British Journal of Pharmacology (2001) 134, 845–852

www.nature.com/bjp

Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide

[1]Tiziana Bisogno, [3]Lumír Hanuš, [2]Luciano De Petrocellis, [3]Susanna Tchilibon, [3]Datta E. Ponde, [1]Ines Brandi, [2]Aniello Schiano Moriello, [4]John B. Davis, [3]Raphael Mechoulam & *,[1]Vincenzo Di Marzo

[1]Endocannabinoid Research Group, Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy; [2]Endocannabinoid Research Group, Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy; [3]Department of Medicinal Chemistry and Natural Products, Faculty of Medicine, Hebrew University, Jerusalem 91120, Israel and [4]Neurology-CEDD, GlaxoSmithKline, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW

1 (−)-Cannabidiol (CBD) is a non-psychotropic component of *Cannabis* with possible therapeutic use as an anti-inflammatory drug. Little is known on the possible molecular targets of this compound. We investigated whether CBD and some of its derivatives interact with vanilloid receptor type 1 (VR1), the receptor for capsaicin, or with proteins that inactivate the endogenous cannabinoid, anandamide (AEA).

2 CBD and its enantiomer, (+)-CBD, together with seven analogues, obtained by exchanging the C-7 methyl group of CBD with a hydroxy-methyl or a carboxyl function and/or the C-5′ pentyl group with a di-methyl-heptyl (DMH) group, were tested on: (a) VR1-mediated increase in cytosolic Ca^{2+} concentrations in cells over-expressing human VR1; (b) [^{14}C]-AEA uptake by RBL-2H3 cells, which is facilitated by a selective membrane transporter; and (c) [^{14}C]-AEA hydrolysis by rat brain membranes, which is catalysed by the fatty acid amide hydrolase.

3 Both CBD and (+)-CBD, but not the other analogues, stimulated VR1 with $EC_{50} = 3.2 - 3.5$ μM, and with a maximal effect similar in efficacy to that of capsaicin, i.e. 67–70% of the effect obtained with ionomycin (4 μM). CBD (10 μM) desensitized VR1 to the action of capsaicin. The effects of maximal doses of the two compounds were not additive.

4 (+)-5′-DMH-CBD and (+)-7-hydroxy-5′-DMH-CBD inhibited [^{14}C]-AEA uptake ($IC_{50} = 10.0$ and 7.0 μM); the (−)-enantiomers were slightly less active ($IC_{50} = 14.0$ and 12.5 μM). CBD and (+)-CBD were also active ($IC_{50} = 22.0$ and 17.0 μM).

5 CBD ($IC_{50} = 27.5$ μM), (+)-CBD ($IC_{50} = 63.5$ μM) and (−)-7-hydroxy-CBD ($IC_{50} = 34$ μM), but not the other analogues ($IC_{50} > 100$ μM), weakly inhibited [^{14}C]-AEA hydrolysis.

6 Only the (+)-isomers exhibited high affinity for CB_1 and/or CB_2 cannabinoid receptors.

7 These findings suggest that VR1 receptors, or increased levels of endogenous AEA, might mediate some of the pharmacological effects of CBD and its analogues. In view of the facile high yield synthesis, and the weak affinity for CB_1 and CB_2 receptors, (−)-5′-DMH-CBD represents a valuable candidate for further investigation as inhibitor of AEA uptake and a possible new therapeutic agent.

British Journal of Pharmacology (2001) **134,** 845–852

Keywords: Cannabinoid; endocannabinoid; vanilloid; receptors; FAAH; anandamide transporter

Abbreviations: AEA, arachidonoylethanolamide; AMT, anandamide membrane transporter; CBD, (−)-cannabidiol; $[Ca^{2+}]_i$, cytosolic calcium concentration; DMH, di-methyl-heptyl; FAAH, fatty acid amide hydrolase; HEK, human hembryonic kidney; HEK-hVR1, HEK cells transfected with human VR1 cDNA; THC, (−)-Δ^9-tetrahydrocannabinol; VR1, vanilloid receptor of type 1

Introduction

Among the bioactive constituents of *Cannabis sativa*, (−)-cannabidiol (CBD, Figure 1) is one of those with the highest potential for therapeutic use (Mechoulam, 1999). Although the pharmacological properties of the other major *Cannabis* component, (−)-Δ^9-tetrahydrocannabinol (THC), have been more thoroughly investigated (Mechoulam, 1999; Pertwee, 1999, for reviews), THC, unlike CBD, exhibits potent psychotropic effects, which have complicated the full assessment of its therapeutic potential. Little is known of the molecular mechanism(s) of action of CBD, which, unlike THC, has very little affinity for either cannabinoid receptor subtypes identified so far, the CB_1 and CB_2 receptors (Pertwee, 1997, for review). Recent studies,

*Author for correspondence; E-mail: vdimarzo@icmib.na.cnr.it

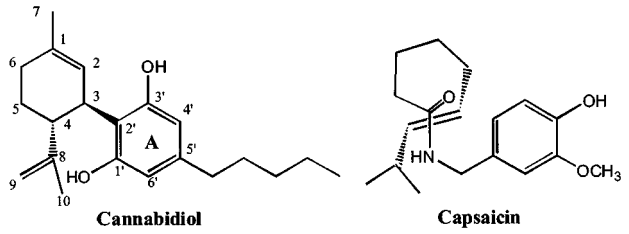


Figure 1 Chemical structures of cannabidiol and capsaicin. The numbering for cannabidiol carbon atoms, and a possible cannabidiol-like conformation for capsaicin are shown.

