Constitutive Activity of the Cannabinoid CB1 Receptor Regulates the Function of Co-expressed Mu Opioid Receptors[*][S]

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The human mu opioid receptor was expressed stably in Flp-In T-REx HEK293 cells. Occupancy by the agonist DAMGO (Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol) resulted in phosphorylation of the ERK1/2 MAP kinases, which was blocked by the opioid antagonist naloxone but not the cannabinoid CB1 receptor inverse agonist SR141716A. Expression of the human cannabinoid CB1 receptor in these cells from the inducible Flp-In T-REx locus did not alter expression levels of the mu opioid receptor. This allowed the cannabinoid CB1 agonist WIN55212-2 to stimulate ERK1/2 phosphorylation but resulted in a large reduction in the capacity of DAMGO to activate these kinases. Although lacking affinity for the mu opioid receptor, co-addition of SR141716A caused recovery of the effectiveness of DAMGO. In contrast co-addition of the CB1 receptor neutral antagonist O-2050 did not. Induction of the CB1 receptor also resulted in an increase of basal [^{35}S]guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) binding and thereby a greatly reduced capacity of DAMGO to further stimulate [^{35}S]GTPγS binding. CB1 inverse agonists attenuated basal [^{35}S]GTPγS binding and restored the capacity of DAMGO to stimulate. Flp-In T-REx HEK293 cells were generated, which express the human mu opioid receptor constitutively and harbor a modified D163N cannabinoid CB1 receptor that lacks constitutive activity. Induction of expression of the modified cannabinoid CB1 receptor did not limit DAMGO-mediated ERK1/2 MAP kinase phosphorylation and did not allow SR141716A to enhance the function of DAMGO. These data indicate that it is the constitutive activity inherent in the cannabinoid CB1 receptor that reduces the capacity of co-expressed mu opioid receptor to function.

G protein-coupled receptors (GPCRs)[2] are the largest family of transmembrane signal-transducing polypeptides found in man, with more than 800 genes encoding these proteins (1). Many cells express a wide range of GPCRs at detectable levels, and the profile of GPCR expression in individual cells is both plastic and tightly regulated during development and differentiation. Activation of a GPCR can often regulate the function of other co-expressed GPCRs. Nevertheless, it remains routine to explore the details of function and pharmacology of individual GPCRs in isolation. The cannabinoid CB1 receptor is both widely distributed and one of the most highly expressed GPCRs in the mammalian central nervous system (2). Many of the actions of the CB1 receptor, including inhibition of adenylyl cyclase activity and regulation of both calcium and potassium channels, are produced via activation of pertussis toxin-sensitive G proteins of the $G\alpha_i/G\alpha_o$ family. These signals are integrated to inhibit the release of neurotransmitters (2–4). The mu opioid peptide (MOP) receptor also functions predominantly via the same group of G proteins, and thus it has many functions that overlap with those of the cannabinoid CB1 receptor (5, 6). The CB1 and MOP receptors not only are expressed in similar brain areas but are co-expressed in individual neurons in rat striatum (7), caudate nucleus (8), and dorsal horn (9).

A substantial body of data has demonstrated the capacity of ligands at these two GPCRs to cause cross-regulation (10–15), and a number of studies have suggested this might relate to receptor heterodimerization (12, 15). Rios *et al.* (15) recently employed co-transfection of bioluminescence resonance energy transfer-competent forms of CB1 and MOP receptors to demonstrate such direct interactions in HEK293 cells. There is growing evidence of the importance of such heterodimeric interactions in modulation of receptor pharmacology and function (16, 17). However, this is clearly only one of a number of mechanisms by which cross-talk might occur between pairs of co-expressed GPCRs. Downstream integration of signals generated from non-associated receptors is also a well established means of cross-regulation (18, 19).

To explore the importance of both GPCR heterodimerization (20, 21) and other avenues for receptor cross-talk (22), we have made considerable use of Flp-In T-REx HEK293

[2] The abbreviations and chemical names used are: GPCR, G protein-coupled receptor; MOP, mu opioid peptide; GRK, G protein receptor kinase; HEK, human embryonic kidney; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; h, human; eYFP, enhanced yellow fluorescent protein; eCFP, enhanced cyan fluorescent protein; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; ANOVA, analysis of variance; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; DAMGO, Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol; LY320135, 4-[[6-methoxy-2-(4-methoxyphenyl)-3-benzofuranyl]carbonyl]benzonitrile; O-2050, (6a*R*,10a*R*)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran; SR141716A, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide]; WIN55212-2, [(*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt].

cells. These allow one GPCR to be expressed stably and constitutively, whereas a second can then be expressed, on demand, in an entirely inducible fashion. It is thus possible to examine the function, regulation, and pharmacology of a GPCR in the absence and presence of a second GPCR in the same cells. In the current study we employed this approach to examine the molecular basis for cross-talk between co-expressed CB1 and MOP receptors. We demonstrate a key role for the ligand-independent, constitutive activity of the cannabinoid CB1 receptor in the control of MOP receptor function.

EXPERIMENTAL PROCEDURES

Materials—All materials for tissue culture were from Invitrogen. DAMGO, naloxone, forskolin, pertussis toxin, cholera toxin, and tetrahydrolipstatin were from Sigma-Aldrich. WIN55212-2, O-2050, and LY320135 were from Tocris (Avonmouth, UK). SR141716A was the kind gift of GlaxoSmithKline. The radiochemicals [³H]adenine and [³H]SR141716A were from GE Healthcare. [³H]Diprenorphine and [³⁵S]GTPγS were from PerkinElmer Life Sciences. Phospho-specific and total anti-ERK1/2 antibodies were from Cell Signaling (Hitchin, Hertfordshire, UK). Antisera directed against the C-terminal decapeptides of G protein α-subunits have been described previously (23–25).

Flp-In Constructs—h-CB1-eCFP and D163N-h-CB1-eCFP in pcDNA5/FRT/TO were obtained by subcloning h-CB1-eCFP from pcDNA3.1 into the pcDNA5/FRT/TO vector (Invitrogen) using the HindIII and NotI restriction sites.

Site-directed Mutagenesis—To introduce the D163N amino acid substitution into the primary structure of the h-CB1 receptor site-directed mutagenesis of the encoding nucleotide sequence was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Cell Culture and Generation of Stable Flp-In T-REx HEK293 Cells—Cells were maintained in Dulbecco's modified Eagle's medium without sodium pyruvate, 4500 mg/liter glucose, and L-glutamine supplemented with 10% (v/v) fetal calf serum, 1% antibiotic mixture, and 10 μg/ml blastacidin at 37 °C in a humidified atmosphere of air/CO_2 (19:1).

To generate Flp-In T-REx HEK293 cells able to inducibly express a receptor of interest, the cells were transfected with a mixture containing the desired receptor cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using Effectene® transfection reagent (Qiagen) according to the manufacturer's instructions. Cell maintenance and selection were as detailed elsewhere (22). Resistant clones were screened for receptor expression by both fluorescence and Western blotting. To induce expression of receptors cloned into the Flp-In locus, cells were treated with 0.1 μg/ml doxycycline for varying periods of time.

Pertussis and Cholera Toxin Treatment—Cells expressing the appropriate receptors were treated overnight with 25 ng/ml pertussis toxin and/or 100 ng/ml of cholera toxin before being processed for the appropriate assay.

Live Cell Epifluorescence Microscopy—Cells expressing the appropriate receptors tagged to enhanced cyan fluorescent protein (eCFP) or enhanced yellow fluorescent protein (eYFP) were grown on poly-D-lysine-treated coverslips. Coverslips were placed into a microscope chamber containing physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM D-glucose, pH 7.4). Fluorescent images of the cells were acquired using an inverted Nikon TE2000-E microscope (Nikon Instruments, Melville, NY) equipped with a ×40 (numerical aperture = 1.3), oil immersion, Plan Fluor lens and a cooled, digital Cool Snap-HQ CCD camera (Roper Scientific/Photometrics, Tucson, AZ) (see Ref. 26 for details).

Cell Lysates and Western Blotting—Cell lysates were obtained by harvesting the cells with ice-cold radioimmune precipitation assay buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate supplemented with 10 mM NaF, 5 mM EDTA, 10 mM NaH_2PO_4, 5% ethylene glycol, and a protease inhibitor mixture (Complete; Roche Diagnostics), pH 7.4). Cellular extracts were then centrifuged for 30 min at 14,000 × g, and the supernatant was recovered.

