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RESEARCH PAPER

The orphan receptor GPR55 is a novel cannabinoid receptor

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Background: The endocannabinoid system functions through two well characterized receptor systems, the CB_1 and CB_2 receptors. Work by a number of groups in recent years has provided evidence that the system is more complicated and additional receptor types should exist to explain ligand activity in a number of physiological processes.
Experimental approach: Cells transfected with the human cDNA for GPR55 were tested for their ability to bind and to mediate GTPγS binding by cannabinoid ligands. Using an antibody and peptide blocking approach, the nature of the G-protein coupling was determined and further demonstrated by measuring activity of downstream signalling pathways.
Key results: We demonstrate that GPR55 binds to and is activated by the cannabinoid ligand CP55940. In addition endocannabinoids including anandamide and virodhamine activate GTPγS binding via GPR55 with nM potencies. Ligands such as cannabidiol and abnormal cannabidiol which exhibit no CB_1or CB_2 activity and are believed to function at a novel cannabinoid receptor, also showed activity at GPR55. GPR55 couples to Gα13 and can mediate activation of rhoA, cdc42 and rac1.
Conclusions: These data suggest that GPR55 is a novel cannabinoid receptor, and its ligand profile with respect to CB_1 and CB_2 described here will permit delineation of its physiological function(s).
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Abbreviations: 2-AG, 2 arachidonoylglycerol; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide; BSA, bovine serum albumin; CP55940, (−)-*cis*-3-[2-hydroxy-4-(1,1–dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; DTT, dithiothreitol; FLIPR, fluorescent imaging plate reader; GDP, guanosine 5′-diphosphate; GTPγS, guanosine 5′-[γ-^{35}S]-triphosphate; HEK, human embryonic kidney; HU210, (−) 11-OH-8-tetrahydrocannabinol-dimethylheptyl; OEA, oleoylethanolamide; PCR, polymerase chain reaction; PEA, palmitoylethanolamide; PEI, polyethylenimine; SR141716, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-pyrazole-3-carboxamide; Δ9-THC, Δ9-tetrahydrocannabinol; WIN55,212-2, *R*(+)-[2,3-di-hydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de] -1,4-benz-oxazinyl]-(1-naphthalenyl)-methanone-mesylate

Introduction

Preparations of *Cannabis sativa* have been used for medicinal and recreational purposes for at least 4000 years and extracts of *C. sativa* contain over 60 different pharmacologically active components the most prominent being Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (Mechoulam, 1970a; Mechoulam *et al.*, 1970b; Howlett, 2002). Cannabinoids exert their effects by binding to specific receptors located in the membrane of the cell. Two types of high-affinity cannabinoid receptors have been identified so far by molecular cloning; CB_1 receptors (Devane *et al.*, 1988; Matsuda *et al.*, 1990), and CB_2 receptors (Munro *et al.*, 1993). Both CB_1 and CB_2 are coupled to the G_i, G-protein signal transduction pathway. Activation of these cannabinoid receptors leads to inhibition of adenylate cyclase and activation of mitogen-activated protein (MAP) kinase. CB_1 receptors can also modulate ion channels, inhibiting N-, and P/R-type calcium channels, stimulating inwardly rectifying potassium channels and enhancing the activation of A-type

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potassium channels (for recent reviews of cannabinoid signal transduction see Howlett, 2004; Demuth and Molleman, 2006).

Cannabinoid type 1 (CB_1) receptors are primarily, but not exclusively expressed in the CNS and are believed to mediate the CNS effects of endogenous (for example, anandamide) and exogenously administered cannabinoids. Peripherally, CB_1 receptor expression is found in the pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, superior cervical ganglion, heart, blood vessels, lung, bladder and adrenal gland (reviewed by Howlett, 2002). Recently, the liver and adipose has been added to the list (Cota *et al*., 2003; Osei-Hyiaman *et al*., 2005). CB_1 receptors are also located on central and peripheral nerve terminals and when activated, seem to suppress the neuronal release of excitatory and inhibitory transmitters for example, acetylcholine, noradrenaline, dopamine, 5-hydroxytryptamine, γ-amino butyric acid, glutamate and aspartate (Pertwee, 1997, 2001; Ong and Mackie, 1999) adding to the complexity of the physiological responses to the endocannabinoids.

CB_2 receptor expression is restricted to the periphery, mainly in immune cells with particularly high levels in B cells and natural killer cells (Galiegue *et al*., 1995) although it has been reported that the CB_2 receptor is expressed in microglia cells of the CNS (Walter *et al*., 2003) and in brain stem neuronal cells (Van Sickle *et al*., 2005).

Some studies suggest that endocannabinoids regulate multiple physiological and pathological reproductive functions (Maccarrone *et al*., 2002) and that endocannabinoids such as 2-arachidonoylglycerol play a role in the progression of the pathophysiology of shock (Cainazzo *et al*., 2002) and act as immunomodulators (Parolaro *et al*., 2002). Others have shown that CB_2 receptors play a very important role in the stimulation of growth in most haematopoietic lineages (Valk *et al*., 1997; Derocq *et al*., 2000). Thus, cannabinoid receptors and endocannabinoids are physiologically or pathophysiologically relevant in a great diversity of tissues and organs like the CNS and cardiovascular, reproductive, endocrine, immune and gastrointestinal systems. Particularly, the CNS and its hypothalamic appetite-regulating control system have attracted much attention over the last ten years and endocannabinoids have classically been shown to play a role in the physiological regulation of food intake (Sofia and Knobloch, 1976; Anderson-Baker *et al*., 1979; Pacheco *et al*., 1993; Berry and Mechoulam, 2002; Fride, 2002), effects that are inhibited by the non-endogenous ligand *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716).

A number of endogenous ligands such as anandamide, 2-arachidonoylglycerol, noladin ether, palmitoylethanolamine, virodhamine and oleoylethanolamide (OEA) have been identified, which are believed to modulate the cannabinoid system via the previously identified CB_1 and CB_2 receptors, or by their action at as-yet unidentified receptors.

In recent years, a number of studies have suggested the existence of additional cannabinoid receptors that function in these processes and these reports have been reviewed by Begg *et al*. (2005). In this study we show that the orphan G-protein-coupled receptor, GPR55, is a novel cannabinoid receptor with an ability to interact with and be modulated by endogenous, plant and synthetic cannabinoid ligands and to be a candidate for one of the non-CB_1/CB_2 receptors, described by others.

Methods

Cloning of hGPR55

hGPR55 (EMBL accession no. BC032694) was amplified from human genomic DNA by polymerase chain reaction (PCR) and sub-cloned into mammalian expression plasmids pIRESneo2 and pcDNA3 using standard techniques.