together with the earlier finding of the anti anxiety (Guimaraes *et al*., 1994), neuro-protective and anti-convulsive activity of CBD and some of its analogues (Consroe *et al*., 1981; Martin *et al*., 1987), indicate that CBD may also exert cyto-protective effects by inhibiting the release of inflammatory cytokines from blood cells (Srivastava *et al*., 1998; Malfait *et al*., 2000), thus producing an anti-inflammatory action, for example against rheumatoid arthritis (Malfait *et al*., 2000). These effects of CBD may be due to its anti-oxidant properties (Hampson *et al*., 1998), to its direct interaction with cytochrome p450-enzymes (Bornheim & Correia, 1989) and other enzymes of the 'arachidonate cascade' (Burstein *et al*., 1985), or to an action at a specific receptor. Recent studies have investigated whether CBD interacts with proteins of the 'endocannabinoid signalling system' other than the CB_1/CB_2 receptors. These proteins are: (i) fatty acid amide hydrolase (FAAH) (Cravatt *et al*., 1996), the intracellular enzyme catalysing the hydrolysis of the endogenous cannabinoid ligand, anandamide (arachidonoylethanolamide, AEA) (Ueda *et al*., 2000, for review); and (ii) the 'anandamide membrane transporter' (AMT) (Di Marzo *et al*., 1994), which facilitates the transport of AEA across the cell membrane and, subsequently, its intracellular degradation (Hillard & Jarrahian, 2000, for review). It was found that CBD inhibits both AEA hydrolysis by FAAH-containing membrane preparations (Watanabe *et al*., 1996), and AEA uptake by RBL–2H3 cells *via* the AMT (Rakhshan *et al*., 2000). Although these effects were observed at high μM concentrations, these findings raised the possibility that some of the pharmacological actions of CBD might be due to inhibition of AEA degradation, with subsequent enhancement of the endogenous levels of this mediator, for which neuroprotective (Hansen *et al*., 1998) and anti-inflammatory (Di Marzo *et al*., 2000a) properties have been previously suggested.

Many pharmacological activities of CBD have been established only *in vivo*, hence some of them may be due to CBD metabolites. The metabolism of CBD is well established. The primary step is hydroxylation on C-7, leading to (−)-7-hydroxy-CBD, followed by further oxidation to (−)-7-carboxy-CBD (Agurell *et al*., 1986). Although the metabolism of the dimethyl-heptyl homologue of CBD and of the (+) enantiomer of CBD has not been investigated, it is reasonable to assume that it follows the same pathways. Hence we prepared these CBD metabolites, their DMH homologues and some of the respective metabolites in the unnatural (+) series. In particular, in the present study we have examined whether the stereochemistry and the presence of certain chemical groups on the C-5′ and C-1 of CBD affect its capability of influencing AEA inactivation *via* the AMT and FAAH. Furthermore, we have addressed the question of the possible molecular transducer of CBD by studying the possibility that this natural compound, its (+)-enantiomer and some of its synthetic analogues, interact with another proposed target for AEA, i.e. the vanilloid receptor type 1 (VR1) for capsaicin (Holzer, 1991, Figure 1). This protein is a ligand-, heat- and proton-activated non-specific cation channel acting as a molecular integrator of nociceptive stimuli (Tominaga *et al*., 1998). Recently, it was discovered that AEA is a full, albeit weak, VR1 agonist (Zygmunt *et al*., 1999; Smart *et al*., 2000) and that synthetic capsaicin analogues can interact with either CB_1 receptors or the AMT, or both (Di Marzo *et al*., 1998). Thus, there appears to be some overlap between the ligand recognition properties of VR1 and CB_1 receptors and, in particular, of VR1 and the AMT (De Petrocellis *et al*., 2000; Szallasi & Di Marzo, 2000). Although VR1, *via* the release of inflammatory and algesic peptides, is involved in inflammatory hyperalgesia (Davis *et al*., 2000; Caterina *et al*., 2000), the stimulation of this receptor by capsaicin and some of its analogues leads to rapid desensitization, with subsequent paradoxical analgesic and anti-inflammatory effects (Holzer, 1991; Szallasi & Blumberg, 1999). As a consequence of this tachyphylactic effect, capsaicin, like CBD, has been used to treat arthritis (Lorton *et al*., 2000) and convulsions (Dib & Falchi, 1996).

We report data suggesting that VR1 is a possible molecular target for CBD, and that inhibitors of the AMT can be developed by chemical modification of this natural product.

Methods

Materials

The synthesis of some of the compounds assayed in this study will be described separately. CBD, whose structure and stereochemistry were described many years ago (Mechoulam & Shvo, 1963; Mechoulam & Gaoni, 1967), was isolated from hashish. (−)-5′-DMH-CBD, (+)-CBD and (+)-5′-DMH-CBD were prepared as described previously (Baek *et al*., 1985; Leite *et al*., 1982). The synthesis of the CBD metabolite, (−)-7-hydroxy-CBD was recently reported (Tchilibon & Mechoulam, 2000). [^{14}C]-AEA (5 mCi mmol^{-1}) was synthesized from [^{14}C]-ethanolamine and arachidonoyl chloride as described (Devane *et al*., 1992b). Capsaicin, ionomycin and capsazepine were purchased from Sigma.

Cytosolic calcium concentration ($[Ca^{2+}]_i$) assay

Over-expression of human VR1 cDNA into human hembryonic kidney (HEK) 293 cells was carried out as described previously (Hayes *et al*., 2000). Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% foetal calf serum and 0.2 mM glutamine, and maintained under 95%/5% O_2/CO_2 at 37°C. The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} (Smart *et al*., 2000; De Petrocellis *et al*., 2000). One day

prior to experiments, cells were transferred into 6-well dishes coated with Poly-L-lysine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment the cells (50–60,000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes) in DMSO containing 0.04% Pluoronic. After the loading, cells were washed with Tyrode pH = 7.4, trypsinized, resuspended in Tyrode and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of the test compounds at various concentrations. Capsazepine (10 μM) was added 30 min before CBD. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The efficacy of the effect was determined by comparing it to the analogous effect observed with 4 μM ionomycin. Response calibration was carried out by measuring the fluorescence intensity of intracellular fluo-3 with known extracellular [Ca^{2+}] (Molecular Probes). The following equation was used to determine an ion dissociation constant (K_d) of 325 nM:

$$[Ca^{2+}]\text{free} = K_d[F - F_{min}]/[F_{max} - F],$$

where F_{min} and F_{max} are the fluorescence intensities of fluo-3 without or with maximal [Ca^{2+}], and F is the fluorescence intensity with an intermediate [Ca^{2+}]. Average F_{EM}/F_{EX} was 200 and this value was increased by 60 ± 7% in the presence of 4 μM ionomycin.

