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THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

The plant cannabinoid Δ^9-tetrahydrocannabivarin can decrease signs of inflammation and inflammatory pain in mice

Daniele Bolognini[1,2], Barbara Costa[3], Sabatino Maione[4], Francesca Comelli[3], Pietro Marini[1], Vincenzo Di Marzo[5], Daniela Parolaro[2], Ruth A Ross[1], Lisa A Gauson[1], Maria G Cascio[1] and Roger G Pertwee[1]

[1]*Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK,* [2]*DBSF, Pharmacology Section and Neuroscience Centre, University of Insubria, Varese, Italy,* [3]*Department of Biotechnology & Bioscience, University of Milano-Bicocca, Milano, Italy,* [4]*Endocannabinoid Research Group, Division of Pharmacology 'L Donatelli', Department of Experimental Medicine, Second University of Naples, Naples, Italy and* [5]*Endocannabinoid Research Group, Institute of Biomolecular Chemistry, CNR, Pozzuoli, Naples, Italy*

Background and purpose: The phytocannabinoid, Δ^9-tetrahydrocannabivarin (THCV), can block cannabinoid CB_1 receptors. This investigation explored its ability to activate CB_2 receptors, there being evidence that combined CB_2 activation/CB_1 blockade would ameliorate certain disorders.
Experimental approach: We tested the ability of THCV to activate CB_2 receptors by determining whether: (i) it inhibited forskolin-stimulated cyclic AMP production by Chinese hamster ovary (CHO) cells transfected with human CB_2 (hCB_2) receptors; (ii) it stimulated [^{35}S]GTPγS binding to hCB_2 CHO cell and mouse spleen membranes; (iii) it attenuated signs of inflammation/hyperalgesia induced in mouse hind paws by intraplantar injection of carrageenan or formalin; and (iv) any such anti-inflammatory or anti-hyperalgesic effects were blocked by a CB_1 or CB_2 receptor antagonist.
Key results: THCV inhibited cyclic AMP production by hCB_2 CHO cells (EC_{50} = 38 nM), but not by hCB_1 or untransfected CHO cells or by hCB_2 CHO cells pre-incubated with pertussis toxin (100 ng·mL^{-1}) and stimulated [^{35}S]GTPγS binding to hCB_2 CHO and mouse spleen membranes. THCV (0.3 or 1 mg·kg^{-1} i.p.) decreased carrageenan-induced oedema in a manner that seemed to be CB_2 receptor-mediated and suppressed carrageenan-induced hyperalgesia. THCV (i.p.) also decreased pain behaviour in phase 2 of the formalin test at 1 mg·kg^{-1}, and in both phases of this test at 5 mg·kg^{-1}; these effects of THCV appeared to be CB_1 and CB_2 receptor mediated.
Conclusions and implications: THCV can activate CB_2 receptors *in vitro* and decrease signs of inflammation and inflammatory pain in mice partly *via* CB_1 and/or CB_2 receptor activation.
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Keywords: Δ^9-Tetrahydrocannabivarin; CP55940; CB_2 receptor; CB_1 receptor; pertussis toxin; pain; inflammation; carrageenan; formalin

Abbreviations: AM630, 6-iodopravadoline; CHO, Chinese hamster ovary; CP55940 (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; DMSO, dimethyl sulphoxide; PMSF, phenylmethylsulphonyl fluoride; rimonabant, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; THCV, Δ^9-tetrahydrocannabivarin

Correspondence: Professor RG Pertwee, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK. E-mail: rgp@abdn.ac.uk
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Introduction

We have reported previously that the plant cannabinoid, Δ^9-tetrahydrocannabivarin (THCV; Figure 1), can behave as a CB_1 receptor antagonist, both *in vitro* and *in vivo* (Pertwee

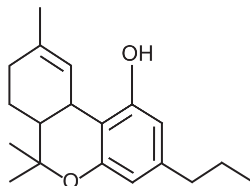

Figure 1 The structure of THCV.

et al., 2007; receptor nomenclature follows Alexander *et al*., 2009), and also that this phytocannabinoid opposes the ability of the cannabinoid receptor agonist, CP55940, to stimulate [^{35}S]GTPγS binding to human CB_2 receptors in Chinese hamster ovary (CHO) cell membranes (Thomas *et al*., 2005). In this paper, we present evidence that THCV can behave *in vitro* as a CB_2 receptor partial agonist when the measured response is inhibition of forskolin-induced stimulation of cyclic AMP production by CHO cells expressing very high densities of human CB_2 receptors or stimulation of [^{35}S]GTPγS binding to membranes obtained either from these cells or from mouse spleen. No such effects were induced by THCV in human CB_1 CHO cells, in mouse whole brain membranes or in mouse spleen membranes obtained from $CB_2^{-/-}$ mice, findings that are in line with previous reports that THCV can behave as a CB_1 receptor antagonist (Thomas *et al*., 2005; Pertwee *et al*., 2007; Dennis *et al*., 2008; Ma *et al*., 2008). Because there is convincing pre-clinical evidence that combined activation of CB_2 receptors and blockade of CB_1 receptors would ameliorate disorders such as chronic liver diseases and stroke (Mallat *et al*., 2007; Zhang *et al*., 2009), our discovery that THCV can behave as a CB_2 receptor agonist *in vitro* prompted us to investigate whether this compound can also produce signs of CB_2 receptor activation *in vivo*. This we did by determining whether THCV shares the ability of established selective CB_2 receptor agonists to reduce signs of inflammation and inflammatory pain in a manner that can be antagonized by a selective CB_2 receptor antagonist (Guindon and Hohmann, 2008). These experiments were performed with mice in which paw oedema and signs of hyperalgesia were induced by intraplantar injections of either carrageenan or formalin.

Methods

Animals

All animal care and experimental procedures complied with Italian (D.L. 116/92) and EEC (O.J. of EC L358/1 18/12/1986) regulations on the protection of laboratory animals, and with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines for the use of experimental animals. Guidelines of the International Association for the Study of Pain were also followed. Brain and spleen tissue was obtained from adult male C57BL/6J mice weighing 25–40 g and maintained on a 12/12 h light/dark cycle with free access to food and water. These animals were either wild-type mice (Harlan UK Ltd, Blackthorn, UK) or mice from which the CB_2 receptor had been genetically deleted as described by Buckley *et al*., 2000. All *in vivo* experiments were also performed with male C57BL/6J mice (Harlan, Milan, Italy). These mice were 9 weeks old and housed three per cage under controlled illumination (12:12 h light : dark cycle; light on at 0600 h) and standard environmental conditions (room temperature 22 ± 1°C; humidity 60 ± 10%) for at least 1 week before experimental use. Mouse chow and tap water were available *ad libitum*. All the *in vivo* experiments were performed in a randomized manner by an experimenter, unaware of the pharmacological treatments.

CHO cells

CHO cells either untransfected or transfected with cDNA encoding human cannabinoid CB_2 or CB_1 receptors were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10% fetal bovine serum and 0.6% penicillin–streptomycin for all cells together with G418 (400 μg·mL^{-1}) for the CHO–hCB_2 cells or with hygromycin B (300 μg·mL^{-1}) and G418 (600 μg·mL^{-1}) for the CHO–hCB_1 cells. All cells were maintained at 37°C and 5% CO_2 in their respective media, and were passaged twice a week using non-enzymatic cell dissociation solution.

