THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 18, pp. 12328–12338, May 1, 2009

Lipid G Protein-coupled Receptor Ligand Identification Using β-Arrestin PathHunter™ Assay

Received for publication, August 22, 2008, and in revised form, March 12, 2009 Published, JBC Papers in Press, March 13, 2009, DOI 10.1074/jbc.M806516200

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A growing number of orphan G-protein-coupled receptors (GPCRs) have been reported to be activated by lipid ligands, such as lysophosphatidic acid, sphingosine 1-phosphate (S1P), and cannabinoids, for which there are already well established receptors. These new ligand claims are controversial due to either lack of independent confirmations or conflicting reports. We used the β-arrestin PathHunter™ assay system, a newly developed, generic GPCR assay format that measures β-arrestin binding to GPCRs, to evaluate lipid receptor and ligand pairing. This assay eliminates interference from endogenous receptors on the parental cells because it measures a signal that is specifically generated by the tagged receptor and is immediately downstream of receptor activation. We screened a large number of newly "deorphaned" receptors (GPR23, GPR92, GPR55, G2A, GPR18, GPR3, GPR6, GPR12, and GPR63) and control receptors against a collection of ~400 lipid molecules to try to identify the receptor ligand in an unbiased fashion. GPR92 was confirmed to be a lysophosphatidic acid receptor with weaker responses to farnesyl pyrophosphate and geranylgeranyl diphosphate. The putative cannabinoid receptor GPR55 responded strongly to AM251, rimonabant, and lysophosphatidylinositol but only very weakly to endocannabinoids. G2A receptor was confirmed to be an oxidized free fatty acid receptor. In addition, we discovered that 3,3′-diindolylmethane, a dietary molecule from cruciferous vegetables, which has known anti-cancer properties, to be a CB_2 receptor partial agonist, with binding affinity around 1 μM. The anti-inflammatory effect of 3,3′-diindolylmethane in RAW264.7 cells was shown to be partially mediated by CB_2.

Over the past few years, a large number of orphan GPCRs[3] have been shown to respond to lipid ligands for which there are already known receptors. For example, LPA was found to activate five more new orphan receptors as follows: GPR23 (1, 2), GPR92 (3, 4), GPR87 (5), $P2Y_5$ (6), and $P2Y_{10}$ (7), which are not very homologous to the three previously known high affinity LPA receptors in the endothelial differentiation gene (EDG) family. Five well established high affinity S1P receptors ($S1P_{1-5}$ receptors) are also part of the EDG receptor family. Now five new receptors, GPR3, GPR6, GPR12, GPR63, and $P2Y_{10}$, have been claimed to be novel S1P receptors (7–10). Similarly, GPR55 was discovered to be a new cannabinoid receptor adding to the two classical cannabinoid receptors CB_1 and CB_2 (11–14). Most of these claims are tentative at this point due to either lack of follow up studies or contradictory reports. For example, a recent study showed that farnesyl pyrophosphate (FPP) might be a more potent endogenous ligand than LPA for GPR92 (15). And GPR55 was also reported to be a lysophosphatidylinositol receptor (16).

Interestingly, lipid molecules that previously were not recognized as GPCR signaling mediators have also been shown to trigger signal transduction pathways via GPCRs. For example, G2A receptor was reported to be activated by oxidized free fatty acid 9*S*-hydroxyoctadecadienoic acid (9-HODE) (17), which was previously thought to activate nuclear hormone receptor peroxisome proliferator-activated receptor γ (18). This finding opens exciting new possibilities, but a lack of follow up reports leaves the original report unconfirmed. G2A was initially reported to be a lysophosphatidylcholine (LPC) receptor, but the report was later retracted (19). Whether it is a proton receptor like its sequence-related family members OGR1, TDAG8, and GPR4 has also been disputed (20, 21). G2A is highly expressed in lymphocytes and macrophages and has been postulated to be involved in cell proliferation control (22) or chemotaxis (23, 24), but some of these conclusions were reached using the retracted ligand LPC. It is essential to establish the true ligand of G2A to fully understand its function.

The pairing of GPCRs and ligands is highly error-prone because of the fact that GPCRs usually cannot be expressed and assayed as purified proteins (with the notable exception of rhodopsin) but instead require alternative complex cell-based assay systems (25). Even though most published papers do contain seemingly satisfactory internal controls, different reports often do not agree with each other, and paper retraction is not uncommon. The most widely used cell-based assays measure G protein-dependent secondary messenger formation, such as [cAMP] changes, Ca^{2+} flux, or reporter gene activation. In these systems, intact cells that express a milieu of endogenous receptors in addition to the overexpressed receptor of interest

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[3] The abbreviations used are: GPCR, G-protein-coupled receptor; 2-AG, 2-arachidonoylglycerol; CHO, Chinese hamster ovary; DIM, 3,3′-diindolylmethane; EDG, endothelial differentiation gene; FFA, free fatty acids; FPP, farnesyl pyrophosphate; FRET, fluorescence-resonance energy transfer; GTPγS, guanosine 5′-*O*-3 thiotriphosphate; HEK293, human embryonic kidney 293; HEK293-BAEA, HEK293 cells stably express β-arrestin2-EA fusion protein; 9-HODE, 9-hydroxyoctadecadienoic acid; 11-HETE, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; HTRF, homogeneous time-resolved fluorescence; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; LPS, lipopolysaccharide; NaGly, *N*-arachidonylglycine; PAF, platelet-activating factor; S1P, sphingosine 1-phosphate; FBS, fetal bovine serum; IL, interleukin; FLIPR, fluorometric imaging plate reader.

are used. The readout of GPCR activation is not receptor-specific (meaning it is a sum of all GPCRs in the assay tube), and may be several or many signaling steps downstream of receptor activation. Promiscuous G proteins such as $G\alpha_{16}$ or G-protein chimeras such as G_{qi5} are often used to artificially reroute the GPCR signaling to the Ca^{2+} pathway, which can also introduce artifacts. The signal difference between the receptor-overexpressed system and the wild type cell system is interpreted as the receptor-specific response. Because most host cell lines do express some combination of EDG receptors that respond to S1P and LPA, getting a silent parental cell response for such lipid molecules in traditional GPCR assays is very difficult. Thus, it is extremely important that multiple assay formats are used to validate claims of receptor-ligand pairing, especially for lipid ligands such as LPA and S1P.

Analogous to the orphan receptors, there are also some "orphan" compounds, compounds that have known *in vivo* effects but lack a defined molecular target. DIM is one such example. It is a dietary indole derived from digestion of indole-3-carbinol, found in *Brassica* vegetables such as broccoli and cauliflower. DIM is in clinical trials as a treatment for numerous forms of cancer because of its safety at high doses and its promising anti-tumor effect *in vitro* and *in vivo* (26, 27). It is also being investigated as a potential treatment for a variety of viral and antibiotic-resistant bacterial infections, because of its immunomodulatory effects (28, 29). The reported cellular effects of DIM are numerous (30), including cell cycle regulation, apoptosis induction, nuclear receptor-mediated gene transcriptional changes, induction of various drug-metabolizing cytochrome P450 enzymes, estrogen metabolism changes resulting in an increase in anticarcinogenic 2-hydroxylation of estrogen, inhibition of mitochondrial H^+-ATP synthase resulting in induction of $p21^{Cip1/Waf1}$ (31), stimulation of interferon-γ production and signaling (32), etc. The molecular target of the molecule is not well established, and it is unclear whether there might be many low affinity effectors or a single high affinity effector mediating the plethora of effects of DIM. DIM has been reported to directly bind (IC_{50} = 50 μM in one report (33) and K_i = 90 nM in another report (34)) and both agonize and antagonize aryl hydrocarbon receptor (35) and in turn modulate cytochrome P450 1A1 and estrogen metabolism and tumorigenesis (36). DIM also directly binds and antagonizes the androgen receptor with an IC_{50} in micromolar range (37). No other molecular target has been reported to our knowledge.

