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Similarities and differences upon binding of naturally occurring Δ^9-tetrahydrocannabinol-derivatives to cannabinoid CB_1 and CB_2 receptors

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ABSTRACT

We have here assessed, using Δ^9-tetrahydrocannabinol (Δ^9-THC) for comparison, the effect of Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and of Δ^9-tetrahydrocannabivarin (Δ9-THCV) that is mediated by human versions of CB_1, CB_2, and CB_1-CB_2 receptor functional units, expressed in a heterologous system. Binding to the CB_1 and CB_2 receptors was addressed in living cells by means of a homogeneous assay. A biphasic competition curve for the binding to the CB_2 receptor, was obtained for Δ^9-THCV in cells expressing the two receptors. Signaling studies included cAMP level determination, activation of the mitogen-activated protein kinase pathway and ß-arrestin recruitment were performed. The signaling triggered by Δ^9-THCA and Δ^9-THCV via individual receptors or receptor heteromers disclosed differential bias, i.e. the bias observed using a given phytocannabinoid depended on the receptor (CB_1, CB_2 or CB_1-CB_2) and on the compound used as reference to calculate the bias factor (Δ^9-THC, a selective agonist or a non-selective agonist). These results are consistent with different binding modes leading to differential functional selectivity depending on the agonist structure, and the state (monomeric or heteromeric) of the cannabinoid receptor. In addition, on studying Gi-coupling we showed that Δ^9-THCV and Δ^9-THCA and Δ^9-THCV were able to revert the effect of a selective CB_2 receptor agonist, but only Δ9-THCV, and not Δ9-THCA, reverted the effect of arachidonyl-2′-chloroethylamide (ACEA 100 nM) a selective agonist of the CB_1 receptor. Overall, these results indicate that cannabinoids may have a variety of binding modes that results in qualitatively different effects depending on the signaling pathway that is engaged upon cannabinoid receptor activation

1. Introduction

The effects of *Cannabis* smoking led to suspect the existence in the central nervous system of receptors that are now known as cannabinoid receptors [1–4]. The two proteins that are considered as cannabinoid receptors are the CB_1 and the CB_2; both belong to the superfamily of G protein-coupled receptors (GPCRs). The two main endogenous agonists of these receptors, 2-arachidonoylglycerol (2-AG), and anandamide (N-arachidonoylethanolamine), have an aliphatic lipid-like structure. Almost any compound derived from *Cannabis sativa* L. with

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terpene-phenolic structure is considered as a cannabinoid; more than 150 phytocannabinoids are already described from Cannabis sativa L. specie [5]. Two of the more abundant phytocannabinoids are Δ^9-tetrahydrocannabinol (Δ^9-THC), (6a*R*,10a*R*)− 6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6 H-benzo[*c*]chromen-1-ol (CAS registry number #1972–08–3) and cannabidiol (CBD), (1′*R*, 2′*R*)− 5′-methyl-4-pentyl-2′-(prop-1-en-2-yl)− 1′,2′,3′,4′-tetrahydro-[1,1′-biphenyl]− 2,6-diol (CBD, CAS registry number #13956–29–1). *Cannabis sativa* L. has gained interest because, apart from Δ^9-THC and CBD, there are other compounds that are bioactive, by interacting with cannabinoid receptors and/or interacting with a variety of other GPCRs, e.g. GPR18, GPR55, and/or with peroxisome proliferator-activated receptor (PPAR) proteins or transient receptor potential ion channels [6–10]. The CB_1 receptor (CB_1R) is considered the most abundant GPCR in the central nervous system (CNS) and is expressed in many different neuronal types. The expression of the CB_2 receptor (CB_2R) is restricted to some neuronal populations, e.g. the globus pallidus of non-human primates [11], but is expressed in non-neuronal cells within the CNS [9,12–16].

Pharmacology of cannabinoids acting on cannabinoid receptors is complex. Recent data that have elucidated the structure of the cannabinoid receptors show that the site of agonist binding is not readily available to extracellular molecules, but the active compounds must enter through the lipid bilayer [17–22]. Also, it has been described the existence of exosites to which agonists of GPCRs may interact and regulate receptor functionality [23] A recent study reports synthetic bivalent compounds able to bind to both the orthosteric site and an exosite of the CB_2R [23,24]. In a previous study, we have characterized the binding and pharmacological properties of a variety of phytocannabinoids from CBD-type and CBG-type at CB_1 and CB_2 receptors and at CB_1/CB_2 receptor heteromers (CB_1-CB_2Hets) [25]. It should be noted that GPCRs may interact to form heteromers whose functional properties are different from those of individually expressed receptors [26,27]. Such heteromerization may lead to differential functional selectivity and, consequently, to biased agonism [28]. CB_1-CB_2Hets have been identified in neurons and glia [11,27,29–32], and both CB_2R and CB_1-CB_2Hets may exert neuroprotective actions in animal models of neurodegenerative diseases [33–35]. Interestingly, a relevant role of the CB_1-CB_2Het in mediating the regulation exerted by cannabinoids on microglial activation has suggested that these heteromers may be target for delaying progression of Parkinson's disease [30]. Δ^9-THC can affect signaling through CB_1R or CB_2R. The psychotropic effects induced by Δ^9-THC are primarily due to the binding on the CB_1R and may be due to biased signaling [36]. Although CBD may bind to the orthosteric center of cannabinoid receptors at high concentrations, its main role seems to be of allosteric nature. For both CB_1R and CB_2R, the compound acts as a negative allosteric modulator [37,38]. CBD also skews agonism at cannabinoid receptors, although the potency to do so is reduced in CB_1-CB_2Hets [39].

Data in animal models suggest potential health benefits for several of the main compounds in *Cannabis sativa* L. Apart from Δ^9-THC and CBD, that are already approved for human use, other cannabinoids can be of therapeutic use in a variety of diseases. A review on the health benefits and therapeutic potential of lesser known phytocannabinoids, which can be divided into acidic, varinic and "minor", has been recently released [40]. A previous study on the mode of action of cannabigerol- and cannabidiol-type phytocannabinoids in CB_1R, CB_2R and CB_1-CB_2Hets showed that all tested compounds were acting as partial agonists, that cannabigerivarin (CBGV) was the most potent compound and that there was a direct correlation between binding mode and biased agonism [25]. The aim of the present paper was to assess the pharmacological properties derived from the action of Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and Δ^9- tetrahydrocannabivarin (Δ^9-THCV) on CB_1R, CB_2R and CB_1-CB_2Hets using innovative receptor binding technologies and whole cell direct signaling pathways studies. We reasoned that Δ^9-THCA would not be a compound able to readily enter into the hydrophobic site of the cannabinoid receptors due to its polar carboxylic group also wanting to replicate the THCV antagonistic properties on CB_1R and CB_2R. Δ^9-THC and synthetic reference agonists were also tested for comparative purposes. The results, which complement those in early pharmacological studies [41] (see also [42]) and in more recent in vitro and in vivo studies [43,44], disclose novel properties of Δ^9-THCA and Δ^9-THCV and provide information on the different binding modes and signaling pathways involved upon cannabinoid receptor activation.

2. Results

2.1. Competition binding assays in living HEK-293 T cells

To analyze the effects of Δ^9-THCA and Δ^9-THCV that are mediated by cannabinoid CB_1 and CB_2 receptors, we first analyzed the binding in living cells. First of all, HEK-293 T cells were transfected with SNAP-CB_1R (see Methods). As shown in Supplementary Fig. S1A, the SNAP-CB_1R show similar functionality as the CB_1R. Then, transfected cells were incubated with the fluorophore-conjugated CELT-335 ligand and homogenous competition assays were performed using increasing concentrations of either Δ^9-THCA or Δ^9-THCV. Fig. 1 shows that competition was similar for Δ^9-THC and Δ^9-THCV. The pK_i values obtained for Δ^9-THC and Δ^9-THCV are 7.2 ± 0.6 and 7.2 ± 0.3, respectively, whereas the affinity for Δ^9-THCA was much lower (pK_i = 5.8 ± 0.6). The data for CB_1R obtained using CELT-335 are reliable since they are comparable with those reported in the literature for the tested compounds (Δ^9-THC, Δ^9-THCA and Δ^9-THCV). Data comparison is summarized in Supplementary tables S1 and S2.

