scale bar is 20 μm. (B) On-Cell Western quantitative measure of CB_1 cell surface expression following 24 h AM251 treatment (10 μM) in 293H cells stably expressing CB_1-GFP^2. ***$P < 0.001$ compared with DMSO vehicle; $n = 4$–6. (C) Confocal images of 293H cells transfected with CB_1-GFP^2 and HA-β_2AR. Left panels show GFP^2 fluorescence, middle panels are anti-HA immunofluorescence utilizing a Cy3-conjugated secondary antibody, and the right panels are the merged images. Scale bar is 20 μm. (D) On-Cell Western quantitative measure of CB_1-GFP^2 cell surface expression in 293H cells stably expressing CB_1-GFP^2 and transiently transfected with pcDNA, HA-β_2AR, HERG-GFP^2 or mGluR6-GFP^2. ***$P < 0.001$ compared with pcDNA transfected cells; $n = 4$–18. (E) Basal pERK levels in CB_1-GFP^2/TreHA-β_2AR cells without and with Dox pretreatment (10 $\mu g \cdot mL^{-1}$, 24 h) to induce expression of HA-β_2AR. ***$P < 0.001$; $n = 20$. Inset shows On-Cell Western using an anti-HA primary antibody to measure HA-β_2 expression without or with Dox. (F) pERK levels in CB_1-GFP^2/TreHA-β_2AR cells treated with 0.05% DMSO vehicle (open bars) or AM251 (1 μM, 10 min, solid bars) in cells without or with Dox pretreatment (10 $\mu g \cdot mL^{-1}$, 24 h). ***$P < 0.001$ compared with respective vehicle controls; $n = 20$–34. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; DMSO, dimethylsulphoxide; Dox, doxycycline; ERK, extracellular signal-regulated kinase; HERG, human *ether-a-go-go* related gene; mGluR6, metabotropic glutamate receptor 6.

(Figure 2B). These results demonstrate that CB_1 receptors are constitutively active, resulting in a constitutive internalization of the receptor, which can be reversed by the inverse agonist AM251.

Co-expression of CB_1-GFP^2 with HA-β_2AR also resulted in increased localization of CB_1-GFP^2 at the cell surface. Immunofluorescence for the HA tag was carried out on HEK 293H cells transiently transfected with both CB_1-GFP^2 and HA-β_2AR (Figure 2C). When cells were successfully transfected with both receptor constructs, CB_1-GFP^2 expression was largely observed at the cell surface (lower panels of Figure 2C), but when CB_1-GFP^2 was expressed alone, the distribution was consistent with an internalized receptor (upper panels of Figure 2C). In order to measure the effect that HA-β_2AR co-expression had on CB_1-GFP^2 cell surface expression, On-Cell Western blots were used in CB_1-GFP^2 cells transiently transfected with HA-β_2AR (Figure 2D). Transient expression of HA-β_2AR in these cells resulted in a significant ($P < 0.001$,

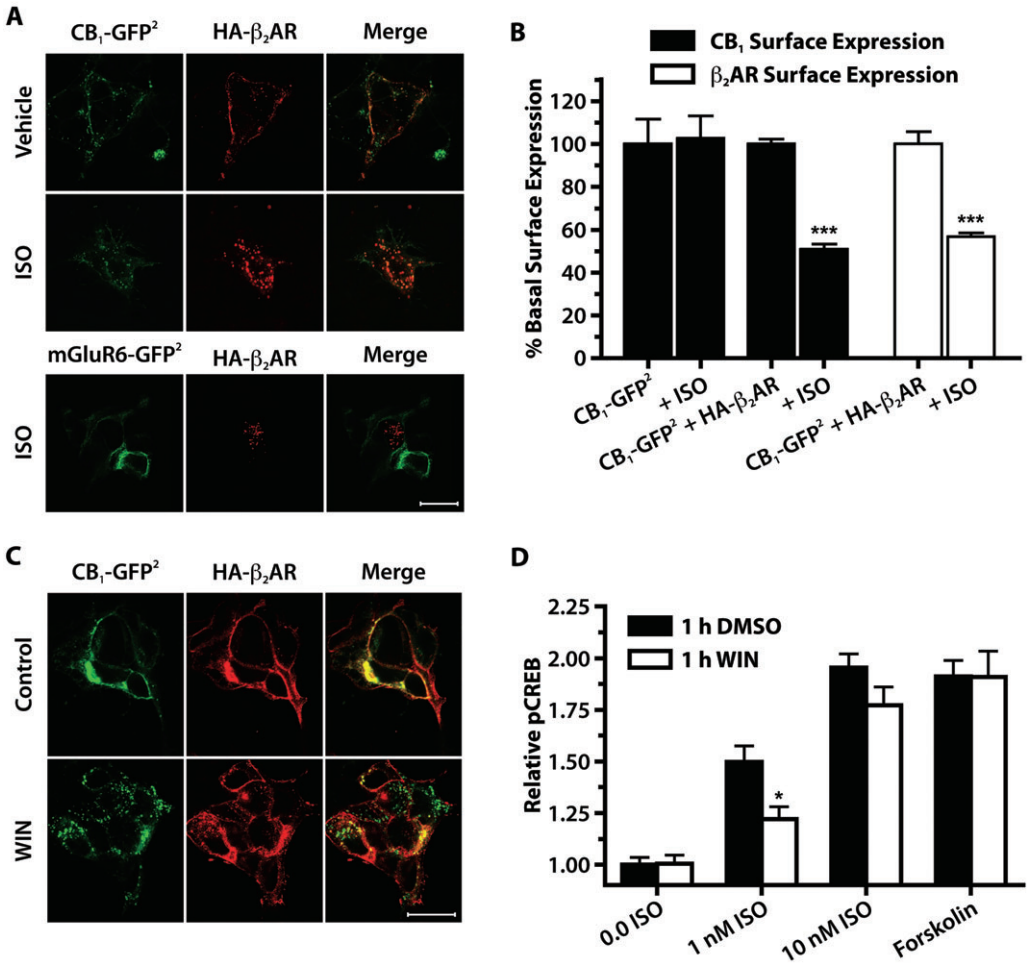

Figure 3 HA-β_2AR and CB_1-GFP^2 are co-internalized when exposed to either isoprenaline (ISO) or WIN. (A) Confocal images of 293H cells transiently transfected with CB_1-GFP^2 or mGluR6-GFP^2 and HA-β_2AR treated for 30 min with vehicle or 10 μM isoprenaline. Left panels show GFP^2 fluorescence, middle panels are anti-HA immunofluorescence utilizing a Cy3-conjugated secondary antibody, and right panels are merged images. Scale bar is 20 μm. (B) On-Cell Western quantitative measure of CB_1-GFP^2 or HA-β_2AR cell surface expression in 293H cells stably expressing CB_1-GFP^2 and transiently transfected with either pcDNA (CB_1-GFP^2 bars) or HA-β_2AR. Cells were treated with either H_2O vehicle (labelled CB_1-GFP^2 + HA-β_2) or isoprenaline (10 μM) for 30 min. ***$P < 0.001$ compared with CB_1-GFP^2 + HA-β_2AR vehicle-treated groups; $n = 6$. (C) Confocal images of HEK 293H cells transiently transfected with CB_1-GFP^2 and HA-β_2AR, upper panels are untreated controls, and lower panels are treated with WIN (10 μM, 30 min). Scale bar is 20 μm. (D) Relative pCREB levels in CB_1-GFP^2/TreHA-β_2AR cells pretreated with Dox (10 μM, 24 h), treated for 1 h with either DMSO (0.05%) or WIN (10 μM), followed by 30 min treatment with isoprenaline (0–10 nM), or forskolin (10 μM). *$P < 0.05$ compared with respective 1 h DMSO treatments; $n = 12$–20. CREB, cyclic AMP response element binding protein; DMSO, dimethylsulphoxide; Dox, doxycycline; HEK, human embryonic kidney; mGluR6, metabotropic glutamate receptor 6; WIN, WIN 55,212-2.