To detect receptor or G protein expression, samples were heated at 65 °C for 15 min, and for detection of ERK1/2, samples were boiled for 5 min. Cell lysates were then subjected to SDS-PAGE analysis using 4–12% bis-Tris gels (NuPAGE; Invitrogen) and MOPS buffer. After electrophoresis, proteins were transferred onto nitrocellulose membranes that were incubated in 5% non-fat milk and 0.1% Tween 20/Tris-buffered saline solution at room temperature on a rotating shaker for 2 h to block nonspecific binding sites. The membrane was incubated overnight with the corresponding antibody (1:5000 goat anti-MOP receptor, 1:5000 rabbit anti-G protein) and detected using a horseradish peroxidase-linked anti-goat (Sigma) or anti-rabbit (Amersham Biosciences) IgG secondary antiserum, respectively. Immunoblots were developed by application of enhanced chemiluminescence solution (Pierce).

ERK1/2 Phosphorylation and Immunoblots—Cells were grown in 12-well plates and serum-starved overnight prior to treatment with ligands as indicated. Cell lysates were prepared as described above with the addition of Na_3VO_4 (1 mM) to prevent dephosphorylation. ERK1/2 phosphorylation was detected by protein immunoblotting using phospho-specific antibodies and horseradish peroxidase-conjugated anti-rabbit IgG as secondary antibody for immunodetection. After visualization of ERK1/2 phosphorylation, the membranes were stripped and reprobed using a total anti-ERK1/2 antibody.

Cell Membrane Preparation—Pellets of cells were resuspended in 10 mM Tris, 0.1 mM EDTA, pH 7.4 (TE buffer) plus protease inhibitor mixture and homogenized using 40 strokes of a glass on Teflon homogenizer. Samples were centrifuged at 1000 × g for 10 min at 4 °C to remove unbroken cells and nuclei. The supernatant fraction was removed and passed through a 25-gauge needle 10 times before being transferred to Ultracentrifuge tubes and subjected to centrifugation at 50,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in TE buffer. Protein concentration was assessed, and membranes were diluted to 1 mg/ml and stored at −80 °C until required.
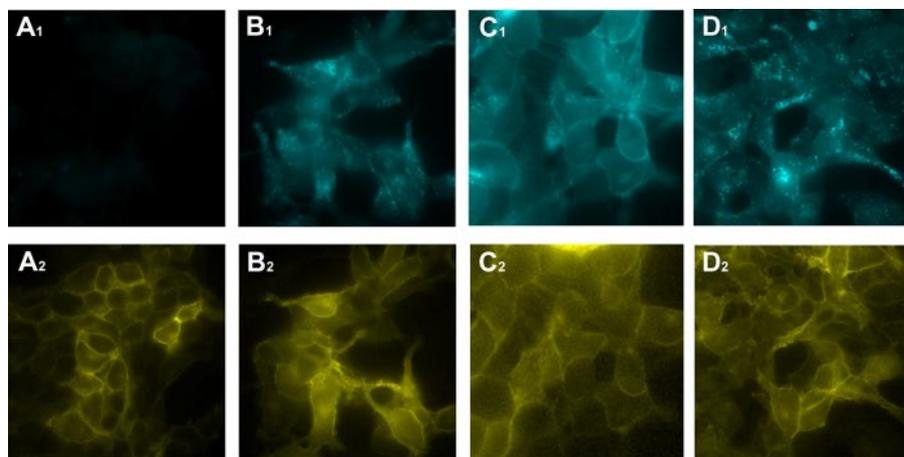
FIGURE 1. **Generation of cell lines stably expressing the h-MOP receptor and harboring the h-CB1 receptor at an inducible locus.** Flp-In T-REx HEK293 cells were transfected with h-CB1-eCFP (*blue*) and pools of cells isolated in which expression of this construct was dependent upon addition of the inducer doxycycline (compare A_1 and B_1). These cells were further transfected to express h-MOP-eYFP (*yellow*) stably and constitutively, and individual clones were selected (A_2 and B_2). Key characteristics are shown. Both in the absence and presence of h-CB1-eCFP, h-MOP-eYFP was present predominantly at the cell surface, whereas h-CB1-eCFP, which was undetectable in the absence of inducer (A_1), was expressed with a predominantly punctate, intracellular distribution subsequent to addition of the inducer (B_1). The addition of the CB1 receptor inverse agonist SR141716A (overnight, 10 nM) resulted in substantial relocation of h-CB1-eCFP to the cell surface (C_1), but treatment with the CB1 receptor neutral antagonist O-2050 (overnight, 100 nM) did not (D_1). Treatment with neither SR141716A nor O-2050 altered the cellular distribution of h-MOP-eYFP (C_2 and D_2). This pattern of receptor distribution was observed in multiple independent clones.

Radioligand Binding—[^{3}H]SR-141716A binding reaction mixtures were established in a volume of 1000 μl containing 20–40 μg of membrane protein in binding buffer (50 mM Tris, 1 mM EDTA, 3 mM MgCl$_2$, and 0.3% bovine serum albumin, pH 7.4) containing a range of concentrations (0.25–8 nM) of [^{3}H]SR-141716A. Potential competing ligands were diluted in binding buffer. Nonspecific binding was determined using the antagonist AM251 (10 μM). Samples were incubated for 90 min at 37 °C prior to filtration through Whatman GF/C filters previously soaked in phosphate-buffered saline/polyethyleneimine 0.1%.

[^{3}H]Diprenorphine binding was performed similarly. Reaction mixtures contained 20 μg of membrane protein in binding buffer (50 mM Tris, 100 mM NaCl and 3 mM MgCl$_2$, pH 7.4) and a range of concentrations (0.05–2 nM) of [^{3}H]diprenornphine. Nonspecific binding was determined using the antagonist naloxone (100 μM). Samples were incubated for 1 h at 25 °C prior to filtration through Whatman GF/C filters.

Data were analyzed using Graphpad Prism, and B_{max} and K_d values were determined via saturation binding analysis.

[^{35}S]GTPγS Binding—To analyze h-CB1 activation, cell membranes (10 μg) were incubated in buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% bovine serum albumin, pH 7.4) containing 30 μM GDP and various concentrations of ligands. All experiments were performed in triplicate. The reaction was initiated by the addition of cell membranes and incubated at 30 °C for 30 min. A 100-μl volume of [^{35}S]GTPγS (0.1 nM final concentration) was then added, and the incubation was continued for a further 30 min. The reaction was terminated by rapid filtration with a Brandel cell harvester and three 4-ml washes with ice-cold phosphate-buffered saline. Radioactivity was determined as described for saturation analysis. A similar procedure was employed to assess h-MOP receptor activation using cell membranes (10 μg) incubated in buffer (20 mM HEPES, 100 mM NaCl, 4 mM MgCl$_2$, pH 7.4) containing 1 μM GDP and various concentrations of ligands.

Intact Cell Adenylyl Cyclase Activity Measurements—Intact cell adenylyl cyclase activity measurements were performed essentially as described previously (27). Cells were split into wells of a poly-D-Lysine coated 12-well plate and allowed to reattach. Cells were then incubated in medium containing [^{3}H]adenine (1.5 μCi/well) for 16 h. The generation of [^{3}H]cAMP in response to the treatment of the cells with various ligands and other reagents was then assessed. Results are presented as the ratio of levels of [^{3}H]cAMP to total [^{3}H]adenine nucleotides.

RESULTS

The cannabinoid CB1 receptor is one of the most highly expressed GPCRs in the mammalian central nervous system. In a number of regions its expression pattern overlaps strongly with that of the MOP receptor (7–9). The human MOP receptor was C-terminally tagged with eYFP (h-MOP-YFP). This construct was expressed stably and constitutively in Flp-In T-REx HEK293 cells that had previously been engineered to harbor at the Flp-In locus, a form of the human cannabinoid CB1 receptor C-terminally tagged with eCFP (h-CB1-eCFP). Expression from this locus is controlled in a tetracycline/doxycycline-dependent fashion. Individual clones were then isolated. h-MOP-eYFP was present predominantly at the plasma membrane in the absence of the CB1 receptor, and the cellular distribution of h-MOP-eYFP was unaffected by induction of h-CB1-eCFP expression (Fig. 1). h-CB1-eCFP was not detectable in the absence of the inducer doxycycline. However, when induced the pattern of distribution of this construct was markedly different from the MOP receptor. At steady state the bulk of this protein was present in punctate, intracellular vesicles (Fig. 1). This distribution pattern was observed in multiple independent clones (data not shown). It has previously been demonstrated that sustained treatment of cells expressing h-CB1-eCFP with the CB1 receptor inverse agonist SR141716A (also called rimonabant) results in an enrichment of h-CB1-eCFP at the cell surface (20). This effect (Fig. 1) appeared to reflect the inverse agonist action of SR141716A, because equivalent treatment of the cells with the ligand O-2050 did not alter the cellular distribution of h-CB1-eCFP (Fig. 1). O-2050 has been described as a CB1 receptor neutral antagonist (28). Quantitation of receptor expression level was achieved via selective saturation ligand binding studies. In such studies the opioid antagonist [^{3}H]diprenorphine indicated that h-MOP-eYFP is expressed at between 1.0 and 1.5 pmol/mg membrane protein in various clones. Expression levels of h-MOP-eYFP were unaltered by the presence or absence of h-CB1-eCFP

TABLE 1

Quantitation of expression of h-CB1-eCFP and h-MOP-eYFP in clones of Flp-In T-REx HEK293 cells

Doxycycline was used at 0.1 μg/ml for 48 h. All data are means $\pm$ S.E. from at least three independent experiments. NA, not applicable.

