Cannabigerol Action at Cannabinoid CB₁ and CB₂ Receptors and at CB₁–CB₂ Heteroreceptor Complexes

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Cannabigerol (CBG) is one of the major phytocannabinoids present in Cannabis sativa L. that is attracting pharmacological interest because it is non-psychotropic and is abundant in some industrial hemp varieties. The aim of this work was to investigate in parallel the binding properties of CBG to cannabinoid CB₁ (CB₁R) and CB₂ (CB₂R) receptors and the effects of the compound on agonist activation of those receptors and of CB₁–CB₂ heteroreceptor complexes. Using [³H]-CP-55940, CBG competed with low micromolar Kᵢ values the binding to CB₁R and CB₂R. Homogeneous binding in living cells, which is only technically possible for the CB₂R, provided a 152 nM Kᵢ value. Also interesting, CBG competed the binding of [³H]-WIN-55,212-2 to CB₂R but not to CB₁R (Kᵢ; 2.7 versus >30 μM). The phytocannabinoid modulated signaling mediated by receptors and receptor heteromers even at low concentrations of 0.1–1 μM. cAMP, pERK, β-arrestin recruitment and label-free assays in HEK-293T cells expressing the receptors and treated with endocannabinoids or selective agonists proved that CBG is a partial agonist of CB₂R. The action on cells expressing heteromers was similar to that obtained in cells expressing the CB₂R. The effect of CBG on CB₁R was measurable but the underlying molecular mechanisms remain uncertain. The results indicate that CBG is indeed effective as regulator of endocannabinoid signaling.

Keywords: cannabinoid receptor, cannabigerol, G-protein-coupled receptor, phytocannabinoid, TR-FRET, partial agonist

Abbreviations: Δ⁸-THC, Δ⁹-tetrahydrocannabinol; Δ⁹-THCA, Δ⁹-tetrahydrocannabinolic acid; Δ⁸-THCV, Δ⁸-tetrahyrocannabivarin; 2-AG, 2-arachidonoyl glicerol; AEA, anandamide; CB₁R, cannabinoid receptor 1; CB₂R, cannabinoid receptor 2; CB₇C, cannabichromene; CBD, cannabidiol; CBDA, cannabidivarin; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinoide; CNS, central nervous system; DMR, dynamic mass redistribution; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; SNAP, protein used as a tag; it contains circa 180 amino acids and may be covalently labeled with different probes; Tb, terbium; TLB, Tag-lite labeling medium.
INTRODUCTION

Cannabinoid compounds bind and activate cannabinoid CB₁ (CB₁R) and CB₂ (CB₂R) receptors, which belong to the superfamily of G-protein-coupled receptors. There are many ways to classify them, but the most used distinguishes between endogenous molecules (endocannabinoids), phytocannabinoids and synthetic cannabinoids. Endocannabinoids and one of the most studied phytocannabinoids, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), are agonists with more or less CB₁R/CB₂R selectivity. Furthermore, synthetic cannabinoids mainly act (as agonists or antagonists) by binding to the orthosteric site of receptors (Mechoulam, 2016). Indeed, there is a limited number of molecules, either synthetic or phytocannabinoids, that behave as allosteric modulators of cannabinoid receptor function.

Anandamide and 2-arachidonoyl glycerol (2-AG) are the two main endocannabinoids, being synthesized from membrane lipids and having an alkyl-amide chemical structure. They are retrograde effectors being produced in the post-synaptic neuron to act in the pre-synaptic neuron where they regulate the release of neurotransmitters (Diana and Marty, 2004).

Phytocannabinoids are phenolic terpenes biosynthesized in nature nearly exclusively in the Cannabis sativa L. plant. In the Cannabis plant, all cannabinoids are biosynthesized in the acid form, mainly Δ⁹-TCHA, CBDA, etc. CBGA is the first molecule formed in the biosynthetic pathway and the substrate of Δ⁹-tetrahydrocannabinol-synthase and CBD-synthase (Fellermeier and Zenk, 1998). The pharmacologic effects of Cannabis components, traditionally consumed through inhalation, are attributed to the decarboxylated neutral products of above mentioned acids: Δ⁹-THC, CBD, and CBG.

Synthetic cannabinoids are very different in chemical structure. For instance, they may be indoles like WIN-55,212-2, AM-1241 or JWH-018, or phenolic, phenols lacking the pyrene ring, like CP-55,940 or HU-308. All these compounds have been used in cannabinoid research and have helped to unveil pharmacological aspects of the endocannabinoid system. It should be noted that some of these compounds have recently arrived at the streets sold as legal highs, thus raising Public Health concerns (Adams et al., 2017; Weinstein et al., 2017).

The endocannabinoid system is constituted by the endogenous cannabinoids, the enzymes that produce and degrade them, and by the receptors that mediate their actions. Whereas endocannabinoids consist of molecules with aliphatic structure, AEA and 2-AG, the structure of natural cannabinoids, derived from C. sativa L., is fairly different [see (Lu and Mackie, 2016) and references therein]. Although it is well established that one of the main active components of the plant and one of the few that are psychoactive, namely Δ⁹-THC, acts via cannabinoid receptors, there is controversy on whether these receptors mediate the action of phytocannabinoids such as CBN, CBD or CBG. As happened the last years for CBD, a new research and revision of the cannabinoid receptor pharmacology must be done with the rest of phytocannabinoids as CBG. A further phenomenon that may be considered to understand the action of molecules from C. sativa L. and its extracts is the fact that cannabinoid receptors may form heteromers, namely CB₁–CB₂ heteroreceptors, which display particular functional properties (Callén et al., 2012). It should be noted that in CNS those heteromers are mainly expressed in pallidal neurons (Lanciego et al., 2011; Sierra et al., 2015) and in activated microglia (Navarro et al., 2018a).