We recently completed an evaluation of a new generic GPCR assay system developed by DiscoveRx that measures β-arrestin translocation to the activated GPCR using β-galactosidase enzyme fragment complementation technology (38). This technology offers a tagged receptor assay whose readout, β-arrestin binding measured by reconstituted β-galactosidase activity, is immediately downstream of receptor activation. Thus, it is clearly advantageous over prior cell-based GPCR assays in proving specificity of ligand-dependent receptor activation, as it eliminates many of the issues mentioned above, such as endogenous receptor signal contamination and indirect readout. Furthermore, it is a generic GPCR assay that works for receptors that couple to all classes of G proteins, as it examines the desensitization and internalization pathway, not G protein-dependent signaling pathway. Compared with some other β-arrestin technologies that measure translocation by imaging, the readout is luminescence signal strength, which makes this an easy quantitative assay that does not require cell imaging, and amenable to high throughput screening.

To study orphan and novel receptor function, we first focused on a panel of recently deorphaned lipid GPCRs. We prepared a panel of 43 lipid molecules, including the various reported lipid ligands and a BioMol lipid collection of 345 compounds, and we tested them against a panel of 16 putative lipid GPCRs in the β-arrestin assay system in a transient transfection format. Our results shed light on the true ligands of these controversial receptors and also uncovered a novel receptor target for dietary molecule DIM.

EXPERIMENTAL PROCEDURES

Reagents—Lipids were purchased from Avanti Polar Lipids, Sigma, Cayman Chemicals, Biomol, or Tocris Bioscience. BioMol compound libraries included bioactive lipids (201 compounds), endocannabinoids (60 compounds), and orphan ligands (84 compounds) libraries. Rimonabant (also known as SR141716) and SR141528 were synthesized at Novartis. Flash detection reagent for β-arrestin assay and coelenterazine for aequorin assay were purchased from DiscoveRx. FuGENE 6 and EDTA-free complete protease inhibitor mixture were from Roche Applied Science; [^{3}H]CP55940 and [^{35}S]GTPγS were from PerkinElmer Life Sciences. β-Arrestin assay technology was licensed from DiscoveRx. Aequorin technology was licensed from Euroscreen SA. CHO-hCB_1 stable cell line was purchased from Euroscreen. CHO-hCB_2 stable cell line was generated in-house. hGPR55-HEK293 stable cell line was generated by Novartis GPCR-EP and kindly provided to us. cAMP High-Range HTRF kits were purchased from CisBio-US Inc.

β-Arrestin Assay—GPCRs of interest were cloned into the Prolink vector (DiscoveRx) for GPCR-prolink fusion protein production. Parental HEK293 cells that stably express β-arrestin2-β-gal-EA fusion protein (HEK293-BAEA) were detached and transiently transfected with the receptor of interest using FuGENE 6 transfection reagent in suspension mode. Transfected cells in assay medium (phenol red-free Dulbecco's modified Eagle's medium with 3% FBS) were plated into white solid 384-well plates at 15,000 cells/25 μl/well. After overnight incubation, 200 nl of test molecules were transferred into the cell plates by PinTool (GNF Systems) followed by 1–2 h of incubation at 37 °C, 5% CO_2. Flash detection reagents were added at 12.5 μl/well. After 5 min to 1 h of incubation at room temperature, the cell plates were read on CLIPR (PerkinElmer Life Sciences) or Acquest (Molecular Devices) for luminescence signal.

Aequorin Assay—Parental CHO cells that stably express aequorin were transiently transfected with the receptor (in pcDNA3.1 vector) using the same protocol as with the β-arrestin assay. The transfected cells were seeded into 384-well black clear bottom plates (Greiner Bio-one) at 10,000 cells/25 μl/well in F-12 medium containing 3% FBS and incubated overnight. Coelenterazine (20 μM final) was added into the cell plates at 25 μl/well. The cell plates were returned to the incubator for 3 h. The compounds were diluted 1:20 into an intermediate plate

with Tyrode buffer (130 mM NaCl, 2 mM $CaCl_2$, 5 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4). 12.5 μl/well of pre-diluted compounds were transferred into the cell assay plate and read on LumiLux (PerkinElmer Life Sciences) for flash luminescence.

An algorithm similar to area under curve was created in-house to analyze the aequorin kinetic data, which was named Slope Threshold (Slope 100). The algorithm defines the beginning and the end of the luminescence intensity peak by comparing the difference in intensity of each time point with an earlier time point, and by determining if the difference exceeds a defined threshold, which was usually set at 100. If it does, then the intensity of the later time point is added into the Slope Threshold sum.

Radioligand Binding Assay—Membrane preparation using hCB_1-CHO and hCB_2-CHO cell lines was described previously (39). Saturation radioligand binding experiments were performed on each batch of the membrane preparation to determine the K_d (dissociation constant of the radioligand to the receptor) and the B_{max} values (maximal receptor binding sites). The K_d value obtained from the saturation binding experiments that was used for the K_i calculation was 0.5 nM for human CB_1 and human CB_2. Competitive binding experiments were done with 0.5–0.8 nM of [^{3}H]CP55940. Each reaction was carried out in round-bottom 96-well polystyrene assay plates, including test compound, membranes (3–10 μg/well), and [^{3}H]CP55940 in assay buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.05% bovine serum albumin, pH 7.4). The reaction was carried out at room temperature for 120 min before the membranes were harvested onto a Unifilter GF/B-96 filter plate using a Packard Filtermate Harvester. After nine washes with wash buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.05% bovine serum albumin, pH 7.4), the filter was dried in a 37 °C oven for 30 min. MicroScint-20 was added and the plate sealed for scintillation counting on a TopCount.

GTPγS Binding Assay—The assay was carried out in 96-well filtration format as described (39).

cAMP Assay—CHO-CB_1 or CHO-CB_2 cells were detached and diluted in cell culture medium (F-12 with 10% FBS and 1% penicillin/streptomycin/glutamine) to a density of 40,000 cells/ml. 10,000 cells (25 μl) were seeded per well into the 384-well assay plate and incubated in 37 °C incubator overnight. 500 nl of compound was then transferred into each assay well, followed by 5 μl of forskolin addition for a final concentration of 60 μM. Cells were returned to a 37 °C incubator for 30 min. Finally, 15 μl each of HiRange d2-cAMP and anti-cAMP cryptate were dispensed per well. After >1 h room temperature incubation, data acquisition was done in the time-resolved fluorescence-resonance energy transfer (FRET) mode on Envision or ViewLux. The ratio between the acceptor fluorescence signal ($A_{665\ nm}$) and donor fluorescence signal ($A_{620\ nm}$) × 10^4, representing the FRET between the conjugated cAMP and the anti-cAMP antibody, was calculated and plotted on *y* axis. The higher the signal, the lower endogenous cAMP concentration is in the sample.

GPR55 Reporter Gene Assay—pGL3 basic vector (Promega) was modified to have three repeats of multiple response element consensus sequence, one cAMP-response element that is from a vasoactive intestinal peptide gene promoter, and serum-response element promoter before the luciferase reporter gene. HEK293-hGPR55 cells were transiently transfected with pGL3-CRE-MRE-SRE-luc reporter gene and plated at 15,000 cells/25 μl/well. After overnight incubation, test compounds were added to the cells with Pintool and incubated for 16–24 h. The reaction was then stopped by Bright-Glo addition (12 μl/well), and luminescence signal was read on CLIPR with 5 s of exposure.