VR1 receptor binding assays

The affinity of CBD and (+)-CBD for human VR1 receptors was assessed by means of displacement assays carried out with membranes (50 μg tube^{-1}) from HEK-hVR1 cells, prepared as described previously (Ross *et al*., 2001), and the high affinity VR1 ligand [^{3}H]-resiniferatoxin (48 Ci mmol^{-1}, NEN-Dupont), using the incubation conditions described previously (Ross *et al*., 2001). Under these conditions the K_d and B_{max} for [^{3}H]-resiniferatoxin were 0.5 nM and 1.39 pmol mg^{-1} protein. The K_i for the displacement of 1 nM [^{3}H]-resiniferatoxin by increasing concentrations of CBD and (+)-CBD was calculated from the IC_{50} values (obtained by GraphPad Software) using the Cheng–Prusoff equation. Specific binding was calculated with 1 μM resiniferatoxin (Alexis Biochemicals) and was 78.1 ± 3.7%.

Cannabinoid CB_1 and CB_2 receptor binding assays

These methods have been described previously by Devane *et al*. (1992a) for CB_1, and Bayewitch *et al*. (1996) for CB_2. For CB_1 receptor binding assays synaptosomal membranes from rat brains were used. Sabra male rats weighing 250–300 g were decapitated and their brains, without the brain stem, were quickly removed. Synaptosomal membranes were prepared from the brains by centrifugations and gradient centrifugation after their homogenization. The synaptosomal proteins thus obtained were used in the binding assay. The CB_2 receptor binding assays were performed with transfected cells. COS-7 cells were transfected with plasmids containing CB_2 receptor cDNA, and crude membranes were prepared. The high affinity CB_1/CB_2 receptor ligand, [^{3}H]-HU-243, with a dissociation constant of 45 pM, was incubated with synaptosomal membranes (3–4 μg), for CB_1 assays, or transfected cells, for CB_2 assays, for 90 min at 30°C with the different cannabidiol derivatives or with the vehicle alone, and then centrifuged at 13,000 r.p.m. for 6 min. Bound and free radioligand were separated by centrifugation. The data were normalized to 100% of specific binding, which was determined with 50 nM unlabelled HU-243. All experiments were repeated 2–3 times and each point performed in triplicate. The K_i values were determined with a GraphPad Prism program version 2.01 (San Diego, CA, U.S.A.) and using the Cheng–Prusoff equation.

Anandamide cellular uptake assay

The effect of compounds on the uptake of [^{14}C]-AEA by rat basophilic leukaemia (RBL–2H3) cells was studied by using 3.6 μM (10,000 c.p.m.) of [^{14}C]-AEA as described previously (Bisogno *et al*., 1997). Cells were incubated with [^{14}C]-AEA for 5 min at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [^{14}C]-AEA in the incubation media after extraction with $CHCl_3$/CH_3OH 2:1 (by vol.), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells (De Petrocellis *et al*., 2000). Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}) calculated by GraphPad.

Fatty acid amide hydrolase assay

The effect of CBD and its analogues on the enzymatic hydrolysis of AEA was studied as described previously (Bisogno *et al*., 1997), using cell membranes from mouse neuroblastoma (N18TG2) cells, incubated with compounds and [^{14}C]-AEA (9 μM) in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [^{14}C]-Ethanolamine produced from [^{14}C]-AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol.). Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}), calculated by GraphPad.

Statistics

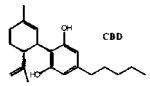
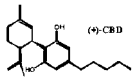
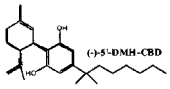
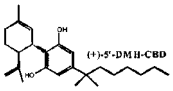
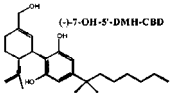
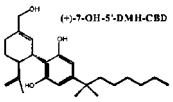
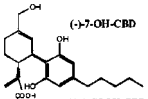
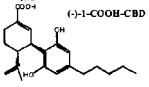
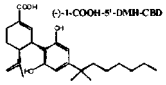
Means were compared by means of analysis of variance followed by Bonferroni's test (ANOVA, StatMost™ software, DataMost Corp.).

Results

Effect of CBD analogues on human vanilloid VR1 receptors

The effects upon $[Ca^{2+}]_i$ in HEK–hVR1 cells of CBD, (+)-CBD, and (−)-7-hydroxy-5′-DMH-CBD (HU-317) are shown in Figure 2. These three compounds all induced an increase in $[Ca^{2+}]_i$ and behaved as full agonists as compared to capsaicin, although only the two former compounds exerted this effect with an $EC_{50} < 10$ μM and independently of

Table 1 Chemical structures of the compounds examined in this study and their activity on the uptake of [^{14}C]-anandamide (AEA) by RBL−2H3 cells (AMT, IC_{50}, μM), the hydrolysis of [^{14}C]-AEA by N18TG2 cell membranes (FAAH, IC_{50}, μM), the binding of [^{3}H]-HU-243 to rat brain synaptosomes (CB_1, K_i, μM) and the binding of [^{3}H]-HU-243 to COS-7 CB_2-transfected cell membranes (CB_2, K_i, μM)

Analogue	*AMT*	*FAAH*	*CB_1*	*CB_2*
 CBD	22.0±1.7	27.5±3.3	>10	>10
 (+)-CBD	17.0±1.6[a]	63.5±4.2[b]	0.842±0.036	0.203±0.016
 (-)-5'-DMH-CBD	14.0±1.3	>100	>10	1.8
 (+)-5'-DMH-CBD	10.0±1.2[a]	>100	0.0174±0.0018	0.211±0.023
 (-)-7-OH-5'-DMH-CBD	12.5±2.0	>100	4.4	0.671±0.012
 (+)-7-OH-5'-DMH-CBD	7.0±1.2[a]	>100	0.0025±0.00003	0.044±0.0031
 (-)-7-OH-CBD	~50	34.0±2.9	>10	>10
 (-)-1-COOH-CBD	>50	>100	>10	>10
 (-)-1-COOH-5'-DMH-CBD	>50	>100	1.9	5.0

Data for AMT and FAAH are means±s.e.mean of $n=4$ experiments, and for CB_1 and CB_2 binding assays are means±s.e.mean of $n=3$ experiments, except for those compounds with little affinity ($K_i>1$ μM), where data are means of $n=2$ experiments. [a]Means significantly lower than those of the (−)-enantiomers ($P<0.05$ by ANOVA). [b]Mean significantly higher than that of the (−)-enantiomer ($P-0.01$ by ANOVA).