Cannabigerol was isolated, characterized and synthetized by the same researchers than reported the structure of the main psychotropic agent of Cannabis, Δ⁹-THC (Gaoni and Mechoulam, 1964). Few years later in vivo assays showed that CBG was non-psychoactive (Grunfeld and Edery, 1969; Mechoulam et al., 1970). The lower concentration and the lack of psychoactivity was probably the cause that CBG was shadowed by Δ⁹-THC. In fact, CBG has attracted less attention than Δ⁹-THC and even than CBD, but nowadays is gaining interest among the scientific community. Some commercial hemp varieties have CBG and CBGA as main cannabinoids and, therefore, CBG is another of the phytocannabinoids to be considered by the unregulated market of hemp oils and derivatives. As recently pointed out, the increased therapeutic potential of C. sativa L. components requires a more in deep understanding of the pharmacology of phytocannabinoids other than Δ⁹-THC, namely CBD, CBG, CBN, Δ⁹-THCV, Δ⁸-THC, CBC and CBDV (Turner et al., 2017).

Preliminary results using membranes from mice brain or from CHO cells expressing the human CB₂R led to postulate that CBG could be a partial agonist at both CB₁R and CB₂R with Kᵢ values in the 300–500 nM range (Gauson et al., 2007; Pertwee, 2008). The first published data on the binding of CBG to human CB₁R and CB₂R were provided by (Rosenthaler et al., 2014) working with [³H]CP-55,940 as radioligand and with preparations from Sf9 cells co-expressing one receptor and the Gα/i3β1y2 protein. The Kᵢ values obtained in competition assays are 897 and 153 nM for CB₁R and CB₂R, respectively. CBG may modulate the activity of transient receptor potential channels of ankyrin type-1; however, the EC₅₀ values lie in the micromolar range (De Petrocellis et al., 2008). It has been reported that CBG binds to CB₁R (Kᵢ = 381 nM) from mouse brain membranes and CB₂R (Kᵢ = 2.6 µM) from CHO cells expressing the human receptor; CBG at high concentrations (10 µM) antagonized [³⁵S]GTPyS binding in mouse brain membranes treated with AEA or CP-55940 (Cascio et al., 2010). Authors also reported CBG as α₂-adrenoceptor agonist at nanomolar levels (EC₅₀ = 0.2 nM), and being also able to antagonize [³⁵S]GTPyS binding upon stimulation of the 5HT₁A receptor by 1 µM 8-OH-DPAT (Cascio et al., 2010). Other findings indicate that CBG can act as (i) agonist/desensitizer of TRPA1 (EC₅₀ = 700 nM), (ii) agonist of TRPV1 (EC₅₀ = 1.3 µM) (iii) agonist of TRPV2 (EC₅₀ = 1.7 µM), (iv) antagonist of TRPM8 channels (IC₅₀ = 160 nM) and v) inhibitor of AEA cell uptake (Kᵢ = 11.3 µM) (De Petrocellis et al., 2011). More recently, the PPARγ has been reported as target of the phytocannabinoid CBG (Kᵢ = 11.7 µM) that at high concentrations, in the 10–25 µM range, may enhance the PPARγ transcriptional activity (Granja...
were calculated as weight (%) versus a commercial standard. All samples were analyzed in duplicate. The results of each experiment, conditions, CBG mainly acts on CB2 receptors and behaves as a partial agonist.

MATERIALS AND METHODS

Reagents

ACEA, JW133, and AEA were purchased from Toarcis Bioscience (Bristol, United Kingdom). CBD and CBG analytical standard solutions were purchased from THCpharm (Frankfurt, DE). Concentrated (10 mM) stock solutions prepared in ethanol (CBG, ACEA, and AEA) or DMSO (JW133 and CM-157) were stored at −20°C. In each experimental session, aliquots of concentrated solutions of compounds were thawed and conveniently diluted in the appropriate experimental solution. For non-radioactive binding assays, TLB was obtained from Cisbio Bioscience (Bristol, United Kingdom), CBD and CBG analytical standard solutions were purchased from THCpharm (Frankfurt, DE).

Cannabidiol was purified from dried leaves and inflorescences of the Cannabis variety SARA (CPVO file number: 20150098), CBG from the variety AIDA (CPVO file number: 20160167) following a previously described method (Nadal, 2016) that provides compounds with >95% purity. An Agilent liquid chromatography set-up (Model 1260, Pittsburgh, PA, United States) consisting of a binary pump, a vacuum degasser, a column oven, an autosampler and a diode array detector (DAD) equipped with a 150 mm length × 2.1 mm internal diameter, 2.7 µm pore size Poroshell 120 EC-C18 column was used for the quality control of the purified cannabinoids. The analysis was performed using water and acetonitrile both containing ammonium formate 50 mM as mobile phases. Flow-rate was 0.2 mL/min and the injection volume was 3 µL. Chromatographic peaks were recorded at 210 nm. All determinations were carried out at 35°C. All samples were analyzed in duplicate. The results of each cannabinoid purity, 96.04% for CBD and 99.9% for CBG, were calculated as weight (%) versus a commercial standard from THCpharm (CBD batch n° L01258-M-1.0; CBG batch n° L01260-M-1.0).

Radioligand Binding Assays

Cell Culture and Membrane Preparation

For radioligand binding experiments CHO cells, stably transfected with cDNA for human CB1 or CB2 cannabinoid receptors, were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and geneticin (G418, 0.4 mg/mL) at 37°C in a humid atmosphere of 5% CO2. Membranes were prepared from cells washed with PBS and scraped off plates in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at 40,000 × g.