IL-1β Taqman Reverse Transcription-PCR in RAW264.7 Cells—RAW264.7 cells were seeded into 6 well plates at 0.5 × 10^6 cells/well and incubated at 37 °C for 24 h. The cells were serum-starved in assay medium (Dulbecco's modified Eagle's medium + 1% FBS + penicillin/streptomycin) for 24 h, before receiving 18 h of compound treatment, followed by 12 h of LPS stimulation (100 ng/ml; Invivogen catalog number tlrl-eklps). Total RNA extraction, purification, and reverse transcription reaction were carried out using Qiagen RNeasy mini kit and ABI high capacity cDNA reverse transcription kit (catalog number 4368814). 200 ng/reaction of cDNA products from the reverse transcription reaction was used for Taqman PCR with TaqMan Universal PCR Master Mix (ABI catalog number 4304437). The primer and probe mix of mouse IL-1β (catalog number Mm01336189_m1) and mouse and tumor necrosis factor-α (Mm00443258_m1) were purchased from ABI. Mouse 36B4 primer 5′-AGA TGC AGC AGA TCC GCA-3′ (forward) and 5′-GTT CTT GCC CAT CAG CAC C-3′ (reverse) and probe 5′VIC-CGC TCC GAG GGA AGG CCG-TAMRA3′ were used as control.

Data Analysis—EC_{50} or IC_{50} values were obtained by fitting the data with the sigmoidal dose-response curve-fitting tool of the GraphPad Prism software. Eight or twelve different concentrations were usually used and two or three data points per concentration. In radioligand binding assays, K_i was calculated using Equation 1 of Cheng and Prusoff,

$$K_i = IC_{50}/(1 + [\text{radioligand}]/K_d) \quad \text{(Eq. 1)}$$

RESULTS

In the evaluation of the β-arrestin PathHunter™ system, we confirmed the reported agonists and antagonists of a large number of known receptors, including G_s-, G_i-, and G_q-coupled receptors (Table 1). Furthermore, we were encouraged by the fact that transient transfection of receptors into β-arrestin-EA parental HEK293 cells gave robust signals, circumventing the need for labor-intensive stable cell line generation and selection. The EC_{50} values obtained in the transient β-arrestin assay for a subset of receptors were significantly higher than the EC_{50} values obtained from other assay formats (Table 1). This appears to be partly due to the low sensitivity resultant from transient transfection method. For example, β_2-adrenergic receptor showed 4.6 nM EC_{50} in stable cell pool but 17–40 nM EC_{50} in transient assays. It is also possible that β-arrestin binding may require a higher degree of receptor activation than G-protein-dependent signaling or that the β-galactosidase detection system has lower sensitivity despite great amplification. Regarding antagonist or inverse agonist, no general trend

TABLE 1

Activities of known receptor agonists and antagonists in β-arrestin assay *versus* other assay formats

The indicated agonist and antagonist compounds were tested on the corresponding receptor in various GPCR assay formats in dose-response curves. EC_{50}, IC_{50}, K_i, or K_b values, or the value ranges obtained in a number of experiments, were determined and tabulated to compare assay sensitivity.

GPCR name	Agonist name	Transient β-arrestin assay, EC_{50}	Other assay format, EC_{50} (stable cell line unless noted)	Antagonist or inverse agonist name	Transient β-arrestin assay, IC_{50}	Other assay format, IC_{50} (stable cell line)
β_2-Adrenergic receptor	Isoproterenol	17–40 nM, 4.6 nM in stable pool	1–9 nM in cAMP-HTRF assay	Propranolol	K_b = 0.79 nM	K_b = 1.5 nM
Orexin receptor 2	Orexin A	8.7–14.5 nM	1.2–15.5 nM in FLIPR assay, 11.9 nM in IP1-HTRF assay, 14–31 nM in pERK Surefire assay	Compound 29 (Ref. 61)	157 nM with 10 nM orexin A	289 nM with 100 nM orexin A in FLIPR assay
CB_2	CP55940	1.6 nM	0.28 nM in GTPγS assay, K_i = 0.22 nM in binding assay, 0.38 nM in cAMP-HTRF assay	SR144528	2.4 nM with no agonist	0.8 nM with no agonist in GTPγS, K_i = 0.6 nM in binding
$S1P_1$	S1P	26–43 nM	0.9–2.6 nM in GTPγS assay	JTE-013	8.9 μM with 10 nM S1P	1.9 μM with 10 nM S1P in GTPγS assay
H4	Histamine	46 nM	55–77 nM in transient aequorin assay with $G\alpha_{16}$			
GPR154	Neuropeptide S	10 nM	19 nM in transient aequorin assay			
LPA1 (EDG2)	LPA	165 nM	5.5 nM in FLIPR assay	Ki16425	120 nM with 10 μM LPA	45–136 nM with 500 nM LPA in FLIPR assay

of rightward shift of potency was observed, even in transient assay format (Table 1).

A number of lipid molecules (see Table 2 for full names of all compounds) were purchased, dissolved into appropriate solvents, and arrayed into compound plates in serial dilutions. Additionally, the bioactive lipid library, endocannabinoid library, and orphan ligand library from BioMol were purchased and arrayed at a single concentration. The 16 selected human GPCRs (Table 2) were cloned into the Prolink expression vector, and β-arrestin assays were performed in transient transfection format to test the activity of compounds on each receptor.

GPR92 Is an LPA Receptor but Not GPR23—LPA2 (EDG4), GPR23, and GPR92 were included in the lipid molecule screen, with the well established LPA2 receptor serving as a positive control. LPA2 and GPR92 responded to LPA as expected (Fig. 1, *A* and *B*). LPA2 was activated more potently by LPA (18:1) with an EC_{50} of 93 nM, and less potently by LPA (16:0) with an EC_{50} of >10 μM. GPR92 was activated by LPA (18:1) and LPA (16:0) with EC_{50} of 339 nM and 5.8 μM, respectively. In contrast, GPR23 showed essentially no response to either form of LPA up to ~100 μM (Fig. 1*C*). The small amount of signal was clearly insignificant as nonspecific responses to platelet-activating factor (PAF), LPC, and lyso-PAF reached much higher levels in the assay.

We noticed that a small number of lipid molecules in the collection, namely LPC, PAF, lysophosphatidylethanolamine, lysophosphatidylglycerol, and lyso-PAF triggered significant signals at close to 100 μM on many receptors, even though they did not trigger responses from all receptors or the parental cells (data not shown). We do not know whether this represents some weak affinity of many receptors toward these molecules.

FPP and *N*-arachidonylglycine (NaGly) were recently reported to be endogenous ligands for GPR92 (15). In the report, LPA consistently showed weaker activity compared with FPP in several assays, including reporter gene assay, inositol phosphate production assay, and cAMP assay (15). We tested FPP, geranylgeranyl diphosphate, NaGly, and LPA using our transient β-arrestin and aequorin assay systems (Fig. 2). Our results showed that LPA is the most potent activator of the receptor, whereas FPP and geranylgeranyl diphosphate showed weaker but significant activities. NaGly showed only a minimal amount of activity, consistent with the published result (15).

3,3′-Diindolylmethane Is a CB_2 Partial Agonist—CB_2 receptor was included in the screen as a positive control for the cannabinoid receptor family. As expected, the two endocannabinoids in the collection, anandamide and 2-arachidonylglycerol (2-AG), showed up as hits for CB_2. Arachidonic acid, a precursor molecule for 2-AG, also activated the receptor weakly, with the EC_{50} in the 10–100 μM range (data not shown). Interestingly, we identified DIM as a confirmed agonist hit for CB_2 from the Biomol bioactive lipids collection.