Similar experiments in cells expressing the SNAP-CB_2R, which shows similar functionality as in cells expressing CB_2R (Supplementary Fig. S1B), were performed with fluorophore-conjugated CM157 ligand, which is a compound that is selective for the CB_2R [45]. Competition assays showed that results for Δ^9-THC and Δ^9-THCV, with pK_i values of 6.4 ± 0.2 and 6.5 ± 0.3 respectively, were similar, whereas the affinity of Δ^9-THCA was lower (pK_i =5.2 ± 0.5) (Fig. 2A). When using HEK-293 T cells expressing SNAP-CB_2R in the presence of CB_1R, the competition curves were similar as for SNAP-CB_2R-expressing cells, with the exception of Δ^9-THCV, whose competition curve can be fitted to a two-site model with pK_i values of 9.9 ± 1.9 for the high-affinity component of the binding and of 6.5 ± 0.3 for the low-affinity one (Fig. 2B).

In summary, Δ^9-THC and Δ^9-THCV showed, in living HEK-293 T cells, a higher affinity for CB_1R than for CB_2R, and Δ^9-THCA consistently showed less affinity than the other two compounds. An interesting

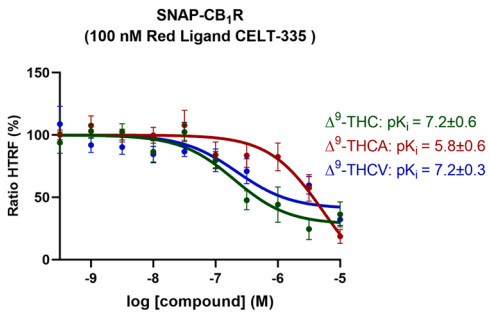

Fig. 1. Competition experiments of binding of fluorophore-conjugated CELT-335 to living HEK-293 T cells expressing the CB_1R. Competition binding experiments were performed in HEK-293 T cells transfected with 1 μg cDNA for SNAP-CB_1R. Tb labelling was performed as described in Methods. Competition binding curves were obtained by HTRF using 100 nM of fluorophore-conjugated CELT-335 and increasing concentrations of Δ^9-THC, Δ^9-THCA and Δ^9-THCV (0–10 μM). HTRF ratio = 665 nm acceptor signal/620 nm donor signal x 10,000; the percentage is calculated by taking the highest value as 100%. Data represent the mean ± SEM (n = 5 in triplicates).

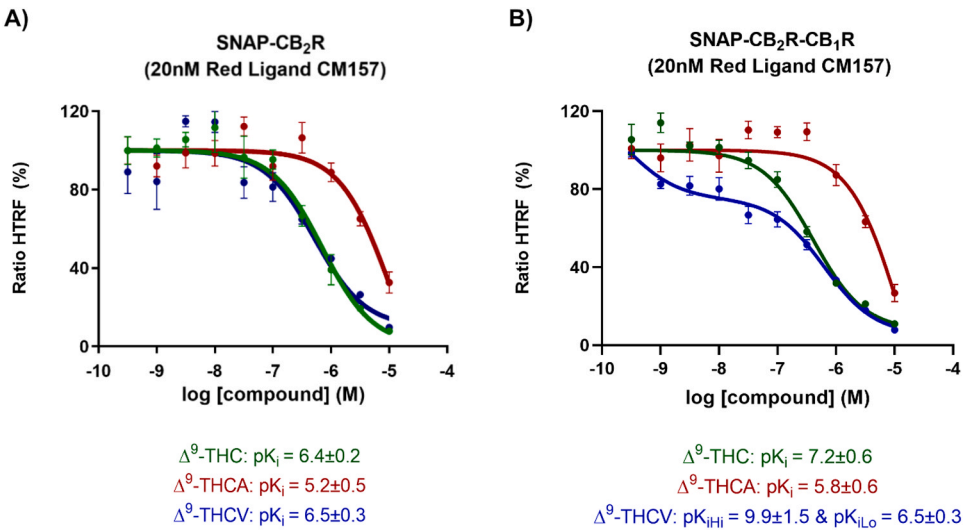

Fig. 2. Competition experiments of binding of fluorophore-conjugated CM157 to living HEK-293 T cells expressing the CB_2R (A) or the $CB_{1/2}RHet$ (B). Competition binding experiments were performed in HEK-293 T cells transfected with 1 μg cDNA for SNAP-CB_2R in the presence or in the absence of 1 μg cDNA for CB_1R. Tb labelling was performed as described in Methods. Competition binding curves were obtained by HTRF using 20 nM fluorophore-conjugated CM157 and increasing concentrations of Δ^9-THC, Δ^9-THCA or Δ^9-THCV (0–10 μM HTRF ratio = 665 nm acceptor signal/620 nm donor signal x 10,000; the percentage is calculated by taking the highest value as 100%. Data from a representative experiment are shown.

observation is the biphasic competition curve of Δ^9-THCV in cells expressing the two receptors.

2.2. Effect of Δ^9-THCA and Δ^9-THCV in cannabinoid receptor functionality

The potential agonistic action of Δ^9-THCA and Δ^9-THCV was measured in cells expressing CB_1R, CB_2R or both receptors. Supplementary Fig. S2 shows that HEK-293 T cells expressing the two receptors may, as previously reported [30,31], form CB_1-CB_2Hets. Three signaling outputs were assayed: cAMP levels determined in cells treated with 0.5 μM forskolin, ERK1/2 phosphorylation and ß-arrestin recruitment. When indicated, CP55940, a nonselective compound, was used as agonist.

In CB_1R-expressing cells only Δ^9-THC, at relatively high concentrations, was able to reduce the forskolin-induced cAMP levels. No effect was found for the other natural cannabinoids (assayed at up to 10 μM). In CB_2R-expressing cells Δ^9-THCA reduced the forskolin-induced cAMP levels with an IC_{50} of circa 1 μM. Neither Δ^9-THC nor Δ^9-THCV provoked any response. Remarkably, in cells expressing the two receptors, Δ^9-THC was inactive whereas the effect of Δ^9-THCA and of Δ^9-THCV were significant (Fig. 3); a higher potency was found for Δ^9-THCV than for Δ^9-THCA (IC_{50} values of 15.1 ± 1.2 and 200 ± 50 nM for, respectively, Δ^9-THCV and Δ^9-THCA). In summary, the agonist effect depends on which receptor is expressed, and it changes qualitatively and quantitatively when the two receptors are expressed together. The use of the nonselective reference compound, CP55940, showed that Δ^9-THC behaved as full agonist in CB_1R-expressing cells and that Δ^9-THCA behaved as a full agonist in CB_2R-expressing cells; it was noticeable that in cells expressing the two receptors the maximal effect of CP55940 was much higher than that of the other two compounds that produced a partial effect, Δ^9-THCA and Δ^9-THCV (Fig. 3).

A complex scenario was also found when the activation of the mitogen-activated protein kinase (MAPK) pathway was determined. HEK-293 T cells expressing CB_1R, CB_2R or both were treated with increasing concentrations of phytocannabinoids for 7 min (time-response was selected after developing time-response curves (Supplementary Fig. S1C, D). The three compounds increased the phosphorylation level of ERK1/2 in cells expressing the CB_1R and in cells expressing the CB_2R. In contrast, in cells expressing the heteromer the effect of Δ^9-THCV was smaller, while Δ^9-THC and Δ^9-THCA provided significant activation of the MAPK signaling pathway with fairly similar dose-response curves (Fig. 4). The Δ^9-THCA- and Δ^9-THCV-induced effects are specific as they were completely blocked by the selective antagonists (SR141716 for CB_1R and AM-630 for CB_2R) (Fig. 6). CP55940 led to a maximal response similar to that of Δ^9-THC in CB_1R-expressing cells. However, Δ^9-THC showed in CB_2R-expressing cells a similar effect to that of Δ^9-THCA and Δ^9-THCV and lower than that of CP55940. The