Membrane preparation

Binding assays with [^{3}H]CP55940 or [^{35}S]GTPγS were performed with CHO–hCB_2 cell membranes (Ross *et al*., 1999a), with mouse whole brain membranes (Viganò *et al*., 2003) or with mouse spleen membranes, the preparation of which was based on a method described by Hillard *et al*. (1999) for preparing rat spleen membranes. The hCB_2 receptor-transfected cells were removed from flasks by scraping and then frozen as a pellet at −20°C until required. Before use in a radioligand binding assay, cells were defrosted, diluted in Tris buffer (50 mM Tris–HCl and 50 mM Tris–base) and homogenized with a 1 mL hand-held homogenizer. Protein assays were performed using a Bio-Rad DC Kit (Hercules, CA, USA). Spleens were cut into several pieces and placed in a Choi lysis buffer (Tris–HCl 20 mM, sucrose 0.32 M, EDTA 0.2 mM, EGTA 0.5 mM, pH 7.5) containing Roche protease inhibitor cocktail (1:40 v/v; Roche Diagnostics, Mannheim, Germany) and phenylmethylsulphonyl fluoride (PMSF; 150 μM), and then homogenized. The homogenate was centrifuged at 500× *g* for 2 min, and the resulting supernatant was re-centrifuged at 16 000× *g* for 20 min. The harvested membranes were resuspended in TME buffer (50 mM Tris–HCl; EDTA 1.0 mM; $MgCl_2$ 3.0 mM; pH 7.4) and stored at −80°C for no more than 1 month. Mouse brains were homogenized in ice-cold Choi lysis buffer containing Roche protease inhibitor cocktail (1:40 v/v) and PMSF (1 mM). The homogenate was centrifuged at 13 500× *g* for 15 min, and the resulting pellet was kept at −80°C for at least 2 h. The pellet was then resuspended in TME buffer, homogenized and stored at −80°C.

Cyclic AMP assay

Adherent CHO–hCB_1 or CHO–hCB_2 cells were washed once with Dulbecco's phosphate-buffered saline (PBS) and detached using non-enzymatic cell dissociation solution.

After centrifugation, cells were resuspended (2×10^6 cells·mL^{-1}) in buffer containing PBS (calcium and magnesium free), 1% BSA and 10 μM rolipram. Cells were incubated for 30 min at 37°C with the cannabinoid under investigation. A further 30 min incubation was carried out with 10 μM of forskolin in a total volume of 500 μL. The reaction was terminated by the addition of 0.1 M HCl, followed by centrifugation to remove cell debris. The pH was then adjusted to between 8 and 9 by the addition of 1 M of NaOH, and cyclic-AMP content was measured using a radioimmunoassay kit (GE Healthcare Amersham Ltd, Little Chalfont, Buckinghamshire, UK). Forskolin and rolipram were dissolved in dimethyl sulphoxide (DMSO) and stored at –20°C as 10 mM stock solutions. Some CHO cells were pretreated overnight with pertussis toxin (100 ng·mL^{-1}; Coutts *et al*., 2001). The pertussis toxin used in these experiments was dissolved in distilled water and stored at 4°C.

Radioligand displacement assay
The assays were carried out with [^{3}H]CP55940 and Tris-binding buffer (50 mM Tris–HCl, 50 mM Tris–base, 0.1% BSA, pH 7.4), total assay volume 500 μL, using the filtration procedure described previously by Ross *et al*. (1999b). Binding was initiated by the addition of transfected hCB$_2$ cells (50 μg protein per well). All assays were performed at 37°C for 60 min before termination by the addition of ice-cold Tris-binding buffer and vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA) and Brandel GF/B filters that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 mL aliquot of Tris-binding buffer. The filters were oven-dried for 60 min and then placed in 5 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabelled CP55940. The concentration of [^{3}H]CP55940 used in our displacement assays was 0.7 nM. The compounds under investigation were stored as stock solutions of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. The binding parameters for [^{3}H]CP55940 were 215 pmol·mg^{-1} (B_{max}) and 4.3 nM (K_d).

[^{35}S]GTPγS binding assay
The method used for measuring agonist-stimulated binding of [^{35}S]GTPγS was based on previously described methods (Hillard *et al*., 1999; Thomas *et al*., 2005). The assays were carried out with GTPγS binding buffer (50 mM Tris–HCl, 100 mM NaCl, 0.1% BSA) in the presence of [^{35}S]GTPγS and GDP, in a final volume of 500 μL. The GTPγS binding buffer also contained 50 mM Tris–base, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EDTA (CHO cell membrane experiments) or 3 mM $MgCl_2$ and 0.2 mM EGTA (brain and spleen membrane experiments). Binding was initiated by the addition of [^{35}S]GTPγS to the wells. Non-specific binding was measured in the presence of 30 μM GTPγS. The drugs were incubated in the assay for 60 min at 30°C. The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer, and the radioactivity was quantified by liquid scintillation spectrometry. In all the [^{35}S]GTPγS-binding assays, we used 0.1 nM [^{35}S]GTPγS, 30 μM GDP and 10 μg (brain membranes), 40 μg (spleen membranes) or 50 μg (cell membranes) protein per well. Additionally, mouse brain and spleen membranes were pre-incubated for 30 min at 30°C with 0.5 U·mL^{-1} adenosine deaminase (200 U·mL^{-1}) to remove any endogenous adenosine.

Carrageenan-induced inflammation
The mice were anaesthetized with sodium pentobarbital (60 mg·kg^{-1} i.p.). Acute inflammation was induced by intraplantar injection of 20 μL of λ-carrageenan (2% w/v in saline) into the right hind paw. The volume of the injected paw, as well as of the contralateral paw, was measured with a plethysmometer (Ugo Basile, Varese, Italy). Data are expressed as oedema (difference in volume between the right and left paws). Responses to thermal stimuli were measured in the same animals used to monitor oedema. After recording baseline withdrawal latencies (s), withdrawal latencies of both hind paws were estimated at different time-points after carrageenan injection, starting 3 h after injection of this inflammatory stimulus, when all mice had recovered from anaesthesia. Heat hypersensitivity was tested according to the Hargreaves procedure (Hargreaves *et al*., 1988) using the plantar test (Ugo Basile). Briefly, the animals were placed in a clear plexiglass box and allowed to acclimatize. A constant intensity radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. The control animals received saline instead of carrageenan by intraplantar injection. All these experiments were performed by the same experimenter.

Formalin test
Formalin injection induces biphasic stereotypical nocifensive behaviour (Dubuisson and Dennis, 1977). Nociceptive responses are divided into an early, short lasting first phase (0–7 min) caused by a primary afferent discharge produced by the stimulus and a subsequent second, prolonged phase (15–60 min) of tonic pain (Sawynok and Liu, 2004). These two phases are separated by a transient quiescent period. The mice received formalin (1.25% in saline, 30 μL) into the dorsal surface of one side of the hind paw. Each mouse was randomly assigned to one of the experimental groups and placed in a plexiglass cage, and allowed to move freely for 15–20 min. A mirror was placed at a 45° angle under the cage to allow full view of the hind paws. Lifting, favouring, licking, shaking and flinching of the injected paw were recorded as nociceptive responses (Abbott and Guy, 1995). Nociceptive responses were measured every 5 min and expressed as their total duration in min (mean ± SEM). Recording of nociceptive behaviour commenced immediately after formalin injection and was continued for 60 min. All these experiments were performed by the same experimenter.

In vivo *drug treatments and experimental design*
Different doses of THCV or an appropriate volume of its vehicle were administered i.p., 30 min before carrageenan

injection or 15 min before formalin injection. THCV was dissolved in a mixture of ethanol : cremophorEL : saline (1:1:18), when tested in the carrageenan model, or in 0.5% DMSO in saline (0.9% aqueous solution of NaCl), when tested using the formalin test. These vehicles were chosen because they have previously been shown to be devoid of activity in the carrageenan and formalin models, respectively (Costa *et al*., 2004; Maione *et al*., 2007). Carrageenan-induced oedema and thermal hyperalgesia were evaluated 2 and 3 h after the injection of phlogogen respectively. The maximal effective dose (0.3 $mg \cdot kg^{-1}$ i.p.) was employed to evaluate the time-course of the anti-inflammatory effects of THCV: oedema was measured at 2, 3, 4, 6 and 24 h and thermal hyperalgesia at 3, 6 and 24 h after carrageenan administration. Some experiments were performed with the selective CB_1 receptor antagonist/inverse agonist, rimonabant (0.5 $mg \cdot kg^{-1}$), or with the selective CB_2 receptor antagonist/inverse agonist, SR144528 (1 $mg \cdot kg^{-1}$). These were administered to the mice i.p. 15 min before THCV in both carrageenan and formalin experiments. Oedema and withdrawal latency were evaluated at 2 and 3 h after carrageenan injection, and nociceptive behaviour was observed for 60 min after formalin administration. Rimonabant and SR144528 were dissolved in a mixture of Tween80 : DMSO : distilled water (1:2:7). To determine the ability of THCV to improve established signs of inflammation and pain, an additional series of experiments was performed in which this compound was administered to the mice at 0.3 $mg \cdot kg^{-1}$ i.p. 30 min after intraplantar injection of carrageenan, and for 3 consecutive days after the induction of inflammation. Behavioural evaluations were performed 2, 24, 48 and 72 h after carrageenan injection. No overt behavioural changes were observed in this study following administration of vehicle or of any of the drugs at the dosage used. The mice remained alert and generally active throughout these experiments.