To further confirm that DIM is indeed a CB_2 agonist and to test its activity on the related CB_1 receptor, we performed the β-arrestin assay, GTPγS binding assay, radioligand binding assay, and also cAMP assay on human CB_1 and CB_2 receptors (Fig. 3 and Fig. 4). The $EC_{50}/IC_{50}/K_i$ values are summarized in Table 3. DIM agonist activity was confirmed in all four assays performed on CB_2, with EC_{50} values ranging from 0.42 to 1.7 μM in different assays. The binding K_i is 1.1 ± 0.3 μM, as determined from four independent radioligand binding experiments. Similar to anandamide, DIM appears to be a partial agonist when compared with small molecule full agonist CP55940 in functional assays (Fig. 3, *A*, *C* and *D*). DIM also binds the CB_1 receptor with a K_i of 4.3 ± 0.3 μM. However, this binding did not result in detectable functional activity in the cAMP assay (Fig. 4*D*). Significant but weak CB_1 inverse agonist activity was detected in the GTPγS assay, with IC_{50} = 11.1 μM, and in the β-arrestin assay, with IC_{50} > 10 μM (Fig. 4, *A* and *C*). In addition, we tested DIM on putative cannabinoid receptor GPR55 and did not detect any activity (data not shown). Thus, we confirmed using multiple assay formats that DIM is a CB_2 receptor partial agonist with ~1 μM binding affinity and that it may also have some weak CB_1 inverse agonist activity.

CB_2 Partially Mediates the Anti-inflammatory Effect of DIM in Murine Macrophages—DIM was reported to suppress inflammatory response to lipopolysaccharide (LPS) in murine monocyte/macrophage RAW264.7 cells (29). As CB_2 is expressed in the cell line (data not shown), we wondered

TABLE 2

List of receptors and lipid molecules used for screening

Each of the 16 human G-protein-coupled receptors was assayed against the 43 lipid molecules on the right. The lipid molecules were dissolved and stored according to the manufacturer's recommendations.

Receptor	Lipid	Full name of lipid
CB_2	(±)11-HETE	11-Hydroxy-5,8,12,14-eicosatetraenoic acid
G2A	(±)12-HETE	12-Hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
GPR12	(±)5-HETE	5-Hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
GPR18	(±)8-HETE	8-Hydroxy-5*Z*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid
GPR23	(±)9-HETE	9-Hydroxy-5,7,11,14-eicosatetraenoic acid
GPR3	(±)9-HODE	9–10*Z*,12*E*-Hydroxyoctadecadienoic acid
GPR34	12(*S*)-HETE	12-Hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
GPR40	2-AG	2-Arachidonoylglycerol
GPR43	5-Oxo-ETE	5-Oxo-6,8,11,14-eicosatetraenoic acid
GPR55	9(*S*)-HODE	(9*S*)-10*Z*,12*E*-Hydroxyoctadecadienoic acid
GPR6	9(*S*)-HpODE	9(*S*)-Hydroperoxy-10*Z*,12*E*-octadecadienoic acid
GPR63	Anandamide	*N*-Arachidonylethanolamine
GPR84	Arachidonic acid (20:4)	Arachidonic acid
GPR92	C10 (10:0)	Decanoic acid
LPA_2	C11 (11:0)	Undecanoic acid
$S1P_1$	C12	Sodium dodecanoate
	C16 (16:0)	Palmitic acid
	C16 (16:3)	γ-Lindenic acid
	C2 (NaAc)	Sodium acetate
	C3 (3:0)	Propanoic acid
	C4 (4:0)	Butanoid acid
	C5 (5:0)	Valeric acid
	C6 (6:0)	Hexanoic acid
	DHA (22:6)	Docosahexaenoic acid
	DoPA (2 × 18:1)	1,2-Dioleoyl-*sn*-glycero-3-phosphate
	Lanostadien	Lanostadien
	Lauric acid (12:0)	Lauric acid or dodecanoic acid
	Linoleic acid (18:2)	Linoleic acid
	LPA (16:0)	Lysophosphatidic acid
	LPA (18:1)	Lysophosphatidic acid
	LPC (16:0)	Lysophosphatidylcholine
	LPC (18:0)	Lysophosphatidylcholine
	LPC (chicken egg) (16:0, 18:0)	Lysophosphatidylcholine
	LPC (liver)	Lysophosphatidylcholine
	LPG (18:1)	Lysophosphatidylglycerol
	Lyso-PAF	Lyso-platelet-activating factor, alkyl lysophosphatidylcholine
	Lyso-PE (18:1)	Lysophosphatidylethanolamine
	Lyso-PS (18:1)	Lysophosphatidylserine
	NaGly	*N*-Arachidonylglycine
	PAF	Platelet-activating factor
	PC (18:1-16:1)	Phosphatidylcholine
	S1P	Sphingosine 1-phosphate
	SPC	Sphingosine phosphorylcholine

whether CB_2 might be the target molecule that mediates the anti-inflammatory effect of DIM. In the absence of LPS, DIM caused a small decrease in IL-1β mRNA level in RAW264.7 cells (Fig. 5). When RAW264.7 cells were stimulated with 100 ng/ml of LPS, there was a dramatic increase in IL-1β and tumor necrosis factor-α mRNA levels, and DIM inhibited this increase (Fig. 5 and data not shown). The inhibition was of small magnitude but reproducible over many experiments. This DIM-dependent decrease in IL-1β mRNA level was significantly, but only partially, inhibited by a CB_2-specific antagonist SR144528 (Fig. 5). Furthermore, CB_1/CB_2 dual agonist CP55940 also caused a significant but lower level of decrease in IL-1β mRNA level compared with DIM (Fig. 5). Thus, CB_2 appears to be partially responsible for anti-inflammatory effect of DIM in this cellular system.

AM251, Rimonabant, and LPI Activate GPR55—In contrast to CB_2, the GPR55 screen did not reveal the two endocannabinoid ligands, 2-AG and anandamide, as positive hits. However, AM251, a known CB_1 antagonist/inverse agonist contained in the Biomol collection, was a confirmed agonist hit. We then tested a large number of cannabinoid receptor-related synthetic compounds or natural ligands on GPR55 in both the β-arrestin assay (Fig. 6*A*) and a reporter gene assay using pGL3-CRE-MRE-SRE-luc reporter gene (Fig. 6*B*). Rimonabant, also known as SR141716, a CB_1 inverse agonist whose structure closely resembles AM251, also triggered significant agonist activity in both assays. Lysophosphatidylinositol (LPI), another reported ligand of GPR55 (16), was tested in the β-arrestin assay and was indeed active (Fig. 6*A*). It did not significantly active CB_1 or CB_2 (data not shown). Anandamide and Δ9-tetrahydrocannabinol (*Δ9-THC*) afforded a very small amount of activity (12% efficacy) in the β-arrestin assay (Fig. 6*A*), and 2-AG showed some activity in the reporter gene assay (Fig. 6*B*). Although the activities of

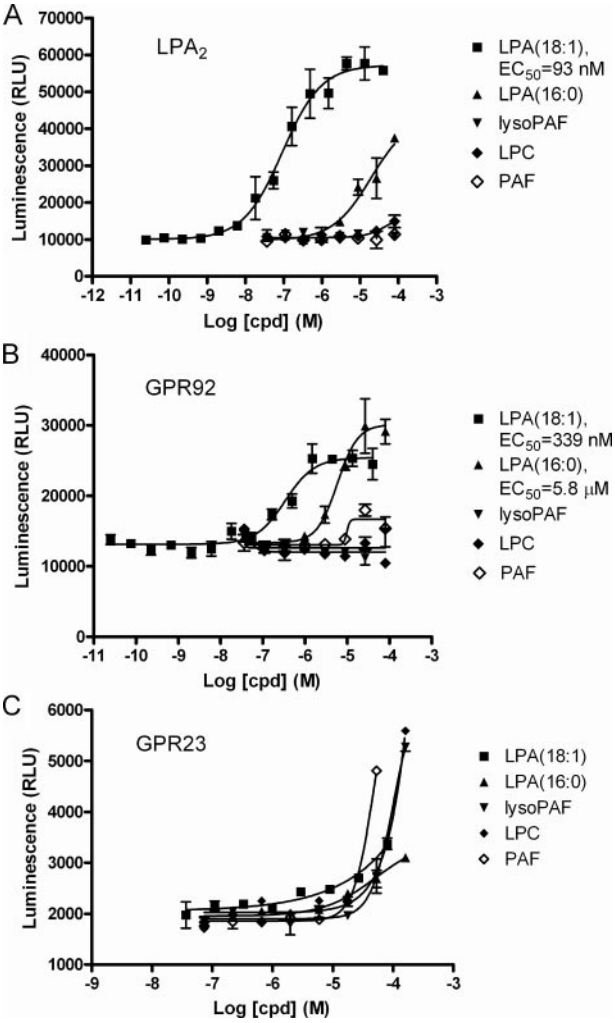

FIGURE 1. **Activation of putative and established LPA receptors by LPA and other lipid molecules.** Indicated human receptors (*A,* LPA2; *B,* GPR92; *C,* GPR23) were transiently transfected into the HEK293-BAEA parental cell line. 16–24 h later, test compounds (*cpd*) were added to the cells. 1 h later, the reaction was stopped by the addition of flash detection reagent, and β-galactosidase activity was measured. Relative luminescence unit (*RLU*) is plotted on the *y* axis, and the data are expressed as mean ± S.E.