130%) increase in CB_1-GFP^2 cell surface expression, while the expression of either HERG-GFP^2 or mGluR6-GFP^2 did not increase CB_1-GFP^2 surface expression.

To assess whether the increased CB_1-GFP^2 surface expression was the result of decreased constitutive activity of CB_1 receptors, basal CB_1 pERK signalling was measured in CB_1-GFP^2/TreHA-β_2AR cells treated with Dox to induce HA-β_2AR expression (Figure 2E). Induction of HA-β_2AR resulted in a significant decrease in the basal pERK level of these cells ($P < 0.001$). In order to show that this decrease in basal pERK was in fact the result of decreased constitutive activity of CB_1 receptors, the ability of AM251 to reduce basal pERK level was then measured in these cells in the absence or presence of co-expressed HA-β_2AR (Figure 2F). AM251 (1 μM, 10 min) significantly reduced basal pERK in the absence of HA-β_2AR ($P < 0.001$), but had no significant effect on cells co-expressing HA-β_2AR. These findings demonstrate that CB_1 receptors are constitutively active in 293H cells, but that co-expression of β_2-adrenoceptors attenuates this constitutive activity.

CB_1 receptors and β_2-adrenoceptors are co-internalized upon addition of a CB_1 receptor or β_2-adrenoceptor agonist

Co-transfection of CB_1-GFP^2 and HA-β_2AR in HEK 293H cells resulted in expression of both receptors primarily at the cell membrane (Figure 3A). When these cells were exposed to the β_2-adrenoceptor agonist, isoprenaline (10 μM, 30 min), internalization of not only HA-β_2AR but also CB_1-GFP^2 was observed. In contrast, when cells were transfected with mGluR6-GFP^2 and HA-β_2AR and treated with isoprenaline,

only HA-β_2AR was internalized, while mGluR6-GFP^2 remained at the cell surface. On-Cell Western analyses were used for a quantitative measure of the ability of isoprenaline to co-internalize CB_1-GFP^2 in cells stably expressing CB_1-GFP^2 and transiently transfected with HA-β_2AR (Figure 3B). In the absence of HA-β_2AR there was no effect on the cell surface distribution of CB_1-GFP^2 following treatment with isoprenaline. However, isoprenaline treatment of CB_1-GFP^2 cells transfected with HA-β_2AR resulted in a significant decrease ($P < 0.001$, 49%) in CB_1-GFP^2 cell surface expression. As expected, HA-β_2AR cell surface expression was also significantly decreased ($P < 0.001$) in CB_1-GFP^2 cells transfected with HA-β_2AR following treatment with isoprenaline. These findings show that CB_1-GFP^2 is co-internalized with HA-β_2AR following treatment with the β-adrenoceptor agonist, isoprenaline.

Similar to the co-internalization observed when cells were co-transfected with CB_1-GFP^2 and HA-β_2AR and treated with the CB_1 receptor agonist WIN (10 μM, 30 min), internalization of both receptors was observed (Figure 3C). To further examine the functional significance of this co-internalization, the phospho-CREB (pCREB) signalling response to activation of β_2-adrenoceptors was assessed in CB_1-GFP^2/TreHA-β_2AR cells pretreated with Dox (10 μM, 24 h) to induce HA-β_2AR expression, before being treated with WIN (10 μM, 1 h) to induce internalization (Figure 3D). In these cells, the ability of 1 nM isoprenaline to increase pCREB was attenuated by WIN treatment ($P < 0.05$), but the ability of 10 nM isoprenaline or 10 μM forskolin to increase pCREB were unaffected, indicating that the effect is not due to physiological antagonism of the pCREB signalling pathway. Taken together, these data demonstrate co-internalization of β_2-adrenoceptors with CB_1 receptors mediated by the agonist WIN, and that this co-internalization has functional consequences in the cross-desensitization of β_2-adrenoceptors.

Co-expression of CB_1 receptors and β_2-adrenoceptors affects their ability to stimulate ERK and CREB phosphorylation

In-Cell Western analyses were used to assess levels of phospho-ERK (pERK) and pCREB in HEK 293H cells expressing CB_1-GFP^2 and/or HA-β_2AR. When cells expressing CB_1-GFP^2 alone were treated with WIN, increases in both pERK (5 min exposure) and pCREB (30 min exposure) were observed. The WIN-dependent pERK response was sensitive to PTx, while the pCREB response was not (data not shown). pERK dose–response measurement following WIN treatment in CB_1-GFP^2/TreHA-β_2AR cells that were not induced with Dox (i.e. no HA-β_2AR expression) yielded a pEC_{50} of 6.85 ± 0.04, E_{max} of 1.74 ± 0.02 and a Hill coefficient of 1.07 ± 0.11 (Figure 4A). Pretreatment of these cells for 48 h with Dox to induce HA-β_2AR expression, resulted in a significantly different WIN pERK dose–response curve ($P < 0.001$), with pEC_{50} values of 6.66 ± 0.03, 1.91 ± 0.02 for E_{max} and a Hill coefficient of 1.64 ± 0.20. However, the PTx sensitivity of the WIN–pERK response was not affected by co-expression of HA-β_2AR with CB_1-GFP^2 (data not shown). This demonstrates that co-expression of β_2-adrenoceptors enhances CB_1 receptor-dependent pERK signalling.

WIN treatment of CB_1-GFP^2/TreHA-β_2AR cells that were not pretreated with Dox resulted in a pCREB dose–response curve with a pEC_{50} of 6.82 ± 0.08, E_{max} of 1.79 ± 0.03 and a Hill coefficient of 1.32 ± 0.29 (Figure 4B). Pretreatment of these cells with Dox (10 μg·mL^{-1}, 48 h) resulted in a significantly different ($P < 0.001$) dose–response curve with values for pEC_{50} of 6.92 ± 0.28, for E_{max} of 1.51 ± 0.07 and a Hill coefficient of 0.69 ± 0.33. To determine the long-term effect of co-expression of CB_1 receptors and β_2-adrenoceptors, a cell line stably expressing both CB_1-GFP^2 and HA-β_2AR was employed. In these cells, the WIN-dependent pCREB response was nearly completely abolished at WIN concentrations of 0.3 and 1.0 μM, compared with cells expressing CB_1 receptors alone (Figure 4C), indicating that co-expression of β_2-adrenoceptors inhibits the CB_1 receptor-pCREB signalling pathway.