Expression profiling

GPR55 mRNA levels in human and mouse tissues were analysed by quantitative real-time PCR analysis using ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Hercules, CA, USA). Primer/probe sets for hGPR55 were: 5′-TCTACATGATCAACCTGGCAGTCT-3′, 5′-CTGGGACAGGACCATCTTGAA-3′ and 5′-FAM-TGACCTGCTGCTGGTGCTCTCCC-TAMRA-3′, and for mGPR55 were: 5′-CTATCTACATGATCAACTTGGCTGTTT-3′, 5′-TGTGGCAGGACCATCTTGAA-3′ and 5′-FAM-CGATTTACTGCTGGTGCTCTCCCTCCC-TAMRA-3′. To determine relative mRNA levels of GPR55, results were normalized to its content of the mRNA encoding the ribosomal protein 36B4 (used as an internal standard).

Cell transfection and membrane preparation

Human embryonic kidney—HEK293s cells (5×10^6) were seeded in T75 flasks and after 24 h, cells were transiently transfected with 10 μg of relevant plasmid using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA). Membranes were prepared after 48 h using standard methods and stored at −80 °C. Protein concentration was measured according to the method of Bradford (Bio-rad Laboratories, Foster City, CA, USA) (Bradford, 1976). CB_1 and CB_2 membranes were commercially available (PerkinElmer).

Radioligand binding assays

Radioligand binding was initiated by the addition of 5 μg of membrane protein to each well of a 96-well plate containing 50 nM [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1–dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940) (Tocris, Ellisville, Missouri, USA), [^{3}H]-SR141716 (Amersham, Piscataway, NJ, USA) or [^{3}H]-*R*(+)-[2,3-di-hydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de] -1,4-benz-oxazinyl]-(1-naphthalenyl)-methanone-mesylate (WIN55,212-2) (Amersham), sufficient volume of buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.4, 0.1% bovine serum albumin (BSA)) to bring the total volume of each well to 200 μl. Non-specific binding was determined in the presence of 10 μM CP55940 (Tocris), SR141716 and WIN55,212-2 (Tocris). The membranes were incubated at 30 °C for 90 min and the reaction was then terminated by the addition of ice-cold wash buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.4) followed by rapid filtration under vacuum

through Printed Filtermat B glass fibre filters (Wallac, Turku, Finland) (0.05% polyethylenimine (PEI)-treated) using a Micro 96 Harvester (Skatron Instruments, Lier, Norway). The filters were dried for 30 min at 50 °C, then a paraffin scintillant pad was melted onto the filters and the bound radioactivity was determined using a 1450 Microbeta Trilux (Wallac) scintillation counter.

[^{35}S]-GTPγS binding assay

[^{35}S]-Guanosine 5′-[γ-^{35}S]-triphosphate (GTPγS) binding assays were conducted at 30 °C for 45 min in membrane buffer (100 mM NaCl, 5 mM, 1 mM EDTA, 50 mM HEPES, pH 7.4) containing 0.025 μg μl^{-1} of membrane protein with 0.01% BSA (fatty-acid free) (Sigma, St Louis, MO, USA), 10 μM guanosine 5′-diphosphate (GDP) (Sigma), 100 μM dithiothreitol (DTT) (Sigma) and 0.53 nM [^{35}S]-GTPγS (Amersham) in a final volume of 200 μl. Non-specific binding was determined in the presence of 20 μM unlabelled GTPγS (Sigma). The reaction was terminated by addition of ice-cold wash buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.4) followed by rapid filtration under vacuum through Wallac GF/B glass-fibre filters using a cell harvester (Skatron). The filters were left to dry for 30 min at 50 °C, then a paraffin scintillant pad was melted onto the filters and the bound radioactivity was determined using a microbeta scintillation counter (Wallac). Antagonist potency was determined versus an EC_{80} concentration of CP55940 that was determined empirically on the day of the experiment. Data were fitted using the equation $y = A + ((B-A)/1 + ((C/x) \wedge D)))$ and the EC_{50} estimated where A is the non-specific binding, B is the total binding, C is the IC_{50} and D is the slope.

Peptide and antibody blocking of [^{35}S]-GTPγS binding assays

[^{35}S]-GTPγS binding assays were performed as above with additional pre-incubation of membranes with and without peptides or antibodies for the G-protein subunits $G\alpha_{13}$, $G\alpha_i$ and $G\alpha_s$ for 15 min at 30 °C (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Data were analysed using paired *t*-test (**$P<0.05$; ***$P<0.01$).

Pertussis toxin treatment

Cell transfections were conducted as described above with the exception that the cells prior to harvesting were pre-incubated with Pertussis toxin (Sigma) overnight (0.1 μg ml^{-1} final concentration). The cells were then harvested and membranes were prepared as described above.

Plate-based FLIPR Ca^{2+} assays

In brief, 1 day before the assay was performed, HEK293 cells expressing GRP55 were plated in 96-well, black-walled, assay plates, at a density of 25 000 cells per well. These plates were then returned to the cell-culture incubator until 1.5 h before the assay when they were removed and the cells were loaded with the Ca^{2+} reporter dye Fluo4 (Invitrogen) for 1 h in a cell-culture incubator. After this, the plates were placed into a fluorescent imaging plate reader (FLIPR) to monitor fluorescence ($\lambda_{ex} = 488$ and $\lambda_{EM} = 540$ nm) before and after the addition of ligands of interest.

Determination of rhoA, rac1 and cdc42 activity

RhoA, rac1 and cdc42 activity was measured according to the manufacturer's instructions (Upstate Biotechnology Inc., Charlottesville, Virginia, USA). HEK293s GPR55-transfected cells were seeded on six-well plates, grown to 80% confluence, and serum-starved for 24 h. Following treatment with selected compounds at 37 °C for 15 min, the cells were washed with phosphate-buffered saline and harvested with 500 μl of lysis buffer provided by the manufacturer, with the addition of a mixture of protease inhibitors (Roche Molecular Biochemicals, Basel, Switzerland). The cell lysates were clarified by centrifugation at 15 000 *g* for 1 min. For a negative control, cell lysate was incubated with 1 mM GDP for 15 min at 30 °C. The cell lysates were then incubated with 10 μg of GST-RBD-agarose (Rho-binding domain of rhotekin) or GST-PBD-agarose (p21-binding domain of human PAK-1) to precipitate GTP-bound rhoA and GTP-bound rac1 and cdc42, respectively. The beads were then washed three times with lysis buffer and samples were prepared for electrophoresis by adding 1 × sodium dodecyl sulphate loading dye. Samples were boiled for 5 min and resolved by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis . Bound rhoA, rac1 and cdc42 were detected by western blot using the appropriate polyclonal antibodies specific for rac1, cdc42 (1:1000; Upstate Biotechnology) and rhoA (1:200; Santa Cruz Biotechnology).

Results

Cloning and sequence determination of GPR55

Nucleotide primers designed against the 5′- and 3′-ends of human, mouse and rat gpr55 were used to isolate open reading frames for GPR55 from the three species using genomic DNA. The sequence of the human gene was similar but not identical to that already described (Sawzdargo *et al*., 1999), however it was consistent with the human genome sequence. The sequence of all clones isolated differed in that there was a nucleotide insertion and deletion at positions 393 and 427 respectively, resulting in a frame shift of the translated sequence, consequently changing 11 amino acids at the predicted junction of intracellular loop 2 and transmembrane helix 4 (Figure 1). Since we could find no evidence for the existence of the previously published sequence, we concluded that the difference originated from a sequencing error by the authors (Sawzdargo *et al*., 1999).

