The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling

Michael Bayewitch[a], Tomer Avidor-Reiss[a], Rivka Levy[a], Jacob Barg[a,b], Raphael Mechoulam[c], Zvi Vogel[a,*]

[a]*Department of Neurobiology, The Weizmann Institute of Science, 76100 Rehovot, Israel*
[b]*Therapuetic Community, Ramot Yehuda, Zoharim, Israel*
[c]*Department of Natural Products, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel*

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Abstract Two cannabinoid receptors, designated neuronal (or CB1) and peripheral (or CB2), have recently been cloned. Activation of CB1 receptors leads to inhibition of adenylate cyclase and N-type voltage-dependent Ca^{2+} channels. Here we show, using a CB2 transfected Chinese hamster ovary cell line, that this receptor binds a variety of tricyclic cannabinoid ligands as well as the endogenous ligand anandamide. Activation of the CB2 receptor by various tricyclic cannabinoids inhibits adenylate cyclase activity and this inhibition is pertussis toxin sensitive indicating that this receptor is coupled to the G_i/G_o GTP-binding proteins. Interestingly, contrary to results with CB1, anandamide did not inhibit the CB2 coupled adenylate cyclase activity and Δ^9-tetrahydrocannabinol had only marginal effects. These results characterize the CB2 receptor as a functional and distinctive member of the cannabinoid receptor family.

Key words: Adenylate cyclase; Anandamide; Cannabinoid receptor; Δ^9-Tetrahydrocannabinol; GTP-binding proteins; Tricyclic cannabinoids

1. Introduction

Cannabinoids have been shown to inhibit adenylate cyclase (AC) activity in rat brain and in $N_{18}TG_2$ neuroblastoma cells, as well as to inhibit the N-type voltage-dependent Ca^{2+} channels in the $N_{18}TG_2$ cells. These cannabinoid receptor activities are pertussis toxin (PTX) sensitive indicating coupling to G_i/G_o GTP-binding proteins (G proteins) [1–5]. The cloning of the neuronal cannabinoid receptor (CB1) [6,7] has allowed for the establishment of cell lines that express this receptor. Using such cell lines, we and others have recently shown that tricyclic cannabinoids, as well as the isolated endogenous cannabinoid ligands, anandamide, docosotetraenylethanolamide and homo-γ-linoenylethanolamide, interact with the CB1 receptor and inhibit AC activity in a dose-dependent manner [5,8,9].

Although the original focus of cannabinoid function has been on the nervous system, it has been noted that cannabinoid binding sites are present in several nonneuronal tissues. Cannabinoid binding sites have been localized to regions of the mouse and rat spleen and it has been proposed that these sites are located on myeloid cells or on B-cells [10,11]. In addition, cannabinoid receptor mRNA expression has been observed in both testis and spleen [7,10]. Functionally, cannabinoids have been shown to have suppressive effects on a variety of macrophage functions in vitro [12] and to inhibit the proliferative and antibody-forming cell responses in spleenocytes [13].

A cannabinoid receptor, designated CB2 (or the peripheral cannabinoid receptor) has been recently cloned from the HL-60 promyelocytic leukemia cell line [14]. It belongs to the seven transmembrane G protein-coupled receptor family and has a 68% amino acid sequence homology to the CB1 receptor in the seven transmembrane domains, which are proposed to confer ligand specificity [14]. CB2 receptor expression was shown to increase when HL-60 cells are driven to differentiate by either phorbol esters or dimethylformamide [14]. Because these materials drive HL-60 to differentiate to either macrophage- or granulocyte-like cells [15], it was suggested that the CB2 receptor could have a role in the terminal differentiation of pluripotent hemopoietic stem cells.

Very little is currently known about the structure–function relationship of cannabinoids with regard to the CB2 receptor, as well as regarding the signal transduction pathways utilized by this receptor. Here we report on the establishment of a Chinese hamster ovary (CHO) cell line that stably expresses the CB2 receptor. We demonstrate that this receptor interacts with a variety of tricyclic cannabinoid ligands and with anandamide and that it mediates the inhibition of AC activity through PTX sensitive G proteins.

2. Materials and methods

2.1. Materials

The cannabinoids HU-243, HU-210, HU-211, Δ^9-tetrahydrocannabinol (Δ^9-THC), and anandamide have been previously described [5,16–18]. The synthesis of the cannabinoids HU-293 and HU-293a will be described separately. For formulae of tricyclic cannabinoids, see Fig. 1. Cannabinoids were kept in ethanol and diluted before use in 50 mg/ml fatty acid free-bovine serum albumin (FAF-BSA) as described [16]. The phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (IBMX) and Ro-20-1724 were from Calbiochem (La Jolla, CA). All other materials were previously described [5].

2.2. Cell transfection and culture

The human CB2 receptor cDNA inserted into the mammalian expression vector pcDNA1 was kindly provided by Dr. Sean Munro (Cambridge, UK). Parental CHO-K1 cells (American Type Tissue Culture Collection, Rockville, MD) were transfected with pcDNA1-CB2 along with the pRC/cmv vector carrying the neomycin resistance gene (Invitrogen Co., San Diego, CA) using the lipofectamine transfection method (Gibco-BRL). Clones resistant to G418 (1 mg/ml) were ana-

*Corresponding author. Fax: (972) (8) 34-4131.

Abbreviations: AC, adenylate cyclase; CHO, Chinese hamster ovary; CTX, cholera toxin; Δ^9-THC, Δ^9-tetrahydrocannabinol; DMEM, Dulbecco's modified Eagle's medium; FAF-BSA, fatty acid-free bovine serum albumin; G proteins, GTP proteins; IBMX, 1-methyl-3-isobutylxanthine; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin.