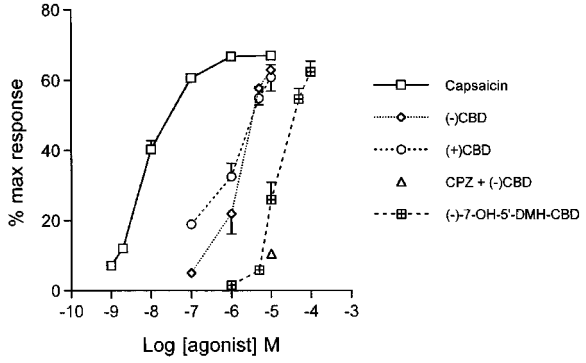


Figure 2 Effect of (−)-CBD, (+)-CBD, (−)-7-OH-5′-DMH-CBD and capsaicin on cytosolic Ca^{2+} concentration in HEK-hVR1 cells. The effect is expressed as per cent of the effect of 4 μM ionomycin, and data represent means±s.d. of $n=4$ experiments. The effect of capsazepine (CPZ, 10 μM) on 10 μM CBD is also shown (open triangle).

their stereochemistry. In fact, the potency (EC_{50} 3.2±0.4) and efficacy (max. effect 68.5±3.1% of the effect of 4 μM ionomycin) of (+)-CBD were indistinguishable from those of CBD (EC_{50} 3.5±0.3, max. effect 64.1±3.9%, means±s.e.mean, $n=4$). The efficacy of both compounds was almost identical to that of a maximal concentration of capsaicin (70.2±3.5% at 10 μM, mean±s.e.mean, $n=4$), which was however 100 fold more potent ($EC_{50}=26\pm9$ nM). The other six CBD analogues examined in this study were all inactive or very weakly active on $[Ca^{2+}]_i$. The effect of CBD was abolished by the VR1 receptor antagonist capsazepine (10 μM, Figure 2) and could not be observed in wild-type HEK cells (data not shown). This strongly suggests that this effect, like that of capsaicin, was due to stimulation of VR1 receptors. Binding assays for the displacement of [^{3}H]-resiniferatoxin from HEK−hVR1 cell membranes by CBD and (+)-CBD confirmed this hypothesis, and showed that CBD and (+)-CBD compete for the binding of [^{3}H]-resiniferatoxin with K_i values (3.6±0.2 and 3.0±0.3 μM, respectively, means±s.d., $n=3$) similar to the EC_{50} values for the effect on $[Ca^{2+}]_i$. Furthermore, capsaicin (0.1 μM) and CBD (10 μM) exhibited cross-desensitization of their effect on $[Ca^{2+}]_i$, providing evidence consistent with these two compounds acting at the same receptor. A 1 h pre-exposure to 0.1 μM capsaicin reduced the effect of 10 μM CBD from 66.7±3.4 to 11.7±1.5% of the effect of 4 μM ionomycin, whereas a 1 h pre-exposure to 10 μM CBD reduced the effect of 0.1 μM capsaicin from 68.1±4.1 to 22.3±3.5% (means±

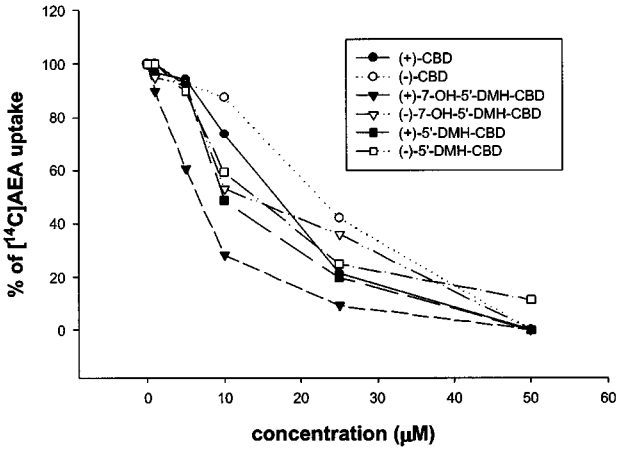


Figure 3 Effect of (−)-CBD and some of its analogues on the uptake of [^{14}C]-anandamide (AEA) by RBL−2H3 cells. The effect is expressed as per cent of [^{14}C]-AEA taken up by cells in the absence of any substance, and data are means of $n=4$ experiments. The s.e.m. bars are not shown for the sake of clarity and were never higher than 5% of the means.

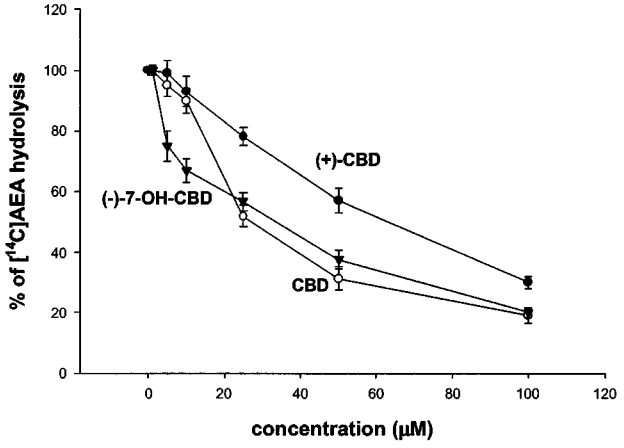


Figure 4 Effect of cannabidiol (CBD) and some of its analogues on the hydrolysis of [^{14}C]-anandamide (AEA) by N18TG2 cell membranes. The effect is expressed as per cent of [^{14}C]-AEA hydrolysed in the absence of any substance, and data are means ± s.e.mean of $n=4$ experiments. ● = (+)-CBD; ○ = CBD; ▲ = (−)-7-hydroxy-CBD.

s.e.mean, $n=4$, $P<0.01$ by ANOVA). Co-treatment of cells with both capsaicin (10 μM) and CBD (10 μM) did not produce any additive effect (72.2 ± 4.1% of the effect of ionomycin, mean ± s.e.mean, $n=4$).