Clone	Without doxycycline		With doxycycline	
	B_{max}	K_d	B_{max}	K_d
	fmol/mg	*nM*	*fmol/mg*	*nM*
[³H]SR141716A binding:				
#o	26 $\pm$ 26	NA	1020 $\pm$ 280	0.44 $\pm$ 0.1
#4	281 $\pm$ 173	NA	3191 $\pm$ 480	0.62 $\pm$ 0.12
[³H]diprenorphine binding:				
#o	1167 $\pm$ 156	0.2 $\pm$ 0.09	1489 $\pm$ 420	0.2 $\pm$ 0.02
#4	1180 $\pm$ 173	0.19 $\pm$ 0.06	1221 $\pm$ 183	0.21 $\pm$ 0.06

(Table 1). As monitored by the specific binding of [³H]SR141716A, h-CB1-eCFP was absent without induction, whereas clones expressing between 1.0 and 3.0 pmol/mg membrane protein were identified following induction (Table 1).

In the absence of induction of h-CB1-eCFP, ERK1/2 MAP kinase phosphorylation was produced by the highly selective MOP receptor agonist DAMGO (Fig. 2 and supplemental Fig. 1). This effect was abolished by overnight pretreatment of the cells with pertussis toxin (supplemental Fig. 1) but not by equivalent treatment with cholera toxin (supplemental Fig. 1). The cannabinoid CB1 receptor agonist WIN55212-2 was without effect in the absence of h-CB1-eCFP induction but stimulated ERK1/2 phosphorylation effectively following treatment of the cells with doxycycline (Fig. 2 and supplemental Fig. 1). Interestingly, treatment with a combination of pertussis toxin and cholera toxin was required to ablate this effect of WIN55212-2 (supplemental Fig. 1). Of equal interest was the markedly reduced capacity of DAMGO to stimulate ERK1/2 MAP kinase phosphorylation in the presence of h-CB1-eCFP (Fig. 2). Combinations of time courses and concentration-response curves demonstrated that the reduced capacity of DAMGO to stimulate phosphorylation of ERK1/2 in the presence of h-CB1-eCFP did not reflect either an alteration in ligand potency or the kinetics of activation, which was rapid and transient (Fig. 2A). Following induction of h-CB1-eCFP expression, WIN55212-2 also produced rapid and transient ERK1/2 phosphorylation (Fig. 2B). Furthermore, the loss of capacity of DAMGO to promote ERK1/2 MAP kinase phosphorylation in the presence of h-CB1-eCFP was observed in multiple, distinct clones (Fig. 2C).

Cells expressing h-MOP-eYFP and harboring h-CB1-eCFP were employed to explore the basis of these observations. Cells were exposed to DAMGO in the presence of the selective receptor blockers SR141716A (CB1 receptor) and naloxone (opioid receptors). As anticipated, naloxone blocked the effect of DAMGO, but SR141716A did not (Fig. 3). SR141716A was, however, clearly active. It was able to block WIN55212-2-stimulated ERK1/2 MAP kinase phosphorylation in cells induced to express h-CB1-eCFP (Fig. 3). Unexpectedly, the addition of SR141716A along with DAMGO resulted in restoration of the capacity of DAMGO to cause ERK1/2 MAP kinase phosphorylation in cells co-expressing the two GPCRs (Fig. 3).

SR141716A is generally accepted to be a CB1 receptor inverse agonist (29, 30). It is therefore able to reduce constitu-

tive, ligand-independent activity of the receptor. We explored the contribution of h-CB1-eCFP constitutive activity to the effect of SR141716A by also employing the ligand O-2050. This has been described as a CB1 receptor neutral antagonist (28). O-2050 did not block the action of DAMGO in cells expressing only h-MOP-eYFP (Fig. 4A). O-2050 was also unable to restore the capacity of DAMGO to stimulate ERK1/2 MAP kinase phosphorylation in cells induced to co-express h-CB1-eCFP and h-MOP-eYFP (Fig. 4A). However, it did block WIN55212-2 stimulation of ERK1/2 phosphorylation (Fig. 4A). The appropriate definition of a compound as a neutral antagonist is potentially system-dependent. The key requirements are that the ligand binds to the appropriate receptor and is able to reverse the effects of both agonist and inverse agonist compounds (30, 31). O-2050 was able to compete with [³H]SR141716A with high affinity (K_i = 3 nM) to bind h-CB1-eCFP (Fig. 4B). Furthermore, O-2050 was able to reverse both WIN55212-2-stimulated [³⁵S]GTPγS binding and SR141716A-mediated inhibition of [³⁵S]GTPγS binding in a concentration-dependent manner (Fig. 4C). O-2050 also had no significant effect on basal [³⁵S]GTPγS binding in membranes of cells induced to express h-CB1-eCFP (Fig. 4D), whereas SR141716A inhibited this constitutive activity (Fig. 4D). O-2050, therefore, acted as a neutral antagonist in this system. The capacity of SR141716A but not O-2050 to reverse the attenuation of DAMGO-mediated ERK1/2 MAP kinase phosphorylation in the presence of hCB1-eCFP is thus consistent with suppression of constitutive activity of this receptor construct. In certain cases, apparent constitutive activity may reflect the presence of an undetected endogenous agonist. Further evidence that hCB1-eCFP displayed constitutive activity was that tetrahydrolipstatin did not produce an effect akin to SR141716A (data not shown). Tetrahydrolipstatin is an inhibitor of diacylglycerol lipase and blocks endogenous generation of the endocannabinoid 2-arachidonyl glycerol (32).

To explore whether the effect of the constitutive capacity of h-CB1-eCFP to inhibit ligand stimulation of ERK1/2 MAP kinase phosphorylation was specific for the h-MOP receptor, we generated further Flp-In T-REx HEK293 cell lines. These harbored h-CB1-eCFP at the Flp-In locus and expressed a C-terminally eYFP-tagged form of the human D2 dopamine receptor stably and constitutively. Like h-MOP, the D2 dopamine receptor functions predominantly via activation of pertussis toxin-sensitive G_i family proteins (33, 34). Saturation binding studies employing the D2 receptor antagonist/inverse agonist [³H]spiperone indicated that h-D2-eYFP was expressed at levels similar to h-MOP-eYFP in the clones analyzed earlier. In the absence of h-CB1-eCFP, dopamine stimulated ERK1/2 MAP kinase phosphorylation (supplemental Fig. 2). Induction of h-CB1-eCFP expression in these cells resulted in a reduction of the extent of ERK1/2 MAP kinase phosphorylation in response to dopamine, but co-addition of SR141716A was unable to reverse this reduction (supplemental Fig. 2).