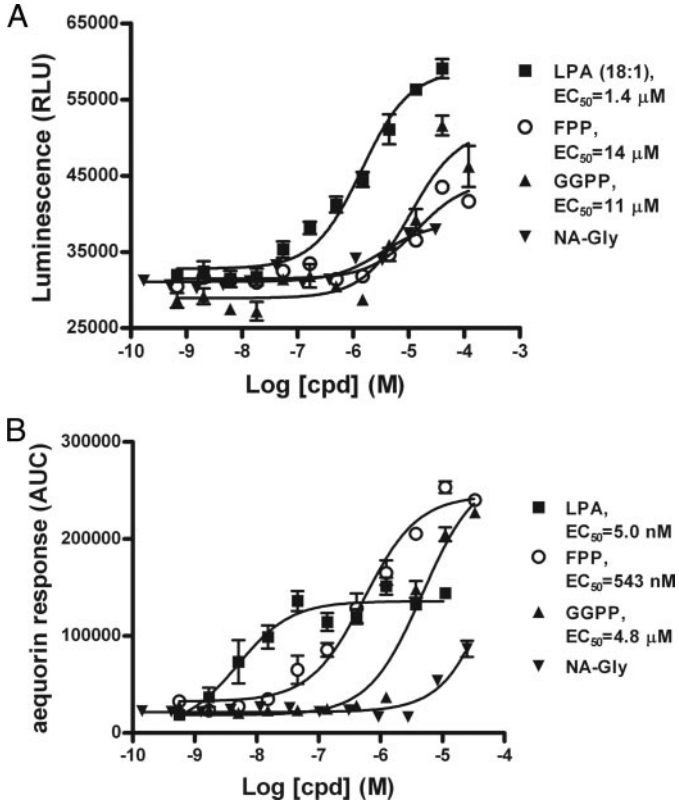

FIGURE 2. **Response of human GPR92 to selected lipid molecules in the β-arrestin assay (*A*) and aequorin assay (*B*).** HEK293-BAEA (*A*) or CHO-aequorin parental cells (*B*) were transiently transfected with GPR92. The next day, they were assayed for their responses to the indicated lipid molecules. *GGPP*, geranylgeranyl diphosphate.

anandamide and Δ9-tetrahydrocannabinol were very weak, we believe that they are real as the responses were enhanced to a more significant level when a low amount of AM251 (5 μM) was included in the assay (data not shown). As controls, the same compounds all triggered expected agonist or antagonist responses from the CB_1 and CB_2 receptors (Figs. 3 and 4 and data not shown). Noticeably, many compounds that were reported to have potent activity on GPR55, such as CP55940, HU210, abnormal cannabidiol (*Abn-CBD*, Fig. 6), and O-1602 (12, 13), did not show any detectable activity in our assays.

G2A Responds to Oxidized Free Fatty Acid 9-HODE and 11-HETE but Not LPC—Consistent with the Obinata *et al.* study (17), our screening revealed oxidized free fatty acids such as 9-HODE as agonist hits (Fig. 7*A*). G2A was also weakly activated by linoleic acid (nonoxidized precursor to 9-HODE), but with a higher EC_{50} value and a lower activation maximum. We found LPC, 13(*S*)-HODE, and lauric acid to be completely inactive on G2A (Fig. 7*A*). Because the EC_{50} values of these compounds appeared to be high in the β-arrestin assay, making accurate EC_{50} determinations difficult, we also tested these compounds in the aequorin assay. The aequorin assay is a more sensitive assay for G2A, giving lower EC_{50} values for all compounds tested. When we compared a variety of oxidized free fatty acids in the aequorin assay, we found that (±)9-HODE, 9(*S*)-HODE, 9-hydroperoxyoctadecadienoic acid (*9-HpODE*), and 11-HETE were the most potent agonists, with EC_{50} values ranging from 247 to 978 nM (Fig. 7*B*). Importantly, they show specific activity on G2A that is lacking in the parental cell line (data not shown). 8-HETE and 12-HETE showed very low or no activity. LPC and linoleic acid triggered massive responses from the parental CHO cell line in the aequorin assay (data not shown), making assessment of the G2A activity of these molecules in the aequorin assay impossible.

GPR3, GPR6, GPR12, GPR63, and GPR18 Remain Orphans—We did not detect any hit for the putative S1P receptors GPR3, GPR6, GPR12, and GPR63. Although control $S1P_1$ receptor responded to S1P very well with an EC_{50} of 34 nM, the above four receptors showed no activity whatsoever at up to 8 μM of S1P and 42 μM of sphingosine phosphorylcholine (data not shown). The assay also revealed anandamide and 2-AG as weak agonists on $S1P_1$ receptor in the micromolar range (data not shown). This cross-reactivity of endocannabinoids on $S1P_1$ is not surprising given the high receptor sequence homology.

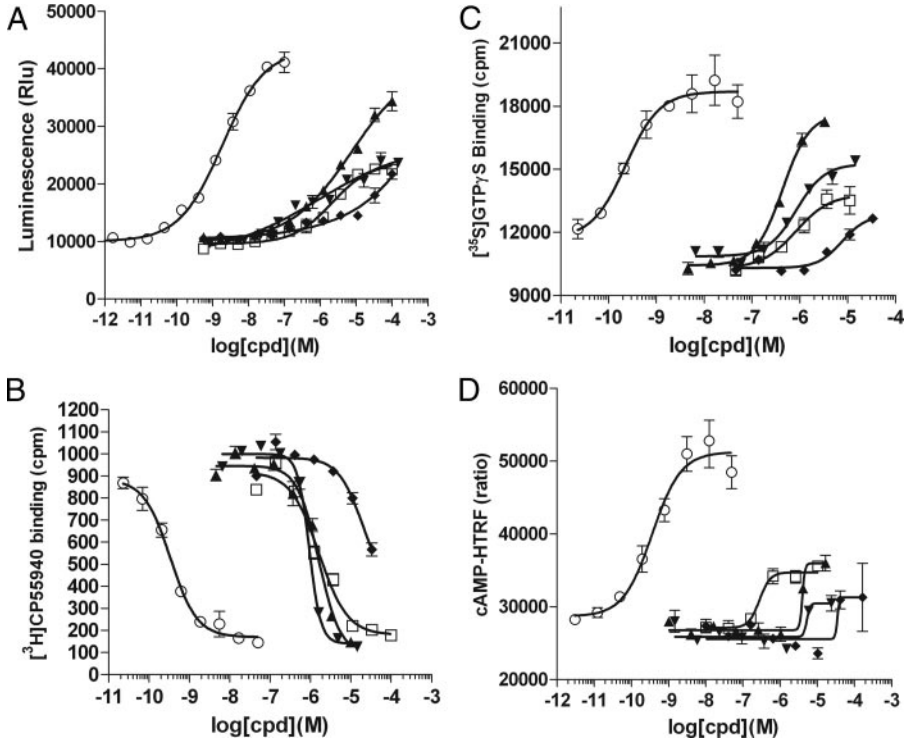

FIGURE 3. **DIM is a CB_2 partial agonist.** Response of the human CB_2 receptor to the test molecules in the β-arrestin assay (*A*), radioligand competition binding assay (*B*), GTPγS binding assay (*C*), and cAMP assay (*D*). ○, CP55940; □, DIM; ▲, 2-AG; ▼, anandamide; and ◆, arachidonic acid. *A*, HEK293-BAEA parental cells were transiently transfected with CB_2 and then assayed the next day. *B* and *C*, membrane preparations from a CB_2-CHO stable cell line were used. *D*, CB_2-CHO stable cell line was seeded and 16 h later stimulated with test compounds along with 60 μM of forskolin. After 30 min of stimulation, cells were lysed, and cAMP level was measured using cAMP-HTRF HighRange kit. The *y* axis plots the FRET ratio which is inversely proportional to the cellular cAMP concentration.