Statistical analysis
Values have been expressed as means and variability as SEM or as 95% confidence intervals (CIs). The concentration of THCV and CP55940 that produced a 50% displacement of [^{3}H]CP55940 from specific binding sites (IC_{50} value) was entered into the equation of Cheng and Prusoff (1973) to calculate the corresponding K_i value. Values obtained *in vitro* for EC_{50} and maximal effect (E_{max}) have been calculated by non-linear regression analysis using the equation for a sigmoid concentration–response curve. Statistical analysis of all *in vivo* data was performed by ANOVA followed by the Newman–Keuls multiple comparison test. All these statistical analyses were performed using GraphPAD software (San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

Materials
THCV, extracted from *Cannabis*, was a gift from GW Pharmaceuticals (Porton Down, Wiltshire, UK). It was 99.4% pure and contained no detectable Δ^9-tetrahydrocannabinol, cannabinol or cannabidiol. Rimonabant and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France). Carrageenan and formalin were purchased from Sigma-Aldrich (Milan, Italy) and (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), from Tocris (Bristol, UK). Pertussis toxin, forskolin and rolipram were supplied by Sigma-Aldrich (Poole, Dorset, UK) and [^{3}H]CP55940 (174.6 $Ci \cdot mmol^{-1}$) by PerkinElmer Life Sciences Inc (Boston, MA, USA).

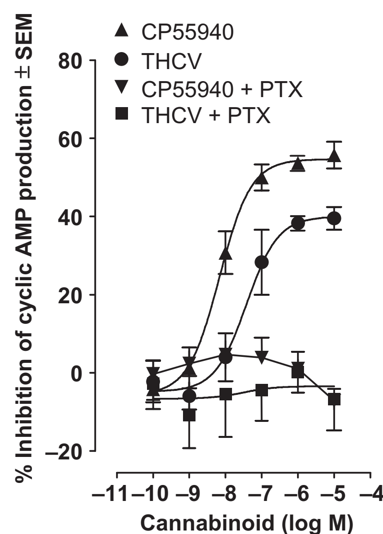

Figure 2 The effect of THCV and CP55940 on forskolin-induced stimulation of cyclic AMP production in CHO cells transfected with hCB_2 receptors that had or had not been pre-incubated overnight with pertussis toxin (PTX; 100 $ng \cdot mL^{-1}$) (n = 4). EC_{50} values determined in cells not pre-incubated with pertussis toxin, with 95% CIs shown in brackets, were 38 nM (12 and 124 nM) for THCV and 6.9 nM (3.5 and 13 nM) for CP55940. The corresponding E_{max} values were 40% (32 and 48%) and 55% (50 and 60%) respectively. Symbols represent mean values ± SEM.

Results

THCV behaves in vitro *as a potent cannabinoid CB_2 receptor agonist*
In our initial experiments, we investigated whether THCV shares the ability of established cannabinoid CB_2 receptor agonists (Pertwee, 1999) to inhibit forskolin-induced stimulation of cyclic AMP production in hCB_2-transfected CHO cells. As shown in Figure 2, we found that at concentrations in the nanomolar range THCV can indeed induce such inhibition in this bioassay, its EC_{50} and E_{max} values with 95% CIs shown in parentheses being 38 nM (12 and 124 nM) and 40% (32 and 48%) respectively. Corresponding values for the established CB_1/CB_2 receptor agonist, CP55940, which is thought to display full (high-efficacy) agonism at the cannabinoid CB_2 receptor were 6.9 nM (3.5 and 13 nM) and 55% (50 and 60%), respectively (Figure 2).

We also obtained some confirmatory evidence that these inhibitory effects of THCV and CP55940 were cannabinoid CB_2 receptor mediated. First, as shown in Figure 2, we established that at concentrations of 0.1 nM to 10 μM, neither of these compounds affected forskolin-stimulated cyclic AMP production in CHO–hCB_2 cells when these had been pre-incubated overnight with pertussis toxin in a manner

expected to eliminate $G_{i/o}$-mediated signalling (Glass and Felder, 1997; Bonhaus *et al.*, 1998; Coutts *et al.*, 2001). Second, we found that at such concentrations, neither THCV nor CP55940 inhibited forskolin-stimulated cyclic AMP production in untransfected CHO cells (n = 4; data not shown). Next, we showed that THCV can displace [^{3}H]CP55940 from specific binding sites on cell membranes from CHO cells transfected with hCB$_2$ receptors, its mean K_i value with 95% CIs shown in brackets being 225 nM (170 and 298 nM; n = 8).

We have found previously that THCV opposes CP55940-induced stimulation of [^{35}S]GTPγS binding to membranes of CHO–hCB$_2$ cells (Thomas *et al.*, 2005), and that by itself THCV does not behave as a CB$_2$ receptor agonist in this bioassay at concentrations ranging from 0.01 nM to 10 μM (Thomas and Pertwee, unpublished). However, the CB$_2$ receptor density determined by [^{3}H]CP55940 saturation binding was three times lower in the CHO cells used in these previous experiments ([^{3}H]CP55940 B_{max} = 72.57 pmol·mg^{-1}) than in the cells used in the present investigation (Methods). We therefore carried out some additional experiments directed at testing the hypothesis that THCV would behave as a CB$_2$ receptor agonist in the [^{35}S]GTPγS assay when this was performed with membranes obtained from the CHO–hCB$_2$ cell line in which this cannabinoid did appear to activate CB$_2$ receptors (Figure 2). We found that THCV can indeed stimulate [^{35}S]GTPγS binding to membranes obtained from these CHO–hCB$_2$ cells. Its EC$_{50}$ and E_{max} values with 95% CIs shown in parentheses were 41.5 nM (10.5 and 164 nM) and 30% (26 and 34%), respectively (n = 6), and hence similar to the EC$_{50}$ and E_{max} values we obtained for THCV in the cyclic AMP assay. Corresponding values for CP55940 were 0.8 nM (0.3 and 2.1 nM) and 45% (40 and 50%), respectively (n = 4).

We also discovered that unlike CP55940, THCV did not inhibit forskolin-stimulated cyclic AMP production in hCB$_1$-transfected CHO cells, behaving instead as an hCB$_1$ cannabinoid receptor inverse agonist (Figure 3A). This ability of THCV to enhance forskolin-induced stimulation of cyclic AMP production was not observed in CHO–hCB$_1$ cells that had been pre-incubated overnight with pertussis toxin (Figure 3B). In contrast, the effect of CP55940 on cyclic AMP production switched from an inhibitory to a stimulatory effect in response to such pre-incubation with pertussis toxin (Figure 3B), presumably reflecting the reported ability of CB$_1$ receptors to activate G_s proteins in the absence of functional $G_{i/o}$ coupling (Glass and Felder, 1997; Bonhaus *et al.*, 1998; Howlett *et al.*, 2002). No attempt was made to determine whether THCV-induced inhibition of forskolin-stimulated cyclic AMP production by CHO–hCB$_2$ cells was susceptible to antagonism by the established selective cannabinoid CB$_2$ receptor competitive antagonists, SR144528 and AM630. This was because, as we have also reported previously (Ross *et al.*, 1999a,b), both these antagonists by themselves produced marked signs of inverse agonism in the cyclic AMP assay performed with our CHO–hCB$_2$ cells (data not shown).