Although SR141716A promoted the capacity of DAMGO to stimulate ERK1/2, and WIN55212-2 also stimulated ERK1/2 phosphorylation in the h-CB1-eCFP plus h-MOP-eYFP co-expressing cells, there was no indication of constitutive levels of phosphorylation of the ERK MAP kinases. We thus explored
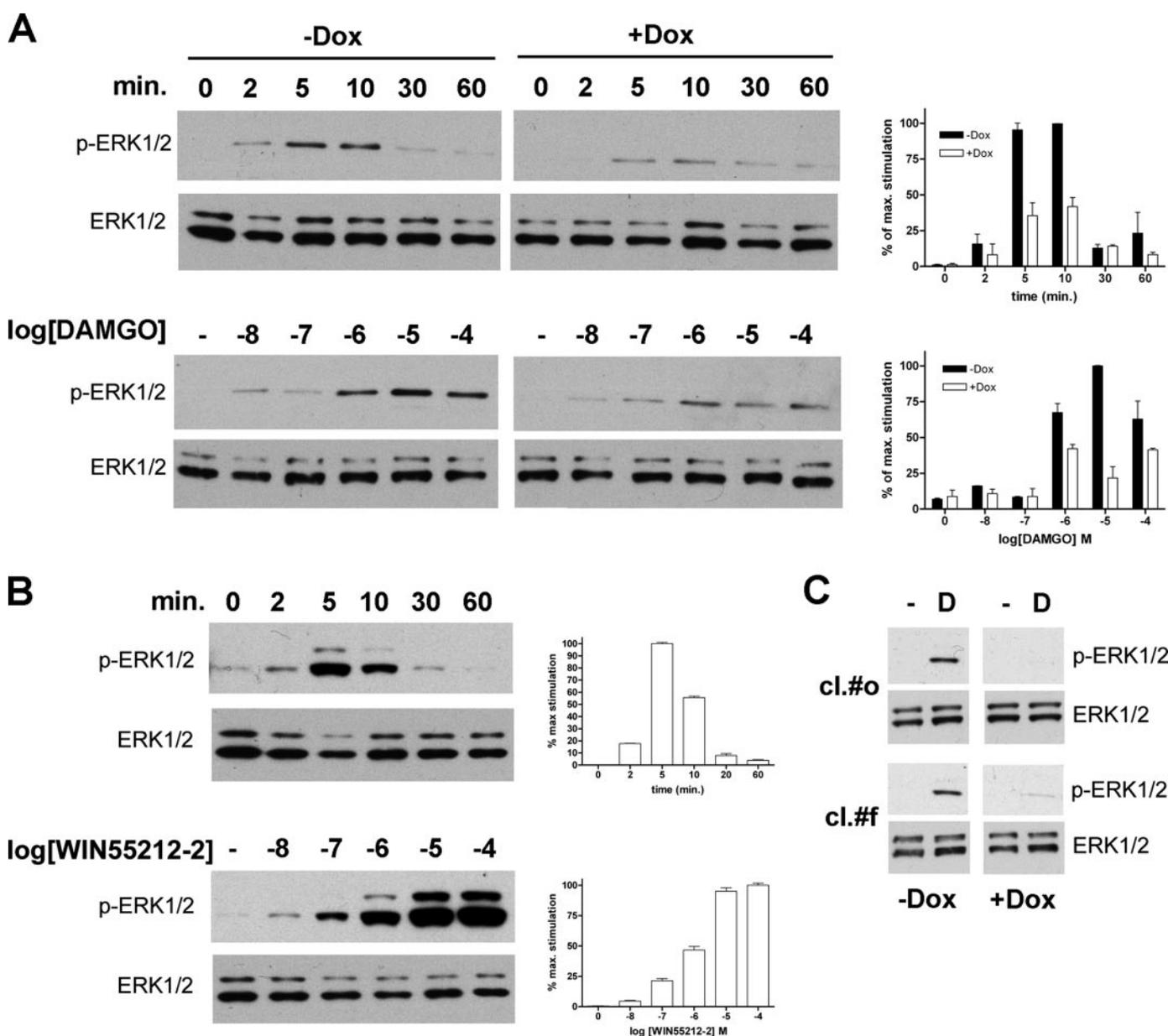
FIGURE 2. **Expression of h-CB1 does not alter the kinetics or concentration dependence of ERK1/2 phosphorylation by DAMGO.** *A*, Flp-In T-REx HEK293 cells expressing h-MOP-eYFP and harboring h-CB1-eCFP (clone 4) were untreated (−*Dox*) or exposed to doxycycline (0.1 μg/ml, 48 h) (+*Dox*). Time courses of DAMGO (1 μM)-induced phosphorylation of ERK1/2 were performed (*upper panels*) as were concentration-response curves for DAMGO (*lower panels*). Data are representative of three experiments performed. Data from each of the three experiments were quantitated (means ± S.E.) (*right-hand panels*). The maximal signal in the absence of doxycycline treatment was set at 100%, and the other samples are presented relative to the 100% effect. *B*, time courses and concentration-response curves of ERK1/2 phosphorylation stimulated by WIN55212-2 (1 μM) were also performed (*left-hand panels*) and quantitated (*right-hand panels*) after doxycycline-induced expression of h-CB1-eCFP. *C*, the marked reduction of DAMGO (*D*)-induced phosphorylation of ERK1/2 after 5 min of treatment in the presence of h-CB1-eCFP (+*Dox*) (see *A*) was observed in multiple independent clones including clone o (*cl.#0*) and clone f (*cl.#f*). In each case blots were reprobed to detect total ERK1/2 levels as loading controls.

the contribution of h-CB1 receptor constitutive activity to the alteration in MOP receptor function at the level of G protein activation. As shown in Fig. 4*D*, simple induction of hCB1-eCFP expression resulted in a large increase in basal [^{35}S]GTPγS binding in membranes from these cells. In these membranes WIN55212-2 was able to enhance further [^{35}S]GTPγS binding (Fig. 4*C*). DAMGO stimulated binding of [^{35}S]GTPγS in a concentration-dependent fashion in both the absence and presence of hCB1-eCFP. However, the high ligand-independent binding of [^{35}S]GTPγS produced by induction of hCB1-eCFP expression greatly reduced the absolute

extent of DAMGO function in this assay (Fig. 5*A*). This effect was particularly pronounced when the effect of DAMGO was presented as "-fold stimulation above basal" binding of [^{35}S]GTPγS (Fig. 5*B*). A second cannabinoid CB1 receptor inverse agonist, LY320135 (Fig. 5*C*), greatly reduced basal binding of [^{35}S]GTPγS in membranes co-expressing h-CB1-eCFP and h-MOP-eYFP but not in the absence of h-CB1-eCFP (Fig. 5*C*). SR141716A produced a similar effect (data not shown). This resulted in a greatly enhanced absolute capacity of DAMGO to stimulate [^{35}S]GTPγS binding (Fig. 5*C*). When presented as -fold over basal, such treatments restored the

effect of DAMGO to that observed in the absence of h-CB1-eCFP (Fig. 5*D*). Despite these observations, the stimulatory effects of DAMGO and WIN55212-2 were unaffected by the co-addition of low concentrations of the reciprocal agonist (10 nM) (Fig. 6), which have been reported by others (15) to cause reduced function of each agonist in [^{35}S]GTPγS binding assays.

h-CB1-eCFP-mediated stimulation of ERK1/2 MAP kinase activity was only attenuated by treatment with a combination of both pertussis and cholera toxins. By contrast, prior pertussis toxin treatment abolished both the enhanced basal and WIN55212-2-mediated elevation of [^{35}S]GTPγS in membranes of cells co-expressing h-CB1-eCFP and h-MOP-eYFP (supplemental Fig. 3). However, it is well appreciated that pertussis toxin-sensitive G$_i$ family G proteins are generally more amenable to analysis via [^{35}S]GTPγS binding studies than G$_s$ (35, 36). We therefore examined the potential for h-CB1-eCFP to generate signals via G$_s$, because interactions of the cannabinoid CB1 receptor with G$_s$ are well established in a range of systems (2, 37, 38). Adenylyl cyclase assays were performed in intact cells in the absence or presence of induced h-CB1-eCFP. In both situations basal adenylyl cyclase activity was unaffected by the addition of 1 μM WIN55212-2 (Fig. 7*A*). However, basal levels increased, and these were now enhanced substantially by WIN55212-2 when the experiments were performed on pertussis toxin-treated cells. This finding suggests an enhanced capacity to interact with G$_s$ in the absence of coupling to pertussis toxin-sensitive G proteins (Fig. 7*A*). This effect of WIN55212-2 was concentration-dependent (Fig. 7*B*), blocked
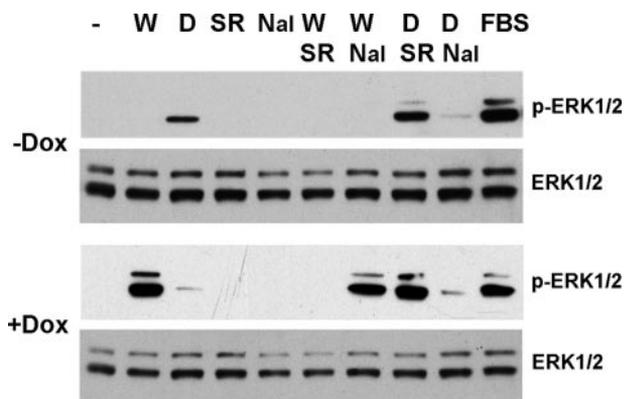