Affinity of CBD analogues for cannabinoid CB_1 and CB_2 receptors

Of the nine compounds tested, the (−) analogues were all weakly active or inactive ($K_i>10$ μM) in binding assays for CB_1 and CB_2 receptor affinity (Table 1), with the exception of (−)-7-hydroxy-5′-DMH-CBD, which exhibited a weak affinity for CB_2 receptors ($K_i=0.7$ μM). By contrast, of the three (+)-analogues tested, (+)-5′-DMH−CBD and (+)-7-hydroxy-5′-DMH−CBD behaved as high affinity CB_1 receptor ligands ($K_i=17.4$ and 2.5 nM), and were 10−20 fold less active as CB_2 receptor ligands ($K_i=211$ and 44.0 nM).

Effect of CBD analogues on the anandamide membrane transporter

Of the nine analogues tested, only (+)- and (−)-CBD, (+)- and (−)-5′-DMH-CBD and (+)- and (−)-7-hydroxy-5′-DMH−CBD inhibited the uptake of [14C]-AEA from RBL−2H3 cells with IC_{50} values lower than 25 μM (Figure 3 and Table 1). Of these six compounds, the (+)-enantiomers were significantly ($P<0.05$ by ANOVA) and consistently more active than the (−)-enantiomers, and, in particular, (+)-5′-DMH-CBD and (+)-7-hydroxy-5′-DMH-CBD were as potent as the AMT inhibitor, AM404 (Khanolkar & Makriyannis, 1999) ($IC_{50}=10.0$, 7.0 and 8.1 μM for the two compounds and AM404, respectively). (−)-7-hydroxy-5′-DMH-CBD and (−)-5′-DMH-CBD were also almost as potent as AM404 ($IC_{50}=12.5$ and 14.0 μM). The IC_{50} of CBD (22.0 μM) was higher than that previously reported for this compound in the same cell line (11.4 μM; Rakhshan *et al*., 2000).

Effect of CBD analogues on fatty acid amide hydrolase

Only (+)- and (−)-CBD, and (−)-7-hydroxy-CBD exhibited a $IC_{50}<100$ μM for the inhibition of [^{14}C]-AEA hydrolysis by N18TG2 cell membrane preparations (Figure 4 and Table 1), which express high levels of FAAH. The (−)-enantiomer was significantly more potent than the (+)-enantiomer, CBD being also the most potent compound found ($IC_{50}=27.5$ μM). However, none of the CBD analogues tested can be considered a potent inhibitor of FAAH (i.e. with an $IC_{50}\leqslant 20$ μM). The activity of CBD in this study was higher than that previously reported for anandamide hydrolysis by mouse brain (Watanabe *et al*., 1996; 1998), where however only a very high concentration of the compound (160 μM) was used.

Discussion

In this study we investigated whether CBD, now being considered as a possible therapeutic agent (Straus, 2000), is capable of interacting with the recently cloned vanilloid VR1 receptor. In fact, some of the pharmacological actions of CBD are similar to those of natural (e.g. capsaicin) and synthetic agonists of VR1. Although stimulation of VR1 receptors leads to vasodilation and inflammation, capsaicin and its long chain analogues exert anti-inflammatory effects by rapidly desensitizing VR1 receptors to the action of nociceptive stimuli and causing depletion of sensory vasoactive neuropeptides (Szallasi & Blumberg, 1999). CBD also induces anti-inflammatory effects, a possible explanation for this property being its capability of modulating the release of anti-inflammatory or pro-inflammatory mediators (Srivastava *et al*., 1998, Malfait *et al*., 2000). CBD and capsaicin also have in common anti-convulsive and anti-rheumatoid-arthritis effects (Consroe *et al*., 1981; Dib & Falchi, 1996; Malfait *et al*., 2000; Lorton *et al*., 2000). Here we found that CBD, compared to capsaicin, is a full, although weak, agonist of human VR1 at concentrations that might be

attained after administration of this compound at the doses often used *in vivo* (10–50 mg kg^{-1} in men), and lower than those required for CBD to bind to cannabinoid receptors. CBD desensitized VR1 to the action of capsaicin, thus opening the possibility that this cannabinoid exerts an anti-inflammatory action in part by desensitization of sensory nociceptors. Future studies with capsazepine (which antagonizes capsaicin effects in rats (Di Marzo *et al*., 2001) but not always in mice (Di Marzo *et al*., 2000b), and VR1 'knockout' mice (Davis *et al*., 2000; Caterina *et al*., 2000), should test the involvement of VR1 in the pharmacological actions of CBD.

We found that insertion in CBD of a DMH instead of an *n*-pentyl chain on the C-5′, or of a carboxyl function instead of the methyl group on the C-1, abolishes the capability of the cannabinoid to induce a VR1-mediated functional response, whereas insertion of both a hydroxy-group on the C-7 and of a 5′-DMH group decreases the potency but not the efficacy of CBD. By contrast, inversion of the stereochemistry does not modify the activity of CBD. These data suggest that the C-1 methyl and the aromatic 'A' ring, which is chemically similar to the vanillyl moiety of capsaicin (Figure 1), are more important than the chiral part of CBD for its interaction with VR1. That CBD binds to the same site as capsaicin is suggested by the finding that both compounds displace [^{3}H]-resiniferatoxin from its specific binding sites in membranes from cells over-expressing VR1 receptors (this study and Ross *et al*., 2001). However, while capsaicin exhibits higher potency than affinity for vanilloid receptors (Szallasi & Blumberg, 1999), CBD is as active in the [^{3}H]-resiniferatoxin binding assay as in the hVR1 functional assay. This suggests that CBD is less capable than capsaicin to induce a VR1-mediated functional response at low concentrations, even though the efficacy of high concentrations of both compounds is the same. It should be noted that the cells used here to assess the functional activity at VR1 express high levels of this receptor, and that the potency and efficacy of CBD in native cells containing lower amounts of vanilloid receptors might be lower than those observed here.