When cells stably expressing HA-β_2AR were treated with isoprenaline, PTx-insensitive increases in both pCREB and pERK were observed (data not shown). In order to determine what influence co-expression of CB_1-GFP^2 had on the isoprenaline-stimulated pCREB and pERK responses, HA-β_2AR cells were transfected with either pcDNA vector control or CB_1-GFP^2 48 h prior to treatment with isoprenaline. Cells transfected with CB_1-GFP^2 showed significantly greater ($P < 0.01$) isoprenaline-stimulated pCREB responses than those transfected with vector (Figure 4D), while there was no difference in the isoprenaline-stimulated pERK responses between cells transfected with pcDNA or CB_1-GFP^2 (data not shown). Again, the PTx insensitivity of the isoprenaline-induced pERK and pCREB responses was not affected by co-expression of CB_1-GFP^2 (data not shown).

Co-application of WIN and isoprenaline results in an additive response for pERK but not pCREB

Isoprenaline-stimulated pERK dose–response curves from CB_1-GFP^2/TreHA-β_2AR cells pretreated with Dox to induce HA-β_2AR expression were generated in the presence of dimethylsulphoxide (DMSO) vehicle, 0.1 and 0.3 μM WIN (Figure 5A). The curves generated in the presence of 0.1 and 0.3 μM WIN were both significantly different from the DMSO vehicle curve ($P < 0.001$). Similar pEC_{50} values of 7.57 ± 0.7, 7.48 ± 0.12 and 7.41 ± 0.14 were obtained for DMSO, 0.1 and 0.3 μM WIN respectively. Baseline pERK levels were increased from 0.91 ± 0.03 in DMSO-treated cells to 1.16 ± 0.04 and 1.30 ± 0.04 in 0.1 and 0.3 μM WIN-treated. Similarly, E_{max} values were also increased from 1.59 ± 0.04 in DMSO-treated cells to 1.84 ± 0.07 and 1.92 ± 0.07 in cells treated with 0.1 and 0.3 μM WIN respectively. This pattern of increased baseline pERK and E_{max}, but unchanged pEC_{50} is consistent with additive WIN- and isoprenaline-induced pERK responses. In contrast, when 1 μM WIN was co-applied with isoprenaline (1 and 10 nM) to these cells, the pCREB response was unchanged compared with isoprenaline application alone (Figure 5B). To determine if the pCREB response was saturated, 10 μM forskolin was co-applied with isoprenaline (1 and 10 nM); it was found that at 1 but not 10 nM isoprenaline, forskolin significantly further elevated pCREB levels over isoprenaline treatment alone ($P < 0.001$). This demonstrates that WIN and isoprenaline show additive pERK but not pCREB responses in cells co-expressing these two receptors.

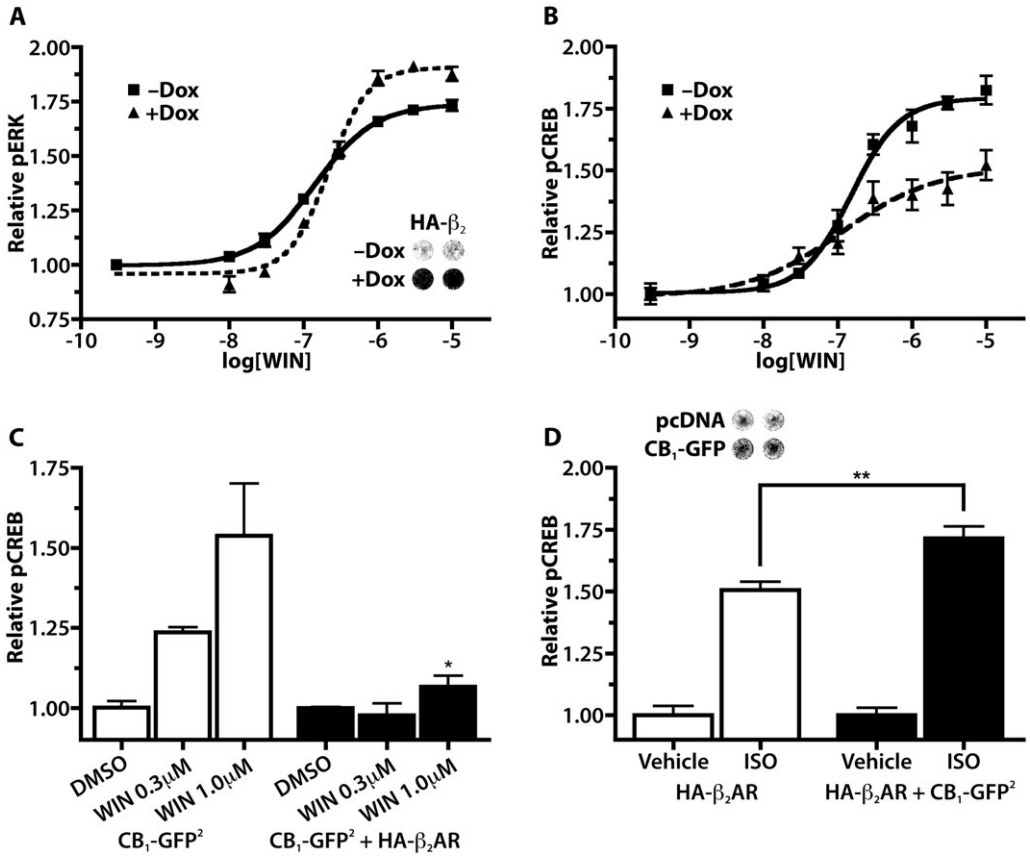

Figure 4 Co-expression of HA-β_2AR and CB_1-GFP^2 alters receptor efficacy to activate ERK and CREB phosphorylation. (A) Dose–response curves for WIN pERK activation in CB_1-GFP^2/TreHA-β_2AR cells pretreated for 48 h without or with Dox (10 $\mu g \cdot mL^{-1}$); $n = 8$. Inset shows On-Cell Western using an anti-HA primary antibody in CB_1-GFP^2/TreHA-β_2AR cells without or with Dox (10 $\mu g \cdot mL^{-1}$, 48 h). (B) Dose–response curves for WIN pCREB activation in CB_1-GFP^2/Tet-ON/HA-β_2AR cells pretreated for 48 h without or with Dox (10 $\mu g \cdot mL^{-1}$); $n = 4$–8. (C) WIN–pCREB responses in 293H cells stably expressing CB_1-GFP^2 alone or CB_1-GFP^2 and HA-β_2AR. *$P < 0.05$ compared with 1.0 μM WIN in CB_1-GFP^2 cells; $n = 3$. (D) Isoprenaline–pCREB responses in 293H cells stably expressing HA-β_2AR and transiently transfected with pcDNA (solid bars) or CB_1-GFP^2 (open bars). **$P < 0.01$; $P = 7$–8. Inset is On-Cell Western using anti-CB_1 primary antibody of cells transfected with pcDNA or CB_1-GFP^2. CREB, cyclic AMP response element binding protein; Dox, doxycycline; ERK, extracellular signal-regulated kinase; WIN, WIN 55,212-2.