Saturation Binding Experiments

[3H]-CP-55940 saturation binding experiments (specific activity 169 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.03 – 10 nM) in binding buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM MgCl2 for CB1R or 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl2 for CB2R) using CHO membranes expressing the human versions of CB1R or CB2R (10 µg protein/sample) at 30°C. Non-specific binding was determined in the presence of 1 µM WIN-55,212-2. At the end of the incubation period (90 min for CB1R or 60 min for CB2R) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

[3H]-WIN-55,212-2 saturation binding experiments (specific activity 48 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.5–100 nM for CB1R or 0.2–40 nM for CB2R) in binding buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl2) with CB1R- or CB2R-containing CHO cell membranes (10 µg protein/sample) at 30°C. Non-specific binding was determined in the presence of 1 µM WIN-55,212-2. At the end of the incubation period (60 min) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

Competition Binding Experiments

[3H]-CP-55940 competition binding experiments were performed incubating 0.3 nM of radioligand and different concentrations of the tested compounds with membranes obtained from CHO cells expressing human CB1 or CB2 receptors (10 µg protein/sample) for 90 min (CB1R) or 60 min (CB2R) at 30°C. Non-specific binding was determined in the presence of 1 µM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).
Competition binding experiments were also performed incubating 3 nM [³H]-WIN-55,212-2 and different concentrations of the tested compounds with membranes obtained from CHO cells transfected with human CB₁ or CB₂ receptors (10 µg protein/sample) for 60 min at 30°C. Non-specific binding was determined in the presence of 1 µM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Homogeneous Binding Assays in Living Cells

Expression Vector
cDNAs for the human version of cannabinoid CB₂R without their stop codon were obtained by PCR and subcloned to SNAP-containing vector (PSNAP; Cisbio Bioassays) using sense and antisense primers harboring unique restriction sites for HindIII and BamHI generating the SNAP tagged CB₂R (CB₂R-SNAP).

Cell Culture and Transfection
For HTRF assays, HEK-293T cells were used. HEK 293T (HEK-293T) cells were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin/streptomycin, and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) [all supplements were from Invitrogen, (Paisley, Scotland, United Kingdom)]. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were passaged, with enzyme-free cell dissociation buffer (13151-014, Gibco®, Thermo Fisher, Waltham, MA, United States), when they were 80–90% confluent, i.e., approximately twice a week. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, Missouri, United States) method as previously described (Navarro et al., 2018b) and used for functional assays 48 h later (unless otherwise stated).

Labeling of Cells Expressing SNAP-Tagged CB₂R
Cell culture medium was removed from the 25-cm² flask and 100 nM SNAP-Lumi4-Tb, previously diluted in 3 mL of TLB 1X, was added to the flask and incubated for 1 h at 37°C under 5% CO₂ atmosphere in a cell incubator. Cells were then washed four times with 2 mL of TLB 1X to remove the excess of SNAP-Lumi4-Tb, detached with enzyme-free cell dissociation buffer, centrifuged 5 min at 1,500 rpm and collected in 1 mL of TLB 1X. Tag-lite-based binding assays were performed 24 h after transfection. Densities in the 2,500–3,000 cells/well range were used to carry out binding assays in white opaque 384-well plates.

Non-radioactive Competition Binding Assays
For competition binding assays, the fluorophore-conjugated CB₂R ligand (labeled CM-157), unconjugated CM-157 and CBG were diluted in TLB 1X. HEK-293T cells transiently expressing Tb-labeled SNAP-CB₂R with or without CB₁R were incubated with 20 nM fluorophore-conjugated CB₂R ligand, in the presence of increasing concentrations (0–10 µM range) of CBG or CM-157. Plates contained 10 µL of labeled cells, and 5 µL of TLB 1X or 5 µL of CBG or 5 µL CM-157 were added prior to the addition of 5 µL of the fluorescent ligand. Plates were then incubated for at least 2 h at room temperature before signal detection. Detailed description of the HTRF assay is found in Martínez-Pinilla et al. (2016).

Signal was detected using an EnVision microplate reader (PerkinElmer, Waltham, MA, United States) equipped with a FRET optic module allowing donor excitation at 337 nm and signal collection at both 665 and 620 nm. A frequency of 10 flashes/well was selected for the xenon flash lamp excitation. The signal was collected at both 665 and 620 nm using the following time-resolution settings: delay, 150 µs; integration time, 500 µs. HTRF® ratios were obtained by dividing the acceptor (665 nm) by the donor (620 nm) signals and multiplying by 10,000. The 10,000-multiplying factor is used solely for the purpose of easier data handling.

Functional Assays

Cell Culture and Transient Transfection
HEK-293T cells were grown in DMEM medium (Gibco, Paisley, Scotland, United Kingdom) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, MEM Non-Essential Amino Acids Solution (1/100) and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO₂ at 37°C. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, Missouri, United States) method as previously described (Navarro et al., 2018b) and used for functional assays 48 h later (unless otherwise stated).

cAMP Determination
Signaling experiments have been performed as previously described (Navarro et al., 2010, 2016, 2018b; Hinz et al., 2018). Two hours before initiating the experiment, HEK-293T cell-culture medium was replaced by serum-starved DMEM medium. Then, cells were detached, resuspended in growing medium containing 50 µM zarzadaverine and placed in 384-well microplates (2,500 cells/well). Cells were pretreated (15 min) with CBG -or vehicle- and stimulated with agonists (15 min) before adding 0.5 µM forskolin or vehicle. Readings were performed after 15 min incubation at 25°C. HTRF energy transfer measures were performed using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, United States). Fluorescence at 665 nm was analyzed in a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab Technologies, Offenburg, Germany).