The endocannabinoid AEA is thought to exert anti-inflammatory and neuroprotective actions (Di Marzo *et al*., 2000a; Hansen *et al*., 1998). As it was found to inhibit the re-uptake and hydrolysis of AEA *in vitro* (Rakhshan *et al*., 2000; Watanabe *et al*., 1996), it is possible that CBD acts in part by interfering with AEA inactivation, thereby enhancing the putative tonic inhibitory action of AEA on inflammation. The fact that the pharmacological actions of CBD are not influenced by CB_1/CB_2 receptor antagonists should not be taken as evidence against this hypothesis, since it is now established that AEA also acts upon non-cannabinoid receptor targets, including VR1 receptors and TASK-1 K^+ channels (Zygmunt *et al*., 1999; Maingret *et al*., 2001). Here we confirmed that CBD inhibits AEA transporter-mediated uptake by cells and enzymatic hydrolysis. We also found that analogues of CBD are inhibitors of the AMT, and that this property is more pronounced with (+)-enantiomers, or when the C-7 and C-5′ are derivatized with a hydroxyl- and a DMH group, respectively. The most potent inhibitor found was (+)-7-hydroxy-5′-DMH-CBD. However, this compound exhibited high affinity for CB_1 and CB_2 receptors. Also (+)-5′-DMH-CBD was more active as a CB_1 and CB_2 receptor ligand than as an AMT inhibitor. By contrast, (−)-7-hydroxy-5′-DMH-CBD and (−)-5′-DMH-CBD, which were almost as potent as AM404 against the AMT, but, unlike AM404, had low affinity for the two cannabinoid receptors subtypes and no activity at VR1, may represent metabolically stable and relatively selective pharmacological tools for the study of AEA inactivation *in vitro*. The (−)-5′DMH-CBD is obtained by a facile, high yield synthesis (Baek *et al*., 1985) and may find application as therapeutic agent for those disorders where AEA exerts an endogenous tone with beneficial effects. The novel AMT inhibitors developed here should be tested also on the cellular uptake of palmitoylethanolamide, a natural anti-inflammatory AEA congener (Lambert & Di Marzo, 1999), since a recent study showed that CBD inhibits the facilitated transport of this compound into RBL–2H3 cells (Jacobsson & Fowler, 2001).

We have mentioned above that the (+)-enantiomers of the CBD analogues tested here on AEA cellular uptake were more potent inhibitors than the (−)-enantiomers. A certain enantio-specificity for the interaction with the AMT has been noted previously also for AEA analogues (see Khanolkar & Makriyannis, 1999, for review). It was also noted that the same stereochemical preference existed for the interaction of these compounds with FAAH, whereas the interaction with CB_1 receptors followed the opposite enantio-selectivity (Khanolkar & Makriyannis, 1999). We noted that CBD inhibits FAAH more potently than the (+)-enantiomer. By contrast, all but one of the (+)-CBD analogues tested exhibited much higher affinity for CB_1 receptors than their (−)-enantiomers. Thus, for CBD analogues, the stereochemical requisites for the interaction with the AMT and CB_1 receptors are the same, and they may be opposite to those necessary for the interaction with FAAH. The binding data were indeed unexpected. In the tetrahydrocannabinol series the (−) (3*R*,4*R*) enantiomers bind to CB_1 and have pharmacological activity in various typical cannabinoid assays, while the (+) (3*S*,4*S*) enantiomers are essentially inactive (Mechoulam *et al*., 1988; Howlett *et al*., 1990; Little *et al*., 1989; Jarbe *et al*., 1989). In the CBD series of compounds we observed here the opposite situation. The reason for this dichotomy is unknown. Further studies investigating the potency and efficacy of the compounds in functional assays of CB_1 receptor-mediated activity need to be performed in order to fully assess the cannabimimetic activity of the compounds in the (+)-CBD series.

In conclusion, the present study has provided novel insights into the possible mechanism(s) of action of the natural cannabinoid CBD by identifying in VR1 receptors a novel potential molecular target for this compound. Furthermore, we have shown that potent inhibitors of AEA cellular uptake can be developed from certain chemical modifications of CBD, and have confirmed that CBD can act in principle also by inhibiting AEA inactivation. Future studies will be needed to address the question of whether vanilloid receptors or endogenous cannabinoids contribute to the anti-inflammatory and neuroprotective actions of CBD.

We thank the U.S. National Institute on Drug Abuse (grant DA 9789, to R. Mechoulam), the Israel Science Foundation (to R. Mechoulan), the Yeshaya Horowitz Association (to R. Mechoulam) and the MURST (3933 to V. Di Marzo) for support.

References

AGURELL, S., HALLDIN, M., LINDGREN, J.E., OHLSSON, A., WIDMAN, M., GILLESPIE, H. & HOLLISTER, L. (1986). Pharmacokinetics and metabolism of delta-1-tetrahydrocannabinol and other cannabinoids with emphasis on man. *Pharmacol. Rev.,* **38,** 21–43.

BAEK, S.H., SREBNIK, M. & MECHOULAM, R. (1985). Borontrifluoride on alumina–a modified Lewis acid reagent. An improved synthesis of cannabidiol. *Tetrahedron Lett.,* **26,** 1083–1086.

BAYEWITCH, M., RHEE, M.H., AVIDOR-REISS, T., BREUER, A., MECHOULAM, R. & VOGEL, Z. (1996). (−)-Delta9-tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J. Biol. Chem.,* **271,** 9902–9905.