The CB_1 inverse agonist AM251 but not the neutral antagonist O-2050 inhibits the isoprenaline-stimulated pERK response

Dose–response curves for pERK were generated in CB_1-GFP^2 cells for the CB_1 receptor agonist WIN, the inverse agonist AM251, and the neutral antagonist O-2050 (Figure 6A). AM251 produced a dose-dependent decrease in pERK from baseline to 0.78 ± 0.03 with a pEC_{50} of 8.2 ± 0.4, while O-2050 had no effect on basal pERK levels. Although both AM251 and O-2050 were capable of completely blocking the WIN–pERK response in these cells (data not shown), AM251 acts as an inverse agonist, while O-2050 acts as a neutral antagonist.

Isoprenaline-mediated pERK dose–response curves were produced in CB_1-GFP^2/TreHA-β_2AR cells pretreated for 24 h with Dox to induce HA-β_2AR expression then 15 min with either DMSO vehicle, AM251 or O-2050 (Figure 6B). Following DMSO pretreatment, the isoprenaline dose–response curve had a pEC_{50} of 8.57 ± 0.15 and an E_{max} of 1.71 ± 0.05. This dose–response curve was significantly altered by pretreatment with AM251 ($P < 0.001$) but not O-2050. AM251 pretreatment did not affect the pEC_{50} producing a value of 8.70 ± 0.28, but did result in a much lower E_{max} value of 1.45 ± 0.06. These findings demonstrate that the CB_1 receptor inverse agonist AM251, but not the neutral antagonist O-2050 attenuates pERK signalling mediated by β_2-adrenoceptors in HEK 293 cells.

Isoprenaline-mediated pCREB dose–response curves were also generated from CB_1-GFP^2/TreHA-β_2AR cells pretreated for 24 h with Dox and 15 min with either DMSO vehicle, AM251 or O-2050 (Figure 6C). The isoprenaline pCREB dose–response curve with DMSO pretreatment had a pEC_{50} of 8.06 ± 0.07 and E_{max} of 1.98 ± 0.05. Neither pretreatment with AM251 or O-2050 significantly altered the isoprenaline pCREB dose–response curve in these cells. In addition, the β_2-adrenoceptor inverse agonist timolol had no effect on CB_1 receptor-mediated pERK or pCREB responses (data not shown).

Interactions between pERK signalling induced by CB_1 receptors and β_2-adrenoceptors in HTM cells

Immunofluorescence studies in HTM cells demonstrated clear labelling of endogenous CB_1 receptors and β_2-adrenoceptors (Figure 7A) when compared with secondary antibody-only controls (data not shown). CB_1 receptor and β_2-adrenoceptor expression was detected at the cell membrane in HTM cells, with co-localization of the two receptors apparent.

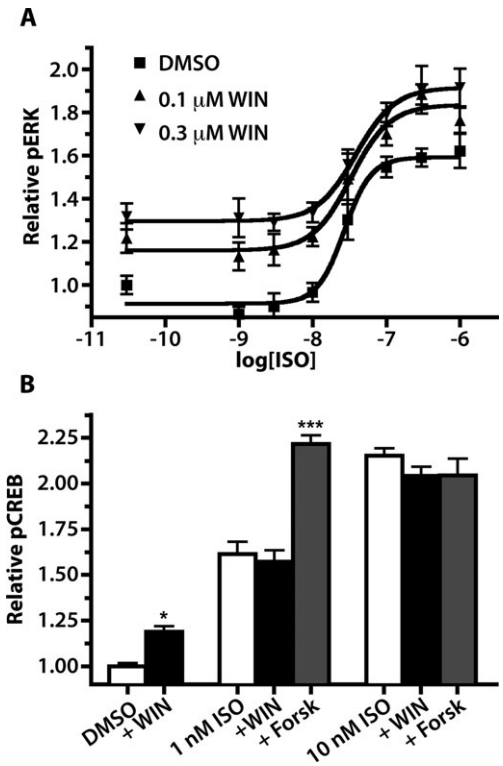

Figure 5 Co-application of isoprenaline (ISO) and WIN results in an additive increase in ERK phosphorylation. (A) Dose–response curves for pERK activation following co-exposure of isoprenaline and 0.05% DMSO vehicle, 0.1 μM WIN or 0.3 μM WIN in CB_1-GFP^2/TreHA-β_2AR pretreated for 24 h with 10 μg·mL^{-1} Dox; $n = 6$. (B) pCREB responses in CB_1-GFP^2/Tet-ON/HA-β_2AR cells pretreated for 24 h with Dox (10 μg·mL^{-1}) then co-exposed to isoprenaline (0, 1 and 10 nM) and DMSO vehicle (0.05%), WIN (1.0 μM) or forskolin (10 μM). *P < 0.05, ***P < 0.001 compared with DMSO, or isoprenaline alone groups, respectively; n = 10–28. CREB, cyclic AMP response element binding protein; DMSO, dimethylsulphoxide; Dox, doxycycline; ERK, extracellular signal-regulated kinase; WIN, WIN 55,212-2.