ERK Phosphorylation Assays
To determine ERK1/2 phosphorylation, 50,000 HEK-293T cells/well were plated in transparent Deltalab 96-well microplates and kept at the incubator for 24 h. 2 to 4 h before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were pre-treated at 25°C for 10 min with vehicle or CBG in serum-starved DMEM medium and stimulated for an additional 7 min with the specific agonists. Cells were then washed twice with cold PBS before addition of lysis
buffer (20 min treatment). 10 µL of each supernatant were placed in white ProxiPlate 384-well microplates and ERK 1/2 phosphorylation was determined using AlphaScreen®SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States).

Dynamic Mass Redistribution Assays (DMR)

Cell mass redistribution induced upon receptor activation was determined by illuminating the underside of a biosensor with polychromatic light and measuring the changes in the wavelength of the reflected monochromatic light. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. HEK-293T cells were seeded in 384-well sensor microplates to obtain 70–80% confluent monolayers constituted by approximately 10,000 cells per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated for 2 h with assay-buffer containing 0.1% DMSO (24°C, 30 µL/well). Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 µL of CBG for 30 min followed by the addition of 10 µL of specific agonists; all test compounds were dissolved in assay buffer. The cell signaling signature was determined using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States) by a label-free technology. Then, DMR responses were monitored for at least 5,000 s. Results were analyzed using EnSpire Workstation Software v 4.10.

β-Arrestin 2 Recruitment

Arrestin recruitment was determined as previously described (Medrano et al., 2017; Navarro et al., 2018b). Briefly, BRET experiments were performed in HEK-293T cells 48 h after transfection with the cDNA corresponding to the CB2-R-YFP or CB2-R-YFP and 1 µg cDNA corresponding to β-arrestin 2-Rluc. Cells (20 µg protein) were distributed in 96-well microplates (Corning 3600, white plates with white bottom) and were incubated with CBG for 15 min and stimulated with the agonist for 10 min prior the addition of 10 µL of specific agonists; all test compounds were dissolved in assay buffer. The cell signaling signature was determined using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States) by a label-free technology. Then, DMR responses were monitored for at least 5,000 s. Results were analyzed using EnSpire Workstation Software v 4.10.

Data Handling and Statistical Analysis

Affinity values (Ki) were calculated from the IC50 obtained in competition radioligand binding assays according to the Cheng and Prusoff equation: \( K_i = \frac{IC_{50}}{1 + [C]/K_D} \), where [C] is the free concentration of the radioligand and \( K_D \) its dissociation constant (Cheng, 2001).

Data from homogeneous binding assays were analyzed using Prism 6 (GraphPad Software, Inc., San Diego, CA, United States). 

\( K_i \) values were determined according to the Cheng and Prusoff equation with \( K_D = 21 \) nM for CM-157 (Cheng, 2001). Signal-to-background (S/B) ratio calculations were performed by dividing the mean of the maximum value (\( \mu_{\text{max}} \)) by that of the minimum value (\( \mu_{\text{min}} \)) obtained from the sigmoid fits.

The data are shown as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Bonferroni’s multiple comparison post hoc test. Significant differences were considered when \( p < 0.05 \).

RESULTS

Saturation and Competition Radioligand-Based Assays in Membranes Expressing CB1R or CB2R

The effect of CBG on radioligand binding to CB1R or CB2R was first tested using the classical radioligand-binding assay in membranes isolated from CHO cells expressing human CB1R or CB2R and incubated with radioligands: [3H]-CP-55940 or [3H]-WIN-55,212-2. Data obtained from binding isotherms using increasing [3H]-CP-55940 or [3H]-WIN-55,212-2 concentrations lead to a monophasic saturation curve. Saturation curves, receptor density (\( B_{\text{max}} \)) and affinity (\( K_D \)) values are shown in Figures 1A–D. The affinity of the two radioligands was in the nanomolar range for both CB1R and CB2R. \( K_D \) for [3H]-CP-55940 to CB1R and CB2R was similar with values around 0.3 nM. \( K_D \) values for WIN-55,212-2 were 9.4 and 3.2 nM for CB1R and CB2R, respectively (Figures 1C,D). Overall the results agree with previously reported data (McPartland et al., 2007; Merighi et al., 2010).

Competition binding assays of WIN-55,212-2 showed similar \( K_i \) values using the two radioligands to CB1R and CB2R and agreed with the \( K_D \) values for [3H]-WIN-55,212-2 binding (Table 1 and Figures 1E,F). Table 1 reports the affinity values of CBG. \( K_i \) values of CBG obtained using [3H]-CP-55940 as radioligand were in the low micromolar range in both CB1R and CB2R. The affinity value of CBG obtained using [3H]-WIN-55,212-2 for CB2R was 2.7 µM, about twofold higher than that obtained using [3H]-CP-55940. Using [3H]-WIN-55,212-2 in competition binding experiments on CB1R, CBG was not able to displace the radioligand (Figures 2A,B). In summary, CBG displayed \( K_i \) values in the low micromolar range when competing for the binding to the CB2R. Surprisingly, significant competition in the binding to the CB1R was only observed when using [3H]-CP-55940 as radioligand.