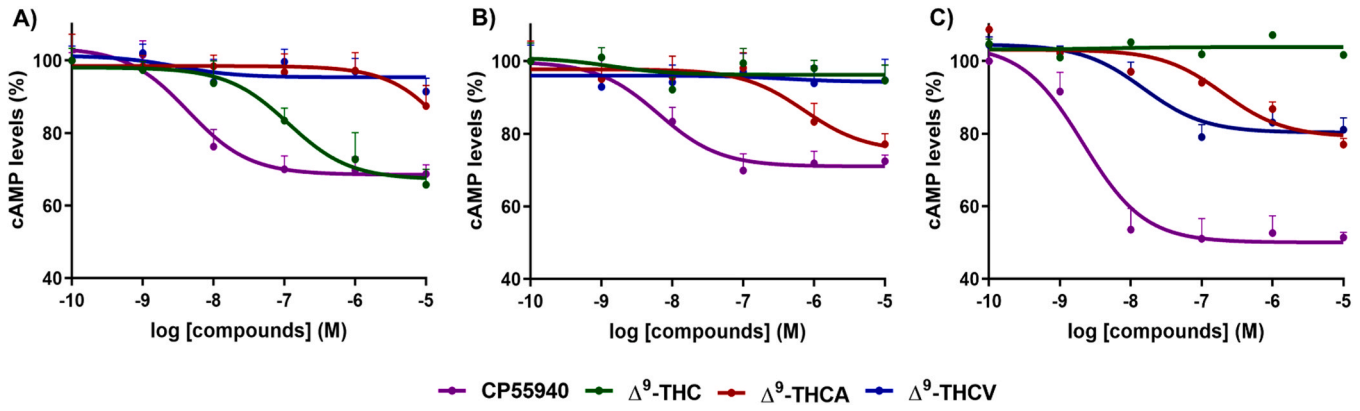

Fig. 3. Effect of Δ^9-THC, Δ^9-THCA and Δ^9-THCV on cannabinoid receptor mediated in cAMP production. HEK-293 T cells were transfected with cDNAs encoding for CB_1R (1.2 μg cDNA) (A), CB_2R (1 μg cDNA) (B), or CB_1R (1.2 μg cDNA) and CB_2R (1 μg cDNA) (C). In each case cells were treated with Δ^9-THC, Δ^9-THCA or Δ^9-THCV. Data in dose–response curves for reduction of forskolin-induced cAMP production (0.1 nM to 10 μM range) are expressed in % respect to the effect of forskolin (0.5 μM, 100%). Data represent the mean ± SEM (n = 5 in triplicates).

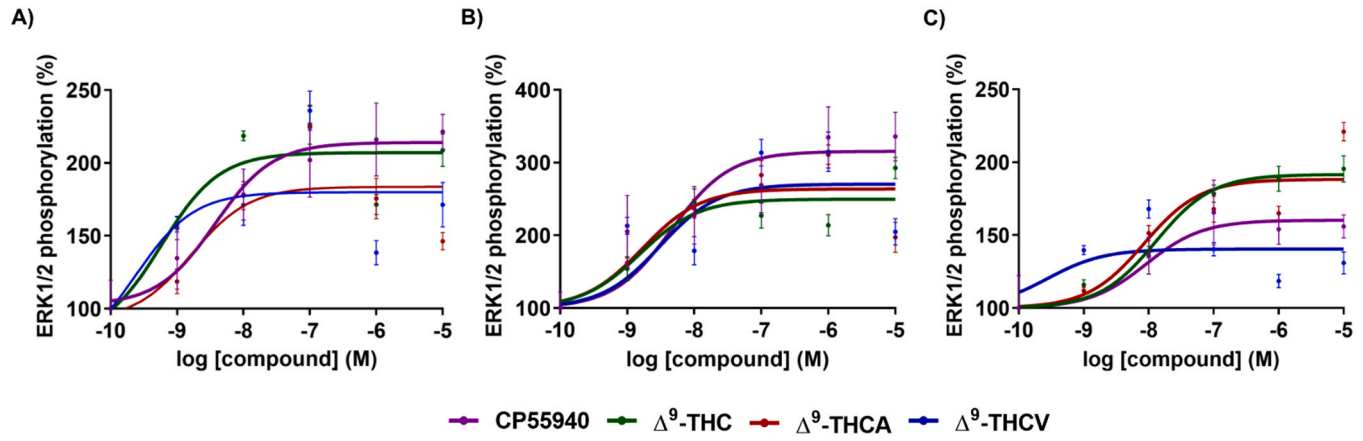

Fig. 4. Effect of Δ^9-THC, Δ^9-THCA and Δ^9-THCV on cannabinoid receptor mediated in ERK 1/2 phosphorylation. HEK-293 T cells were transfected with cDNAs encoding for CB_1R (1.2 μg cDNA) (A), CB_2R (1 μg cDNA) (B) or CB_1R (1.2 μg cDNA) and CB_2R (1 μg cDNA) (C). In each case cells were treated with Δ^9-THC, Δ^9-THCA or Δ^9-THCV. Dose-response curves for ERK1/2 phosphorylation (0.1 nM to 10 μM range) was analyzed and data are expressed as increases in % over basal. Data represent the mean ± SEM (n = 5 in triplicates).

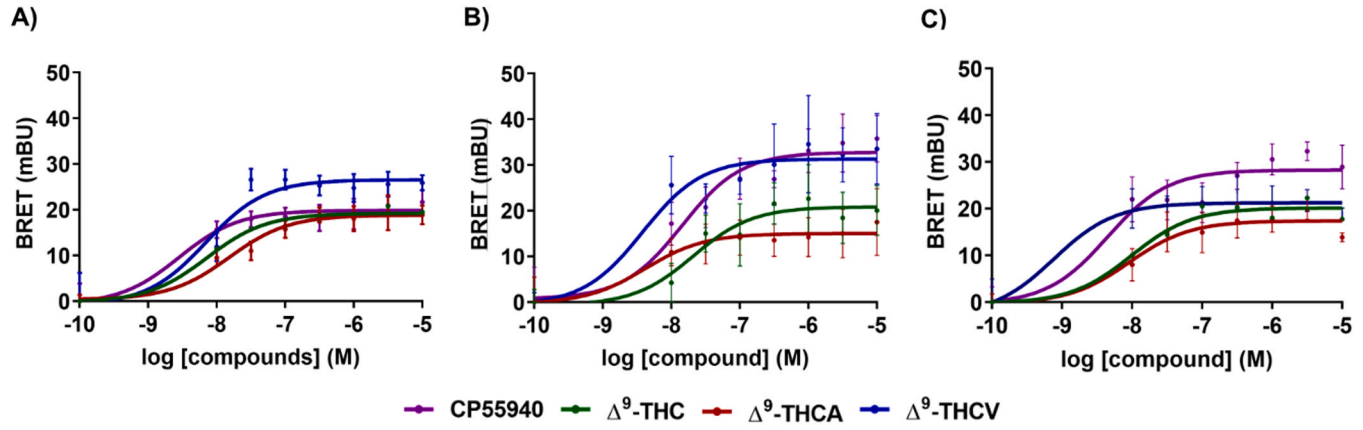

Fig. 5. Effect of Δ^9-THC, Δ^9-THCA and Δ^9-THCV on cannabinoid receptor mediated in β-arrestin recruitment. HEK-293 T cells were transfected with cDNAs encoding for CB_1R (1.2 μg cDNA) (A), CB_2R (1 μg cDNA) (B), or CB_1R (1.2 μg cDNA) and CB_2R (1 μg cDNA) (C). In all cases β-arrestin-RLuc (1 μg cDNA) was also expressed. In each case cells were treated with Δ^9-THC, Δ^9-THCA or Δ^9-THCV. Dose-response curves for β-arrestin recruitment (0.1 nM to 1 μM range) was analyzed and data are expressed as increases in % over basal. Data represent the mean ± SEM (n = 10 in triplicates).

CP55940-induced effect is specific of cannabinoid expressing cells as it is not observed in non-transfected cells (Supplementary Fig. S1E). Interestingly, the effect of the nonselective reference compound in cells coexpressing the two receptors was modest, lower than that of Δ^9-THC and Δ^9-THCA (Fig. 4). These results may be due to allosteric interactions within the heteromer. Finally, in ß-arrestin recruitment assays we observed that the maximal effect produced by Δ^9-THCV in cells expressing CB_1R was higher than that of CP55940, Δ^9-THCA or Δ^9-THC, while in cells expressing CB_2R or the heteromer, both Δ^9-THCV and CP55940 showed similar potency and it was higher than that of Δ^9-THC and of Δ^9-THCA. Overall, the maximal effect of Δ^9-THCV was higher than that of CP55940 in CB_1R-expressing cells, similar in cells expressing the CB_2R and lower than that of CP55940 in cells coexpressing the two receptors (Fig. 5).