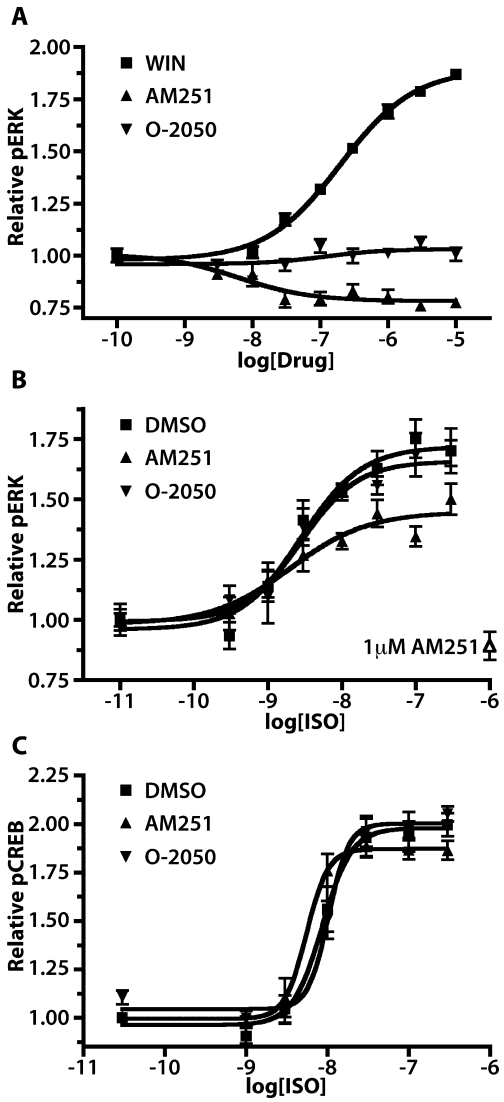

Figure 6 The CB_1 inverse agonist AM251 but not the CB_1 neutral antagonist O-2050 attenuates isoprenaline (ISO)-induced ERK phosphorylation. (A) pERK dose–response curves for WIN, AM251 and O-2050 in 293H cells stably expressing CB_1-GFP^2. WIN exposures were for 5 min, while AM251 and O-2050 exposures were for 15 min; n = 6–16. (B) Isoprenaline pERK dose–response curves for CB_1-GFP^2/Tet-ON/HA-β_2AR cells pretreated for 24 h with Dox (10 μg·mL^{-1}) and 15 min with 0.05 % DMSO (squares), AM251 (triangles) or O-2050 (inverted triangles); n = 9–12. Each curve is normalized to the pERK level in cells untreated with isoprenaline but pretreated with the DMSO, AM251 or O-2050. The effect of 20 min AM251 exposure on the basal pERK level in these cells is indicated by the open triangle. (C) Isoprenaline pCREB dose–response curves in CB_1-GFP^2/TreHA-β_2AR cells pretreated for 24 h with Dox (10 μg·mL^{-1}) and 15 min with 0.05% DMSO, AM251 or O-2050; n = 6–9. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CREB, cyclic AMP response element binding protein; DMSO, dimethylsulphoxide; Dox, doxycycline; ERK, extracellular signal-regulated kinase; O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; WIN, WIN 55,212-2.

In order to assess the role CB_1/β_2-adrenoceptors heterodimerization might play in HTM cells, cross-desensitization of pCREB signalling induced by activation of β_2-adrenoceptors, following treatment with the CB_1 receptor agonist WIN was measured (Figure 7B). In these cells following a 1 h pretreatment with WIN (10 μM) the isoprenaline–pCREB response was significantly (P < 0.01) reduced. In contrast, WIN pretreatment had no effect on the forskolin-stimulated pCREB in these cells, indicating that the effect is not due to a non-specific effect on the pCREB signalling pathway by CB_1 receptors. These findings indicate that, as in HEK 293 cells, the CB_1 receptor agonist WIN produces a cross-desensitization of β_2-adrenoceptors.

To demonstrate function of CB_1 receptors and β_2-adrenoceptors in HTM cells, the abilities of adrenergic and CB receptor ligands to affect pERK levels were examined using In-Cell Western analysis (Figure 7C). In these cells, isoprenaline treatment (100 nM) produced a significant decrease in basal pERK (P < 0.001) that was blocked by the selective β_2-adrenoceptor antagonist ICI 118,551 (1 μM, P < 0.001), but not by the selective β_1-adrenoceptor antagonist CGP 20712 (1 μM), or by PTx (100 ng·mL^{-1}). In contrast, WIN treatment (10 μM) of HTM cells resulted in a significant increase in pERK (P < 0.001) that was blocked by the selective CB_1 receptor inverse agonist AM251 (1 μM, P < 0.001) as well as by PTx (100 ng·mL^{-1}, P < 0.001), but not by the selective CB_2 receptor