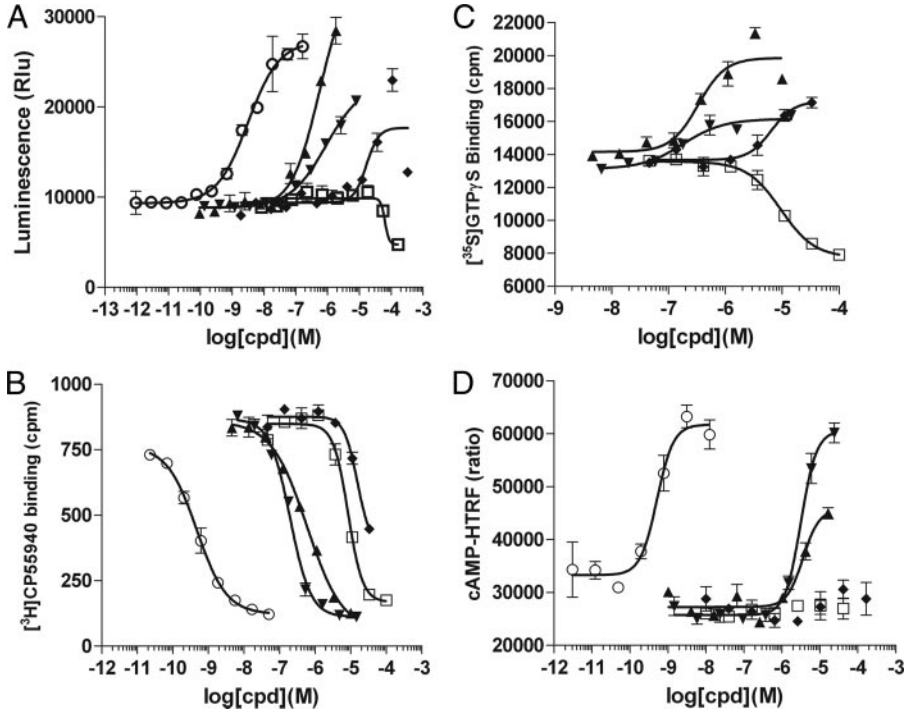

FIGURE 4. **DIM is a weak CB_1 inverse agonist.** Response of the human CB_1 receptor to the test molecules in the β-arrestin assay (*A*), radioligand competition binding assay (*B*), GTPγS binding assay (*C*), and cAMP assay (*D*). ○, CP55940; □, DIM; ▲, 2-AG; ▼, anandamide; and ◆, arachidonic acid. *A*, HEK293-BAEA parental cells were transiently transfected with CB_1 and then assayed the next day. *B* and *C*, membrane preparations from a CB_1-CHO stable cell line were used. *D*, CB_1-CHO stable cell line was seeded and 16 h later stimulated with test compounds along with 60 μM of forskolin. After 30 min of stimulation, cells were lysed, and cAMP level was measured using cAMP-HTRF HighRange kit. The *y* axis plots the FRET ratio that is inversely proportional to the cellular cAMP concentration.

FTY720, a potent $S1P_{1,3,4,5}$ receptor agonist, has been shown to have weak activity on the CB_1 receptor (40).

We screened three reported free fatty acid (FFA) receptors GPR43 (41), GPR40 (42), and GPR84 (43) against the lipid collection. GPR43 responded to short chain FFA as expected. C2 and C3 carboxylic acids most potently activated the receptor and C4 fatty acid moderately activated the receptor, whereas C6 was almost inactive (data not shown). Mid-chain FFAs caused a decrease in signal at high concentrations (~100 μM), likely due to nonspecific effects on cells at high concentrations, which may explain why we did not observe any positive response of C10-C12 on GPR40 and GPR84 (data not shown).

GPR18 and GPR34 were also included in the lipid screen; however, no positive hits were identified. The reported ligand for GPR18, NaGly (44), was not part of the original lipid collection and was later tested on GPR18 and found to be inactive (data not shown). We could not find any commercial source for the reported ligand for GPR34, lysophosphatidylserine 16:0 (45). Lysophosphatidylserine (18:1) did not trigger any signal increase at concentrations as high as 100 μM (data not shown).

DISCUSSION

We used the β-arrestin PathHunter™ assay as the main assay system to evaluate some of the recent deorphanization reports on lipid receptor and ligand pairs. This assay system is a relatively new technology that was not used in any of the previous reports on these orphan receptors. The distinct features of the system are as follows: 1) it is a universal, G protein-independent assay; and 2) it generates a receptor-specific signal proximal to receptor activation. Because of these two features, it is perfectly suited to tackle orphan receptors with little G protein signaling information. In Table 4, we summarize

TABLE 3

The activity of DIM on CB_1 and CB_2 receptors in comparison with CP55940, anandamide, 2-AG, and arachidonic acid

The data are expressed as average ± S.D. of three or four experiments. Percentage of efficacy is calculated by normalizing the response range to that of a standard compound (CP55940 or 2-AG); the value shown is the average value of all the experiments.

Receptor	Assay type	CP55940, EC/IC_{50} or K_i	DIM, EC/IC_{50} or K_i	Anandamide, EC/IC_{50} or K_i	2-AG, EC/IC50 or K_i	Arachidonic acid, EC/IC_{50} or K_i
		μM	μM	μM	μM	μM
CB_2	β-Arrestin assay (% of efficacy)	0.0016 ± 0.0004 (100%)	1.7 ± 1.3 (47%)	0.74 ± 0.27 (51%)	4.4 ± 3.2 (82%)	>10
	Ligand binding assay	0.00022 ± 0.00008	1.1 ± 0.3	0.34 ± 0.13	0.59 ± 0.26	13 ± 3
	GTPγS assay (% of efficacy)	0.00028 ± 0.00005 (100%)	0.98 ± 0.22 (37%)	0.62 ± 0.32 (49%)	0.32 ± 0.17 (95%)	10 ± 4 (43%)
	cAMP-HTRF assay (% of efficacy)	0.00038 ± 0.00008 (100%)	0.42 ± 0.23 (43%)	? (<20%)	5.3 ± 2.3 (57%)	>10
CB_1	β-Arrestin assay (% of efficacy)	0.00045 (100%)	>30, inhibition	0.81 (67%)	0.85 (>100%)	>10
	Ligand binding assay	0.00024 ± 0.00008	4.3 ± 0.3	0.09 ± 0.01	0.25 ± 0.05	12 ± 5
	GTPγS assay (% of efficacy)	?	11.1, inhibition	0.44 (56%)	0.32 (100%)	4.2 (40%)
	cAMP-HTRF assay (% of efficacy)	0.0005	>30	3.5 (125%)	3.4 (69%)	>10