BISOGNO, T., MAURELLI, S., MELCK, D., DE PETROCELLIS, L. & DI MARZO, V. (1997). Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J. Biol. Chem.,* **272,** 3315–3323.

BORNHEIM, L.M. & CORREIA, M.A. (1989). Effect of cannabidiol on cytochrome P-450 isozymes. *Biochem. Pharmacol.,* **38,** 2789–2794.

BURSTEIN, S., HUNTER, S.A. & RENZULLI, L. (1985). Prostaglandins and cannabis XIV. Tolerance to the stimulatory actions of cannabinoids on arachidonate metabolism. *J. Pharmacol. Exp. Ther.,* **235,** 87–91.

CATERINA, M.J., LEFFLER, A., MALMBERG, A.B., MARTIN, W.J., TRAFTON, J., PETERSEN-ZEITZ, K.R., KOLTZENBURG, M., BASBAUM, A.I. & JULIUS, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science,* **288,** 306–313.

CONSROE, P., MARTIN, A. & SINGH, V. (1981). Antiepileptic potential of cannabidiol analogs. *J. Clin. Pharmacol.,* **21,** 428S–436S.

CRAVATT, B.F., GIANG, D.K., MAYFIELD, S.P., BOGER, D.L., LERNER, R.A. & GILULA, N.B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature,* **384,** 83–87.

DAVIS, J.B., GRAY, J., GUNTHORPE, M.J., HATCHER, J.P., DAVEY, P.T., OVEREND, P., HARRIES, M.H., LATCHAM, J., CLAPHAM, C., ATKINSON, K., HUGHES, S.A., RANCE, K., GRAU, E., HARPER, A.J., PUGH, P.L., ROGERS, D.C., BINGHAM, S., RANDALL, A. & SHEARDOWN, S.A. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature,* **405,** 183–187.

DE PETROCELLIS, L., BISOGNO, T., DAVIS, J.B., PERTWEE, R.G. & DI MARZO, V. (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.,* **483,** 52–56.

DEVANE, W.A., BREUER, A., SHESKIN, T., JARBE, T.U.C., EISEN, M. & MECHOULAM, R. (1992a). A novel probe for the cannabinoid receptor. *J. Med. Chem.,* **35,** 2065–2069.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D. & MECHOULAM, R. (1992b). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* **258,** 1946–1949.

DIB, B. & FALCHI, M. (1996). Convulsions and death induced in rats by Tween 80 are prevented by capsaicin. *Int. J. Tissue React.,* **18,** 27–31.

DI MARZO, V., BISOGNO, T., MELCK, D., ROSS, R., BROCKIE, H., STEVENSON, L., PERTWEE, R. & DE PETROCELLIS, L. (1998). Interactions between synthetic vanilloids and the endogenous cannabinoid system. *FEBS Lett.,* **436,** 449–454.

DI MARZO, V., BREIVOGEL, C., BISOGNO, T., MELCK, D., PATRICK, G., TAO, Q., SZALLASI, A., RAZDAN, R.K. & MARTIN, B.R. (2000b). Neurobehavioral activity in mice of N-vanillyl-arachidonyl-amide. *Eur. J. Pharmacol.,* **406,** 363–374.

DI MARZO, V., FONTANA, A., CADAS, H., SCHINELLI, S., CIMINO, G., SCHWARTZ, J.C. & PIOMELLI, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature,* **372,** 686–691.

DI MARZO, V., LASTRES-BECKER, I., BISOGNO, T., DE PETROCELLIS, L., MILONE, A., DAVIS, J.B. & FERNANDEZ-RUIZ, J.J. (2001). Hypolocomotor effects in rats of capsaicin and two long chain capsaicin homologues. *Eur. J. Pharmacol.,* **420,** 123–131.

DI MARZO, V., MELCK, D., DE PETROCELLIS, L. & BISOGNO, T. (2000a). Cannabimimetic fatty acid derivatives in cancer and inflammation. *Prostaglandins Other Lipid Mediat.,* **61,** 43–61.

GUIMARAES, F.S., DE AQUIAR, J.C., MECHOULAM, R. & BREUER, A. (1994). Anxiolytic effect of cannabidiol derivatives in the elevated plus-maze. *Gen. Pharmacol.,* **25,** 161–194.

HAMPSON, A.J., GRIMALDI, M., AXELROD, J. & WINK, D. (1998). Cannabidiol and (−) Delta-9-tetrahydrocannabinol are neuroprotective. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 8268–8273.

HANSEN, H.S., LAURITZEN, L., MOESGAARD, B., STRAND, A.M. & HANSEN, H.H. (1998). Formation of N-acyl-phosphatidylethanolamines and N-acetylethanolamines: proposed role in neurotoxicity. *Biochem. Pharmacol.,* **55,** 719–725.

HAYES, P., MEADOWS, H.J., GUNTHORPE, M.J., HARRIES, M.H., DUCKWORTH, D.M., CAIRNS, W., HARRISON, D.C., CLARKE, C.E., ELLINGTON, K., PRINJHA, R.K., BARTON, A.J., MEDHURST, A.D., SMITH, G.D., TOPP, S., MURDOCK, P., SANGER, G.J., TERRETT, J., JENKINS, O., BENHAM, C.D., RANDALL, A.D., GLOGER, I.S. & DAVIS, J.B. (2000). Cloning and functional expression of a human orthologue of rat vanilloid receptor-1. *Pain,* **88,** 205–215.

HILLARD, C.J. & JARRAHIAN, A. (2000). The movement of N-arachidonoylethanolamine (anandamide) across cellular membranes. *Chem. Phys. Lipids,* **108,** 123–134.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.,* **43,** 143–201.

HOWLETT, A.C., CHAMPION, T.M., WILKEN, G.H. & MECHOULAM, R. (1990). Stereochemical effects of 11-OH-delta-8-tetrahydrocannabinol-dimethylheptyl to inhibit adenylate cyclase and bind to the cannabinoid receptor. *Neuropharmacology,* **29,** 161–165.