2.3. Addressing biased agonism

The bias factor reflects the functional selectivity arising from different interaction modes of agonists and/or from receptors differentially coupled to the signaling machinery. Bias factors of Δ^9-THCA and Δ^9-THCV shown in Fig. 7 were calculated taking as reference the canonical G_i-mediated signal, i.e. the reduction in the cAMP levels induced by forskolin, and either the effect of Δ^9-THC, of a non-selective agonist (CP55940) or of a selective agonist (JWH-133 for CB_2R-expressing cells, or arachidonyl-2′-chloroethylamide (ACEA) for CB_1R- and for CB_1-CB_2Het-expressing cells). Taking Δ^9-THC as reference, radar plots show that Δ^9-THCV produces a bias towards the pERK1/2 response, but only in CB_1R-expressing cells; Δ^9-THCA and Δ^9-THCV produces a bias towards ß-arrestin recruitment in (only) CB_2R-expressing cells. The plots displayed using the selective agonists are more informative as the three phytocannabinoids produce bias towards the MAPK signaling pathway in all cell models. Interestingly, Δ^9-THCV in cotransfected cells displayed a marked bias factor for both MAPK pathway activation and ß-arrestin recruitment. When the non-selective agonist, CP55940, was taken as reference, Δ^9-THC, Δ^9-THCA and Δ^9-THCV were biased towards the MAPK pathway activation in CB_2R-expressing cells; while in CB_1R-expressing cells all phytocannabinoids except Δ^9-THCA were biased towards the MAPK signaling pathway. Finally, in cells coexpressing the two receptors Δ^9-THC and Δ^9-THCA led to virtually identical bias towards MAPK pathway activation and Δ^9-THCV displayed a bias for both MAPK pathway and β-arrestin recruitment. (Fig. 7).

2.4. Addressing potential antagonism/partial agonism in G_i coupling

Finally, the capability of Δ^9-THCA and Δ^9-THCV to reverse the action of agonists on reducing the levels of cAMP, i.e. to act as antagonists

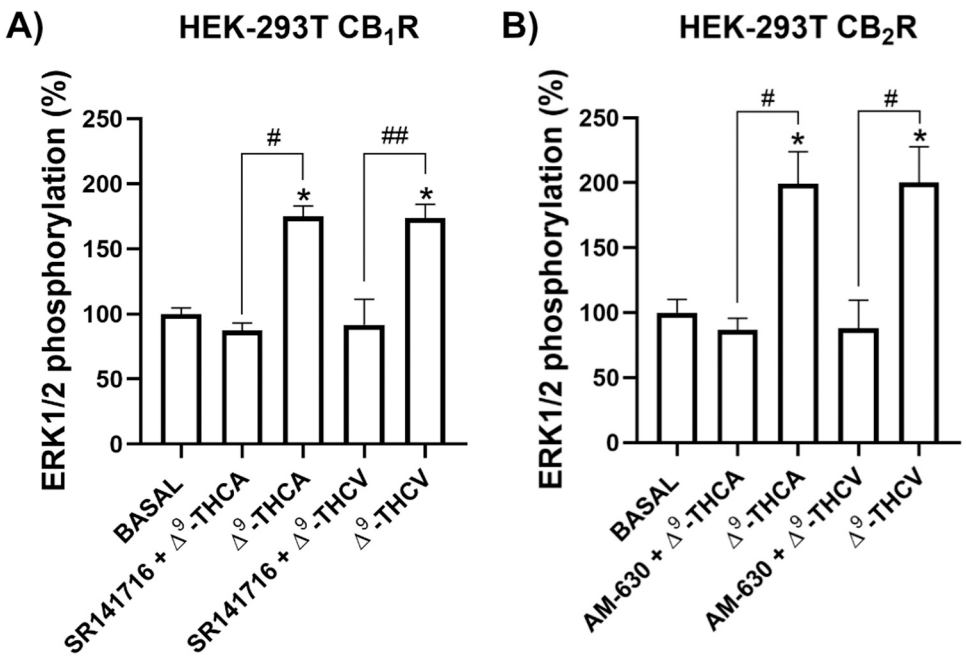

Fig. 6. Selective antagonists block Δ^9-THCA- and Δ^9-THCV- induced effect on cannabinoid receptors. HEK-293 T cells were transfected with cDNAs encoding for CB_1R (1.2 μg cDNA) (A) or CB_2R (1 μg cDNA) (B). In each case cells were pretreated with the specific antagonists SR141617 for CB_1R or AM-630 for CB_2R (1 μM) followed by treatment with Δ^9-THCA or Δ -THCV (20 nM). Data are expressed as increases in % over basal. Data represent the mean ± SEM (n = 5). One-way ANOVA followed by Tukey's multiple comparison post hoc test were used for statistical analysis (*$p < 0.05$ versus basal condition; #$p < 0.05$, ##$p < 0.01$ versus the data obtained in the presence of the selective antagonist).

or partial/inverse agonists, was studied in single transfected cells pretreated with Δ^9-THCA or Δ^9-THCV and subsequently treated (prior to treatment with forskolin) with selective agonists, ACEA for the CB_1R and JWH-133 for the CB_2R. A fixed non-maximal concentration of each selective agonist was used and dose response assays were performed. Data in Fig. 8 shows that Δ^9-THCV but not Δ^9-THCA reverted the effect of 100 nM ACEA, the selective the CB_1R agonist, whereas both compounds reverted the effect of 100 nM JWH-133, the selective CB_2R agonist. Considering the data in Fig. 3, it seems that in the CB_2R, Δ^9-THCV acts as antagonist whereas Δ^9-THCA acts as a partial agonist.

3. Discussion

Often, phytocannabinoids act via CB_1 and CB_2 receptors, but also are capable of modulating the function of a variety of other proteins (see details in Introduction). Due to the complex pharmacology of cannabinoid receptors, a given molecule may appear, depending on the study, as agonist, antagonist or allosteric modulator of cannabinoid receptors. A prototypic example is CBD, which is very abundant in different varieties of the plant; it has been reported that the compound is an allosteric modulator of the CB_1R and of the CB_2R, and an antagonist of the CB_1R [37,38,46]. In our own experience, CBD binds to the CB_2R in, at least, two different sites, one being the orthosteric and another being allosteric [47]. In fact, diversity when measuring binding with different labelled compounds is likely due to the structural properties of cannabinoid receptors, whose entrance to the binding site is located within the lipid bilayer and, also, to a different positioning of labelled compounds in the orthosteric cavity [48]. In that sense, we have detected a particular exosite in the CB_2R to which one of the moieties of symmetric bitopic ligands binds. Such homobivalent compounds bind to the orthosteric site and to an exosite located at the entrance of the wormhole-like structure that connects the orthosteric site with the lipid bilayer [24]. These differences in binding translates into functional selectivity, which is very marked in the case of cannabinoid receptors. The interest in cannabinoids as potential tools to combat a variety of diseases (e.g., metabolic, neurological, etc. see [40] for review), makes necessary to advance in understanding the pharmacology of phytocannabinoids at cannabinoid receptors.

This paper describes that Δ^9-THCV and Δ^9-THCA interact with cannabinoid receptors expressed in a heterologous system. Δ^9-THC, a well-established partial agonist of both cannabinoid receptors (see [10, 42] for review), and CP55940 were used for comparison. HTRF-based competition assays in cells expressing CB_1R, CB_2R or CB_1-CB_2Hets showed that Δ^9-THC and Δ^9-THCV had similar K_i values, whereas the K_i for Δ^9-THCA was significantly higher (Figs. 1–2). This would be consistent with the restraint posed by the charge due to the carboxylic acid in Δ^9-THCA; they are in agreement with previous studies [49,50] although there are studies with divergent data [43,51]. The binding characteristics of radiolabelled compounds used to measure the binding to cannabinoid receptors vary from compound to compound, for instance comparing data obtained with [^{3}H]WIN-55,212–2 and with [^{3}H]CP55940; cannabigerol displays in the CB_1R a K_i of 1 μM using [^{3}H]CP55940 and unspecific binding using [^{3}H]WIN-55,212–2 (K_i >30 μM) (differences are approximately two-fold in the case of the CB_2R) [48].