The rat and mouse genes were cloned using a similar approach and their sequences were found to be identical to those found in GenBank (AC119315 (position 129078–130085) AC107707 (position 31198–32181)) demonstrating 75 and 78% identity to the human sequence respectively (Figure 1). Both the rat and mouse sequences are consistent with the human genome sequence in the region of the intracellular loop 2—transmembrane helix 4 region rather than the published sequence (Sawzdargo *et al*., 1999) containing the insertion and deletion (Figure 1). Despite

```
          1                    TM1------------------TM1    IC1    TM2-----------------TM2
hGPR55    MSQQNTSGDCLFDGVNELMKTLQFAVHIPTFVLGLLLNLLAIHGFSTFLKNRWPDYAATSIYMINLAVFDLLLVLSLPFK
mGPR55    MSQPE-RDNCSFDSVDKLTRTLQLAVHIPTFLLGLVLNLLAIRGFSAFLKKRKLDYIATSIYMINLAVFDLLLVLSLPFK
rGPR55    MSQLD-SNNCSFVFVDNLTKTLQLAVHIPTFLLGLVLNLLAIRGFGAFLKRRQLEYMATSIYMINLAVFDLLLVLSLPFK

          81                                                                          160
                  EC1        TM3---------------TM3        IC2       TM4----------------TM4
                                                          SGPPGRSLGSA
hGPR55    MVLSQVQS-PFPSLCTLVECLYFVSMYGSVFTICFISMDRFLAIRYPLLVSHLRSPRKIFGICCTIWVLVWTGSIPIYSF
mGPR55    MVLPQVES-PLPSFCTLVECLYFISMYGSVFTICFISLDRFLAIQYPILASHLRSPRKTFGICCIIWMLVWIGSIPIYTF
rGPR55    MILPQVKSSPSLVFCTFVECLYFISMYGSVFTICFISLDRLLAIQYPLLVNHFRSPRKTFGICCIIWMLVWVGSIPIYTF

          161                                                                         240
                   EC2         TM5----------------TM5        IC2                  TM6-----
hGPR55    HGKVEKYMCFHNMSDDTWSAKVFFPLEVFGFLLPMGIMGFCCSRSIHILLGRRDHTQDWVQQK---------ACI-YSIA
mGPR55    HREVERYKCFHNMSDVTWSASVFFPLEIFGFLLPMGIMGFCSYRSIHILLRRPDSTEDWVQQRDTKGWVQKRACI-WTIA
rGPR55    HRGVEGYKCFHNMSDSTWSARVFFPLEIFGFLLPMGIMGFCSYRSIHILLSIQGDDQ----------WVRKRACIIWTIA

          241
          -------------------TM6     EC3      TM7-----------------TM7
hGPR55    ASLAVFVVSFLPVHLGFFLQFLVRNSFIVECRAKQSISFFLQLSMCFSNVNCCLDVFCYYFVIKEFRMNIRAHRPSRVQL
mGPR55    TNLVIFVVSFLPVHLGFFLQYLVRNRFILDCRMKQGISLFLQLSLCFSNINCCLDVFCYYFVIKEFRMRIKAHRPSTIKL
rGPR55    TNLVVFVVSFLPVHLGLFLQFLVRNGFILNCRVKQGISLFLQLSLCFSNINCCLDVFCYYFAIKEFRMGIKVHRPSQVQL

          321
hGPR55    VLQDTTISRG
mGPR55    VNQDTMVSRG
rGPR55    VHQDSMVSRA
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Figure 1 Alignment between human (hGPR55), mouse (mGPR55) and rat (rGPR55) GPR55 protein sequences. The putative positions of the transmembrane regions (TM1-7), extracellular loops (EC1-3) and intracellular loops (EC1-3) are shown. The amino-acid differences in the previously published sequence (Sawzdargo *et al*., 1999) for human GPR55 at the IC2/TM4 boundary are shown above the sequences.

the low level of identity between the human and rodent forms of GPR55, the genomic linkage confirms that the rodent genes are orthologues of the human gpr55. Phylogenetically, the GPR55 sequence belongs to a cluster of receptors that are either orphans (GPR35, GPR92, P2Y5) or have been recently deorphanized (P2Y9 (Noguchi *et al*., 2003), GPR40 (Briscoe *et al*., 2003), GPR41 and GPR43 (Brown *et al*., 2003)).

Expression profile of GPR55

We next investigated the expression pattern of GPR55 in a panel of mouse tissues using quantitative PCR (Figure 2). GPR55 mRNA is found in a number of tissues with the highest mRNA levels detected in the adrenals, parts of the gastrointestinal tract, as well as in the CNS. As seen with CB_1 receptors, a broad distribution of GPR55 mRNA is found in brain tissue, however the levels are significantly lower than those for CB_1 (Figure 2, inset).

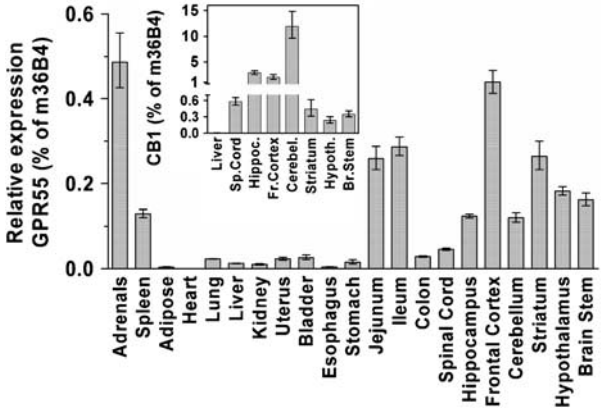

Figure 2 mRNA expression levels of GPR55 and CB_1 receptors in mouse tissues measured by quantitative PCR relative to m36B4. Tissues were dissected from C57BL/6 female mice. Samples from different mice were processed individually in all subsequent steps; RNA preparation, cDNA synthesis and quantitative PCR. Data are mean values ± s.e.m. using tissues from eight (GPR55) or four mice (CB_1) and presented as per cent of the ubiquitously and homogenously expressed ribosomal protein 36B4.

GPR55 binds and is activated by cannabinoid ligands

To test the possibility that GPR55 maybe a cannabinoid receptor, we generated an N terminus FLAG-tagged human GPR55 and transiently transfected the plasmid containing the cDNA into HEK293s cells. Cell-surface expression of the recombinant receptor was confirmed using an anti-FLAG antibody (Figure 3). We then examined the ability of the cannabinoid receptor radioligands [^{3}H]-CP55940, [^{3}H]-SR141716 and [^{3}H]-WIN55,212-2 to bind to membranes prepared from the transiently transfected cells. No specific binding was observed using 50 nM of each radioligand in membranes prepared from untransfected HEK293s cells. However, in membranes prepared from HEK293s cells transfected with the FLAG-tagged cDNA for human GPR55, a clear specific binding for [^{3}H]-CP55940 was observed (Figure 4). In addition, a small specific binding for [^{3}H]-SR141716 was seen whereas there was no binding for [^{3}H]-WIN55,212-2 (Figure 4). Subsequent experiments repeated these findings using alternative ligands as unlabelled competitors to confirm specificity. As a consequence of these observations, we generated a HEK293s cell line stably expressing the FLAG-tagged human GPR55. Cell-surface