Our finding that THCV appears to activate human cannabinoid CB$_2$ receptor *in vitro* prompted us to investigate its ability to activate CB$_2$ receptors *in vivo* in mouse models of inflammation and inflammatory pain, there being evidence that CB$_2$ receptor activation constitutes a potential therapeutic strategy for ameliorating symptoms of this kind (Pertwee, 2009). Whereas human and mouse CB$_1$ receptors have almost identical amino acid sequences, there are significant differences between human and mouse CB$_2$ receptors with regard both to their primary amino acid composition and to the manner in which they respond to at least some ligands (Howlett *et al.*, 2002; Bingham *et al.*, 2007). Thus, for example, the selective ligand for CB$_2$ receptors, *S*-AM1241, has been reported by Bingham *et al.* (2007) to behave as an agonist at the hCB$_2$ receptor, but as an inverse agonist at mouse and rat CB$_2$ receptors. Moreover, our evidence that THCV can activate hCB$_2$ receptors came from experiments with tissue obtained from a cell line in which these receptors are expressed at an extremely high level. Consequently, before proceeding to any *in vivo* experiments, it was first important to establish whether THCV can activate mouse CB$_2$ receptors *in vitro* in a tissue in which these receptors are expressed at physiologically relevant levels. The tissue selected for these experiments was mouse spleen as this has been reported to be well populated with CB$_2$ receptors (Pertwee, 1997). We found that THCV can indeed stimulate [^{35}S]GTPγS binding to these membranes. Its EC$_{50}$ and E_{max} values in these experiments with 95% CIs shown in parentheses were 69 nM (2.6 and 1804 nM) and 23% (3.8 and 42%), respectively (n = 6). Mouse spleen has been reported to express CB$_1$ as well as CB$_2$ receptors (Pertwee, 1997). Even so, it is unlikely that THCV was stimulating [^{35}S]GTPγS binding by activating CB$_1$ receptors in our spleen experiments because concentrations of THCV ranging from 1 to 10 μM produced no detectable stimulation of [^{35}S]GTPγS binding to spleen membranes obtained from CB$_2^{-/-}$ mice (n = 6; data not shown). Moreover, THCV (n = 8; data not shown) did not share the ability of the established CB$_1$/CB$_2$ receptor agonist, CP55940, to stimulate [^{35}S]GTPγS binding to membranes obtained from mouse brain, a tissue in which CB$_1$ receptors are highly expressed (Howlett *et al.*, 2002). The

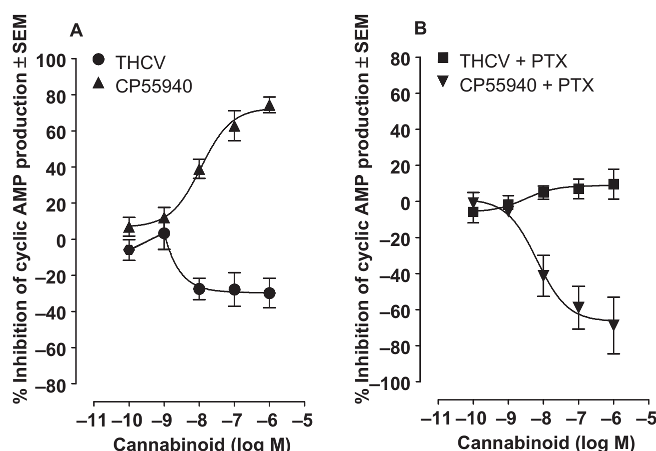

Figure 3 The effect of THCV and CP55940 on forskolin-induced stimulation of cyclic AMP production in CHO cells transfected with hCB$_1$ receptors that (A) had not been or (B) had been pre-incubated overnight with pertussis toxin (PTX; 100 ng·mL^{-1}) (n = 4). The mean E_{max} value of THCV in (A), with 95% CIs shown in brackets, was −29.6% (16.8 and 42.4%). The mean E_{max} value of THCV (8.8%) in (B) did not differ significantly from zero. Its 95% CIs were −0.8 and 18.5%. Symbols represent mean values ± SEM.

EC_{50} displayed by CP55940 in these experiments with its 95% CIs shown in parentheses was 10.7 nM (6.3 and 18.4 nM; $n = 6$).

THCV ameliorates signs of inflammation and hyperalgesia induced in mice by carrageenan or formalin

Having obtained evidence that THCV can activate naturally expressed mouse CB_2 receptors *in vitro*, we went on to investigate its ability to activate mouse CB_2 receptors *in vivo*. This we did by performing experiments directed at determining whether THCV shares the ability of established selective CB_2 receptor agonists (Whiteside *et al*., 2007; Guindon and Hohmann, 2008) to ameliorate signs of inflammation and thermal hyperalgesia induced in rats or mice by intraplantar injection of carrageenan or formalin.

As expected, carrageenan caused the volume of each injected ipsilateral hind paw to increase relative to the volume of the contralateral uninjected paw. At 2 h after carrageenan administration, this ipsilateral hind paw oedema was significantly less in mice that had been pretreated with THCV at a dose of 0.3 mg·kg^{-1} than in vehicle-pretreated animals (Figure 4A). Carrageenan injection also induced marked thermal hyperalgesia, as indicated by a decreased withdrawal latency of the injected paw in response to a thermal stimulus, and pretreatment with THCV at doses of either 0.3 or 1 mg·kg^{-1} was found to reduce this thermal hypersensitivity (Figure 4B). Because carrageenan-induced oedema and thermal hyperalgesia remained at a high level throughout an observation period of 24 h (Figure 5), the anti-inflammatory and anti-nociceptive responses elicited by THCV at 0.3 mg·kg^{-1} were investigated at additional time-points. We found that the anti-oedema effect of THCV was still both present and unreduced at 3 and 4 h after carrageenan administration, slightly reduced but still present 6 h after carrageenan, and absent 24 h after carrageenan (Figure 5A). Similarly, the anti-hyperalgesic effect of THCV decreased progressively over this same observation period and was no longer detectable 24 h after carrageenan administration (Figure 5B). At no dose or time-point did THCV affect the volume or withdrawal latency of the contralateral, uninjected hind paws (data not shown).

We also investigated the ability of rimonabant, a selective CB_1 receptor antagonist/inverse agonist, and SR144528, a selective CB_2 receptor antagonist/inverse agonist, to block the anti-oedema and anti-nociceptive effects of THCV. These compounds were administered 15 min before THCV (0.3 mg·kg^{-1}), and evaluations of oedema and thermal hypersensitivity were made at 2 and 3 h after carrageenan respectively. Figure 6A shows that only SR144528 was able to reverse the anti-oedema effect of THCV, suggesting that this phytocannabinoid induced its anti-inflammatory effect primarily through activation of the CB_2 receptor. However, rimonabant elicited a partial, although statistically not significant, reversal of THCV-induced anti-hyperalgesia, whereas SR144528 induced no sign of any such reversal (Figure 6B). Importantly, the doses of rimonabant (0.5 mg·kg^{-1}) and SR144528 (1 mg·kg^{-1}) used in these experiments did not affect carrageenan-induced paw oedema or thermal hypersensitivity when administered alone (data not shown). Nor did THCV at any of the doses used in this investigation affect the volume or withdrawal latency of paws that had been injected with vehicle instead of carrageenan.

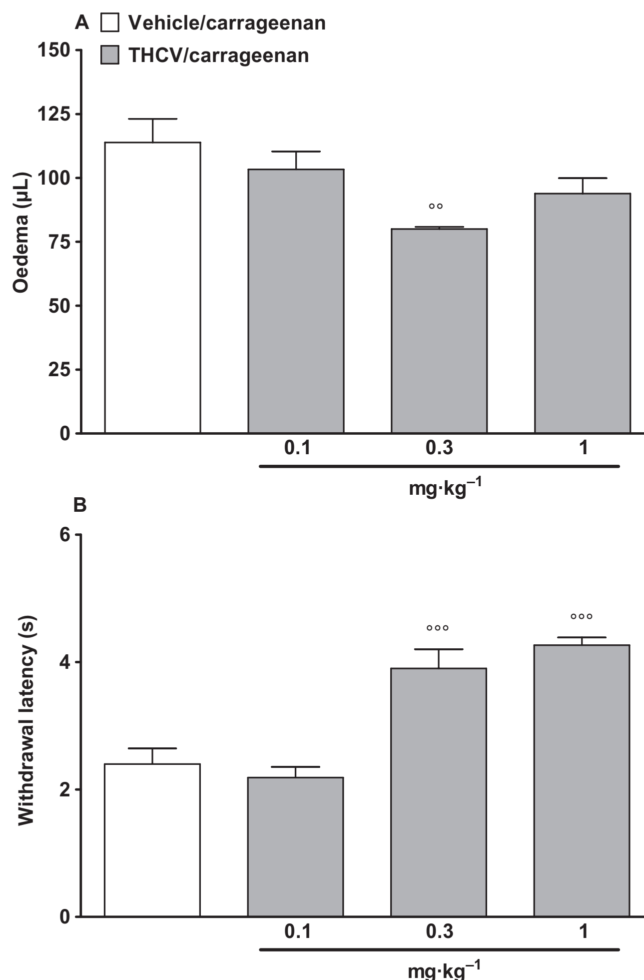

Figure 4 Effect of THCV, administered i.p. 30 min before carrageenan (2%, 20 μL intraplantar), on (A) oedema evaluated 2 h after carrageenan, and (B) thermal hypersensitivity, evaluated 3 h after carrageenan. The basal hind paw withdrawal latency displayed by vehicle-treated mice was 10 ± 0.45 s. Data represent mean values ± SEM ($n = 9$). °°$P < 0.01$, °°°$P < 0.001$ versus mice treated with vehicle/carrageenan.