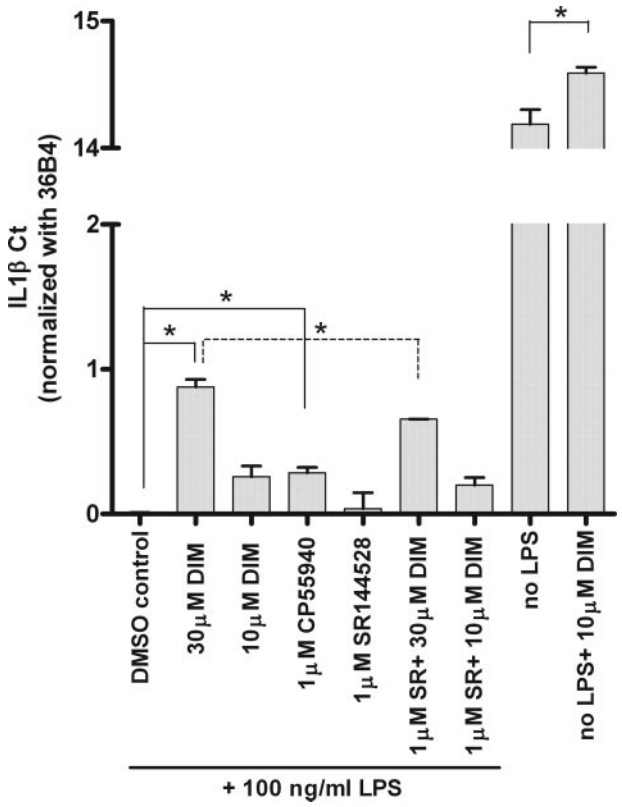

FIGURE 5. **CB_2 partially mediates the anti-inflammatory effect of DIM in RAW264.7 cells.** RAW264.7 cells were serum-starved for 24 h, treated with test compound for 18 h, and followed by 12 h of LPS (100 ng/ml) stimulation when indicated. Total RNA was isolated and Taqman PCR performed for IL-1β and control gene 36B4 in each sample. IL-1β *Ct* values, after subtracting 36B4 *Ct* values, were base-line adjusted and graphed. *Ct* values are inversely proportional to the mRNA levels (*Ct* change of 1 equals 2-fold mRNA level change). CP55940 and SR144528 (*SR*) are CB_1/CB_2 dual agonist and CB_2-specific antagonist, respectively. Each *bar* represents the mean ± S.E., $n = 3$. *, $p < 0.05$.

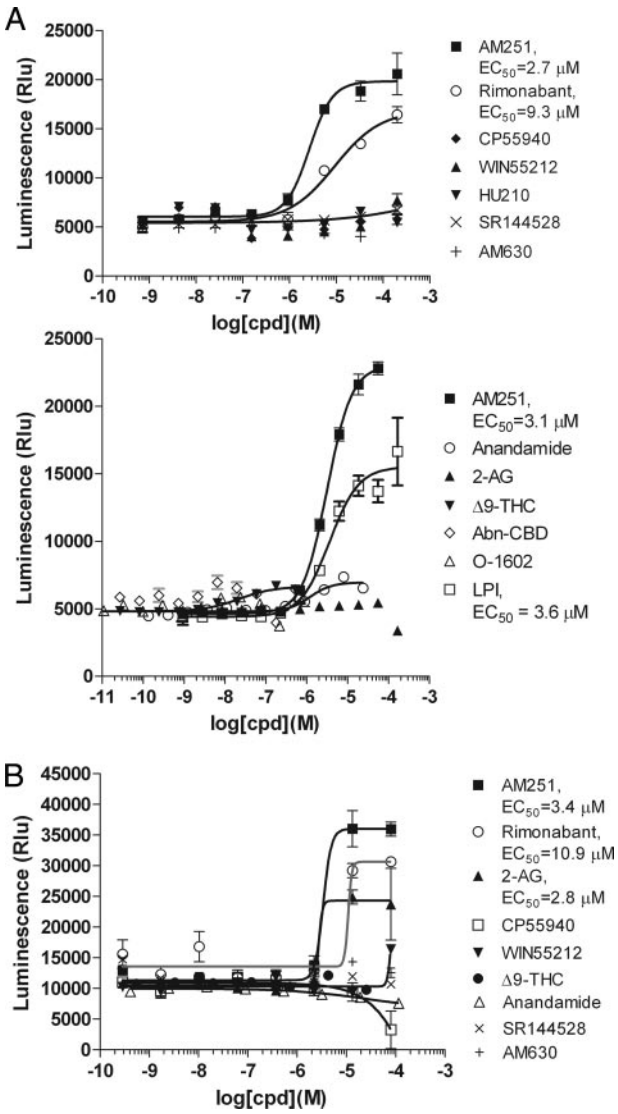

FIGURE 6. **Response of human GPR55 to test molecules in the β-arrestin assay (*A*) and reporter gene assay (*B*).** *A,* HEK293-BAEA parental cells were transiently transfected with GPR55. After 16–24 h, the cells were assayed for their responses to the indicated lipid molecules. Two experiments were shown with different sets of compounds tested. *B,* GPR55-HEK293 stable cell line was transiently transfected with pGL3-SRE-MRE-CRE-luc. Test compounds were added 24 h later. Luciferase activity was assayed 16–24 h after compound stimulation using Bright-Glo and read on CLIPR for luminescence signal. *Δ9-THC*, Δ9-tetrahydrocannabinol; *Abn-CBD*, abnormal cannabidiol.

our findings, either in support of or arguing against published receptor and ligand pairs.

Although a positive result in the β-arrestin system, combined with previous data from different assay formats, leaves little doubt on the authenticity of the receptor-ligand pair, a negative result in the β-arrestin system does not definitely prove that the receptor is not activated by the ligand. There are several reasons why an experiment could theoretically fail to pick up a true ligand. They include the following. 1) The receptor is not expressed or trafficked properly to the cell surface in our transient HEK293 transfection system; we did not observe this problem for any well established receptors so far, but it remains a theoretical possibility. 2) The receptor expression is down-regulated due to the presence of ligand in the serum-

containing growth medium; we routinely used charcoal-filtered serum as an alternative serum source to check for this possibility. 3) The receptor happens to belong to rare cases where the activated receptor does not bind to β-arrestin2 and get internalized. 4) The ligand tested was not soluble or appropriately prepared to activate the receptor. Because a 5–30-fold right shift of the agonist titration curve has been observed (Table 1), it is possible that we may not have observed a weak ligand activation because of limitation on solubility of certain lipid ligands.

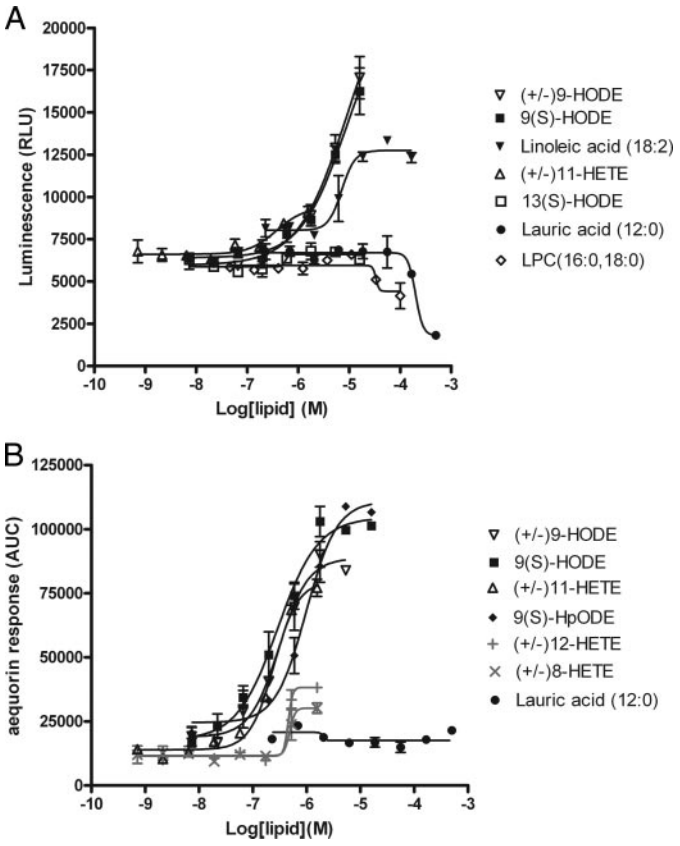

FIGURE 7. **Response of human G2A to lipid molecules in the β-arrestin assay (*A*) and aequorin assay (*B*).** HEK293-BAEA or CHO-aequorin parental cells were transiently transfected with G2A. 16–24 h later, they were assayed for their responses to the indicated lipid molecules. *9-HpODE*, 9-hydroperoxyoctadecadienoic acid.