A comparison of data from HTRF assays with those reported/reviewed in the literature [42,43,49,50] is found in Supplementary tables S1 and S2. In such comparison, a maximum difference of 1.1 in pK_i values is observed between data obtained by radioligand binding assays (reported in literature) and data obtained by HTRF binding assays (published herein). A difference of pK_i of about 1 (10-fold difference in K_i) is tolerable considering that 2 different experimental approaches have been undertaken. A table comparing the main features of radioligand-based and of HTRF-based are shown in Supplementary table S3. Of note are the high affinity reported for Δ^9-THCA by Rosenthaler et al., [51] for CB_1 (K_i= 23 nM) and CB_2 (K_i = 56 nM) and by Zagzoog et al., [43] for the CB_2R (K_i =1.3 nM). The structural features of the cannabinoid receptors orthosteric binding site do not readily explain such increase in affinity when to t the aromatic ring of Δ^9-THC, a carboxylic acid is added.

The three compounds whose binding and pharmacology were here compared bind to greater or lesser extent to cannabinoid receptors expressed in living cells. Binding data in our homogenous assays partially agreed with data presented in a very recent report in which isolated membranes were used [43]. In our assays of binding to cannabinoid receptors in living HEK-293 T cells, all compounds significantly competed for the binding of the reference compound. In membranes isolated from CHO-K1 cells expressing CB_1R or CB_2R receptors and the nonselective radioligand, [^{3}H]CP55940, Δ^9-THCA competed a 30% for the binding to the CB_1R with a K_i of 620 nM, whereas we found a complete competition with a similar K_i, 1.6 μM, (pK_i =5.8). Previous

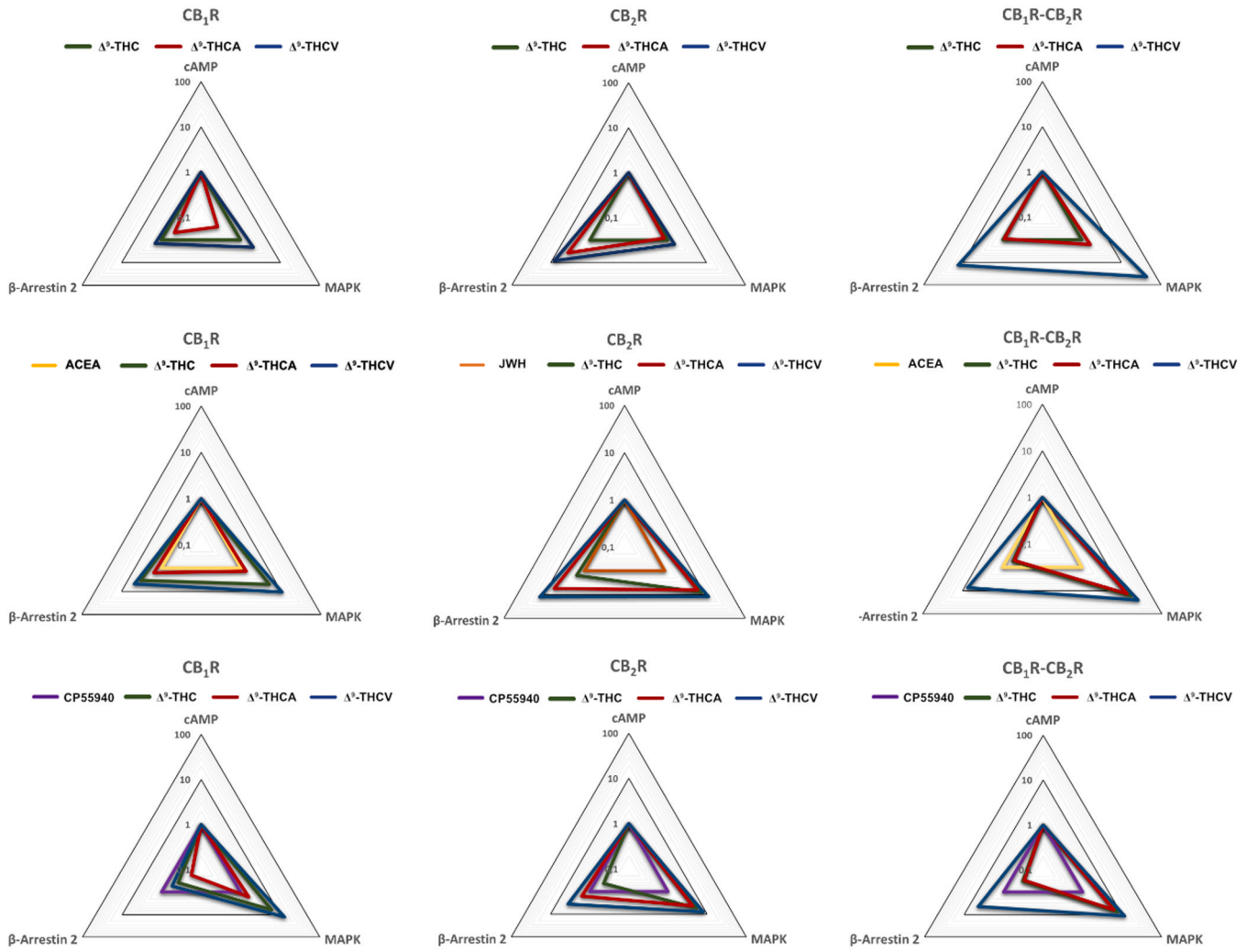

Fig. 7. Biased agonism on CB_1R, CB_2R and $CB_{1/2}$Hets. Radar plots showing the bias factors of the Δ^9-THC, Δ^9-THCA and Δ^9-THCV in cAMP, MAPK and β-arrestin recruitment functional outcomes in HEK-293 T cells expressing CB_1R (left), CB_2R (vertical middle) or both (right). Data were calculated taking Δ^9-THC (top) or the selective agonists, JWH-133 or ACEA (horizontal middle), or the non-selective agonist, CP55940 (bottom) as reference compounds. In all cases the response of reference was forskolin-induced cAMP production.

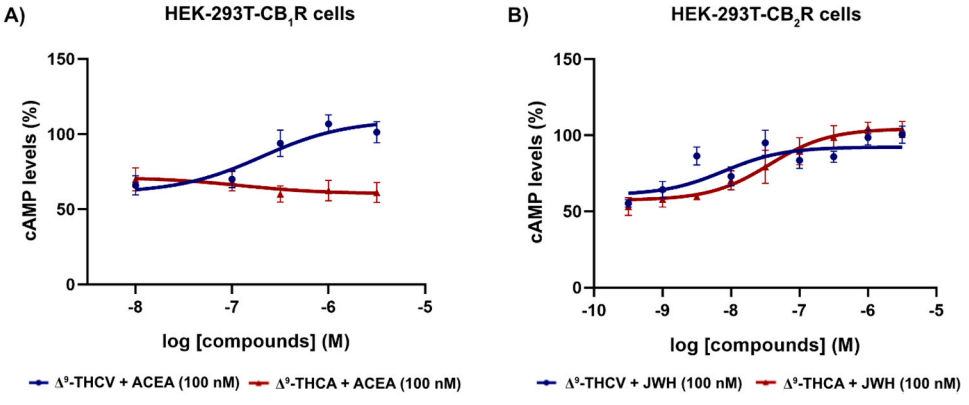

Fig. 8. Potential antagonistic effect of Δ^9-THC, Δ^9-THCA and Δ^9-THCV. HEK-293 T cells expressing CB_1R (1.2 μg cDNA) (A), CB_2R (1 μg cDNA) (B) were treated with 1 μM of Δ^9-THC, Δ^9-THCA or Δ^9-THCV 15 min before addition of the selective CB_1R (ACEA) or CB_2R (JWH-133) agonist. Dose–response curves for decreases in forskolin-induced cAMP production using fixed concentrations of ACEA (100 nM) or JWH-133 (100 nM) and increasing concentrations of Δ^9-THCA or Δ^9-THCV. Data are expressed in % respect to the effect of 0.5 μM forskolin (100%). Data represent the mean ± SEM (n = 5 in triplicates).