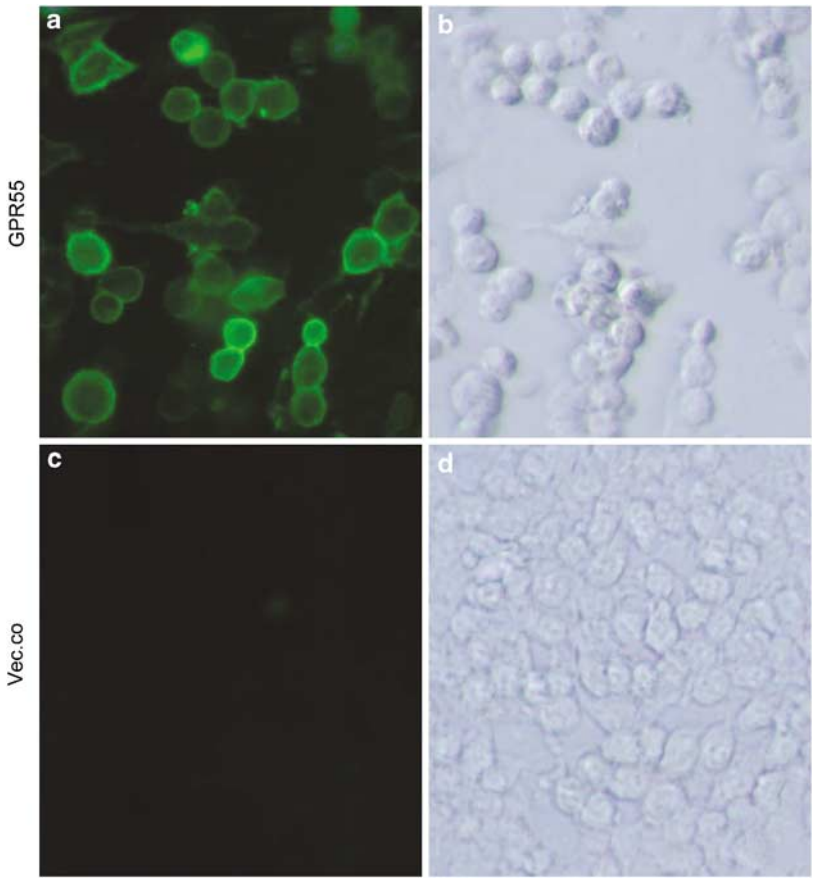

Figure 3 Cell-surface expression of FLAG-tagged hGPR55. Immunofluorescence images of anti-FLAG-stained HEK293s cells transiently transfected with FLAG-hGPR55 (**a**) or empty vector (Vec.co; (**c**)). Corresponding phase-contrast images are shown in (**b**) and (**d**).

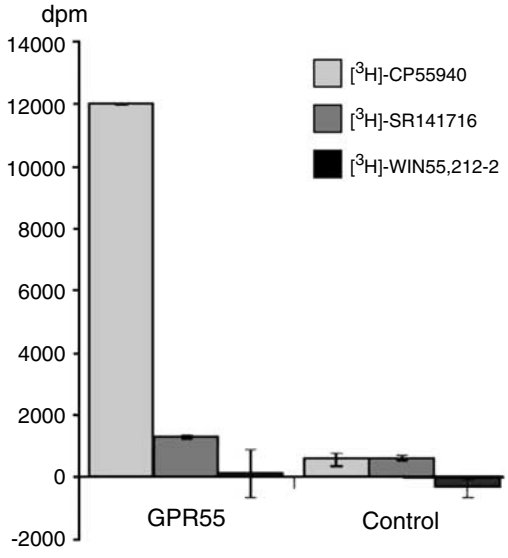

Figure 4 Radioligand binding to GPR55. Membranes prepared from cells transiently transfected with hGPR55 or vector control were incubated with 50 nM [^{3}H]-CP55940, [^{3}H]-SR141716 or [^{3}H]-WIN55,212-2. Specific binding was determined by the addition of 10 μM unlabelled ligand as competitor. The bars show the specific binding (mean ± s.e.m.; $n = 5$) determined for each ligand.

expression was confirmed using an anti-FLAG antibody and this cell line was used for further studies.

We, next determined whether the interaction of CP55940 with GPR55 had a functional consequence. Since GTPγS has the potential to pick up activation of most heterotrimeric G proteins if the experimental conditions are appropriate, we tested membranes expressing GPR55 using a factorial design strategy with and without 1 μM CP55940 varying GDP, $MgCl_2$, NaCl and saponin. A number of the conditions tested generated an increased GTPγS binding in the GPR55-containing membranes, but not with control membranes, in the presence of CP55940 (data not shown). Using the optimum condition identified (see Methods), we found that CP55940 stimulated GTPγS binding with an EC_{50} of 5 nM (Figure 5a and Table 1). With this finding we went on to evaluate other cannabinoid ligands for their ability to promote GTPγS binding via GPR55.

A number of endogenous cannabinoid ligands have been identified and characterized to date and we therefore examined their effect upon GPR55. The endocannabinoid anandamide stimulated GTPγS binding with an EC_{50} of 18 nM (Figure 5b and Table 1). The other endocannabinoids, 2 arachidonoylglycerol (2-AG), noladin ether, palmitoylethanolamide, virodhamine and OEA all stimulated GTPγS binding with EC_{50} values of 3, 10, 4, 12 and 440 nM respectively

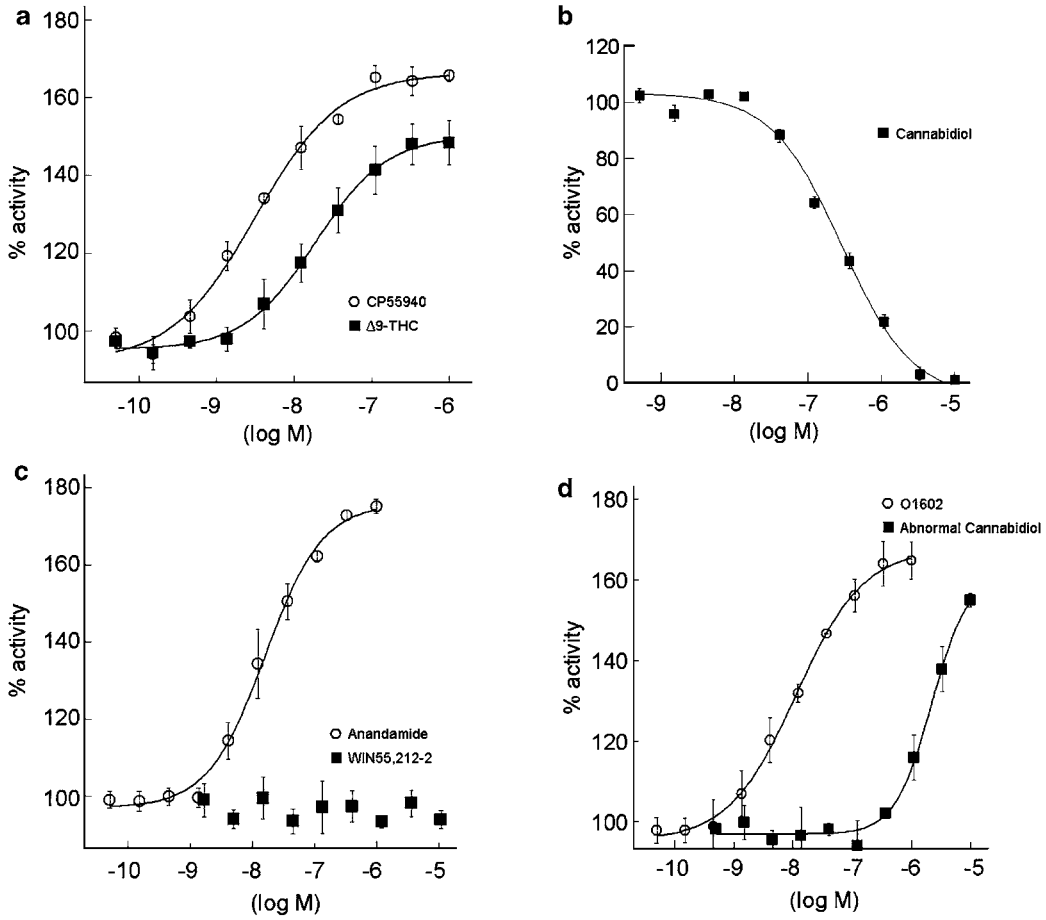

Figure 5 (**a**) Concentration–response curves for various ligands at GPR55 determined using a GTPγS assay: (**a**) CP55940 and Δ^9-THC; (**b**) cannabidiol antagonism of O1602 activation; (**c**) anandamide and WIN55,212-2; (**d**) O1602 and abnormal cannabidiol. Values shown are mean±s.e.m.; $n=5$.

Table 1 Profile of agonist activities of ligands at GPR55, CB_1 and CB_2 receptors

Ligand	*GPR55 EC_{50} (nM) GTPγS binding*	*GPR55 E_{max} (%)*	*CB_1 EC_{50} (nM) GTPγS binding*	*CB_1 E_{max}(%)*	*CB_2 EC_{50} (nM) GTPγS binding*	*CB_2 E_{max}(%)*
Anandamide	18±3	73±5	31±6	66±4	27±6	58±5
Noladin ether	10±1	95±7	37±5	89±5	>30 000	
2-Arachidonoylglycerol	3±1	99±2	519±48	92±6	618±45	87±5
Virodhamine	12±3	160±10	2920±325	75±9	381±34	91±10
Palmitoylethanolamide	4±1	92±1	>30 000		19 800±2821	93±12
Oleoylethanolamide	440±145	92±3	>30 000		>30 000	
Δ^9-THC	8±1	92±5	6±1	61±5	0.4±0.1	67±3
Cannabidiol	Antagonist		>30 000		>30 000	
Cannabinol	>30 000		>30 000		>30 000	
Abnormal cannabidiol	2523±579	76±17	>30 000		>30 000	
AM281	>30 000		Antagonist		Antagonist	
AM251	39±3	88±4	Antagonist		Antagonist	
WIN55,212-2	>30 000		18±3	101±14	1±0.2	97±8
HU210	26±7	78±3	0.2±0.03	91±2	0.5±0.1	99±6
O1602	13±2	99±4	>30 000		>30 000	
CP55940	5±1	100±2	0.2±0.01	100±2	0.3±0.01	100±4

Values shown are the means±s.e.m. derived from five independent experiments.

(Table 1). In parallel experiments, all these compounds generated the expected activities at CB_1 and CB_2 receptors (Table 1). None of these ligands had any effect when tested under identical conditions against membranes prepared from untransfected cells. Of note is the efficacy of virodhamine which under the assay conditions used is approximately 160% that of the other endocannabinoid ligands, noladin ether and 2-AG and double the efficacy of anandamide.

Table 2 Profile of antagonist activities of ligands at GPR55, CB_1 and CB_2 receptors

Ligand	*GPR55 IC_{50} (nM) GTPγS binding*	*CB_1 IC_{50} (nM) GTPγS binding*	*CB_2 IC_{50} (nM) GTPγS binding*
Cannabidiol	445±67	>30 000	>30 000
Cannabinol	>30 000	>30 000	>30 000
AM281	>30 000	7±0.6	2600±463
AM251	Agonist	8±1	2915±102
WIN55,212-2	>30 000	Agonist	Agonist

Data obtained using an EC_{80} concentration of CP55940 as agonist for each receptor. Values shown are the means±s.e.m. derived from five independent experiments.