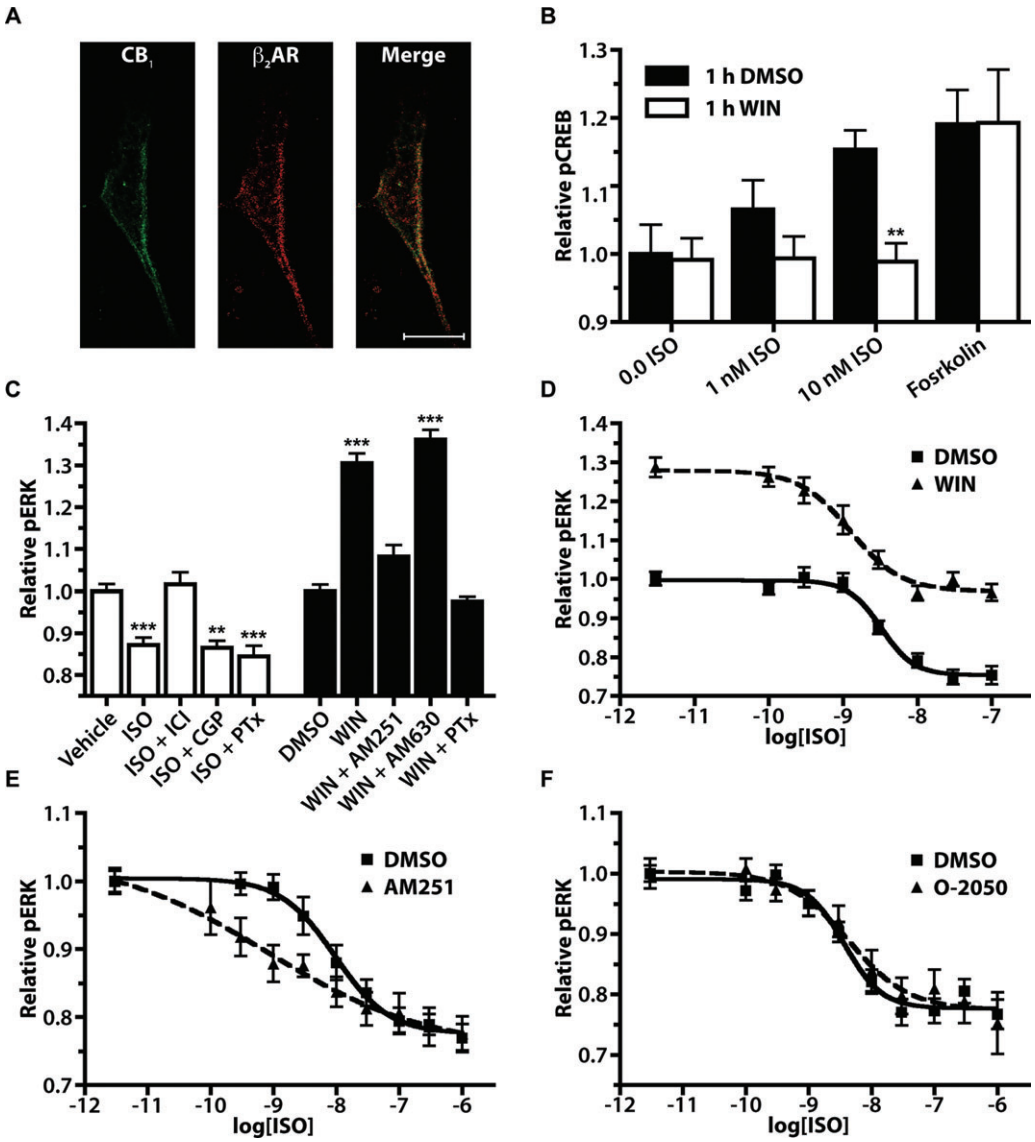

Figure 7 Interactions between CB_1 receptor and β_2-adrenoceptor pERK signalling in HTM cells. (A) Confocal immunofluorescence images of HTM cells using an anti-CB_1 primary antibody with a FITC-conjugated secondary antibody (left panel), an anti-β_2-adrenoceptor primary antibody with a Cy3-conjugated secondary antibody (middle panel) and the merged image (right panel). Scale bar is 20 μM. (B) Isoprenaline (ISO, 1 and 10 nM, 30 min) and forskolin (10 μM, 30 min) pCREB responses in HTM cells following 1 h DMSO or WIN (10 μM) pretreatment. $^{**}P < 0.01$ compared with corresponding isoprenaline concentration pretreated with DMSO; n = 18–24. (C) pERK responses in HTM cells following a 10 min exposure to either aqueous vehicle and isoprenaline (100 nM) treatments, or to 0.05% DMSO vehicle and WIN (10 μM). Pretreatment with ICI (1 μM), CGP (1 μM), AM251 (1 μM) or AM630 (1 μM) was for 15 min and with PTx (100 ng·mL^{-1}) for 24 h before isoprenaline or WIN application. PTx, ICI, CGP and AM251 bars are expressed as relative pERK level normalized to vehicle-treated cells that were also pretreated with the same antagonist or toxin to eliminate any affect these compounds may have had on the basal pERK levels. $^{**}P < 0.01$ compared with vehicle, $^{***}P < 0.001$ compared with vehicle or DMSO; n = 8–27. (D) pERK dose–response in HTM cells following 10 min co-application of isoprenaline with 0.05% DMSO or 1 μM WIN; n = 13–16. (E) Isoprenaline pERK dose–response curves in HTM cells following 15 min pretreatment with either 0.05% DMSO or 1 μM AM251, followed by 5 min treatment with isoprenaline; n = 12–24. (F) Isoprenaline pERK dose–response curves in HTM cells following 15 min pretreatment with 0.05% DMSO or 1 μM O-2050 and 5 min exposure to isoprenaline; n = 12–16. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CREB, cyclic AMP response element binding protein; DMSO, dimethylsulphoxide; ERK, extracellular signal-regulated kinase; HTM, human trabecular meshwork; O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; PTx, *Pertussis* toxin; WIN, WIN 55,212-2.

antagonist AM630 (1 μM). Treatment of HTM cells with AM251 did not alter basal pERK levels in HTM cells, suggesting CB_1 receptors are not constitutively coupled to pERK signalling in these cells (data not shown). Together these findings demonstrate that both CB_1 and β_2AR are functionally expressed in HTM cells, and that activation of these receptors are capable of modulating pERK.

Isoprenaline-mediated pERK dose–response curves in HTM cells were generated in the presence of either DMSO vehicle or WIN (10 μM) (Figure 7D). The two isoprenaline dose–response curves were significantly different ($P < 0.001$) with values for pEC_{50} of 8.49 ± 0.08 and 8.91 ± 0.12; baseline pERK of 1.00 ± 0.01 and 1.28 ± 0.02; E_{max} of 0.75 ± 0.01 and 0.97 ± 0.02 for the curves in the presence of DMSO and WIN

respectively. These data show that the WIN increase and the isoprenaline decrease in pERK inhibit each other to produce no net response when WIN and isoprenaline are applied together.

Isoprenaline-mediated pERK dose–response curves were generated in HTM cells following 15 min pretreatment with either DMSO vehicle, AM251 (1 μM),\ or O-2050 (1 μM) (Figure 7E and F). Pretreatment with AM251 resulted in a significantly altered isoprenaline dose–response curve in HTM cells ($P < 0.001$), which was unaffected by pretreatment with O-2050 ($P > 0.05$). Specifically, AM251 pretreatment resulted in an isoprenaline dose–response curve with a pEC_{50} of 9.10 ± 0.65 and a Hill coefficient of –0.34 ± 0.25, compared with the pEC_{50} of 8.02 ± 0.16 and Hill coefficient of –1.01 ± 0.32 for the DMSO vehicle-pretreated cells. These curves demonstrate that, as observed in HEK 293 cells, the CB_1 receptor inverse agonist AM251 but not the CB_1 receptor neutral antagonist O-2050 was capable of altering the pERK response to β_2-adrenoceptors in HTM cells.

Discussion and conclusion

The ability of rhodopsin-like family A GPCRs to interact with each other as dimers or higher order oligomers has generated considerable interest in recent years. These complexes have received much attention, not only because they are formed by many different GPCRs, but also because they appear to influence nearly every aspect of GPCR function (Terrillon and Bouvier, 2004; Pfleger and Eidne, 2005). The findings presented here demonstrating that the CB_1 receptor and β_2-adrenoceptor BRET pair produce significantly increased $BRET_{Eff}$ that is saturable and can be diluted by an untagged β_2-adrenoceptor construct strongly suggests that CB_1 receptors and β_2-adrenoceptors can physically interact with each other. Similarly, the observed increased $BRET_{Eff}$ values for CB_1/CB_1 receptor and β_2/β_2-adrenoceptor BRET pairs demonstrates that consistent with previous reports both of these receptors can also form homodimers in these cells (Hebert *et al.*, 1996; Angers *et al.*, 2000; Wager-Miller *et al.*, 2002). In comparing the BRET saturation curves for the CB_1 receptor homodimer with the CB_1/β_2-adrenoceptor heterodimer, it is apparent that the $BRET_{50}$ value for the homodimer is lower than that of the heterodimer. This suggests that if these two receptors are expressed at similar levels, the CB_1 receptor will preferentially form the homodimer over the heterodimer, although caution should be exercised in drawing such direct comparisons with two distinct BRET pairs (Mercier *et al.*, 2002). In addition, the observation that treatment with the CB_1 receptor inverse agonist AM251 altered $BRET_{Eff}$ compared with either the agonist WIN or neutral antagonist O-2050, further supports the conclusion of a specific interaction, and implies that AM251 either facilitates increased interactions between the two receptors or alters the conformation of the heterodimer in such a way as to increase the $BRET_{Eff}$ (Ayoub and Pfleger, 2009).

Functional interactions between CB_1 receptors and β_2-adrenoceptors were assessed by first examining the trafficking of these two receptors. When expressed in HEK 293 cells, CB_1 receptors are highly constitutively active, resulting in constitutive internalization of the receptor (Leterrier *et al.*, 2004; Ellis *et al.*, 2006; Bohn, 2007). This is consistent with the punctate expression pattern observed when CB_1-GFP^2 was expressed in HEK 293H cells, which reverts to a cell surface expression pattern upon addition of the CB_1 receptor inverse agonist AM251. In contrast, when β_2-adrenoceptors are expressed in HEK 293 cells, they are predominantly localized to the cell surface (von Zastrow and Kobilka, 1992; Sunaguchi *et al.*, 2003). This non-overlapping distribution of CB_1-GFP^2 and β_2-adrenoceptors indicates that in order for these receptors to physically interact with each other, the subcellular expression of one receptor must be affected by the presence of the other. Our results demonstrate that when cells co-expressed CB_1-GFP^2 and HA-β_2AR, CB_1-GFP^2 localization was indeed shifted towards the cell surface compared with when CB_1-GFP^2 was expressed alone, thus allowing for the physical interaction to take place. One possible mechanism for this would be that β_2-adrenoceptors tempered the constitutive activity of CB_1 receptors, as has been previously shown to occur in the β_1/β_2-adrenoceptor heterodimer (Zhu *et al.*, 2005). This could explain why β_2-adrenoceptors shift CB_1 receptor expression towards the cell membrane, which does not seem to be the case when CB_1 receptors interact with other GPCRs. For example, heterodimerization of CB_1 receptors with the orexin-1 receptor did not affect the internalized localization of CB_1 receptors, but instead altered the distribution of the orexin-1 receptor (Ellis *et al.*, 2006). In addition, when CB_1 receptors and another interacting GPCR, the μ-opioid receptor (Rios *et al.*, 2006), were co-expressed neither CB_1 nor μ-opioid receptor localization was affected, CB_1 receptors remaining largely internalized while the μ-opioid receptors stayed at the cell surface (Ellis *et al.*, 2006; Canals and Milligan, 2008).