FIGURE 3. **Treatment of cells co-expressing h-MOP and h-CB1 with a CB1 receptor inverse agonist enhances the capacity of DAMGO to promote ERK1/2 phosphorylation.** Flp-In T-REx HEK293 cells, clone 4, expressing h-MOP-eYFP and harboring h-CB1-eCFP were untreated (−*Dox*) or exposed to doxycycline (0.1 μg/ml, 48 h) (+*Dox*). These were challenged for 5 min with the CB1 agonist WIN55212-2 (*W*, 1 μM), the h-MOP agonist DAMGO (*D*, 1 μM), CB1 inverse agonist SR141716A (*SR*, 0.1 μM), the opioid receptor antagonist naloxone (*Nal*, 10 μM), or various combinations of these ligands. Fetal bovine serum (*FBS*) was employed as a positive control. Cell lysates were immunoblotted to detect phosphorylation of ERK1/2 (*upper panels*). In each case total immunodetectable levels of ERK1/2 are shown as loading controls (*lower panels*). Data are representative of three experiments performed.
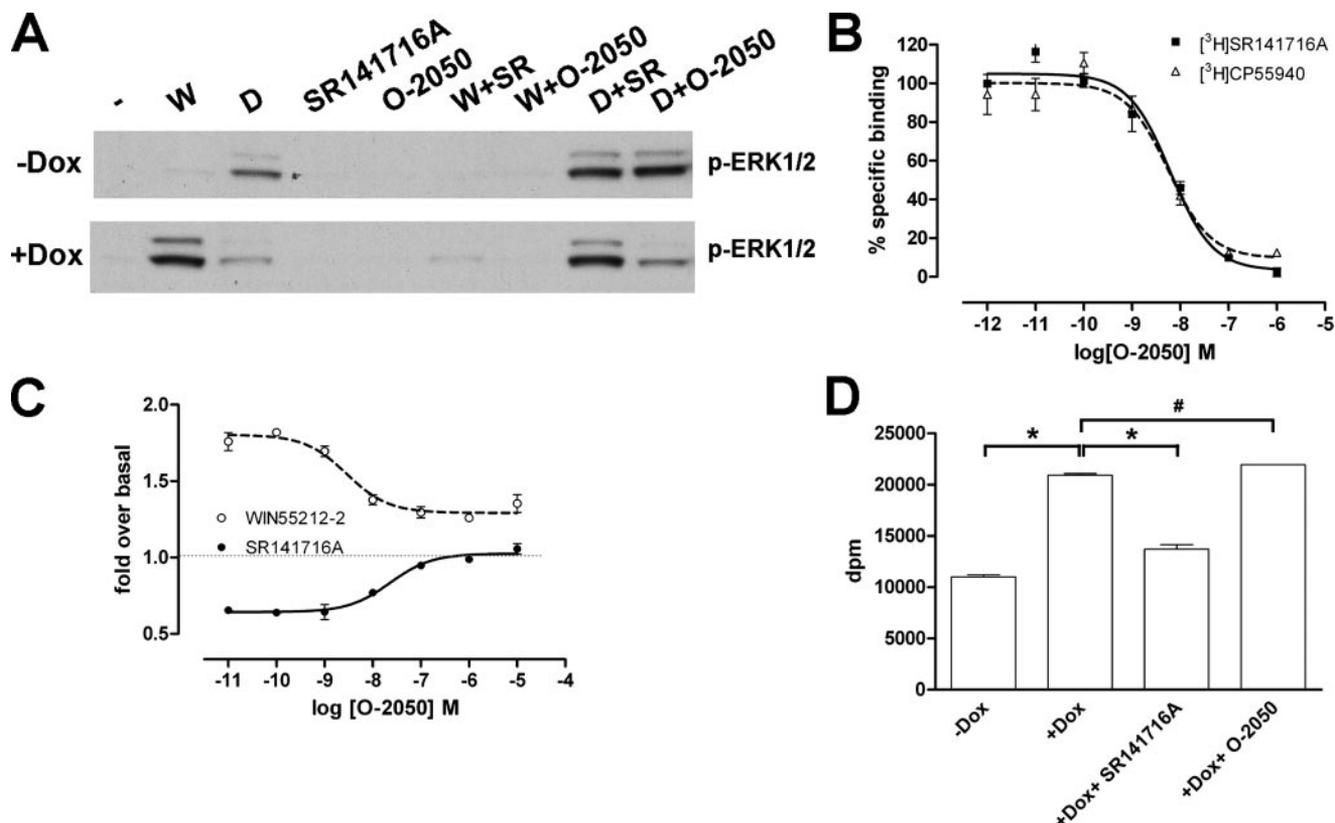

FIGURE 4. **The CB1 receptor neutral antagonist O-2050 fails to enhance the capacity of DAMGO to promote ERK1/2 phosphorylation in cells co-expressing h-MOP and h-CB1.** *A*, ERK1/2 phosphorylation studies were performed as described in the legend for Fig. 3, except that in some situations O-2050 (0.1 μM) replaced SR141716A. *W*, WIN55212-2; *D*, DAMGO. *B*, the capacity of O-2050 to compete for binding of either [^{3}H]SR141716A (1 nM) (*filled symbols*) or the CB1 receptor agonist [^{3}H]CP55940 (0.5 nM) (*open symbols*) was assessed in membranes of clone 4 cells co-expressing h-CB1-eCFP and h-MOP-eYFP. *C*, O-2050 was able to block both WIN55212-2 (0.1 μM) stimulation (*open symbols*) and SR141716A (0.1 μM) inhibition (*filled symbols*) of basal [^{35}S]GTPγS binding in membranes of cells co-expressing h-CB1-eCFP and h-MOP-eYFP. *D*, SR141716A (0.1 μM) but not O-2050 (0.1 μM) was able to block constitutive loading of [^{35}S]GTPγS produced by induction (+*Dox*) of h-CB1-eCFP expression. *, $p < 0.001$, and #, $p > 0.05$ by one-way ANOVA, Tukey's post-test.
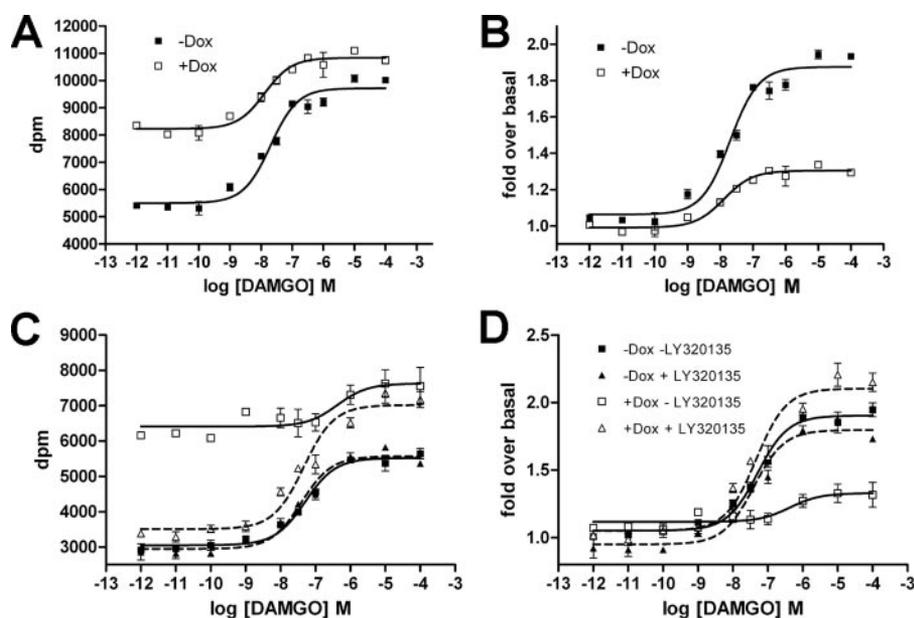
FIGURE 5. **In [³⁵S]GTPγS binding assays, h-CB1 displays high constitutive activity, masking the function of h-MOP, which can be recovered by treatment with a CB1 receptor inverse agonist.** *A* and *B*, Flp-In T-REx HEK293 cells expressing h-MOP-eYFP and harboring h-CB1-eCFP were untreated (−*Dox, filled symbols*) or exposed to doxy-cycline (0.1 μg/ml, 48 h) (+*Dox, open symbols*). Membranes prepared from these cells were employed in [³⁵S]GTPγS binding assays using various concentrations of DAMGO. Data are presented as dpm [³⁵S]GTPγS bound (*A*) or -*fold over basal* stimulation [³⁵S]GTPγS binding (*B*). DAMGO enhanced [³⁵S]GTPγS binding with similar potency in both conditions. However, greatly enhanced binding of [³⁵S]GTPγS was observed in the absence of ligand following induction of h-CB1-eCFP expression (*A*). This resulted in an apparent large reduction in h-MOP stimulated [³⁵S]GTPγS binding when this was calculated and presented as -fold stimulation over basal (*B*). *C* and *D*, basal binding of [³⁵S]GTPγS and the capacity of varying concentrations of DAMGO to stimulate this were measured in the presence (*triangles*) or absence (*squares*) of the cannabinoid CB1 receptor inverse agonist LY320135 (1 μM) in untreated membranes of Flp-In T-REx HEK293 cells expressing h-MOP-eYFP and harboring h-CB1-eCFP (−*Dox, filled symbols*) or exposed to doxycycline (0.1 μg/ml, 48 h) (+*Dox, open symbols*). Data are presented as dpm [³⁵S]GTPγS bound (*C*) or -fold stimulation over basal [³⁵S]GTPγS binding (*D*). The suppression by LY320135 of basal, constitutive [³⁵S]GTPγS binding in membranes of cells induced to express h-CB1-eCFP resulted in an increase in the absolute [³⁵S]GTPγS binding promoted by DAMGO and a restoration of the extent of signal when data were presented as -fold stimulation over basal (*D*).
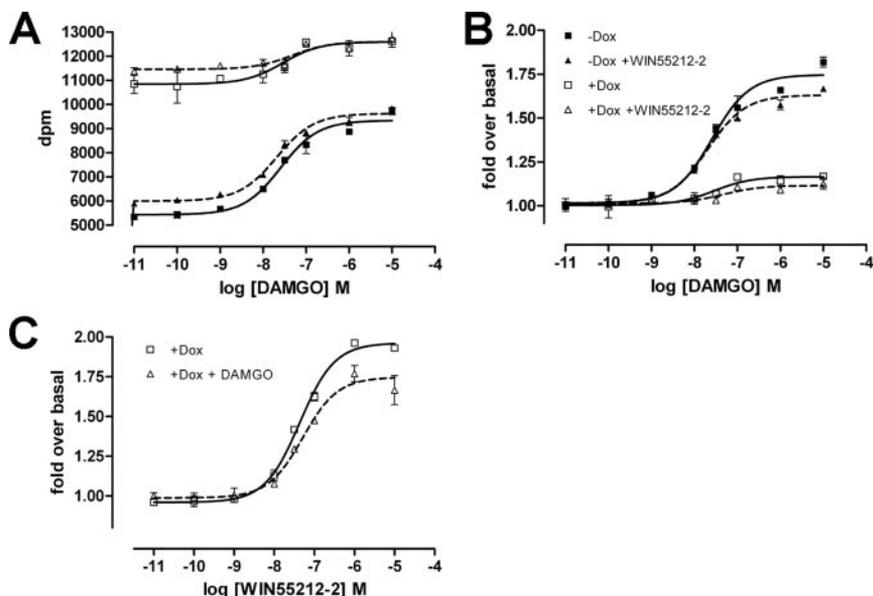