To test the ability of THCV to counteract established inflammation, the compound was administered at 0.3 mg·kg^{-1}, 30 min after carrageenan injection, and then again once daily over the next 3 days. As shown in Figure 7A,B, THCV significantly diminished oedema and thermal hyperalgesia after its first injection, and these anti-oedema and anti-hyperalgesic effects of THCV remained undiminished after each of its subsequent injections.

THCV also displayed dose-dependent activity against formalin-induced nociceptive behaviour (Figure 8A). More specifically, it reduced pain behaviour in both phases of the formalin test at a dose of 5 mg·kg^{-1}, decreased pain behaviour in the second but not the first phase of this test at 1 mg·kg^{-1} and did not significantly affect pain behaviour in either phase at 0.1 mg·kg^{-1}. The ameliorating effect of the higher dose of THCV (5 mg·kg^{-1}) on the first and second phases of the for-

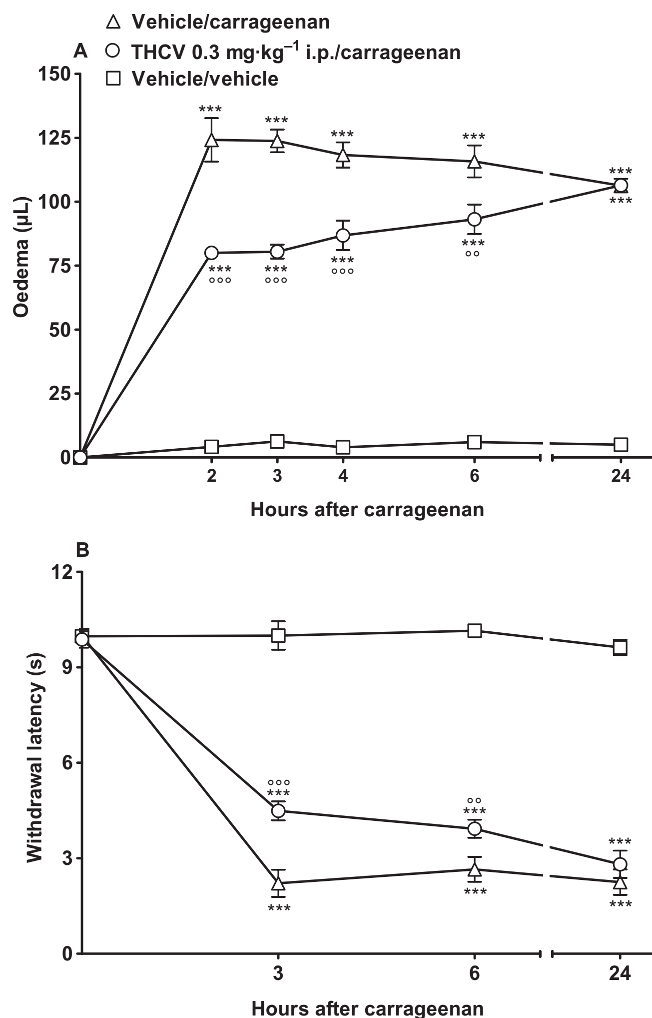

Figure 5 Effect of THCV (0.3 mg·kg^{-1} i.p.), administered 30 min before carrageenan (2%, 20 μL intraplantar), on (A) oedema and (B) thermal hypersensitivity evaluated at different times after carrageenan. Data represent mean values ± SEM (n = 5). ***P < 0.001 versus mice treated with vehicle/vehicle. °°P < 0.01, °°°P < 0.001 versus mice treated with vehicle/carrageenan.

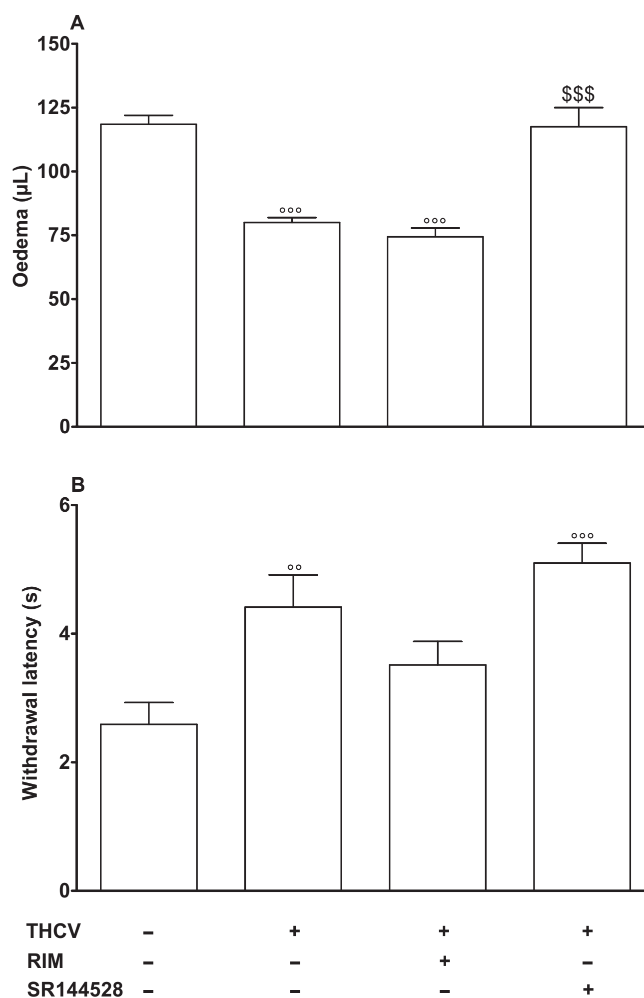

Figure 6 Effect of rimonabant (RIM; 0.5 mg·kg^{-1} i.p.) and SR144528 (1 mg·kg^{-1} i.p.) on (A) anti-oedema and (B) anti-nociceptive effects evoked by THCV (0.3 mg·kg^{-1} i.p.). Antagonists were administered 15 min before THCV, and behavioural evaluations were made 2 h (oedema) and 3 h (thermal hypersensitivity), after carrageenan. Data represent mean values ± SEM (n = 8–10). °°P < 0.01, °°°P < 0.001 versus mice treated with vehicle/carrageenan. $$$$P$ < 0.001 versus mice treated with vehicle/THCV/carrageenan.

malin response (Figure 8B) and of the lower dose of THCV (1 mg·kg^{-1}) on the second phase (Figure 8C) was attenuated by pretreatment both with rimonabant (0.5 mg·kg^{-1}) and with SR144528 (1 mg·kg^{-1}). The doses of rimonabant and SR144528 used in these experiments did not affect formalin-induced pain behaviour when administered alone (data not shown).