studies [49] using the same radioligand in HEK-293 T cell membranes have been found a lower affinity of Δ^9-THCA for CB_1R with a 62% reduction of the specific binding and a K_i 3.1 μM, ($pK_i = 5.5$). It should be noted that another laboratory using HEK-293 cells and [^{3}H]CP55940 found higher binding affinity of Δ^9-THCA for CB_1R (K_i of 252 nM) [50]. The parameters for the binding to the CB_2R were also different to ours, as competition was only partial and with a very low K_i, 1.3 nM (pK_i =8.9). The reasons for such discrepancies, the degree of competition in binding to the two receptors and the very different K_i values in the case of the binding to the CB_2R, are not readily evident. However, it is unlikely that Δ^9-THCA binds to the CB_2R with low nanomolar affinity [43], as a similar assay using the same radioligand, [^{3}H]CP55940, and the

same human version of the receptor leads to much higher K_i values, from 506 nM (pK_i =6.3) [50] to 12.5 μM (pK_i =4.9) [49]. Those low affinity data are more similar to the one obtained by us in the homogenous TR-FRET-based assay, 6.3 μM (pK_i =5.2), using a well-characterized orthosteric ligand [45]. Also difficult to explain is the biphasic curve for competition to CB_2R binding found for Δ^9-THCV in cells expressing CB_1-CB_2Hets, but not in cells expressing one of the receptors. However, the homogenous HTRF method has previously served to identify two populations of the CB_2R displaying different affinities for a given cannabinoid ligand [45].

The results related to G_i coupling showed that only Δ^9-THC in CB_1Rs and only Δ^9-THCA in CB_2Rs were able to decrease forskolin-induced cAMP levels. Our results of cAMP assays in cells expressing the CB_1R agree in the data for Δ^9-THC reported elsewhere [43,50], although in our hands we did not find any response when cells were incubated with Δ^9-THCV or Δ^9-THCA. In agreement with recent data from Palomares et al., [50] but not with Zagzoog et al., [43] we found response to Δ^9-THCA in CB_2R-expressing cells; we did not detected any effect in response to Δ^9-THC or Δ^9-THCV. As Δ^9-THCA did produce effects even when Δ^9-THC failed to do so, results concerning Δ^9-THCA cannot be due to contamination by Δ^9-THC as in data reported elsewhere [49]. Pioneering studies of pharmacological characterization of the "peripheral" cannabinoid receptor, CB_2R, showed that using 1 μM forskolin, Δ^9-THC has negligible effect being even suggested as a weak antagonist of the CB_2R [52–54]. In our assays in HEK-293 T cells we used a relatively low concentration of forskolin, 0.5 μM, which allows us to detect differential actions of the ligands that act on the G_i-coupled receptors. We cannot be sure of the actual factors influencing the differences between studies, although a significant factor is most likely the concentrations of forskolin used. Discovery of the reasons of such inter-study discrepancy may hopefully lead to a better understanding of the mode of action of Δ^9-THC and of other phytocannabinoids. Advantages and disadvantages may be disclosed for any method of cAMP determination, isolated membranes versus intact cells, direct measurement of cAMP versus indirect measure of surrogate marker/reported activity, etc. We prefer the use of intact cells versus the use of isolated membranes and of the method here used over the one that is radioactivity-based. In addition, we measure [cAMP] itself and not any surrogate marker, at 15 min after agonist stimulation, in the presence of an adenylyl cyclase inhibitor and at a temperature that minimizes receptor desensitization and internalization. It should be noted that, in G_i coupling assays, Δ^9-THCV acted as antagonist of both CB_1 and CB_2 receptors.

In our study we performed two more types of functional assays. On the one hand, the link to the MAPK pathway provided meaningful information. In fact, all compounds robustly activated this relevant signaling pathway with potencies that did not correlate with binding parameters obtained in competition binding assays; the potency was in the nanomolar range in all cases. The maximum effect was comparable for the three compounds tested in cells expressing the CB_1R and in cells expressing the CB_2R. The potency of all three compounds was similar in either CB_1 or CB_2 receptors when activation of the MAPK signaling pathway was measured. The reduced response achieved by Δ^9-THCV when activating the receptor in the heteromeric context is of note and probably reflect some constraint due to the inter-protomer interactions in the CB_1-CB_2Het (Fig. 4). On the other hand, radar plots taking as reference Δ^9-THC and the canonical G_i-mediated pathway assessed by determining reductions in forskolin-induced cAMP levels, showed marked bias toward ß-arrestin 2 recruitment for Δ^9-THCV and Δ^9-THCA in the case of the CB_2R (Fig. 7). However, when the selective agonists for each receptor were selected as compounds of reference, the bias pattern changed in a way that every phytocannabinoid behaved differently depending on which was the receptor under study. A differential pattern was also observed when the nonselective agonist, CP55940 was used as reference compound (Fig. 6). What these results confirm is that the binding mode of JWH133, of CP55940 or of phytocannabinoids conditions the response; reciprocally, depending on the compound used as a reference for bias factor calculation, the bias pattern may significantly change leading to apparent bias to either ß-arrestin recruitment or activation of the MAPK signaling cascade. Worth mentioning that our results are consistent with ß-arrestin 2 recruitment to cannabinoid receptors. The controversy that, unlike other class A GPCRs, one or both cannabinoid receptors are not able to recruit the protein upon activation and/or that phyto/endo-cannabinoids are not able to recruit arrestins, may be due to the experimental setup and/or to a particular spatial distribution of the complex that does not allow to easy detection of energy transfer [43,55–59]. In fact, energy transfer decreases rapidly upon increasing donor/acceptor distance; the efficiency is inversely proportional to the sixth power of the distance. In our experimental conditions, the dynamic range of the BRET due to the formation of complexes between ß-arrestin-Rluc and receptor-YFP fusion proteins is small, thus suggesting that the distance between Rluc and YFP is in the limit of energy transfer detection. ß-arrestin recruitment data has been, however, instrumental to understand how CB_1R functional selectivity may affect neuronal viability in a Huntington's disease cell model [58]. Potency of the compounds also affects the possibility to detect ß-arrestin recruitment; it has been described an analytical technique based of the recruitment of truncated ß-arrestin 2 to either CB_1 or CB_2 receptors may detect, in human serum or plasma, subnanomolar concentrations of synthetic agonists whereas > 12 ng/mL were needed to detect Δ^9-THC [60]. Such a report is consistent in that truncating the protein establishes a closer distance between ß-arrestin 2 (truncated) and the CB_1 or the CB_2 receptor.

Until recently, Δ^9-THCA, which was discovered in the late sixties (reviewed in [61,62]), was more investigated as a metabolic precursor of Δ^9-THC than as a molecule with potential health benefits. Apart from lacking the psychotropic activity of Δ^9-THC (see [63] for review), Δ^9-THCA is now considered as an attractive possibility to fight against, among others, neurodegenerative diseases, non-alcoholic liver fibrosis, obesity and metabolic syndromes of diverse etiology via PPAR-ß or cannabinoid receptors [7,44,50]. However, a major problem when trying to use Δ^9-THCA as a therapeutic drug is its stability. In vivo, Δ^9-THCA can be significantly decarboxylated to Δ^9-THC and it may be difficult to identify the actual therapeutic agent, whether it is Δ^9-THCA itself or a metabolic product [44,64,65]. Our results are important from a pharmacological point of view; the product added to the cells was Δ^9-THCA of high purity and with negligible amounts of Δ^9-THC, meaning that results were not likely due to contamination by Δ^9-THC. The actual effect of Δ^9-THCA is important to better delineate, at the molecular level, the interactions of natural cannabinoids with the CB_1R or the CB_2R orthosteric/allosteric (exo) sites. Also our results are relevant from a therapeutic point of view as Δ^9-THCA is not completely degraded in vivo, indeed it is found at significant amounts in blood, urine and hair of Cannabis smokers [66–73].