We next tested Δ^9-THC, the psychoactive component of the cannabis plant *C. sativa*, for its activity at GPR55. Δ^9-THC activated GTPγS binding with an EC_{50} of 8 nM (Figure 5a and Table 1). We also examined the effect of cannabinol, cannabidiol and related compounds. Cannabidiol was without effect as an agonist in the GTPγS assay. However, cannabidiol was able to antagonize the agonist effect of CP55940 with an IC_{50} of 445 nM (Figure 5b and Table 2). Abnormal cannabidiol functioned as an agonist with an EC_{50} of 2.5 μM while a similar compound O1602, was significantly more potent at 13 nM. (−) 11-OH-8-Tetrahydrocannabinol-dimethylheptyl (HU210) is a highly potent CB_1 agonist and also demonstrated agonist activity at GPR55 with a potency of 26 nM, which is more than a 100-fold less potent than that found in parallel experiments at the CB_1 receptor (Table 1). A commonly used tool ligand of the cannabinoid system is WIN55,212-2. Consistent with the demonstrated lack of binding activity of this compound in our initial experiments, we observed no functional activity of WIN55,212-2 as either an agonist or antagonist (Figure 5c and Table 1). Finally, we tested the ability of known antagonists of CB_1 receptors for their effect at GPR55. 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281) was without effect as either an agonist or antagonist whereas 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide (AM251) behaved as an agonist with an EC_{50} of 39 nM. In all the experiments described above, the data were the same whether the receptor was expressed with or without the FLAG epitope.

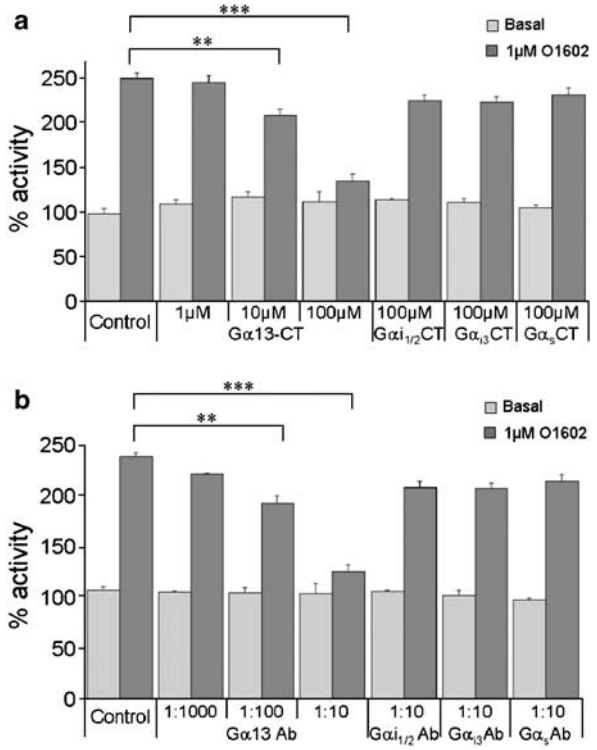

Figure 6 Mapping G-protein coupling of GPR55. (**a**) Basal and 1 μM O1602 stimulated GTPγS binding (% activity, mean±s.e.m.) in human GPR55-expressing membranes in the absence and presence of various concentrations of peptides equivalent to the C termini of $G\alpha_{13}$, $G\alpha_{i1/2}$, $G\alpha_{i3}$ and $G\alpha_s$. (**b**) Basal and 1 μM stimulated GTPγS binding (% activity, mean±s.e.m.) in human GPR55-expressing membranes in the absence and presence of various dilutions of antibodies that bind to the C termini of $G\alpha_{13}$, $G\alpha_{i1/2}$, $G\alpha_{i3}$ and $G\alpha_s$. Data were analysed using paired *t*-test (**$P<0.05$; ***$P<0.01$; $n=5$).

G-protein coupling of GPR55

We next investigated the nature of the signalling pathway activated by GPR55 by examining the G-protein coupling. In the first instance, we examined the effect of Pertussis toxin on the ability of GPR55 to mediate GTPγS binding. Membranes prepared from cells treated with toxin were still able to mediate a robust response to compounds shown to be agonists of GPR55 (data not shown), suggesting that G_i G-proteins are not involved downstream of GPR55. We also tested GPR55-expressing HEK293s cells using FLIPR to determine whether there was evidence of a calcium signal that could be indicative of G_q coupling. No agonist-mediated calcium signalling was detected when compared to untransfected cells suggesting that G_q was not coupling to GPR55. To further investigate the G-protein signalling pathway downstream of GPR55 we took an antibody and peptide blocking approach in the GTPγS assay. Peptides equivalent to the last 12 amino acids of $G\alpha_{i1/2}$, $G\alpha_{i3}$, $G\alpha_s$ and $G\alpha_{13}$ were incubated with GPR55-containing membranes for 15 min prior to performing GTPγS assays. The peptides equivalent to $G_{i1/2}$, G_{i3} and G_s had no effect upon the GTPγS signal consistent with the lack of effect of Pertussis toxin (Figure 6a). However, the G_{13} peptide dose dependently inhibited GTPγS binding suggesting that this peptide makes a specific interaction with GPR55 and prevents the receptor coupling to and activating G_{13} (Figure 6a). A similar experiment was then performed using antibodies raised against the C-terminal peptides of the different G proteins. Consistent with the peptide studies anti-$G_{i1/2}$, anti-G_{i3} and anti-$G\alpha_s$ had no effect upon GTPγS binding mediated by GPR55 (Figure 6b). At the same time, anti-$G\alpha_{13}$ prevented GTPγS binding in a dose-dependent manner, demonstrating

that the GTPγS signal being measured as a consequence of agonist activity at GPR55 was a result of G_{13} activation (Figure 6b).

To further demonstrate that the signalling of GPR55 was $G\alpha_{13}$–mediated, we performed additional studies. Cells stably expressing human GPR55 were transiently transfected with plasmid DNA containing the human $G\alpha_{13}$ gene or with vector control. As shown in Figure 7, while the vector control did not change GTPγS readout, the membranes prepared from the $G\alpha_{13}$-transfected cells showed an augmented signal in response to cannabinoid ligands, indicative of increased expression of the coupling G protein.

Downstream signalling by GPR55

Assuming therefore that GPR55 is $G\alpha_{13}$-coupled, it is reasonable to expect that downstream signalling pathways

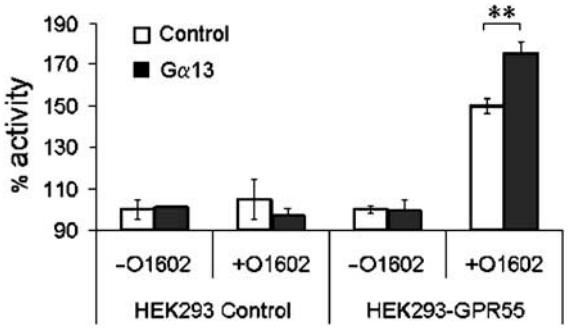

Figure 7 Transfection of $G\alpha_{13}$ into GPR55-expressing HEK293 cells leads to an increased GTPγS signal via GPR55. Membranes prepared from HEK293s cells and HEK293s-GPR55-expressing cells were transfected with transfected with either control or $G\alpha_{13}$-containing plasmids and tested in a GTPγS with and without 1 μM O1602. Membranes containing GPR55 demonstrate a clear increase in GTPγS binding as a result of overexpression of $G\alpha_{13}$. Data (mean ± s.e.m.) were analysed using paired *t*-test (**$P<0.05$; $n=5$).