CBG Binds to the Orthosteric Site of Cannabinoid CB2R at Nanomolar Concentrations

Competition experiments were performed using 20 nM of a fluorophore-conjugated selective CB2R agonist (CM-157) and
FIGURE 1 | Radioligand binding assays to CB$_1$R and CB$_2$R. (A–D) Saturation curves of either $[^{3}H]$-CP-55940 or $[^{3}H]$-WIN-55,212-2 binding on membranes from CHO cells stably expressing human CB$_1$R (A,C) or CB$_2$R (B,D). (E,F) Competition curves for WIN-55,212-2 in radioligand-based assays using either $[^{3}H]$-CP-55940 (E) or $[^{3}H]$-WIN-55,212-2 (F) binding on membranes from CHO cells stably expressing human CB$_1$R or CB$_2$R. Data are expressed as the mean ± SEM of five independent experiments performed in duplicate. $K_D$ (obtained from saturation isotherms) are shown in Table 1.

a homogeneous non-radioactive method performed in living cells expressing SNAP-CB$_2$R (details in Martínez-Pinilla et al., 2016; Figure 2C). Unfortunately, the equivalent fluorophore-conjugated selective CB$_1$R ligand is not available to perform HTRF assays in SNAP-CB$_1$R-expressing living cells. Competition assays were performed in HEK-293T cells expressing Lumi4-Tb-labeled CB$_2$R fused to the SNAP protein and incubated with a fixed amount of the fluorophore-conjugated agonist and different CBG concentrations. As observed in Figure 2, both the unlabelled selective agonist (CM-157) and CBG decreased the binding to SNAP-CB$_2$R in monophasic fashion and with $K_i$ values in the nanomolar range (16 nM for CM-157 and of 152 nM for CBG; Figures 2D,E). The $K_i$ obtained for CM-157 matches with previously reported dissociation constant $K_D$ values (Martínez-Pinilla et al., 2016). These results indicate that CBG can significantly bind to the orthosteric site of cannabinoid CB$_2$R at nanomolar concentrations.

Similar experiments were carried out in HEK-293T cells expressing SNAP-CB$_2$R fusion protein and a similar amount of CB$_1$R, i.e., in cells that express CB$_2$R in a CB$_1$–CB$_2$ receptor heteromer context. In the presence of cannabinoid CB$_1$R the $K_i$ for CM-157 was 19 nM (Figure 2F) and $K_i$ for CBG was reduced
TABLE 1 | Affinity values of CB compounds obtained from radioligand binding assays.

<table>
<thead>
<tr>
<th>[3H]-CP-55940 competition binding experiments</th>
<th>[3H]-WIN-55,212-2 competition binding experiments</th>
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<tbody>
<tr>
<td>CB1 – Kᵦ (nM)</td>
<td>CB2 – Kᵦ (nM)</td>
</tr>
<tr>
<td>0.29 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>CB1 – Kᵦ (nM)</td>
<td>CB2 – Kᵦ (nM)</td>
</tr>
<tr>
<td>8.08 ± 0.65</td>
<td>3.22 ± 0.31</td>
</tr>
<tr>
<td>CBG</td>
<td>CBG</td>
</tr>
<tr>
<td>1.045 ± 0.07</td>
<td>1.225 ± 0.85</td>
</tr>
<tr>
<td>CBD</td>
<td>CBD</td>
</tr>
<tr>
<td>1.690 ± 0.11</td>
<td>1.714 ± 0.70</td>
</tr>
<tr>
<td>CB1 – Kᵦ (nM)</td>
<td>CB2 – Kᵦ (nM)</td>
</tr>
<tr>
<td>9.43 ± 0.83</td>
<td>3.16 ± 0.24</td>
</tr>
<tr>
<td>CBG</td>
<td>CBG</td>
</tr>
<tr>
<td>9.86 ± 0.84</td>
<td>3.48 ± 0.27</td>
</tr>
<tr>
<td>&gt;30,000</td>
<td>2,656 ± 130</td>
</tr>
<tr>
<td>&gt;30,000</td>
<td>4,019 ± 342</td>
</tr>
</tbody>
</table>

Kᵦ values were obtained from saturation isotherms and Kᵦ from data in competition assays using the indicated radiolabelled compounds ([3H]-CP-55940 or [3H]-WIN-55,212-2).

(56 nM, Figure 2G). These results indicate that in cells expressing both cannabinoid receptors, CB₁ and CB₂, CBG shows higher affinity for cannabinoid CB₂R.

**CBG Effects on Cannabinoid Receptor-Agonist-Induced Effects**

Previous reports Gauson et al. (2007), Cascio et al. (2010) suggest that CBG may be a partial agonist of cannabinoid receptors. To investigate this possibility, HEK-293T cells expressing CB₁R or CB₂R were treated with increasing concentrations of CBG (1 nM to 10 µM) and cAMP, MAPK, β-arrestin recruitment and dynamic mass cell redistribution (DMR) assays were developed. Interestingly, it was observed that in cells expressing CB₁R (Figure 3, blue curves), CBG induced a small decrease in forskolin induced cAMP levels and a small increase in β-arrestin recruitment (Figures 3A,C), while having no significant action on MAPK phosphorylation assay (Figure 3B). Consequently, CBG in label-free assays induced a slight effect in the DMR signal (Figure 3D) that is consistent with a G protein-dependent action on cAMP levels; label-free signal is based on optical detection of DMR following receptor activation and mainly reflects G-protein-coupling (Kebig et al., 2009; Schröder et al., 2009; Hamamoto et al., 2015). On the other hand, in HEK-293T cells expressing CB₂R (Figure 3, red curves), the action on forskolin-induced cAMP levels and on the DMR signal was small and similar to that exerted in CB₁R-expressing cells (Figure 3A). On the contrary, the activation of the MAP kinase pathway was notable (Figure 3B). Also noteworthy was the CBG-induced β-arrestin recruitment (Figure 3C). Taken together these data suggest that CBG is a poor agonist of CB₁R, whereas it acts as a partial agonist in some of the signaling pathways analyzed in cells expressing CB₂R.