Discussion

Results from our *in vitro* experiments indicate that THCV exhibits significant potency and efficacy as a cannabinoid CB_2 receptor agonist. Thus, THCV shared the ability of the established CB_1/CB_2 receptor agonist, CP55940, both to inhibit forskolin-induced stimulation of cyclic AMP production by CHO–hCB_2 cells, and to stimulate [^{35}S]GTPγS binding to membranes obtained from these cells. In addition, neither THCV nor CP55940 inhibited cyclic AMP production either in untransfected CHO cells or in CHO–hCB_2 cells that had been pre-incubated overnight with pertussis toxin in order to eliminate $G_{i/o}$-mediated signalling. THCV is most likely an hCB_2 receptor partial agonist because, as predicted by classical drug receptor theory for an agonist of this kind (Kenakin, 1997; 2001), the efficacy that THCV displays at the hCB_2 receptor appears to be greatly influenced by the expression level of these receptors and to be lower than the efficacy displayed by an established high-efficacy CB_1/CB_2 receptor agonist, CP55940. Thus, THCV seems to undergo conversion from an apparent neutral hCB_2 receptor antagonist (Thomas *et al.*, 2005) to an apparent hCB_2 receptor agonist in the [^{35}S]GTPγS binding assay when the expression level of these receptors is increased (Results), and its E_{max} for the activation of hCB_2 receptors both in this assay (Results) and in the cyclic AMP assay (Figure 2) is less than that of CP55940. Some CB_2 receptor ligands, for example AM1241 (Yao *et al.*, 2006; Mancini *et al.*, 2009), display mixed agonist–antagonist–inverse

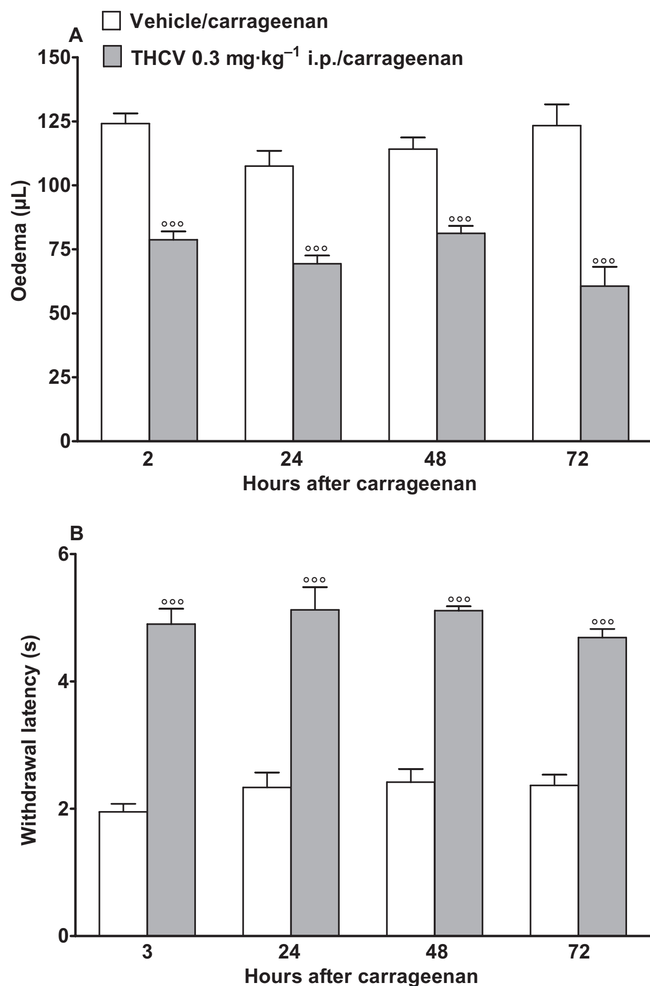

Figure 7 Effect of THCV (0.3 mg·kg^{-1} i.p.), administered after carrageenan once daily for 4 days, on (A) oedema and (B) thermal hypersensitivity. Data represent mean ± SEM (n = 6–8). °°°P < 0.001 versus mice treated with carrageenan/vehicle.

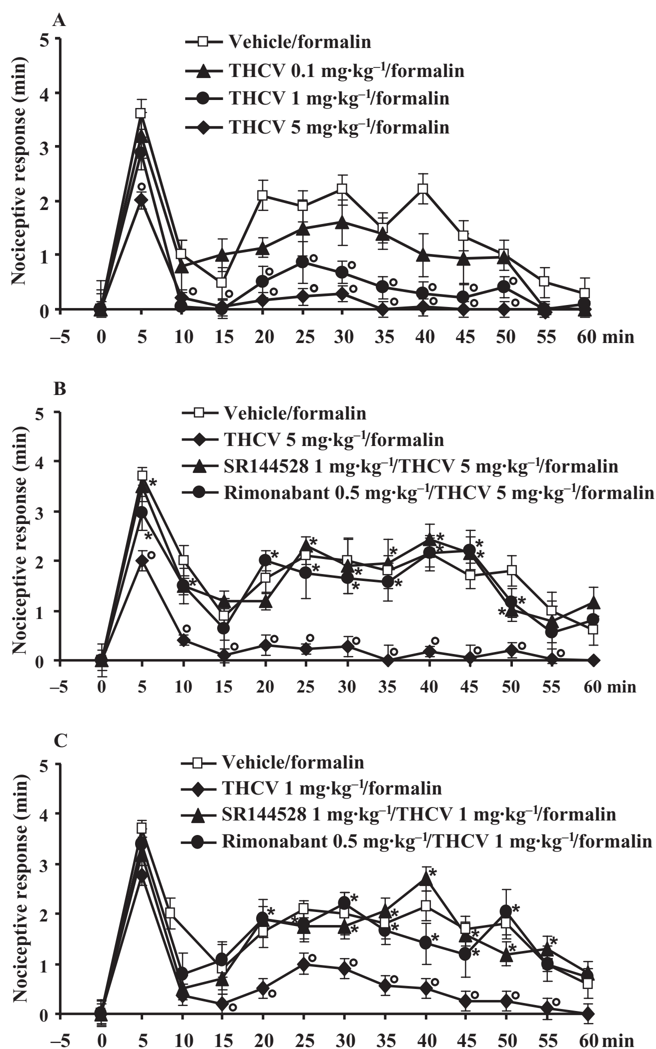

Figure 8 Effect on nociceptive behaviour evoked by formalin (1.25%, 30 µL s.c.) of THCV when administered at different doses by itself (A) or at 5 mg·kg^{-1} (B) or 1 mg·kg^{-1} (C) in combination with SR144528 (1 mg·kg^{-1} i.p.) or rimonabant (0.5 mg·kg^{-1} i.p.). THCV was administered intraperitoneally 15 min before formalin. Antagonists were given 15 min prior to THCV. Data represent mean values ± SEM (n = 8). °P < 0.05 versus vehicle. *Nociceptive responses to formalin that were significantly greater after SR144528 + THCV or rimonabant + THCV than after THCV alone (P < 0.05).

agonist activity and have been classified as protean agonists. These are ligands that can produce signs of agonism, neutral antagonism or inverse agonism at the receptor they are targeting, the effect produced depending on whether the active receptor conformation that they induce is of higher, similar or lower efficacy than the spontaneously formed active conformation of the receptor (Kenakin, 2001). There is as yet no evidence that THCV can induce signs of CB_2 receptor inverse agonism, and, as already discussed, its ability to display mixed agonist–antagonist activity at the hCB_2 receptor could simply be an indication that it is a partial agonist. Even so, the possibility that THCV is a protean agonist does warrant further research.

The mean EC_{50} of THCV for inhibition of cyclic AMP production by CHO–hCB_2 cells (Figure 2) was significantly less than its mean apparent K_i value (225 nM) for displacement of [^{3}H]CP55940 from specific sites on CHO–hCB_2 cell membranes (Results). However, this does not necessarily constitute evidence against our proposed classification of THCV as a CB_2 receptor partial agonist. This is because it could well be that the apparent K_i value we obtained in our experiments was significantly above the true K_i of THCV for the hCB_2 receptor. More specifically, the CB_2 receptor density in the cells used in the present investigation was rather high, and one likely effect of a large receptor concentration is to reduce the potency with which a tritiated ligand is displaced from its specific binding sites by an unlabelled compound (Kenakin, 1997). Such an effect of high receptor density is in line with our finding that the potency with which THCV displaced [^{3}H]CP55940 from hCB_2 receptors was 3.6-fold lower in the present investigation than in experiments performed previously (apparent K_i = 62.8 nM) with membranes obtained from cells with a threefold lower hCB_2 receptor expression level (Thomas *et al*., 2005).