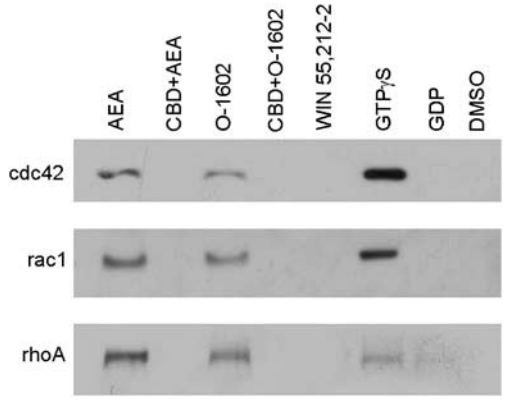

Figure 8 Activation of GPR55 leads to activation of rhoA, cdc42 and rac1. Cells transfected with GPR55 demonstrated O1602-(1 μM) and anandamide (1 μM)-mediated activation of the small G proteins rhoA, cdc42 and rac1 while the non-GPR55-activating ligand WIN55,212-2 had no effect. The activation was blocked by cannabidiol (10 μM) while the positive control GTPγS and negative controls (GDP and dimethyl sulphoxide) generated the expected responses. The blots shown are representative of three independent experiments.

of the G protein will be activated in a GPR55-dependent manner. To this effect, we looked at the activation of rhoA, cdc42 and rac1 in response to various ligands in GPR55-expressing and control HEK293s cells. Figure 8 shows that both anandamide and O1602 but not WIN55,212-2 treatment induced the activation of rhoA, cdc42 and rac1. This effect was blocked by the GPR55 antagonist, cannabidiol.

Discussion and conclusions

In recent years, it has been suggested that there are cannabinoid receptors in addition to CB_1 and CB_2 in brain (Di Marzo *et al*., 2000; Hajos *et al*., 2001; Monory *et al*., 2002), vascular endothelium (Jarai *et al*., 1999) and vascular smooth muscle (Ho and Hiley, 2003) as well as in the immune system (Kaplan *et al*., 2003). In this study, we describe that the orphan G-protein-coupled receptor, GPR55, is expressed in these tissues and is liganded by a range of endogenous, plant-derived and synthetic cannabinoid ligands.

GPR55 was specifically bound and activated by the synthetic cannabinoid ligand CP55940 (Table 1). CP55940 interacts with GPR55 at a potency 25-fold lower than at CB_1 in the comparable experimental system used here. [^{3}H]-CP55940 has been used in several studies (Zimmer *et al*., 1999; Buckley *et al*., 2000) to examine cannabinoid receptor distribution. Because GPR55 binds the radioligand [^{3}H]-CP55940 it may be expected that this radioligand would detect the presence of GPR55, especially in CB_1 and CB_2 knockout mice, but this has not been the case (Zimmer *et al*., 1999). We conclude that the lower affinity of CP55940 for GPR55 without suitably adapted conditions may prevent the detection of GPR55. Taken together, these findings imply that the detection of GPR55 using [^{3}H]-CP55940 in CB_1 knockout mice should be possible if sufficient concentrations of radioligand are used. We have also shown that WIN55,212-2 does not display any activity towards GPR55. Since WIN55,212-2 has been used to define specific binding of [^{3}H]-CP55940 in some of the studies reported, a specific binding to GPR55 would not be detectable. WIN55,212-2 has however been reported to influence activity at a novel cannabinoid receptor in the CNS (Hajos *et al*., 2001) and, as WIN55,212-2 does not bind to or induce activity of GPR55, this receptor is not the brain receptor described, pointing to the presence of at least two novel non-CB_1/CB_2 receptors in the CNS, one of which is GPR55.

Another area of non-CB_1/CB_2 pharmacology relevant for GPR55 is control of vascular tone. We have shown that WIN55,212-2 is not a ligand for GPR55 while abnormal cannabidiol is an agonist and cannabidiol is an antagonist. WIN55,212-2 has been shown to be without effect at novel CB receptors in the vasculature while abnormal cannabidiol behaves as an agonist and cannabidiol is an antagonist (Jarai *et al*., 1999). The finding that cannabidiol is an antagonist of GPR55 is interesting since until recently (Thomas *et al*., 2007) it has not been shown to have any significant effect on CB_1 and CB_2 receptor signalling (Pertwee, 1997), as confirmed by our studies (Table 1). Clearly, the precise pharmacology of this ligand remains to be determined. In addition, O1602, an analogue of abnormal cannabidiol

reported to be active in vaso-relaxation (Jarai *et al*., 1999) was found by us to be a potent agonist of GPR55. Another aspect of the GPR55 pharmacology consistent with a novel cannabinoid receptor in the vasculature is the potent activation by virodhamine (Ho and Hiley, 2004) which appears to be more selective for GPR55 versus CB_1 and CB_2 receptors compared with anandamide (Table 1). Taken together, these findings suggest that GPR55 is a prime candidate for a cannabinoid vascular tone-controlling receptor. Other aspects of the GPR55 receptor may seem inconsistent with a role in vascular tone control. HU210, widely used in the study of cannabinoids, has been shown to affect many physiological processes including vascular tone control and this activity has been attributed to its activity at CB_1 receptors since no effect is observed in CB_1 knockout mice. However, it needs to be considered if appropriate concentrations have been selected to conclusively say that HU210 has no effect through non-CB_1-mediated processes (Jarai *et al*., 1999), since HU210 is more than 100 times less potent at GPR55 than at CB_1 receptors (Table 1)