THCV has been more closely investigated and shows potential, in vitro and/or in animal models, for type II diabetes, for obesity and for reducing neuroinflammation due to ischemia-reperfusion [74–76]. Both Δ^9-THCA and Δ^9-THCV are able to interact with cannabinoid receptors although it is known that they may impact other GPCRs; in the case of Δ^9-THCA the peroxisome proliferator-activated receptor (PPAR) family may be involved in some of its actions [7]. To what extent the health benefits of these compounds are due to interaction with cannabinoid receptors, CB_1-CB_2Hets, or to non-cannabinoid receptor targets will require further experimental effort. Overall, the results agree with the appreciation that the binding mode of each ligand leads to specific cannabinoid receptor conformations underlying biased signaling [25]. Functional selectivity can be achieved by mechanisms other than biased signaling [77,78]. On the one hand, particular receptor environments, elicited by receptor-receptor or receptor-protein interactions, can qualitatively and quantitatively affect signal transduction. On the other hand, it has become clear that cannabinoid receptors have binding sites other than the orthosteric site; even at the orthosteric site there are different forms of ligand/receptor interactions. The development of

bitopic ligands has been fundamental in learning that the modes of interaction are diverse and lead to functional diversity [24,79,80]. In such a scenario, the data presented in this paper and previously reported in vitro and in vivo data suggest that Δ^9-THCV may act on the CB_1R as agonist/inverse agonist/antagonist depending on the concentration [41, 42,81]. Although our in vitro results are not consistent with the possibility that Δ^9-THCV acts as a CB_2R full agonist, in their informative and sound study Bolognini et al., (2010) showed anti-inflammatory actions of the compound that were seemingly due to CB_2R activation and inhibition of CB_1R-mediated signaling [82].

In conclusion, the diverse pharmacology of Δ^9-THCV and Δ^9-THCA and the bias they produce in cannabinoid receptor signaling may serve to better understand their properties in order to design therapeutic interventions for a variety of diseases. We here report Δ^9-THCV as a CB_1R and CB_2R antagonist and Δ^9-THCA being able to act as a partial agonist able to antagonize the effects of full agonists. Such diversity depending on the binding to the orthosteric/allosteric centers and the selective functionality they produce is attractive per se. In fact, just taking into account cannabinoid receptors, different phytocannabinoids may lead to a wide variety of signaling outputs via multiple potential mechanisms, via CB_1R, via CB_2R or via cannabinoid-receptor-containing heteromers. Our results from cells expressing CB_1-CB_2Hets constitute an example of particular properties derived from the interaction between the two cannabinoid receptors. However, they can interact with other GPCRs to form functionally diverse macromolecular complexes [83].

4. Materials and methods

4.1. Reagents

Arachidonyl-2′-chloroethylamide (ACEA), JWH-133, CP55940, SR141716, AM-630 and forskolin were purchased from Tocris Bioscience (Bristol, UK).Δ^9-THC, Δ^9-THCA and Δ^9-THCV were provided by Phytoplant Research S.L.U, Córdoba, Spain. To avoid spontaneous decarboxylation of Δ^9-THCA and to ensure the stability of the used phytocannabinoids all compounds were aliquoted and stored at − 80 °C until used only once.

4.2. Cannabinoid isolation and purification

Δ^9-THC and Δ^9-THCA were purified from the Cannabis variety MONIEK (CPVO/20160114), Δ^9-THCV was purified from the variety RAQUEL (CPVO/20180114) following a previously described liquid-liquid chromatography method [84] that provides compounds with > 95% purity. For purity analysis an Agilent liquid chromatography set-up (Model 1260, Pittsburgh, PA, USA) 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 x 2.1 mm internal diameter, 2.7 μm pore size Poroshell 120 EC-C18 column was used. 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 35ºC. All samples were analyzed in duplicate. The results of each cannabinoid were calculated as weight (%) versus these commercial standards: Δ^9-THC batch nº L01144-M-10.0 purchased from THCpharm, (Frankfurt, Germany), and Δ^9-THCV batch n° FE06011601 and Δ^9-THCA batch n° L01201-M-0.1c purchased from Cerilliant (Round Rock, Texas). The purity of each compound isolated and used in the study was Δ^9-THC = 95.51%, Δ^9-THCA 98.38% (Δ^9-THC impurity 0.49% -under the area peak-) and Δ^9-THCV = 95.86% (Δ^9-THC impurity below detection limit).

4.3. cDNAs and expression vectors

cDNAs for the human version of cannabinoid CB_1R and CB_2R (GenBank accesion number AY242132.1) lacking the stop codon were obtained by PCR and subcloned to a RLuc-containing vector (pRLuc-N1; PerkinElmer, Wellesley, MA) using sense and antisense primers harboring unique restriction sites for *Hin*dIII and *Bam*HI or subcloned to a pEYFP-containing vector (pEYFP-N1; Clontech, Heidelberg, Germany) or a $pGFP^2$-containing vector (Clontech, Heidelberg, Germany) using sense and antisense primers harboring unique restriction sites for *Bam*HI and *Kpn*I generating CB_1R-Rluc, CB_2R-Rluc, CB_1R-YFP, CB_2R-YFP,and CB_2R-GFP^2 fusion proteins. The human version of cDNAs of cannabinoid CB_1R and CB_2R 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 *Hin*dIII and *Bam*HI generating the SNAP tagged CB_1R (SNAP- CB_1R) and CB_2R (SNAP- CB_2R). Human β-arrestin 2–RLuc6 cDNA was cloned in pcDNA3.1 RLuc6 vector (pRLuc–N1; PerkinElmer Life and Analytical Sciences).

4.4. Cell culture and transfection

Human embryonic Kidney HEK-293 T (lot 612968) cells were acquired from the American Type Culture Collection (ATCC). They were amplified and frozen in liquid nitrogen in several aliquots. Cells from each aliquot were used until passage 12. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin/streptomycin, and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). Cells were maintained at 37º C in a humidified atmosphere of 5% CO_2 and were passaged when they reached 80–90% confluent, i.e. approximately twice a week. HEK-293 T cells growing in 35-mm-diameter six-well plates were transiently transfected with the corresponding fusion protein cDNAs using ramified PEI (poly-ethylenimine, Sigma–Aldrich, St. Louis, MO, USA). Cells were incubated with the corresponding cDNA together with PEI (5 mL of 10 mM PEI for each mg of cDNA) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was exchanged to a fresh complete culture medium and used for functional assays 48 h after (unless otherwise stated).

By means of radioligand binding experiments using [^{3}H]CP55940 the expression levels of receptors in total membranes were in the range of 0.4–0.7 pmol/mg protein. These data demonstrate that the fusion proteins are not strongly over-expressed and that the receptors were quantitatively expressed in similar amounts. Expression of fusion proteins was also in the 0.4–0.7 pmol/mg protein range. For BRET assays in ß-arrestin recruitment experiments, the level cDNA used was adjusted to have of ß-arrestin-Rluc-derived luminescence of approximately 100,000 units for receptor-YFP expression levels of approximately 30,000 fluorescence units (30,000 units are equivalent to expression of 0.4–0.7 pmol/mg protein in total cell membranes).

β-arrestin 2 recruitment.

β-arrestin 2 recruitment was determined as previously described [38]. Briefly, BRET experiments were performed in HEK-293 T cells transfected with the cDNA for either CB_1R-YFP, CB_2R-YFP or CB_2R-YFP and CB_1R, 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 incubated with compounds for 10 min prior to the addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR). 1 min after coelenterazine H addition, BRET readings corresponding to β-arrestin 2-Rluc and receptor-YFP were quantified. The readings were collected using a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) that allows the integration of the signals detected in the short-wavelength filter (485 nm) and the long-wavelength filter (530 nm). To quantify protein-RLuc expression, luminescence readings were performed 10 min after the addition of 5 μM coelenterazine H.