FIGURE 6. **Concentrations of h-MOP and h-CB1 agonists that are too low to cause significant stimulation of [³⁵S]GTPγS binding directly do not alter the function of the reciprocal agonist.** Flp-In T-REx HEK293 cells expressing h-MOP-eYFP and harboring h-CB1-eCFP were untreated (−*Dox, filled symbols*) or exposed to doxycycline (0.1 μg/ml, 48 h) (+*Dox, open symbols*). Membranes prepared from these cells were employed in [³⁵S]GTPγS binding assays performed as described in the legend for Fig. 5 and employing varying concentrations of either DAMGO (*A* and *B*) or WIN55212-2 (*C*). These experiments were also conducted in the absence (*squares*) or presence (*triangles*) of a concentration of the reciprocal agonist (10 nM) that was insufficient to stimulate [³⁵S]GTPγS binding on its own. The low amount of the reciprocal agonist was, in each case, without effect on the maximal signal or the potency of the agonist studied. These data should be contrasted with those of Rios *et al.* (15).

by the co-addition of SR141716A (Fig. 7*C*), and mimicked by the distinct CB1 receptor agonist CP 55,940 (Fig. 7*C*), confirming that the effect was mediated by h-CB1-eCFP. Expression of the CB1 receptor was, however, without effect on the ability of DAMGO to inhibit for-skolin-stimulated adenylyl cyclase activity (supplemental Fig. 4). We also assessed whether induction of h-CB1-eCFP expression or pertussis toxin pretreatment altered levels of Gα$_s$ or pertussis toxin-sensitive Gα$_i$ species. Both in the absence and presence of h-MOP-eYFP, induction of h-CB1-eCFP actually reduced immunodetected levels of Gα$_s$ (Fig. 7*D*), but this was without effect on the levels of other Gα subunits (Fig. 7*D*). Pertussis toxin treatment appeared to increase the levels of both the pertussis toxin-sensitive Gα$_i$ and Gα$_o$ G proteins (Fig. 7*D*). However, detailed analysis using a range of antisera directed against distinct epitopes within the sequence of Gα$_o$ indicated that the increased signals following pertussis toxin treatment were, as described previously (39), an artifact due to certain antisera binding the modified protein more effectively than the unmodified form (Fig. 7*D*).

To further confirm the importance of constitutive signaling of the CB1 receptor to the detected alterations in h-MOP-eYFP function, we generated D163N-hCB1-eCFP. This point mutant of the CB1 receptor is reported to lack constitutive activity and lose affinity and function for some (but not all) agonists (40, 41). Clones were generated that expressed h-MOP-eYFP constitutively and harbored D163N-h-CB1-eCFP at the Flp-In locus (Fig. 8, Table 2). Following induction D163N-h-CB1-eCFP was also located predominantly in punctate intracellular vesicles and did not alter the distribution (Fig. 8*A*) or expression of (Table 2) h-MOP-eYFP. D163N-h-CB1-eCFP bound [³H]SR141716A with similar affinity as h-CB1-eCFP (Table 2) but was unable to stimulate the phosphorylation of the ERK1/2 MAP kinases in
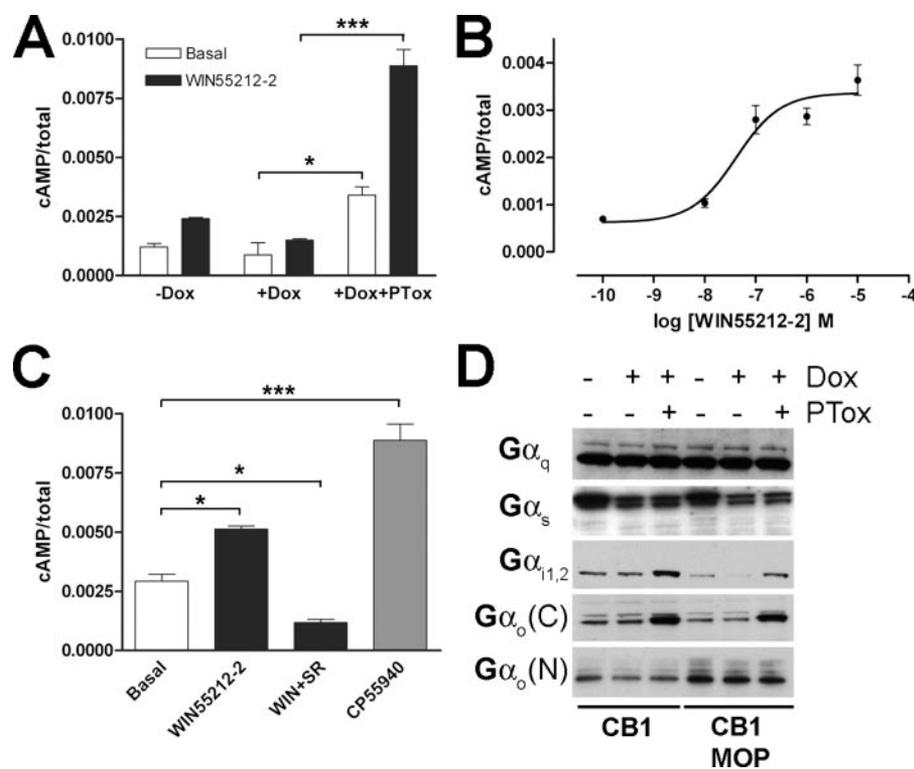
FIGURE 7. **h-CB1 stimulates adenylyl cyclase activity following pertussis toxin treatment.** *A*, Flp-In T-REx HEK293 cells expressing h-MOP-eYFP and harboring h-CB1-eCFP were untreated (−*Dox*) or exposed to doxycycline (0.1 μg/ml, 48 h) (+*Dox*) to induce h-CB1-eCFP expression; these cells were treated with either vehicle or pertussis toxin (25 ng/ml. 24 h) (+*PTox*). During this period, cells were loaded with [³H]adenine, and intact cell adenylyl cyclase assays performed in the absence (*open bars*) or presence (*filled bars*) of 1 μM WIN55212-2 (*A*). *, $p < 0.01$, and ***, $p < 0.001$ by one-way ANOVA, Tukey's post-test. *B* and *C*, the effect of WIN55212-2 in pertussis toxin-pretreated cells was concentration-dependent (*B*) and was both blocked by SR141716A (0.1 μM) and mimicked by the alternate cannabinoid CB1 agonist CP55940 (1 μM); *, $p < 0.05$, and ***, $p > 0.001$, by one-way ANOVA, Tukey's post test (*C*). *D*, although induction of h-CB1 expression was without significant effect on G protein α-subunit expression levels (as measured in immunoblots employing antisera that identify the C-terminal decapeptides of the appropriate G protein) except to cause a down-regulation of levels of $G\alpha_s$, it appeared that pertussis toxin treatment resulted in up-regulation of levels of $G\alpha_i$ and $G\alpha_o$. This, however, was an artifact reflecting that antibodies to the C terminus of these polypeptides display greater avidity to bind ADP-ribosylated forms of these G proteins than the unmodified forms (39). This was confirmed by parallel blots performed with an antiserum raised against the N-terminal domain of $G\alpha_o$ (compare $G\alpha_o$(N) and $G\alpha_o$(C)).