Yet another aspect of non-CB_1/CB_2 pharmacology that is relevant to GPR55 based on its expression profile, is immune cell function and cell migration. We show that palmitoylethanolamide (PEA) is a potent and selective agonist of GPR55. PEA has been reported to affect inflammatory activities (Lambert *et al*., 2002) and microglial cell migration (Franklin *et al*., 2003) and it has been accepted that these effects, at least in part, are via CB_2 receptors. Nevertheless, PEA has also been demonstrated to be activating anti-inflammatory activities through peroxisome proliferator-activated receptor α mediation (Lo Verme *et al*., 2005) and thus such contributions to an anti-inflammatory effect have to be considered. However, PEA activity in microglial-cell migration also overlaps with an activity of abnormal cannabidiol at the so-called abnormal cannabidiol-sensitive receptors in the same cells (Franklin and Stella, 2003), and could be said to advocate GPR55 as a target for its function. (Table 2).

It is also noteworthy that anandamide, the predominant circulating endocannabinoid, activated GPR55 with a potency equivalent to that activating CB_1 and CB_2 receptors, demonstrating that this ligand has the potential to influence signalling by all three receptors equally. Anandamide has been found to be active at non-CB_1/CB_2 receptors (Begg *et al*., 2005) and GPR55 should now be considered a candidate for these receptors. In contrast, PEA, 2-AG and virodhamine show significantly more potent action through GPR55 than through either CB_1 or CB_2, suggesting that GPR55 is more likely to be the cognate receptor for these ligands.

Most of the reports describing non-CB_1/CB_2 receptors suggest that several, though not all (for example Vaccani *et al*. (2005) of these receptors are G_i-coupled, since they appear to be Pertussis toxin sensitive (Begg *et al*., 2005). In contrast, GPR55 appears to be G_{13} coupled at least in the recombinant systems we have tested. This observation may be taken to disqualify GPR55 for a role in the Pertussis toxin-sensitive cannabinoid-mediated activities. However, the mechanism of Pertussis toxin action results in preventing G_i G proteins interacting with their receptors. Since G_i G proteins are highly abundant and the levels of G_{13} are considered to be lower, it should be considered that the Pertussis toxin effect may also be a consequence of G_{13} being bound and sequestered by G_i-coupled receptors resulting in a dominant-negative effect. Furthermore, we have demonstrated that GPR55 also mediates activation of the small G proteins rhoA, cdc42 and rac1. Such an observation is consistent with the G13 coupling we have described and fits well with the cannabidiol-mediated effects on cell migration that are Pertussis toxin insensitive and described for non-CB_1/CB_2 cannabinoid receptors in glial cells (Vaccani *et al*., 2005).

The results presented herein demonstrate that the orphan G-protein-coupled receptor, GPR55, binds a range of endogenous, plant-derived and synthetic cannabinoid ligands. While the data themselves do not, at this stage, point to an unequivocal role for this receptor in any particular cannabinoid function, the comparative ligand profile that we have described provides the tools to start dissecting the functions of GPR55.

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Conflict of interest

The authors state no conflict of interest.

References

Anderson-Baker WC, McLaughlin CL, Baile CA (1979). Oral and hypothalamic injections of barbiturates, benzodiazepines and cannabinoids and food intake in rats. *Pharmacol Biochem Behav* **11**: 487–491.

Begg M, Pacher P, Bátkai S, Osei-Hyiaman D, Offertáler L, Ming Mo F *et al*. (2005). Evidence for novel cannabinoid receptors. *Pharmacol Ther* **106**: 133–145.

Berry EM, Mechoulam R (2002). Tetrahydrocannabinol and endocannabinoids in feeding and appetite. *Pharmacol Ther* **95**: 185–190.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM *et al*. (2003). The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. [see comment]. *J Biol Chem* **278**: 11303–11311.

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D *et al*. (2003). The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278**: 11312–11319.

Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC *et al*. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur J Pharmacol* **396**: 141–149.

Cainazzo MM, Ferrazza G, Mioni C, Bazzani C, Bertolini A, Guarini S (2002). Cannabinoid CB(1) receptor blockade enhances the

protective effect of melanocortins in hemorrhagic shock in the rat. *Eur J Pharmacol* **441**: 91–97.
Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M *et al.* (2003). The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. [see comment]. *J Clin Invest* **112**: 423–431.
Demuth DG, Molleman A (2006). Cannabinoid signalling. *Life Sci* **78**: 549–563.
Derocq JM, Jbilo O, Bouaboula M, Segui M, Clere C, Casellas P (2000). Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. *J Biol Chem* **275**: 15621–15628.
Devane WA, Dysarz III FA, Johnson MR, Melvin LS, Howlett AC (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34**: 605–613.