4.5. Homogeneous competition binding assays

SNAP-CB_2R and SNAP-CB_1R were expressed in HEK-293 T cells using the elsewhere described procedure [38]. Tag-lite-based binding assays were performed 24 h after transfection. For SNAP protein labelling, cell culture medium was removed from the 25 cm^2 flask and 100 nM SNAP-Lumi4-Tb, previously diluted in 3 mL of TLB ("Tag-lite buffer" -Cisbio Bioassays-), was added to the flask and incubated for 1 h at 37 °C under 5% CO_2 atmosphere in a cell incubator. Cells were then washed four times with 2 mL of TLB to remove the excess of SNAP-Lumi4-Tb, detached, centrifuged 5 min at 1500 rpm and treated with 1 mL TLB. Densities of 2500 − 3000 cells/well were used to perform binding assays in white opaque 384-well plates. For competition binding assays, the fluorophore-conjugated CB_2R ligand (labelled CM157), the fluorophore-conjugated CB_1R ligand (CELT-335), Δ^9-THC, Δ^9-THCA and Δ^9-THCV were diluted in TLB. HEK-293 T cells transiently expressing Tb-labelled SNAP-CB_2R with or without CB_1R were incubated with 20 nM fluorophore-conjugated CB_2R ligand in the presence of increasing concentrations (0–10 μM range) of cannabinoid compounds. HEK-293 T cells transiently expressing Tb-labelled SNAP-CB_1R were incubated with 100 nM fluorophore-conjugated CB_1R ligand in the presence of increasing concentrations (0–10 μM range) of cannabinoid compounds. Plates were then incubated for at least 2 h at room temperature before signal detection. Homogeneous time-resolved fluorescence energy transfer (HTRF) was detected using a PHERAstar Flagship microplate reader (Perkin-Elmer, Waltham, MA, USA) equipped with a fluorescence resonance energy transfer (FRET) optic module allowing donor excitation at 337 nm and signal collection at both 665 and 620 nm. CELT-335 is a dual (CB_1/CB_2) fluorescent ligand, developed by Celtarys Research SL (Spain), suitable to perform HTRF binding assays. This fluorescent ligand is a full agonist which binds the orthosteric site of hCB receptors, bearing a highly hydrophilic fluorophore compatible with HTRF technology. CELT-335 shows high affinity for CB_1 and CB_2 cannabinoid receptor subtypes (K_i values of 44.8 nM and 7.4 nM respectively in radioligand binding assays using [^{3}H]CP55940). Extensive validation of CELT-335 as optimal fluorescent ligand for HTRF and other fluorescence-based assays has been performed, the results will be published in a separate paper.

cAMP level determination using a time-resolved (TR)-FRET-based homogeneous assay.

Two hours before initiating the experiment, growth medium was replaced by serum-starved DMEM medium. Then, cells were detached and resuspended in DMEM medium containing an inhibitor of adenylyl cyclase, zardaverine (50 μM). Subsequently, cells were placed in 384-well microplates (2500 cells/well), pretreated (15 min) with the corresponding antagonists -or vehicle- and stimulated with agonists (15 min) before adding 0.5 μM forskolin or vehicle for 15 more min. Finally, reaction was stopped by addition of the Eu-cAMP tracer and the ULight-cAMP monoclonal antibody prepared in the "cAMP detection buffer" (PerkinElmer). All steps were performed at 25º. Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed after 60 min incubation using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, USA). Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab technologies, Offenburg, Germany).

4.6. ERK phosphorylation determination

To determine ERK1/2 phosphorylation, 40,000 cells/well were plated in transparent Deltalab 96-well microplates and kept at the incubator for 24 h. 2–4 h before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were stimulated at 25 °C for 7 min with compounds or vehicle in serum-starved DMEM medium. The time (7 min) was selected upon ad hoc time-response experiments (see Supplementary Fig. S1C, D). Cells were then washed twice with cold PBS before the addition of "Ultra lysis buffer" -Perkin Elmer- (20 min treatment). 10 μL of each supernatant were placed in white ProxiPlate -Perkin Elmer- 384-well microplates and ERK 1/2 phosphorylation was determined using the AlphaScreen®SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). When indicatedm cells were incubated for 10 min with the ad hoc receptor antagonist before adding the agonist compound to be tested.

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CRediT authorship contribution statement

Iu Raïch: Investigation, Data analysis, Approving the final version of the manuscript. **Rafael Rivas-Santisteban:** Data Analysis and design of graphic material. Production of Fig. 6. Approving the final version of the manuscript. **Alejandro Lillo:** Investigation. Approving the final version of the manuscript. **Jaume Lillo:** Investigation. Approving the final version of the manuscript. **Irene Reyes-Resina:** Data Analysis and design of graphic material. Production of part of the supplementary Material. Approving the final version of the manuscript. **Xavier Nadal:** Isolation and purification of phytocannabinoids. Writing. Production of part of the supplementary Material. Approving the final version of the manuscript. **Carlos Ferreiro-Vera:** Control quality and determination of purity of phytocannabinoids. Approving the final version of the manuscript. **Verónica Sánchez de Medina:** Conceptualization, supervision, isolation and purification of phytocannabinoids. Approving the final version of the manuscript. **Maria Majellaro:** Design and synthesis of reagents. Production of part of the supplementary Material. Approving the final version of the manuscript. **Eddy Sotelo:** Design and synthesis of reagents. Writing. Production of part of the supplementary Material. Approving the final version of the manuscript. **Gemma Navarro:** Conceptualization, supervision. Writing. Approving the final version of the manuscript. **Rafael Franco:** Conceptualization, supervision, writing the first draft. Approving the final version of the manuscript.

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Author contributions

RF, GN, VSM, and XN coordinated the entire process. IR made Figs. 1–7 and supplementary Figs. RRS and AL participated in obtaining data in Figs. 1–2 and JL participated in obtaining data in Fig. 4. VSM and XN isolated the phytocannabinoids and CFV analysed the purity. MM and ES designed and prepared the CELT-335 fluorescent ligand. RF prepared a first version, IR, ES, VSM, XN and GN revised the initial version and prepared a second version, which was subsequently edited by RRS, IRR and CFV. All authors have approved the final version of the manuscript.

Summary

Cannabinoid CB_1 and CB_2 receptors are expressed in a variety of cells and when expressed together in a given cell they may form CB_1-CB_2 heteromeric complexes. To complete previous studies we have here assessed, using Δ^9-tetrahydrocannabinol (Δ^9-THC) for comparison, the effect of Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and of Δ^9-tetrahydrocannabivarin (Δ^9-THCV) that is mediated by human versions of CB_1, CB_2 and CB_1-CB_2 receptor functional units, expressed in a heterologous system. Binding to the CB_1 and CB_2 receptors was addressed in living cells by means of a homogeneous assay. A biphasic competition curve for the binding to the CB_2 receptor was obtained for Δ^9-THCV in cells expressing the two receptors. Signaling studies included cAMP level determination, activation of the mitogen-activated protein kinase pathway and ß-arrestin recruitment were perform. The signaling of Δ^9-THCA and Δ^9-THCV via individual receptors or receptor heteromers disclosed differential bias, i.e. the bias for a given phytocannabinoid depended on the receptor (CB_1, CB_2 or CB_1-CB_2) and on the compound used as reference to calculate the bias factor (Δ^9-THC, a selective agonist or a non-selective agonist). These results are consistent with different binding modes leading to differential functional selectivity depending on the agonist structure and the state (monomeric or heteromeric) of the cannabinoid receptor. In addition, on studying G_i-coupling, we showed that Δ^9-THCV and Δ^9-THCA were able to revert the effect of a selective CB_2 receptor agonist, but only Δ^9-THCV, and not Δ^9-THCA, reverted the effect of arachidonyl-2′-chloroethylamide (ACEA, 100 nM) a selective agonist of the CB_1 receptor. Overall, these results indicate that cannabinoids may have a variety of binding modes that results in qualitatively different effects depending on the signaling pathway engaged upon cannabinoid receptor activation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: IR, RRS, AL, JL, IRR, MM, GN and RF declare no conflict of interests. CFV and VSM declare that they work for Phytoplant Research SL, a research company that does not directly sell any product (web page: https://www.phytoplantresearch.com/). XN worked for Phytoplant Research SL and is not currently linked to the company; now he is owner and work for Ethnophytotech Research & Consulting S.L.U. a research company that does not directly sell any product. Neither CFV, VSM nor XN have shares of Phytoplant Research S.L.U. or any cannabinoid-related for-profit company. ES declares that he has shares of Celtarys Research SL, a company that has provided the CELT-335 compound without any participation in financing the research. MM works for Celtarys Research SL.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2021.105970.

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