widely accepted that individual GPCRs homodimerize (17, 42), and there are clear examples in which pairs of co-expressed GPCRs undoubtedly are able to physically interact with one another and by so doing alter their function, pharmacology, and structure (43, 44). Nevertheless, there are a host of mechanisms that allow cross-regulation between pairs of co-expressed GPCRs that do not require direct protein-protein interactions (18, 19).

Studies ranging from the molecular to the behavioral level have demonstrated that ligands at the MOP and cannabinoid CB1 receptors have the capacity to cause cross-regulation (9–11) and that these two GPCRs are co-expressed in a range of neurones. In association with the direct demonstration of the ability of these receptors to interact when transiently co-expressed in HEK293 cells (15), it is certainly possible that cross-regulation between these two receptors may reflect their heterodimerization. However, in recent studies we demonstrated that the steady-state distribution pattern of the CB1 receptor and the MOP receptor are markedly different in HEK293 cells in which h-MOP-eYFP is expressed stably and constitutively and in which expression of h-CB1-eCFP

response to WIN55212-2 (Fig. 8*B*). Induction of D163N-h-CB1-eCFP expression did not inhibit DAMGO-mediated phosphorylation of these kinases, and co-addition of SR141716A was now without effect on the extent of stimulation produced by DAMGO (Fig. 8*B*). Equally, induction of expression of D163N-h-CB1-eCFP in the presence of h-MOP-eYFP did not result in a significant increase in basal [³⁵S]GTPγS binding in membranes from these cells (Fig. 8*C*). Furthermore, the extent of stimulation of [³⁵S]GTPγS binding by DAMGO was indistinguishable in the absence or presence of the mutated h-CB1 receptor (Fig. 9). DAMGO is well established as causing rapid and extensive internalization of the MOP receptor from the surface of cells. Induction of expression of h-CB1-eCFP did not substantially alter either the concentration dependence or the kinetics of DAMGO-induced internalization of h-MOP-eYFP (supplemental Fig. 5).

DISCUSSION

In recent times it has become common to ascribe functional interactions between pairs of co-expressed GPCRs to their propensity to heterodimerize (16, 17). In part, this reflects that it is

can be induced on demand (20). In these cells h-MOP-eYFP was located predominantly at the cell surface, whereas h-CB1-eCFP was present predominantly in punctate intracellular vesicles. This finding was in marked contrast to the effect of induction of h-CB1-eCFP on the distribution of h-orexin-1 receptor-eYFP. Although predominantly located at the cell surface in the absence of h-CB1-eCFP, h-orexin-1 receptor-eYFP adopted the punctate, intracellular distribution of the h-CB1-eCFP when expression of this GPCR construct was induced (20). Furthermore, the addition of antagonists selective for either the h-CB1 or h-orexin-1 receptor caused redistribution of both co-expressed receptors back to the cell surface, despite the ligands having no direct affinity for the alternate GPCR (20). These data are strongly supportive of heterodimerization between co-expressed h-CB1 and h-orexin-1 receptors but do not provide support for the presence of a substantial fraction of h-CB1-h-MOP receptor heterodimers. However, it is important to note that functional interactions between the cannabinoid CB1 receptor and both the orexin-1 receptor (45) and the dopamine D2 receptor (46) have also been indicated to reflect receptor heterodimerization. In the current study we have
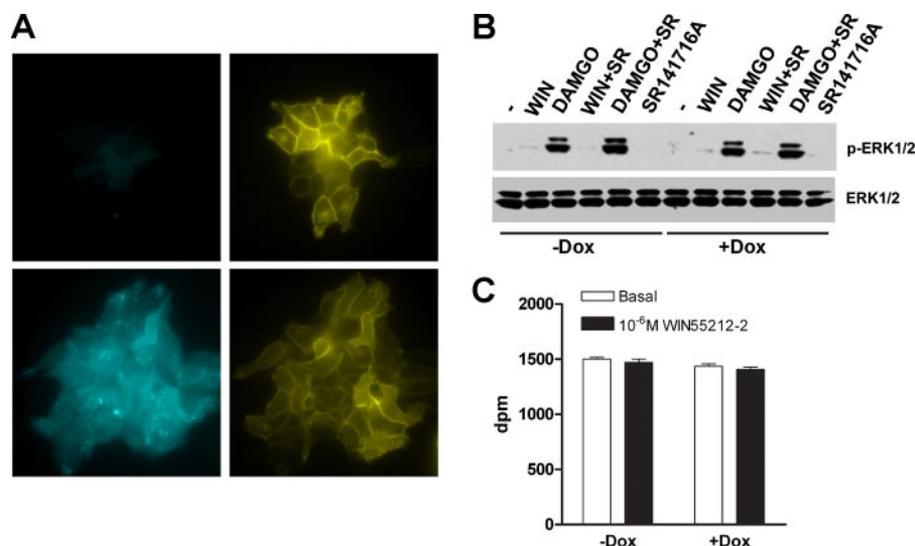
FIGURE 8. **Generation and characterization of cell lines stably expressing the h-MOP receptor and harboring D163N-h-CB1 receptor at an inducible locus.** *A*, Flp-In T-REx HEK293 clones akin to those in Fig. 1, but in which h-CB1-eCFP was replaced by D163N-h-CB1-eCFP, were generated. *Blue*, h-CB1-eCFP; *yellow*, h-MOP-eYFP. *Upper panels*, absence of doxycycline; *lower panels*, exposed to doxycycline (0.1 μg/ml, 48 h). *B*, phosphorylated ERK1/2 (*upper panel*) was detected with a phospho-ERK1/2-specific antibody after exposure of these cells to a range of CB1 receptor and MOP receptor ligands at the concentrations described in Fig. 3. Parallel studies detected total levels of ERK1/2 as loading controls (*lower panel*). Data are representative of three experiments performed. *Dox*, doxycycline; *WIN*, WIN55212-2; *SR*, SR141716A. *C*, [^{35}S]GTPγS binding studies indicated that D163N-h-CB1-eCFP lacks both constitutive activity and the capacity to respond to WIN55212-2.

TABLE 2

Quantitation of expression of D163N-h-CB1-eCFP and h-MOP-eYFP in clones of Flp-In T-REx HEK293 cells

Doxycycline was used at 0.1 μg/ml for 48 h. All data are means ± S.E. from at least three independent experiments. NA, not applicable.

Clone	Without doxycycline		With doxycycline	
	B_{max}	K_d	B_{max}	K_d
	fmol/mg	*nM*	*fmol/mg*	*nM*
[^{3}H]SR141716A binding:				
NM3		NA	850 ± 122	0.16 ± 0.01
[^{3}H]diprenorphine binding:				
NM3	898 ± 76	0.11 ± 0.01	1760 ± 400	0.10 ± 0.01

explored this more fully and also examined the importance of the well appreciated constitutive activity of the h-CB1 receptor on function of co-expressed h-MOP.