A very interesting observation from the screen was the identification of DIM, an anti-cancer compound, as a novel CB_2 receptor agonist. This molecule is known to directly modulate intracellular aryl hydrocarbon receptor (33–35) and androgen receptor (37), but to our knowledge, no literature has linked it with a cell-surface GPCR before. Our results suggest that the activation of CB_2 by DIM is likely the molecular mechanism behind some of the reported anti-inflammatory effects of DIM. CB_2 receptor is highly expressed in the immune cells in the periphery, such as macrophages and T cells. Interestingly, a number of human leukemia and lymphoma cell lines were also reported to express CB_2 (46). These cell lines, which include Jurkat, Molt-4, and Sup-T1, are susceptible to apoptosis induced by a variety of cannabinoids. In fact, cannabinoids have been shown to inhibit growth in several tumor xenograft models, to curb growth or induce apoptosis in a number of transformed cell lines, and inhibit tumor angiogenesis and metastasis (47, 48). Thus, whether CB_2 could also mediate anti-tumor effect of DIM warrants further investigation. CB_2 has been implicated in a large number of physiological functions. Emerging areas of investigation on CB_2 include pain (49), neuroinflammation (50), hepatic fibrosis (51), gastrointestinal motility and inflammation (52), atherosclerosis (53), immune function (54), demyelinating disease (55), ischemia (56), bone metabolism (57), and reproduction (58). Our study not only raises the possibility that CB_2 could be mediating some of the beneficial effects of DIM *in vivo* but also suggests that perhaps DIM, a marketed nutritional supplement, might be useful for treating many more diseases than what is currently appreciated.

Among all the reported novel LPA and S1P receptors, we could only confirm GPR92 to be a true LPA receptor. This is an interesting example where divergent receptors bind the same ligand; GPR92 has a low sequence homology with the classical LPA1–3 receptors (21–22%). The affinity of GPR92 for LPA has not been directly compared with classical LPA receptors before. Kotarsky *et al.* (3) reported a 6.4 nM binding affinity and 9.3 μM EC_{50} of LPA on GPR92 in the cell-based reporter gene assay. Our study directly compared GPR92 and EDG4 (LPA2) in the same assay format and found GPR92 to be almost as potent. GPR92 is highly expressed in gastrointestinal $CD8^+$ cytotoxic lymphocytes (3), small and medium diameter neurons in dorsal

TABLE 4
Summary of lipid receptor-ligand test results

Receptor	Reported ligands	Confirmed	Refs.
CB_1	Various cannabinoids	CP55940, anandamide, 2-AG	
CB_2	Various cannabinoids	CP55940, anandamide, 2-AG, DIM	
GPR55	Various cannabinoids, AM251, rimonabant, LPI, CP55940, HU210, Abn-CBD, O-1602	AM251, rimonabant, LPI, endocannabinoids very weak activity	11–14
$S1P_1$	S1P	Yes	
LPA_2	LPA	Yes	
GPR92	LPA, FPP	LPA, FPP, geranylgeranyl diphosphate	3, 4, 15
G2A	9-HODE, 11-HETE	Yes	17
GPR43	Short chain FFA	Yes	41
GPR3	S1P	No	8
GPR6	S1P	No	8, 9
GPR12	S1P, sphingosine phosphorylcholine	No	8, 62
GPR18	NaGly	No	44
GPR23	LPA	No	1, 2
GPR63	S1P	No	10
GPR34	Lysophosphatidylserine (16:0)	Unable to test	45
GPR40	Mid and long chain FFA	Unable to test	42
GPR84	Mid chain FFA	Unable to test	43

root ganglion, and also embryonic stem cells (4). In recombinant systems, it influences cellular cytoskeletal arrangement, such as stress fiber formation and neurite retraction (4). Its exact function *in vivo* awaits further studies.

There have been several papers and patents reporting that GPR55 is the third cannabinoid receptor (11–14). Although all reports agreed that GPR55 is an unusual G_{13}-coupled receptor, the exact activity and EC_{50} values of various compounds differ in different reports, perhaps reflecting the differences in assay formats and stable cell lines, as well as the lack of a good standard assay for G_{13}-coupled receptors. Consistent with our finding, a patent from GlaxoSmithKline reported AM251 to be a hit in a yeast-based GPR55 screen (WO01/86305). It is interesting to note that AM251 and rimonabant are both inverse agonist compounds that have no agonist activity on CB_1; however, they are purely agonist compounds on GPR55. In the study of Ryberg *et al.* (12), a large number of CB_1- and CB_2-related synthetic or naturally occurring molecules were shown to be potent low nanomolar activators of GPR55 in the GTPγS assay. AM251 was also active with an EC_{50} of 39 nM in their study. However, many of these molecules were found to be inactive in a separate study using intracellular Ca^{2+} as the readout (14). In our β-arrestin assay system and reporter gene assay system, AM251, rimonabant, and LPI are strong agonists, whereas endocannabinoids showed only very weak activity. CP55940, HU210, O-1602, and abnormal cannabidiol showed no activity, possibly reflecting differences between intact cell-based assays and membrane-based GTPγS assay. The function of GPR55 is under active investigation. A recent report (59) suggested that it plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain.

G2A, OGR1, TDAG8, and GPR4 are close sequence homologues constituting a receptor family. Whereas the latter three receptors are now established as pH-sensing receptors, the natural ligand for G2A remains uncertain. Our result is consistent with the Obinata *et al.* study (17) in showing that G2A has no activity whatsoever toward LPC, and that oxidized FFAs such as 9-HODE are its true ligands. This finding expands the known naturally occurring signaling lipids to include oxidized FFAs. Plasma FFAs and low density lipoprotein are continuously exposed to oxidative stress to generate hydroxyl species. Cell-associated phospholipids can also be subjected to oxidative pressure by cellular stress. Interestingly, G2A receptor expression was previously shown to be up-regulated by stress-inducing and cell-damaging agents (60). Thus, G2A might serve as a stress sensor in the immune system to trigger appropriate responses in a stressful environment. 9-HODE was previously reported to directly bind peroxisome proliferator-activated receptor γ with a K_d of 10–20 μM (18). The EC_{50} of 9-HODE on G2A was in hundreds of nanomolar range in the aequorin assay and low micromolar range in the β-arrestin assay. Thus, it is possible that some of the effects of oxidized low density lipoprotein or oxidized FFAs that were previously thought to be mediated via nuclear hormone receptors are actually mediated by GPCR G2A.

In conclusion, we used a novel GPCR assay system to test the authenticity of lipid receptor-ligand pairs that generated a number of interesting findings, including support for and against various published ligands. This information may serve as a critical first step in elucidating the functions of these orphan receptors, as well as helping to understand the mechanism of action of emerging medicinal compounds such as DIM.

Acknowledgments—We are grateful to Xiang-Ju Gu for providing the BioMol libraries; Novartis GPCR-EP for providing GPR55-HEK293 stable cell line; Cecilia Jiang for providing the pGL3-CRE-MRE-SRE-luc construct; Shin-Shay Tian and Jing Li for providing certain lipids and GPR40/43 cDNA clones; GNF gene core and Novartis GPCR-EP for providing GPCR cDNA clones; and GNF Advanced Automation Technology group for technical assistance. We specifically thank Tim R. Smith for assistance with the aequorin assays, and Mitch Hull for writing software for the aequorin data analysis.

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