To further examine the CBG effect over CB₁R, HEK-293T cells expressing CB₁R were treated with the endocannabinoid agonist, AEA, or with ACEA in the presence or in the absence of 100 nM or 1 µM CBG. In forskolin-induced cAMP assays we found that 100 nM or 1 µM CBG pretreatment induced a significant decrease in both, AEA and ACEA induced effects (Figure 4A). In contrast, CBG (100 nM or 1 µM) was unable to modify the agonist-induced MAPK phosphorylation and β-arrestin recruitment (Figures 4B,C). In label-free DMR assays the results were similar to those obtained in cAMP determination assays, i.e., CBG reduced the effect of the agonists (Figure 4D).

Cannabigerol (100 nM or 1 µM) was also tested in HEK-293T cells expressing CB₂R and using AEA and a receptor selective agonist, JWH133. Pretreatment with CBG reduced the effects of AEA and JWH133 in experiments of forskolin-induced cAMP levels, ERK1/2 phosphorylation and in label-free DMR read-outs (Figure 4). In contrast, CBG did not affect the recruitment of β-arrestin induced by agonists (Figure 4G). This last result may be due to the low sensitivity of the assay as β-arrestin recruitment BRET signal was virtually negligible. Energy transfer techniques completely depend on the correct orientation of the fusion proteins and the reduced signal may be due to poor recruitment of β-arrestin and/or to a high distance between BRET donor/acceptor in the putative β-arrestin-Rluc/CB₂R-YFP complex. Thus, CBG in cells activated by endocannabinoids or by selective agonists behaves as a partial agonist of the CB₂R.

**CBG Effect in HEK-293T Cells Expressing CB₁R and CB₂R**

Experiments were finally performed in cells co-expressing the two cannabinoid receptors, which are able to form heteromeric complexes. A CB₁-CB₂ receptor heteromer print consists of a negative cross-talk observed in Akt phosphorylation and neurite outgrowth; i.e., activation of one receptor reduces the signaling originated upon partner receptor activation (Callén et al., 2012). To characterize the CBG effect, experiments were performed in HEK-293T cells expressing the two cannabinoid receptors. Dose-effect curves were provided for cAMP level determination and DMR assays was additive (Figure 5), i.e., the presence of CBG blunted the negative cross-talk in these signaling pathways. However, the negative cross-talk was still evident in both ERK1/2 phosphorylation and β-arrestin recruitment experiments (Figures 5B,C).

Finally, the effect of 100 nM CBG (100 nM) on AEA, ACEA and/or JWH133 actions was investigated in cells co-expressing CB₁R and CB₂R. CBG pretreatment led to significant effects, always reducing the effect of the agonists, in cAMP-related assays (Figure 5E). However, the effect in the other assay types was
FIGURE 2 | Competition by CBG of agonist binding to CB1R and/or CB2R. (A,B) Competition curves for CBG in radioligand-based assays using either [3H]-CP-55940 (A) or [3H]-WIN-55,212-2 (B) binding on membranes from CHO cells stably expressing human CB1R or CB2R. (C) Scheme of the HTRF-based competitive binding assay. The GPCR of interest with the SNAP-tagged enzyme fused to its N-terminal domain is expressed at the cell surface. SNAP is a commercially available tag consisting of circa 180 amino acids, that can be labeled with fluorophores or other probes in a covalent fashion. The GPCR–SNAP-tagged cells are subsequently labeled with a Tb-containing probe (SNAP-Lumi4-Tb) through a covalent bond between the Tb and the reactive side of the SNAP enzyme. The Tb acts as FRET donor of an acceptor covalently linked to a selective CB2 receptor ligand. Thus, upon binding of a fluorophore-conjugated ligand (FRET acceptor) on the donor-labeled SNAP-tagged/GPCR fusion protein, an HTRF signal from the sensitized acceptor can be detected since the energy transfer can occur only when the donor and the acceptor are in close proximity. In competition binding assays using CM-157, the unlabelled specific ligand competes for receptor binding site with the fluorophore-conjugated ligand, leading to a decrease in the HTRF signal detected. (D–G) HEK-293T were transiently transfected with 1 µg cDNA for SNAP-CB2R in the absence (D,E) or presence of 0.5 µg cDNA for CB1R (F,G). Competition curves of specific binding of 20 nM fluorophore-conjugated CM-157 using CM-157 (0–10 µM) (D,F) or of CBG (0–10 µM) (E,G) as competitors are shown. Data represent the mean ± SEM of five experiments in triplicates.
negligible except for the negative modulation of the ACEA effect on ERK1/2 phosphorylation and DMR, and of the AEA effect on DMR read-outs (Figures 5F–H). Therefore, CBG either blunted the cAMP-dependent signaling or did not significantly alter the negative cross-talk when other CB1/CB2-mediated signaling read-outs were determined (see Figures 5B, C). It should be noted that cross-talk at the intracellular signaling level, cannot be ruled out to partly explain some of the findings (Bayewitch et al., 1995; Wartmann et al., 1995; Mcguinness et al., 2009; Peters and Scott, 2009; Van Der Lee et al., 2009).