Further support for the notion that CB_1/β_2-adrenoceptor dimerization attenuates CB_1 receptor constitutive activity comes from the observation that basal pERK levels in CB_1-GFP^2/TreHA-β_2AR cells were decreased following the induction of HA-β_2AR expression, as was the ability of the CB_1 receptor inverse agonist AM251 to decrease the basal pERK level. In addition, the fact that either CB_1 or β_2-adrenoceptor agonists were able to produce co-internalization of both receptors, a phenomenon reported for several other GPCR dimers (Terrillon and Bouvier, 2004), indirectly suggests that β_2-adrenoceptors suppress constitutive activity of CB_1 receptors. Specifically, as WIN produced co-internalization of β_2-adrenoceptors and the constitutive trafficking of GPCRs generally follows a similar mechanism to agonist-induced receptor internalization (Leterrier *et al.*, 2004; Marion *et al.*, 2004), it should be expected that constitutive internalization of CB_1 receptors would produce co-internalization of β_2-adrenoceptors, as was the case when CB_1 receptors were co-expressed with the orexin-1 receptor (Ellis *et al.*, 2006). The fact that this was not observed, and that instead CB_1 receptors were redistributed towards the cell surface, suggests that CB_1 receptors are more likely to have a reduced constitutive internalization when β_2-adrenoceptors are present.

G protein-coupled receptor heterodimerization may also influence the signalling pathways activated by the receptors present in the complex. In HEK 293H cells stably expressing CB_1-GFP^2 the cannabinoid agonist WIN produced both a PTx-

sensitive increase in pERK and a PTx-insensitive increase in pCREB, indicating that CB_1-GFP[2] couples to both $G_{i/o}$ and non-$G_{i/o}$ pathways in these cells. Based on previous reports that in the presence of PTx, CB_1 activates G_s to increase cAMP production (Maneuf and Brotchie, 1997; Jarrahian *et al.*, 2004; Kearn *et al.*, 2005), it is likely that the observed non-$G_{i/o}$ pCREB–WIN response in these cells occurs via G_s. The fact that the induction of HA-β_2AR expression in cells stably expressing CB_1-GFP[2] increased both the E_{max} and Hill coefficient of the WIN-mediated pERK dose–response, while decreasing the E_{max} and Hill coefficient of the WIN-mediated pCREB response suggests that HA-β_2AR alters the G protein coupling preference of CB_1 receptors. Specifically, the presence of β_2-adrenoceptors shifts the $G_{i/o}$ to G_s coupling ratio of CB_1 receptors towards increased $G_{i/o}$, but decreased G_s coupling. GPCR heterodimerization has previously been shown to alter G protein coupling and similar signalling effects have previously been reported for both CB_1 receptors as well as β_2-adrenoceptors (Breit *et al.*, 2004; Kearn *et al.*, 2005; Mcgraw *et al.*, 2006). It therefore is plausible that the physical interaction between CB_1 receptors and β_2-adrenoceptors directly affects CB_1 receptor–G protein coupling, although it also cannot be ruled out that the shift in CB_1 receptor coupling could be the result of G_s sequestration by β_2-adrenoceptors (Vasquez and Lewis, 2003).

Similar to the changes observed in CB_1 receptor signalling, β_2-adrenoceptor signalling pathways were also affected by the presence of CB_1 receptors. Co-expression of CB_1 receptors and β_2-adrenoceptors resulted in an increase in the isoprenaline-stimulated pCREB response with no change in the isoprenaline-mediated pERK response. The enhanced pCREB response is consistent with increased β_2-adrenoceptor–G_s coupling that may result either directly from the physical interaction with CB_1 receptors, or indirectly from decreased β_2-adrenoceptor–G_i coupling caused by CB_1 receptor–G_i sequestration (Vasquez and Lewis, 1999).

Co-application of WIN and isoprenaline to cells expressing both CB_1-GFP[2] and HA-β_2AR resulted in an additive effect on pERK levels, but not on pCREB. The additive pERK response might be expected as WIN and isoprenaline activate pERK through different signalling pathways, WIN through PTx-sensitive $G_{i/o}$ and isoprenaline through PTx-insensitive G_s or β-arrestin (Demuth and Molleman, 2006; Shenoy *et al.*, 2006). However, the lack of an additive effect on pCREB is more interesting. Although activation of CB_1 receptors has previously been shown to increase CREB phosphorylation (Casu *et al.*, 2005), the underlying pathway has not been examined. Our observations suggest that the WIN-mediated pCREB response is not $G_{i/o}$-mediated, as it was insensitive to PTx, and that instead it is likely to be the result of CB_1 receptor–G_s coupling. As β_2-adrenoceptors also activates pCREB via G_s, the lack of an additive effect may be due to overlap in the two pathways. However, the fact that the pCREB response is not additive even at sub-maximal concentrations of isoprenaline suggests that instead it may arise from physiological antagonism resulting from CB_1 receptor-induced activation of G_i, resulting in inhibition of adenylyl cyclase.

Another possible functional consequence of GPCR heterodimerization is altered receptor pharmacology of one or both receptors in the complex (Milligan, 2004; Terrillon and Bouvier, 2004). Our results demonstrate that the CB_1 receptor inverse agonist AM251 inhibits β_2-adrenoceptor-mediated pERK signalling, but had no effect on pCREB signalling, while the CB_1 neutral antagonist O-2050 did not affect either β_2-adrenoceptor-mediated pERK or pCREB activation. Recently, a similar result was reported where the CB_1 receptor inverse agonist SR141716A enhanced pERK signalling of the μ-opioid receptor, while the neutral antagonist O-2050 had no effect (Canals and Milligan, 2008). In this study the result was attributed to the inverse agonist blocking constitutive activity of CB_1 receptors and not to CB_1/μ-opioid receptor heterodimerization because the two receptors were not detected in the same subcellular location. However, in the present study AM251 inhibited rather than enhanced the β_2-adrenoceptor-mediated pERK response, suggesting that the AM251 effect is not simply the result of blocking CB_1 receptor constitutive activity. Instead, our results are best explained by the concept that in a heterodimer, one GPCR can allosterically modulate the second receptor in the complex (Milligan and Smith, 2007). As an inverse agonist, such as AM251, will drive CB_1 receptors from a constitutively active to an inactive state, it is conceivable that such a conformational change would, through heterodimerization, allosterically influence β_2-adrenoceptors and their subsequent signalling. If so, it follows that the CB_1 receptor neutral antagonist O-2050 should not alter the conformation of CB_1 receptors, as a neutral antagonist shows no preference for either the active or inactive states of the receptor, and therefore should not influence β_2-adrenoceptor signalling. Additional support for this conclusion may be drawn from the observation that AM251 treatment resulted in a change in the $BRET_{Eff}$ compared with O-2050. Although alterations in $BRET_{Eff}$ by ligands may be caused by changes in the number of receptors interacting as heterodimers, such ligand-mediated changes in $BRET_{Eff}$ are now believed to be more representative of conformational changes within the heterodimer (Ayoub *et al.*, 2002; Ayoub and Pfleger, 2009). Therefore, our observation that AM251 produces a change in $BRET_{Eff}$ compared with O-2050 supports the notion that this inverse agonist is allosterically modulating the function of β_2-adrenoceptors through the CB_1/β_2-adrenoceptor heterodimer.