THCV also affected forskolin-induced stimulation of cyclic AMP production by CHO–hCB_1 cells, producing signs of

inverse agonism (Figure 3A). No THCV-induced inverse agonism was observed either in untransfected CHO cells or in CHO–hCB_1 cells that had been pre-incubated with pertussis toxin, suggesting this effect was indeed CB_1 receptor mediated. That THCV seems to alter CB_1 receptor signalling in this way is unexpected. Thus, in previous experiments performed with mouse whole brain membranes, synthetic THCV was found to lack detectable agonist or inverse agonist activity (Pertwee *et al*., 2007). Instead, it behaved as a reasonably potent CB_1 receptor neutral antagonist, as indicated by its ability to antagonize CP55940-induced stimulation of [^{35}S]GTPγS binding to brain membranes and by its failure, when administered alone, to stimulate or inhibit [^{35}S]GTPγS binding to these membranes. It is possible that THCV induces signs of inverse agonism in CHO–hCB_1 cells, but not in brain membranes because CB_1 receptors were more highly expressed in the CHO cells and/or because CB_1 receptor signalling is less amplified in the GTPγS binding assay than in the cyclic AMP assay (Pertwee, 1999). Whether THCV can induce inverse agonism *in vivo* remains to be investigated. There is already evidence, however, that it can behave *in vivo* as a CB_1 receptor antagonist. Thus, THCV has been reported to suppress food intake and weight gain in mice (Riedel *et al*., 2009), and to attenuate several *in vivo* effects of Δ^9-THC, including Δ^9-THC-induced anti-nociception in the tail flick test and catalepsy in the ring test (Pertwee *et al*., 2007).

THCV stimulated [^{35}S]GTPγS binding not only to membranes prepared from CHO cells expressing hCB_2 receptors at a very high density, but also to membranes obtained from mouse spleen, a tissue that is thought to express CB_2 receptors naturally (Pertwee, 1997). The spleen is thought also to express CB_1 receptors. However, it is unlikely that THCV stimulated [^{35}S]GTPγS binding to spleen membranes by activating CB_1 receptors as it did not produce any such stimulation either in spleen membranes obtained from $CB_2^{-/-}$ mice or in whole brain membranes obtained from wild-type mice (present results). These mouse spleen findings are in line with a previous report that THCV (10 μM) can stimulate fibroblastic colony formation by rat bone marrow cells in a manner that seems to be mediated by naturally expressed CB_2 receptors (Scutt and Williamson, 2007).

THCV induced signs of cannabinoid CB_2 receptor activation not only *in vitro*, but also *in vivo* as indicated by an ability to suppress carrageenan-induced hind paw oedema (Figures 4–6) and formalin-induced hyperalgesia in mice (Figure 8). Both these effects were significantly attenuated by the CB_2 receptor-selective antagonist, SR144528. Importantly, these are effects that have been shown in previous investigations with rats or mice to be induced by established CB_2 receptor agonists in a manner that can be prevented by CB_2 receptor antagonists (Whiteside *et al*., 2007; Guindon and Hohmann, 2008).

THCV may also have suppressed signs of hyperalgesia in mice through one or more CB_2 receptor-independent mechanisms. Thus, the ability of THCV to suppress formalin-induced hyperalgesia was significantly attenuated not only by the CB_2 receptor-selective antagonist, SR144528, but also by the CB_1 receptor-selective antagonist, rimonabant (Figure 8B,C). Furthermore, the ability of THCV to suppress carrageenan-induced hyperalgesia was not significantly attenuated either by SR144528, at a dose that has been found to block the effect of an established CB_2 receptor in this assay (Guindon and Hohmann, 2008), or by rimonabant (Figure 6B). Moreover, in contrast to previous findings with established CB_2 receptor agonists which have been reported in several investigations to suppress the second phase but not the first phase of formalin-induced nocifensive behaviour (Whiteside *et al*., 2007; Guindon and Hohmann, 2008), THCV suppressed both phases of formalin-induced pain behaviour when administered at a dose of 5 mg·kg^{-1} (Figure 8A). It did, however, affect only the second of these phases when administered at the lower dose of 1 mg·kg^{-1}. The possibility that THCV or, indeed, one or more of its metabolites, might induce anti-nociceptive and/or anti-inflammatory effects at least in part by interacting with pharmacological targets other than CB_1 or CB_2 receptors warrants further investigation, especially because there is already evidence that its structural analogue, Δ^9-THC, has a number of non-CB_1, non-CB_2 sites of action (Pertwee, 2008; Ross, 2009). It should also be borne in mind that although rimonabant and SR144528 are established cannabinoid receptor antagonists, it remains possible that either or both of these compounds could have reduced the ability of THCV to suppress signs of hyperalgesia or paw oedema in a CB_1 or CB_2 receptor-independent manner. Thus, several non-CB_1, non-CB_2 targets have been identified for rimonabant (Fong *et al*., 2009; Ross, 2009), although not yet for SR144528.

Both rimonabant and SR144528 were found to reduce the ability of THCV to suppress formalin-induced hyperalgesia. Although this could have been because all three of these compounds were targeting non-CB_1, non-CB_2 receptors, a more likely explanation for this finding is that THCV produced its anti-nociceptive effect in this assay by activating both CB_1 and CB_2 receptors. It is noteworthy therefore that THCV has been found to behave *in vivo*, although not *in vitro*, as a CB_1 receptor agonist at doses above those at which it produces signs of CB_1 receptor blockade (Pertwee *et al*., 2007). Combined CB_1 and CB_2 receptor activation provides a possible explanation for our finding that THCV-induced suppression of carrageenan-induced hyperalgesia is not antagonized by SR144528 at a dose at which this CB_2 receptor-selective antagonist does antagonize THCV-induced suppression of carrageenan-induced oedema (Figure 6). It might also explain why we found the highest dose of THCV that we tested to suppress both phases of formalin-induced pain behaviour, there being evidence that, in contrast to CB_2 receptor-selective agonists, established mixed CB_1/CB_2 receptor agonists such as Δ^9-THC and CP55940 also act in this way (Pertwee, 2001). Why the relatively low doses of THCV we used in this investigation would activate CB_1 receptors in addition to CB_2 receptors remains to be established, one possible explanation being that it is a consequence of carrageenan- and/or formalin-induced up-regulation of the CB_1 receptor in pain pathways, there already being evidence that such up-regulation does occur in primary afferent neurons in at least one rodent model of inflammatory pain (Amaya *et al*., 2006).

Having established that THCV can suppress carrageenan-induced signs of inflammation and inflammatory pain in mice when it was injected 30 min before carrageenan, we

went on to investigate whether it would also suppress these signs when administered repeatedly after carrageenan, once daily for 4 days. We found that THCV was indeed effective when administered in this way, and also, that no tolerance seemed to develop (Figure 7). That THCV can, at least in mice, counteract signs of established inflammation and inflammatory pain, strengthens the case for investigating its therapeutic potential for the management of inflammation and inflammatory pain as such symptoms are most often targeted, in humans, only after they have appeared. Because the main objective of these additional experiments was to investigate the ability of THCV to suppress signs of established inflammation and inflammatory pain, no attempt was made to determine whether THCV suppressed these signs by acting through CB_1 or CB_2 receptors.

In conclusion, this investigation has demonstrated that, in mice, THCV can display anti-oedema activity in a carrageenan model of acute inflammation, and anti-hyperalgesic activity in carrageenan and formalin models of inflammatory pain. It has also provided evidence that this plant cannabinoid can activate human CB_2 receptors *in vitro* and mouse cannabinoid CB_2 receptors both *in vitro* and *in vivo*. Although THCV only produced detectable *in vitro* activation of human CB_2 receptors when these were expressed at a very high density, it did seem to activate mouse CB_2 receptors expressed in mouse spleen membranes at physiologically relevant levels, an indication that the mouse CB_2 receptor may be more sensitive to THCV than the hCB_2 receptor. Although the anti-oedema activity exhibited by THCV appeared to be CB_2, but not CB_1 receptor mediated, its anti-hyperalgesic activity seemed to be mediated by both CB_1 and CB_2 receptors in the formalin model, but by neither of these receptors in the carrageenan model. Clearly, therefore, further research directed at identifying the mechanisms underlying these *in vivo* effects of THCV, particularly its anti-hyperalgesic effects in the carrageenan model, is warranted. Our finding that THCV reduced carrageenan-induced hyperalgesia at both 0.3 and 1 $mg{\cdot}kg^{-1}$ (Figure 4B), but was effective in reducing carrageenan-induced oedema only at the lower of these two doses (Figure 4A), also merits further investigation. In addition, it will be important to establish more conclusively whether, as has been proposed for CB_2 receptor agonists (Guindon and Hohmann, 2008; Pertwee, 2009), THCV has therapeutic potential both as an anti-inflammatory agent and for the relief of inflammatory, or indeed, neuropathic pain. Because there is evidence that THCV can behave as a CB_1 receptor antagonist *in vivo* (Pertwee *et al*., 2007; Riedel *et al*., 2009), it would also be of interest to explore the possibility that this compound can suppress unwanted symptoms in animal models of disorders in which there is evidence that symptoms can be ameliorated by a combination of CB_2 receptor activation and CB_1 receptor blockade (see Introduction).