**DISCUSSION**

The aim of this paper was to comparatively address CBG pharmacology and effects on CB1 and CB2 receptors, and on CB1–CB2 heteroreceptor complexes. The binding experiments using radiolabelled- and non-radiolabelled-based approaches have provided relevant results. The results on CB2R are clear and indicate that CBG acts as a competitive partial agonist ligand. There is, however, an interesting observation as the Ki values for competing both [3H]-CP-55940 and [3H]-WIN-55,212-2 are in the low micromolar range (Table 1), whereas displaying a value of 152 mM in HTRF-based assays. As pointed out in previous reports, the conditions of the approach using a fluorescent-conjugated CM-157 allows identification of different states of the receptor. Irrespective of the molecular mechanism, the marked differences in affinity constants suggest different ways to accommodate the ligand within the orthosteric center. To our knowledge this is the first report performed in parallel binding assays using three different ligands that reportedly bind to the orthosteric center of the CB2R ([3H]-CP-55940, [3H]-WIN-55,212-2 and fluoresecence-conjugated-CM-157). In summary, the most reasonable assumption is that CBG binds to the orthosteric center of CB2R but with marked differences in affinity depending on the assay. It should be noted that differences in affinity may result from the fact that HTRF binding is performed in living cells whereas radioligand binding assays are performed in isolated membranes. The already existing data concerning CBG affinity for CB1 and CB2 receptors, all performed using [3H]-CP-55940 also indicate that the affinity may vary depending on the context of the receptor, by inter alia the constraints of the membrane, heteromerization or interaction with G-proteins. Comparing our results with similar data using [3H]-CP-55940, the affinity is higher for receptors expressed in HEK-293 cells or in brain membranes (Gauson et al., 2007; Pertwee, 2008; Pollastro et al., 2011) that in receptors expressed in CHO cells (Table 1). In competition assays of radioligand binding to CB1R or to CB2R, affinity for CBG is similar to that previously published (Gauson et al., 2007; Pertwee, 2008), except in the case of Sf9 cells (K<sub>i</sub> : 897 and 153 mM for, respectively, CB1R and CB2R). This piece of data would indicate conformational changes induced by third molecules that affect the binding of the radioligand and/or of CBG. In fact, Sf9 are insect cells that do not express the cognate G<sub>i</sub> protein and, therefore, Gai3β1γ2 was heterologously expressed to perform the
binding assays that led to different affinities for CBG (897 and 153 nM for, respectively, CB1R and CB2R) (Rosenthaler et al., 2014).

The results from binding to the CB1R are not very robust and more difficult to interpret. Unfortunately, there are no ligands available to perform HTRF binding to SNAP-CB1R-expressing living cells, whereas the data from competition assays using [3H]-CP-55940 or [3H]-WIN-55,212-2 were contradictory. On the one hand, the Ki for binding to the CB1R using [3H]-CP-55940 was in the low micromolar range, as it occurred with data from radioligand binding to the CB2R. However, CBG was unable to compete [3H]-WIN-55,212-2 binding to the CB1R. Taking into account that recognition sites for CP-55940 or WIN-55,212-2 were identical in the CB1R and CB2R, one possibility is that CBG binds to the orthosteric center but displaying different equilibrium binding parameters depending on the radioligand. It was early observed that Lys192 in the CB1R third transmembrane domain (TM3) was crucial for binding of CP-55940 and AEA but not for WIN-55,212-2 (Bonner et al., 1996; Chin et al., 1998). Later, in silico models pointed to an hydrophobic pocket for CP-55940 binding that involved residues in different transmembrane domains (not only in TM3) and in the second extracellular loop (Shim et al., 2003). Those models showed that WIN-55,212-2 not only binds to the hydrophobic pocket described for CP-55940 but to another hydrophobic region involving residues in TM2 and TM3 (Shim and Howlett, 2006). The structure of CBG is more similar to CP-55940 than to WIN-55,212-2, bearing an OH in the A ring that may interact with the TM3 Lys192 residue. In brief, CBG binds to the orthosteric center of CB1R as indicated by the fact that CBG affects CP-55940 binding without affecting the binding of [3H]-WIN-55,212-2. In other words, CBG was able to distinguish between two subregions of the CB1R orthosteric center. We therefore suggest that pharmacological studies concerning the CB1R should be run in parallel using radiolabelled CP-55940 and WIN-55,212-2. Interestingly CP-55940 and WIN-55,212-2 are able to fix the CB1R in two different conformations (Georgieva et al., 2008) and, therefore, CBG would affect more the conformation and signaling arising from occupation of the CP-55940 binding site. Other possibilities cannot be ruled out and, in this respect,
Taking together all results, an allosteric action of CBG on the living cells (Martínez-Pinilla et al., 2016), cannot be ruled out. In the case of CBG action of CBG on a particular state of the receptor, which, the binding of H-α[3]WIN-55,212-2 to the CB1R, the phenomenon is known as functional selectivity or biased agonism. In cells expressing CB1R, CBG effect is skewed toward the CB1R-mediated functional assays were easier to interpret. First of all, the efficacy was lower compared to selective synthetic agonists and endocannabinoids. Also, CBG led to biased agonism as the effect on cAMP levels was small while being quite marked in ERK phosphorylation and β-arrestin recruitment. Therefore, CBG acted as a partial agonist and, as such, it was able to reduce the effects of other cannabinoid agonists. At 1 µM the effect of CBG on receptor activation by other agonists was similar to that exerted by 100 nM JWH133 (Figure 4) thus suggesting that the effective affinity in living cells is that obtained in HTRF non-radioactive-based assays.

Due to the complex pharmacology of cannabinoids this research was undertaken to investigate whether CBG could be exerting a differential action on the CB1–CB2 receptor heteromers. Previous data have shown that the interplay between the two receptors in an heteromeric context is also complex. Whereas Callén et al. (2012) showed a negative cross-talk in signaling. Another possibility, which was suggested for AM630, a previously considered CB2R antagonist (Bolognini et al., 2012), is that CBG is a protean agonist displaying biased agonism.