JACOBSSON, S.O. & FOWLER, C.J. (2001). Characterization of palmitoylethanolamide transport in mouse Neuro-2a neuroblastoma and rat RBL-2H3 basophilic leukaemia cells: comparison with anandamide. *Br. J. Pharmacol.,* **132,** 1743–1754.

JARBE, T.U.C., HILTUNEN, A.J. & MECHOULAM, R. (1989). Stereospecificity of the discriminative stimulus functions of the dimethylheptyl homologs of 11-OH-delta-8-tetra-hydrocannabinol in rats and pigeons. *J. Pharmacol. Exper. Ther.,* **250,** 1000–1005.

KHANOLKAR, A.D. & MAKRIYANNIS, A. (1999). Structure-activity relationships of anandamide, an endogenous cannabinoid ligand. *Life Sci.,* **65,** 607–616.

LAMBERT, D.M. & DI MARZO, V. (1999). The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? *Curr. Med. Chem.,* **6,** 757–773.

LEITE, J.R., CARLINI, E.A., LANDER, N. & MECHOULAM, R. (1982). Anticonvulsant effect of (−) and (+) isomers of CBD and their dimethyl heptyl homologs. *Pharmacol.,* **124,** 141–146.

LITTLE, P.J., COMPTON, D.R., MECHOULAM, R. & MARTIN, B. (1989). Stereochemical effects of 11-OH-delta-8-THC-dimethylheptyl in mice and dogs. *Pharmacol. Biochem. Behavior,* **32,** 661–666.

LORTON, D., LUBAHN, C., ENGAN, C., SCHALLER, J., FELTEN, D.L. & BELLINGER, D.L. (2000). Local application of capsaicin into the draining lymph nodes attenuates expression of adjuvant-induced arthritis. *Neuroimmunomodulation,* **7,** 115–125.

MAINGRET, F., PATEL, A.J., LAZDUNSKI, M. & HONORE, E. (2001). The endocannabinoid anandamide is a direct and selective blocker of the background K(+) channel TASK-1. *EMBO J.,* **20,** 47–54.

MALFAIT, A.M., GALLILY, R., SUMARIWALLA, P.F., MALIK, A.S., ANDREAKOS, E., MECHOULAM, R. & FELDMANN, M. (2000). The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 9561–9566.

MARTIN, A.R., CONSROE, P., KANE, V.V., SHAH, V., SINGH, V., LANDER, N., MECHOULAM, R. & SREBNIK, M. (1987). Structure-anticonvulsant activity relationships of cannabidiol analogs. *NIDA Res. Monogr.*, **79,** 48–58.

MECHOULAM, R. (1999). Recent advantages in cannabinoid research. *Forsch. Komplementarmed,* **6,** 16–20.

MECHOULAM, R. & GAONI, Y. (1967). The absolute configuration of delta-1-tetrahydrocannabinol, the major active constituent of hashish. *Tetrahedron Lett.,* 1109–1111.

MECHOULAM, R. & SHVO, Y. (1963). The structure of cannabidiol. *Tetrahedron,* **19,** 2073–2078.

MECHOULAM, R., FEIGENBAUM, J.J., LANDER, N., SEGAL, M., JARBE, T.U.C., HILTUNEN, A.J. & CONSROE, P. (1988). Enantiomeric cannabinoids: stereospecificity of psychotropic activity. *Experientia,* **44,** 762–764.

PERTWEE, R.G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther.,* **74,** 129–180.

PERTWEE, R.G. (1999). Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.,* **6,** 635–664.

RAKHSHAN, F., DAY, T.A., BLAKELY, R.D. & BARKER, E.L. (2000). Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J. Pharmacol. Exp. Ther.,* **292,** 960–967.

ROSS, R.R., GIBSON, T.M., BROCKIE, H.C., LESLIE, M., PASHMI, G., CRAIB, S.J., DI MARZO, V. & PERTWEE, R.G. (2001). Structure-activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. *Br. J. Pharmacol.,* **132,** 631–640.

SMART, D., GUNTHORPE, M.J., JERMAN, J.C., NASIR, S., GRAY, J., MUIR, A.I., CHAMBERS, J.K., RANDALL, A.D. & DAVIS, J.B. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.,* **129,** 227–230.

SRIVASTAVA, M.D., SRIVASTAVA, B.I. & BROUHARD, B. (1998). Delta9 tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology,* **40,** 179–185.

STRAUS, S.E. (2000). Immunoactive cannabinoids: therapeutic prospects for marijuana constituents. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 9363–9364.

SZALLASI, A. & BLUMBERG, P.M. (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159–212.

SZALLASI, A. & DI MARZO, V. (2000). New perspectives on enigmatic vanilloid receptors. *Trends Neurosci.,* **23,** 491–497.

TCHILIBON, S. & MECHOULAM, R. (2000). Synthesis of a primary metabolite of cannabidiol. *Org. Lett.,* **2,** 3301–3303.

TOMINAGA, M., CATERINA, M.J., MALMBERG, A.B., ROSEN, T.A., GILBERT, H., SKINNER, K., RAUMANN, B.E., BASBAUM, A.I. & JULIUS, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron,* **21,** 531–543.

UEDA, N., PUFFENBARGER, R.A., YAMAMOTO, S. & DEUTSCH, D.G. (2000). The fatty acid amide hydrolase. *Chem. Phys. Lipids,* **108,** 107–121.

WATANABE, K., KAYANO, Y., MATSUNAGA, T., YAMAMOTO, I. & YOSHIMURA, H. (1996). Inhibition of anandamide amidase activity in mouse brain microsomes by cannabinoids. *Biol. Pharm. Bull.,* **19,** 1109–1111.

ZYGMUNT, P.M., PETERSSON, J., ANDERSSON, D.A., CHUANG, H., SORGARD, M., DI MARZO, V., JULIUS, D. & HOGESTATT, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* **400,** 452–457.

(Received May 22, 2001
Revised July 19, 2001
Accepted August 3, 2001)