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Conflict of interest

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References

Abbott FV, Guy ER (1995). Effects of morphine, pentobarbital and amphetamine on formalin-induced behaviours in infant rats: sedation versus specific suppression of pain. *Pain* **62**: 303–312.

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). *Br J Pharmacol* **158** (Suppl 1): S1–S254.

Amaya F, Shimosato G, Kawasaki Y, Hashimoto S, Tanaka Y, Ji R-R *et al*. (2006). Induction of CB_1 cannabinoid receptor by inflammation in primary afferent neurons facilitates antihyperalgesic effect of peripheral CB_1 agonist. *Pain* **124**: 175–183.

Bingham B, Jones PG, Uveges AJ, Kotnis S, Lu P, Smith VA *et al*. (2007). Species-specific *in vitro* pharmacological effects of the cannabinoid receptor 2 (CB_2) selective ligand AM1241 and its resolved enantiomers. *Br J Pharmacol* **151**: 1061–1070.

Bonhaus DW, Chang LK, Kwan J, Martin GR (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther* **287**: 884–888.

Buckley NE, McCoy KL, Mezey É, Bonner T, Zimmer A, Felder CC *et al*. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. *Eur J Pharmacol* **396**: 141–149.

Cheng Y-C, Prusoff WH (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108.

Costa B, Colleoni M, Conti S, Parolaro D, Franke C, Trovato AE *et al*. (2004). Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw. *Naunyn Schmiedebergs Arch Pharmacol* **369**: 294–299.

Coutts AA, Anavi-Goffer S, Ross RA, MacEwan DJ, Mackie K, Pertwee RG *et al*. (2001). Agonist-induced internalization and trafficking of cannabinoid CB_1 receptors in hippocampal neurons. *J Neurosci* **21**: 2425–2433.

Dennis I, Whalley BJ, Stephens GJ (2008). Effects of Δ⁹-tetrahydrocannabivarin on [^{35}S]GTPγS binding in mouse brain cerebellum and piriform cortex membranes. *Br J Pharmacol* **154**: 1349–1358.

Dubuisson D, Dennis SG (1977). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* **4**: 161–174.

Fong TM, Shearman LP, Stribling DS, Shu J, Lao J, Huang CR-R *et al*. (2009). Pharmacological efficacy and safety profile of taranabant in preclinical species. *Drug Dev Res* **70**: 349–362.

Glass M, Felder CC (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a G_s linkage to the CB1 receptor. *J Neurosci* **17**: 5327–5333.

Guindon J, Hohmann AG (2008). Cannabinoid CB_2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. *Br J Pharmacol* **153**: 319–334.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77–88.

Hillard CJ, Manna S, Greenberg MJ, Dicamelli R, Ross RA, Stevenson LA *et al*. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J Pharmacol Exp Ther* **289**: 1427–1433.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA *et al*.

(2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54**: 161–202.

Kenakin T (1997). *Pharmacologic Analysis of Drug-receptor Interaction*. Lipincott – Raven Publishers: Philadelphia, PA.

Kenakin T (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15**: 598–611.

Ma Y-L, Weston SE, Whalley BJ, Stephens GJ (2008). The phytocannabinoid Δ^9-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum. *Br J Pharmacol* **154**: 204–215.

Maione S, De Petrocellis L, de Novellis V, Schiano Moriello A, Petrosino S, Palazzo E *et al*. (2007). Analgesic actions of *N*-arachidonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors. *Br J Pharmacol* **150**: 766–781.

Mallat A, Teixeira-Clerc F, Deveaux V, Lotersztajn S (2007). Cannabinoid receptors as new targets of antifibrosing strategies during chronic liver diseases. *Expert Opin Ther Targets* **11**: 403–409.

Mancini I, Brusa R, Quadrato G, Foglia C, Scandroglio P, Silverman LS *et al*. (2009). Constitutive activity of cannabinoid-2 (CB_2) receptors plays an essential role in the protean agonism of (+)AM1241 and L768242. *Br J Pharmacol* **158**: 382–391.

Pertwee RG (1997). Pharmacology of cannabinoid CB_1 and CB_2 receptors. *Pharmacol Ther* **74**: 129–180.

Pertwee RG (1999). Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* **6**: 635–664.

Pertwee RG (2001). Cannabinoid receptors and pain. *Prog Neurobiol* **63**: 569–611.

Pertwee RG (2008). The diverse CB_1 and CB_2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. *Br J Pharmacol* **153**: 199–215.

Pertwee RG (2009). Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br J Pharmacol* **156**: 397–411.

Pertwee RG, Thomas A, Stevenson LA, Ross RA, Varvel SA, Lichtman AH *et al*. (2007). The psychoactive plant cannabinoid, Δ^9-tetrahydrocannabinol, is antagonized by Δ^8- and Δ^9-tetrahydrocannabivarin in mice *in vivo*. *Br J Pharmacol* **150**: 586–594.

Riedel G, Fadda P, McKillop-Smith S, Pertwee RG, Platt B, Robinson L (2009). Synthetic and plant-derived cannabinoid receptor antagonists show hypophagic properties in fasted and non-fasted mice. *Br J Pharmacol* **156**: 1154–1166.

Ross RA (2009). The enigmatic pharmacology of GPR55. *Trends Pharmacol Sci* **30**: 156–163.

Ross RA, Brockie HC, Stevenson LA, Murphy VL, Templeton F, Makriyannis A *et al*. (1999a). Agonist-inverse agonist characterization at CB_1 and CB_2 cannabinoid receptors of L759633, L759656 and AM630. *Br J Pharmacol* **126**: 665–672.

Ross RA, Gibson TM, Stevenson LA, Saha B, Crocker P, Razdan RK *et al*. (1999b). Structural determinants of the partial agonist–inverse agonist properties of 6′-azidohex-2′-yneΔ^8-tetrahydrocannabinol at cannabinoid receptors. *Br J Pharmacol* **128**: 735–743.

Sawynok J, Liu XJ (2004). The formalin test: characteristics and usefulness of the model. *Rev Analg* **7**: 145–163.

Scutt A, Williamson EM (2007). Cannabinoids stimulate fibroblastic colony formation by bone marrow cells indirectly via CB_2 receptors. *Calcif Tissue Int* **80**: 50–59.

Thomas A, Stevenson LA, Wease KN, Price MR, Baillie G, Ross RA *et al*. (2005). Evidence that the plant cannabinoid Δ^9-tetrahydrocannabivarin is a cannabinoid CB_1 and CB_2 receptor antagonist. *Br J Pharmacol* **146**: 917–926.

Viganò D, Rubino T, Di Chiara G, Ascari I, Massi P, Parolaro D (2003). μ Opioid receptor signaling in morphine sensitization. *Neuroscience* **117**: 921–929.

Whiteside GT, Lee GP, Valenzano KJ (2007). The role of the cannabinoid CB2 receptor in pain transmission and therapeutic potential of small molecule CB2 receptor agonists. *Curr Med Chem* **14**: 917–936.

Yao BB, Mukherjee S, Fan Y, Garrison TR, Daza AV, Grayson GK *et al*. (2006). *In vitro* pharmacological characterization of AM1241: a protean agonist at the cannabinoid CB_2 receptor? *Br J Pharmacol* **149**: 145–154.

Zhang M, Martin BR, Adler MW, Razdan RJ, Kong W, Ganea D *et al*. (2009). Modulation of cannabinoid receptor activation as a neuroprotective strategy for EAE and stroke. *J Neuroimmune Pharmacol* **4**: 249–259.