Data from CB2R-mediated functional assays were easier to interpret. First of all, the efficacy was lower compared to selective synthetic agonists and endocannabinoids. Also, CBG led to biased agonism as the effect on cAMP levels was small while being quite marked in ERK phosphorylation and β-arrestin recruitment. Therefore, CBG acted as a partial agonist and, as such, it was able to reduce the effects of other cannabinoid agonists. At 1 µM the effect of CBG on receptor activation by other agonists was similar to that exerted by 100 nM JWH133 (Figure 4) thus suggesting that the effective affinity in living cells is that obtained in HTRF non-radioactive-based assays.

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**FIGURE 5** Effect of CBG in cells expressing CB1 and CB2 receptors. (A–D) Effect of CBG in HEK-293T cells transfected with 0.75 µg cDNA for CB1R and 1 µg cDNA for CB2R (A,B,D) or 1 µg cDNA for β-arrestin-Fluc, 0.75 µg cDNA for CB1R and 1 µg cDNA for CB2R-YFP (C). Dose-effect curves for cAMP production are expressed as % of levels obtained by 0.5 µM forskolin treatment (A). Dose-effect curves for ERK1/2 phosphorylation are expressed as % respect of basal levels (B). Dose-effect curves for β-arrestin recruitment (C) and label-free (D) assays are expressed, respectively, in mBRET units and pm. Dotted lines (red and blue) are the same than those shown in Figure 3 and serve as a reference for differential effects in cells coexpressing both receptors. Data are the mean ± SEM of a representative experiment in triplicates (n = 6). (E–H) HEK-293T cells transfected with 0.75 µg cDNA for CB1R and 1 µg cDNA for CB2R or 1 µg cDNA for CB2R-R-YFP (G) were treated with 100 nM AEA or a selective CB2R ligand (100 nM JWH133 in the absence (black bars) or presence (white bars) of 100 nM CBG. cAMP production (E) is expressed as % of levels obtained by 0.5 µM forskolin. ERK1/2 phosphorylation data are expressed as % respect of basal levels (F). Data for β-arrestin recruitment (G) and label-free (H) assays are expressed, respectively, in mBRET units and pm. Data represent the mean ± SEM of six different experiments performed with three replicates. One-way ANOVA and Bonferroni’s multiple comparison post hoc test were used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001; versus treatment with AEA, ACEA, or JWH133 alone).
a heterologous expression system, the allosteric interaction in the CB$_1$–CB$_2$ heteroreceptor complex is synergistic in primary cultures of activated microglia activated with LPS and interferon gamma and in primary cultures of microglia from a transgenic model of Alzheimer’s disease (Navarro et al., 2018a). Dose-effect experiments here undertaken in the HEK-293T-based heterologous expression system showed that CBG treatment in the absence of any other agonist, led to additive/synergistic effects on cAMP and label-free read-outs. In contrast, in ERK phosphorylation and β-arrestin recruitment, we found the negative cross-talk already described for this heteromer when full agonists are used to activate the receptors (Callén et al., 2012). These results suggest that partial agonism on the CB$_2$R is regulated by the presence of CB$_1$R; however, more complex alternative scenarios cannot be ruled out as CBG may act on the orthosteric site of the CB$_2$R protomer and as protein agonist of the CB$_1$R protomer. In cells expressing the two receptors, the overall effect of 100 nM CBG on agonist-induced activation is more consistent with acting on CB$_2$R than on CB$_1$R. In fact, the results in co-expressing cells, which likely express heteromers, are similar to those encountered in CB$_2$R-expressing cells. In summary, CBG significantly modulates CB$_2$R- or CB$_1$R/CB$_2$R-mediated endocannabinoid action, while the effects are weak in CB$_1$R-expressing cells. Our findings demonstrating the action of CBG on the cannabinoid receptors are in complete agreement and may explain the in vitro results, reporting the protection of macrophages against oxidative stress (Giacoppo et al., 2017), and the beneficial in vivo effects in a model of inflammatory bowel disease (Borrelli et al., 2013). In the first of these two studies CBG-mediated protection is blocked by AM630, a selective CB$_2$R ligand, whereas the CB$_1$R antagonist, SR141716A, had no effect on CBG action (Giacoppo et al., 2017). The second study reported that CBG may both reduce the histological and molecular changes of experimental colitis and nitrite release from macrophages after LPS stimulation; again these effects were seemingly mediated by CB$_1$R (Borrelli et al., 2013). These results can be explained by our findings; CBG acting as a partial agonist and exerting actions via CB$_2$R in macrophages (Giacoppo et al., 2017) or “antagonizing” the effects of endogenous or synthetic cannabinoids, as in LPS-stimulated macrophages (Borrelli et al., 2013). In conclusion, the results presented in this study reveal that the non-psycho tropic phytocannabinoid, CBG, may exert beneficial actions with therapeutic potential via cannabinoid receptors.

**AUTHOR CONTRIBUTIONS**

XN and RF had the original idea, designed and coordinated actions in the different participating institutions, and wrote the initial manuscript. GN performed non-radiolabelled-based homogeneous binding assays, participated in the signaling experiments, and significantly contributed to manuscript preparation. IR-R participated in the signaling experiments and in writing methods. RR-S actively participated in data analysis and parameter calculation. EC supervised data analysis, provided pharmacological expertise, and insight into data interpretation. FV performed the radioligand binding experiments. KV and PB performed the radioligand binding data analysis and interpretation. SC selected the Cannabis varieties and supervised the production of the vegetal raw material used for the isolation and purification of cannabinoids. VSM performed the isolation and purification of cannabinoids. CS-CC and CF-V performed the analytical quality control to the purified cannabinoids. All co-authors critically revised, contributed to the editing, and approved the manuscript.

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**Conflict of Interest Statement:** Authors declare that this research was undertaken in collaboration with Phytoplant Research S.L. Co-authors working in the Spanish and Italian public institutions do not receive honoraria from the company and do not have any participation in the company (stock shares or similar).

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