A number of studies have reported that the cannabinoid CB1 receptor internalizes and recycles to the cell surface spontaneously and in the absence of added agonist ligands. Such a phenotype has been observed both in transfected cells (20, 47) that do not endogenously express this receptor and in neurones (47, 48). However, such a distribution pattern has not been observed in all studies (45). This pattern is not restricted to the CB1 receptor, and in a range of examples, including certain virally encoded chemokine receptors (49), such a phenotype has been attributed to high-level constitutive activity of the receptors (50, 51). In the case of the CB1 receptor, mutation to ablate constitutive activity has been reported to block spontaneous recycling (52). However, we have been unable to replicate such observations in the current studies despite using the equivalent mutation and confirming the lack of constitutive activity of the D163N-h-CB1 receptor. It should be noted that other studies

have also reported differences in the ability of this mutant to bind and respond to the CB1 receptor agonists WIN55212-2 and CP 55940 (40, 41, 52). The basis for these discrepancies is unclear but worthy of more careful reevaluation.

The importance of ligand-independent or constitutive activity to receptor function has been debated widely, particularly in relation to physiology and therapeutic drug treatment (31, 53, 54). Although it appears that most receptor "antagonists" are actually inverse agonists (53), the importance of this for their clinical effectiveness remains uncertain.

In addition to the lack of co-distribution following induction of expression of h-CB1-eCFP in the presence of h-MOP-eYFP, a key early observation in these studies was that in the presence of h-CB1-eCFP the ability of the MOP receptor agonist DAMGO to stimulate phosphorylation and hence activation of the ERK1/2 MAP kinases was reduced substantially. This did not reflect a reduction in h-MOP-eYFP expression or an alteration in either agonist potency or the time course of the agonist effect. However, it did apparently reflect CB1 receptor constitutive activity, because co-treatment of cells with DAMGO and the CB1 receptor inverse agonist SR141716A restored the capacity of DAMGO to phosphorylate these kinases. Although the measured efficacy of a ligand can be system- and end point-dependent (55, 56), in the current studies O-2050 behaved as a neutral antagonist at the CB1 receptor and was unable to replicate the effects of SR141716A, whereas induction of the D163N mutant of h-CB1-eCFP, which lacked constitutive G protein activation, also failed to alter DAMGO function.

Phosphorylation of the ERK1/2 MAP kinases often provides marked signal amplification and can be promoted by concentrations of GPCR agonists that are expected to occupy only a small proportion of the available receptors (57). However, this was not evident in the current studies where ligand concentrations necessary to stimulate ERK1/2 phosphorylation were similar to those required to promote binding of [^{35}S]GTPγS. It is now appreciated that activation of ERK1/2 phosphorylation can reflect an integrated group of signals that may include G protein-independent events (58, 59). β-Arrestin-dependent ERK1/2 phosphorylation might be anticipated to display agonist concentration-response curves similar to receptor agonist-occupancy curves, because agonist occupancy is generally required to promote interactions between GPCRs and β-arrestins. However, responses measured herein were ablated by treatment of the cells with combinations of pertussis and cholera toxins. Although this is often considered definitive evidence of direct G protein involvement, it is not conclusive. Certain
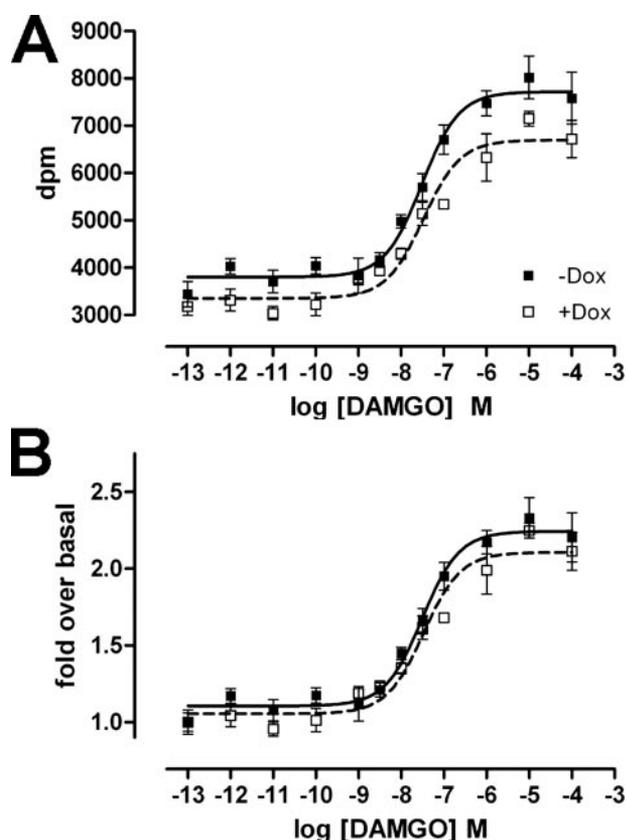
FIGURE 9. **DAMGO stimulation of [^{35}S]GTPγS binding is unaltered by the presence of D163N-h-CB1-eCFP.** Flp-In T-REx HEK293 Cells expressing h-MOP-eYFP and harboring D163N-h-CB1-eCFP were uninduced (*filled symbols*) or induced by treatment with doxycycline (*open symbols*). The ability of varying concentrations of DAMGO to elevate [^{35}S]GTPγS binding was then measured in membranes prepared from these cells. Data are presented as dpm [^{35}S]GTPγS bound (*A*) or -fold stimulation over basal binding (*B*).

members of the G protein receptor kinase (GRK) family require β/γ complexes, produced by G protein activation, for anchorage at the plasma membrane in proximity to a GPCR. Receptor phosphorylation by these kinases is often a key step in promoting interactions with β-arrestins. The agonist effects observed were, however, both rapid and transient in duration. Although the transient kinetics of ERK1/2 MAP kinase activation cannot be used in isolation to eliminate the contribution of a β-arrestin-mediated component, use of silencing RNA-induced knockdown of β-arrestins in HEK293 cells has indicated that G protein-mediated ERK1/2 activation is rapid and transient, whereas ERK1/2 activation via a β-arrestin is slower in onset and prolonged (60, 61). Despite this finding, it has recently been shown that DAMGO-induced phosphorylation of ERK1/2 in striatal neurons is dependent on both β-arrestins and the activity of GRK3 (62). The contribution of GRK3 to this process is interesting because ERK1/2 has been reported to control the transcription of GRK3 in neuronal cells (63), potentially providing a mechanism for feedback control. It appears likely that the mechanism of MOP receptor-mediated activation of the ERK1/2 MAP kinases differs among cell systems.

β-Arrestins also play key roles in ligand-mediated internalization of GPCRs. The CB1 receptor constitutively internalizes and recycles in a β-arrestin-dependent manner. We therefore also explored whether expression of h-CB1-eCFP might limit the availability of β-arrestins and hence reduce the effectiveness of DAMGO in promoting internalization of h-MOP-eYFP. We were unable to record substantial differences in DAMGO-mediated h-MOP-eYFP internalization in these cells in the absence or presence of h-CB1-eCFP. Indeed, it could be argued that treatment of the receptor co-expressing cells with SR141716A might provide a greater pool of available β-arrestins by restricting the cycling of h-CB1-eCFP.

Because the toxin pretreatment data is at least consistent with ERK1/2 phosphorylation proceeding via G protein activation, we also performed a wide range of studies to interrogate receptor cross-regulation directly at the level of G protein activation. These studies also provided strong support for the hypothesis that constitutive activity inherent to the CB1 receptor provides direct regulation of MOP receptor function. It is interesting to note that the manner in which results are presented can influence data interpretation. As noted previously by Rios *et al.* (15), co-expression of the cannabinoid CB1 receptor influences the capacity of agonists at the MOP receptor to stimulate binding of [^{35}S]GTPγS. However, in our hands this was simply a reflection that the high constitutive loading of [^{35}S]GTPγS onto pertussis toxin-sensitive G proteins in the presence of the CB1 receptor limited the capacity of MOP receptor agonists to enhance this further. Blockade of this constitutive activity by addition of CB1 receptor inverse agonists restored the measured activity of the MOP receptor. As this was observed in cell membrane preparations, it cannot reflect a heterologous desensitization of the MOP receptor via second messenger-regulated kinase activity. Such observations reiterate the benefit of presenting raw data, to allow the widest range of interpretations.

There remain uncertainties as to the importance of receptor constitutive activity in native systems (54) and the relevance of the inverse agonist activity of many clinically used receptor blockers (53). Despite these uncertainties, the current studies demonstrate the extent to which constitutive activity can contribute to receptor cross-regulation and the usefulness of the Flp-In T-REx HEK293 cell system in exploring the activity and regulation of a GPCR in the absence and presence of a second GPCR with which it is known to be co-expressed in native tissues.

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