Di Marzo V, Breivogel CS, Tao Q, Bridgen DT, Razdan RK, Zimmer AM *et al.* (2000). Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. *J Neurochem* **75**: 2434–2444.
Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, Stella N (2003). Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* **23**: 7767–7775.
Franklin A, Stella N (2003). Arachidonylcyclopropylamide increases microglial cell migration through cannabinoid CB2 and abnormal-cannabidiol-sensitive receptors. *Eur J Pharmacol* **474**: 195–198.
Fride E (2002). Endocannabinoids in the central nervous system—an overview. *Prostaglandins Leukot Essent Fatty Acids* **66**: 221–233.
Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P *et al.* (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232**: 54–61.
Hajos N, Ledent C, Freund TF (2001). Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience* **106**: 1–4.
Ho WS, Hiley CR (2003). Vasodilator actions of abnormal-cannabidiol in rat isolated small mesenteric artery. *Br J Pharmacol* **138**: 1320–1332.
Ho WS, Hiley CR (2004). Vasorelaxant activities of the putative endocannabinoid virodhamine in rat isolated small mesenteric artery. *J Pharm Pharmacol* **56**: 869–875.
Howlett A (2004). Efficacy in CB1 receptor-mediated signal transduction. *Br J Pharmacol* **142**: 1209–1218.
Howlett AC (2002). The cannabinoid receptors. *Prostaglandins Other Lipid Mediat* **68-69**: 619–631.
Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR *et al.* (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci USA* **96**: 14136–14141.
Kaplan BL, Rockwell CE, Kaminski NE (2003). Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes. *J Pharmacol Exp Ther* **306**: 1077–1085.
Lambert DM, Vandevoorde S, Jonsson KO, Fowler CJ (2002). The palmitoylethanolamide family: a new class of anti-inflammatory agents? *Curr Med Chem* **9**: 663–674.
Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A *et al.* (2005). The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* **67**: 15–19.
Maccarrone M, Falciglia K, Di Rienzo M, Finazzi-Agro A (2002). Endocannabinoids, hormone-cytokine networks and human fertility. *Prostaglandins Leukot Essent Fatty Acids* **66**: 309–317.
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. [see comment]. *Nature* **346**: 561–564.
Mechoulam R (1970a). Marihuana chemistry. *Science* **168**: 1159–1166.
Mechoulam R, Shani A, Edery H, Grunfeld Y (1970b). Chemical basis of hashish activity. *Science* **169**: 611–612.
Monory K, Tzavara ET, Leximе J, Ledent C, Parmentier M, Borsodi A *et al.* (2002). Novel, not adenylyl cyclase-coupled cannabinoid binding site in cerebellum of mice. *Biochem Biophys Res Commun* **292**: 231–235.
Munro S, Thomas KL, Abu-Shaar M (1993). Molecular characterization of a peripheral receptor for cannabinoids. [see comment]. *Nature* **365**: 61–65.
Noguchi K, Ishii S, Shimizu T (2003). Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**: 25600–25606.
Ong WY, Mackie K (1999). A light and electron microscopic study of the CB1 cannabinoid receptor in primate brain. *Neuroscience* **92**: 1177–1191.
Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S *et al.* (2005). Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. [see comment]. *J Clin Invest* **115**: 1298–1305.
Pacheco MA, Ward SJ, Childers SR (1993). Identification of cannabinoid receptors in cultures of rat cerebellar granule cells. *Brain Res* **603**: 102–110.
Parolaro D, Massi P, Rubino T, Monti E (2002). Endocannabinoids in the immune system and cancer. *Prostaglandins Leukot Essent Fatty Acids* **66**: 319–332.
Pertwee RG (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74**: 129–180.
Pertwee RG (2001). Cannabinoid receptors and pain. *Prog Neurobiol* **63**: 569–611.
Sawzdargo M, Nguyen T, Lee DK, Lynch KR, Cheng R, Heng HH *et al.* (1999). Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Res Mol Brain Res* **64**: 193–198.
Sofia RD, Knobloch LC (1976). Comparative effects of various naturally occurring cannabinoids on food, sucrose and water consumption by rats. *Pharmacol Biochem Behav* **4**: 591–599.
Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, Pertwee RG (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists *in vitro*. *Br J Pharmacol* **150**: 613–623.
Vaccani A, Massi P, Colombo A, Rubino T, Parolaro D (2005). Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. *Br J Pharmacol* **144**: 1032–1036.
Valk P, Verbakel S, Vankan Y, Hol S, Mancham S, Ploemacher R *et al.* (1997). Anandamide, a natural ligand for the peripheral cannabinoid receptor is a novel synergistic growth factor for hematopoietic cells. *Blood* **90**: 1448–1457.
Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K *et al.* (2005). Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* **310**: 329–332.
Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G *et al.* (2003). Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* **23**: 1398–1405.
Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI (1999). Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. [see comment]. *Proc Natl Acad Sci USA* **96**: 5780–5785.