CB_1 receptors and β_2-adrenoceptors are co-expressed in many tissues and cells, including parts of the cardiovascular system, female reproductive tract, brain, eye and bone (Jampel *et al.*, 1987a; Wanaka *et al.*, 1989; Tsou *et al.*, 1998; Stamer *et al.*, 2001; Wang *et al.*, 2004; Pacher and Hasko, 2008). However, few studies have directly examined interactions between these two receptors *in vivo*. Instead, most work on interactions between the cannabinoid and adrenergic systems has focused on the presynaptic ability of CB receptors to inhibit noradrenergic neurotransmission (Schlicker *et al.*, 1997; Schultheiss *et al.*, 2005; Pakdeechote *et al.*, 2007; Tam *et al.*, 2008). We therefore examined the potential impact of a CB_1/β_2-adrenoceptor interaction on pERK signalling in primary HTM cells.

The trabecular meshwork represents the primary route of aqueous humour outflow in the human eye and as a result represents a key therapeutic target for the regulation of IOP and for the development of novel treatments for glaucoma (Kaufman *et al.*, 1999; Ferrer, 2006). HTM cells have been

shown to express β_2-adrenoceptors, activation of which leads to increased cAMP levels and facilitates increased aqueous humour outflow (Jampel *et al.*, 1987b; Erickson-Lamy and Nathanson, 1992). In addition, trabecular meshwork cells also express CB_1 receptors, activation of which appears to increase aqueous humour outflow through activation of pERK (Straiker *et al.*, 1999; Njie *et al.*, 2006, Njie *et al.*, 2008). Consistent with this previous work, our demonstration that HTM cells express both CB_1 receptors and β_2-adrenoceptors, that these receptors are functionally active in pERK signalling, and that they are co-localized at the cell membrane, suggests that any physical or functional interaction between these receptors may be relevant in HTM, and as a result in the regulation of aqueous humour outflow.

To determine if the co-internalization of CB_1 receptors and β_2-adrenoceptors observed in HEK 293H cells was also important to the function of these receptors in HTM cells, the ability of WIN to desensitize the isoprenaline-induced pCREB response in these cells was examined. As in HEK 293H cells, when HTM cells were pretreated with WIN, their isoprenaline–pCREB response was attenuated, while their response to forskolin was unaffected. Such cross-desensitization has previously been reported for other GPCR heterodimers (Pfeiffer *et al.*, 2002) and supports the concept that CB_1 receptors and β_2-adrenoceptors form heterodimers in these cells, and further, that this heterodimer leads to cross-desensitization of β_2-adrenoceptors.

To examine the functional interaction between CB_1 receptors and β_2-adrenoceptors in HTM cells, the ability of these receptors to affect pERK signalling was examined. In these cells, β_2-adrenoceptor activation resulted in a PTx-insensitive decrease in pERK, while CB_1 receptor activation led to a PTx-sensitive increase in pERK. As seen in the HEK 293H cells, co-application of the CB_1 receptor and β_2-adrenoceptor agonists to HTM cells produced an additive response; although given that the CB_1 receptor response was positive while the β_2-adrenoceptor response was negative in HTM cells, the overall results was net inhibitory. Also similar to that observed in HEK 293H cells, it was found that the CB_1 receptor inverse agonist AM251 but not the neutral antagonist O-2050 altered the pERK response to the β_2-adrenoceptor agonist isoprenaline. The combination of an increased pEC_{50}, yet decreased Hill coefficient for the isoprenaline–pERK response when AM251 was present resulted in an increased ability of lower doses of isoprenaline to reduce pERK levels. Although this potentiation of the isoprenaline–pERK response by AM251 in HTM cells would seem to conflict with the inhibition AM251 observed in HEK 293H cells, as the HEK 293H–β_2AR response was a positive pERK response, while the HTM–β_2AR pERK response was negative it is conceivable that inhibition of a positive response and potentiation of a negative one could in fact be mediated by a similar mechanism.

Another interesting finding from our study is that the natures of ligand-induced effects differ depending on the cellular context. Other studies have demonstrated that both β_2-adrenoceptor agonists and inverse agonists can activate the ERK pathway in HEK cells through multiple mechanisms (Azzi *et al.*, 2003; Baker *et al.*, 2003; Shenoy *et al.*, 2006). However, to our knowledge, our findings in HTM cells are the first demonstration that a β_2-adrenoceptor agonist can inhibit ERK phosphorylation, given the proper cellular context. Our observation that the CB_1 receptor inverse agonist AM251 alters β_2-adrenoceptor signalling in cells endogenously expressing both CB_1 receptors and β_2-adrenoceptors may be particularly relevant *in vivo* due to the interest in CB_1 receptor inverse agonists for the clinical treatment of obesity, metabolic syndrome, type II diabetes and addiction (Xie *et al.*, 2007; Le foll *et al.*, 2008; Van diepen *et al.*, 2008; Vemuri *et al.*, 2008). Our results suggest that these CB_1 receptor inverse agonists may exert their effects not only by directly blocking CB_1 receptors, but also by indirectly affecting β_2-adrenoceptor function in cells that co-express these receptors. In keeping with this, AM251 was found in one study to block the regional haemodynamic response to lipopolysaccharide in rats, a response that was attributed to β-adrenoceptor activation and was also blocked by the selective β_2-adrenoceptor antagonist ICI 118,551 (Gardiner *et al.*, 2005). In addition, the recent observation that CB_1 receptors are expressed in murine cardiomyocytes (Mukhopadhyay *et al.*, 2007) suggests that some of the cardiovascular effects of CB_1 receptor inverse agonists (Van diepen *et al.*, 2008) may be the result of indirect actions on β_2-adrenoceptors in addition to the direct blockade of CB_1 receptors.

The present study demonstrates a physical and functional interaction between CB_1 receptors and β_2-adrenoceptors. These results suggest a more complex picture than previously surmised in which interactions between the cannabinoid and the adrenergic systems were primarily attributed to CB_1 receptor-mediated presynaptic inhibition of noradrenergic transmission. We suggest, in light of our findings and the wide biological and pharmacological importance of the β-adrenoceptor and endocannabinoid systems, a re-evaluation of the nature of previously reported functional interactions between the cannabinoid and β-adrenoceptor systems in cells and tissues that co-express these two receptors.

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Statement of conflict of interests

The authors report no conflict of interest.

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