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lyzed for their capacity to bind [^{3}H]HU-243. The CHO-CB2 receptor cell line isolated was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, 0.2 mg/ml G418, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°C.

2.3. Cannabinoid receptor-binding and AC assays

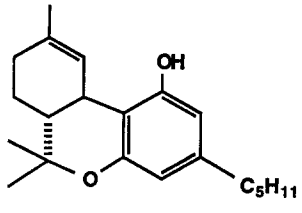
The binding of the labeled high affinity cannabinoid ligand [^{3}H]HU-243 (54 Ci/mmol) was carried out in siliconized Eppendorf tubes as described [5,17]. AC assay was performed by labeling cells in 24-well plates for 3 h with 5 μCi/ml of [^{3}H]adenine (10.3 Ci/mmol). Medium was replaced with 0.485 ml of DMEM containing 20 mM HEPES (pH 7.4), 1 mg/ml FAF-BSA, 0.1 mM IBMX and 0.5 mM Ro-20–1724. Cannabinoids were added in 5 μl containing 50 mg/ml FAF-BSA. Forskolin was added (in 10 μl) to a final concentration of 1 μM. Cells were maintained at 37°C for 10 min. Medium was removed and reaction terminated with 1 ml of 2.5% perchloric acid followed by neutralization and column separation of the [^{3}H]cAMP. AC activity was determined according to the amount of [^{3}H]cAMP detected. For additional information see Vogel et al. [5]. Individual values were calculated in terms of percentage of control (i.e. in the absence of ligand) for each specific experiment. Results are expressed as means ± S.E.M. of these normalized values. Generation of plots, determination of binding parameters (K_d, K_i, IC_{50}, and B_{max}) and EC_{50} values were performed as described [5,9].

3. Results

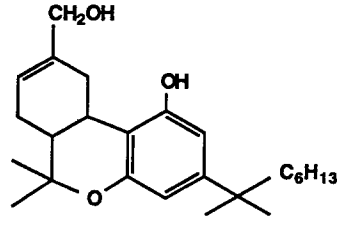
To a establish a stable cell line expressing the CB2 receptor, CHO-K1 cells were transfected with the complete cDNA sequence encoding for the CB2 receptor in pcDNA1 simultaneously with the vector pRC/cmv to provide drug resistance. G418 resistant colonies were screened for binding of the labeled high affinity cannabinoid ligand [^{3}H]HU-243. One positive clone (with B_{max} of 525 ± 6 fmol/mg of protein) was chosen and used for this study.

The Scatchard analysis of the binding of [^{3}H]HU-243 to crude membranes of the cells of the clone revealed a K_d value of 61 ± 5 pM. The competition binding assays performed with a variety of tricyclic cannabinoid ligands and with the endogenous ligand anandamide are shown in Fig. 2. The most effective displacer of [^{3}H]HU-243 binding to CHO-CB2 cell membranes was HU-243 itself with a K_d value of 47 ± 7 pM. This value is similar to that obtained by the Scatchard analysis described above. The other ligands investigated, HU-210, HU-293a, HU-293, Δ^9-THC, and anandamide gave K_i values of 147 ± 7 pM, 20 ± 4 nM, 30 ± 4, 39 ± 5 nM, and 85 ± 13 nM, respectively. HU-211, the enantiomer of HU-210, found to be inactive on the CB1 receptor [5] was also inactive as a displacer of [^{3}H]HU-243 from the CB2 receptor. No binding was observed to nontransfected CHO-K1 cells (data not shown).

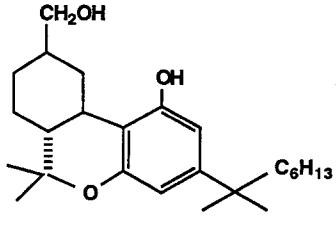
Activation of the cannabinoid receptor of brain and of $N_{18}TG_2$ cells inhibits the forskolin or secretin stimulation of AC [1–3,5,6,20]. We have therefore investigated if the CB2 receptor would have a similar activity. We have assessed the ability of several tricyclic cannabinoid derivatives to inhibit the forskolin-stimulated AC activity in CHO-CB2 cells (Fig. 3). Indeed, about 50% of the forskolin-stimulated AC activity could be inhibited by several tricyclic cannabinoids. HU-210 had the strongest agonistic activity with an EC_{50} value of 1 ± 0.6 nM. HU-293a was less active than HU-210 (EC_{50} of 8 ± 3 nM) while HU-293, the acetate of HU-293a, was slightly less active with an EC_{50} value of 47 ± 4 nM. The inhibition of AC by HU-210 and by HU-293a was not affected by the addition of 10 μM of the CB1 antagonist, SR141716A, [9,21] (15–20 s before agonist addition), demonstrating that this antagonist is selective for CB1 and does not interact with CB2 (data not shown). Interestingly, Δ^9-THC and anandamide, materials that bind to both CB2 and CB1 receptors and which have been shown to inhibit AC in $N_{18}TG_2$ and in CHO-CB1 cells [5,8,9,20], did not significantly inhibit AC activity in the forskolin activated CHO-CB2 cells.



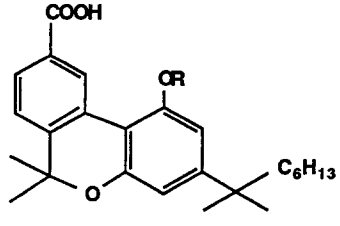
Δ^9-THC



HU-210, (-) enantiomer
HU-211, (+) enantiomer



HU-243



HU-293, R = COCH$_3$
HU-293a, R = H

Fig. 1. Formulae of tricyclic cannabinoids.

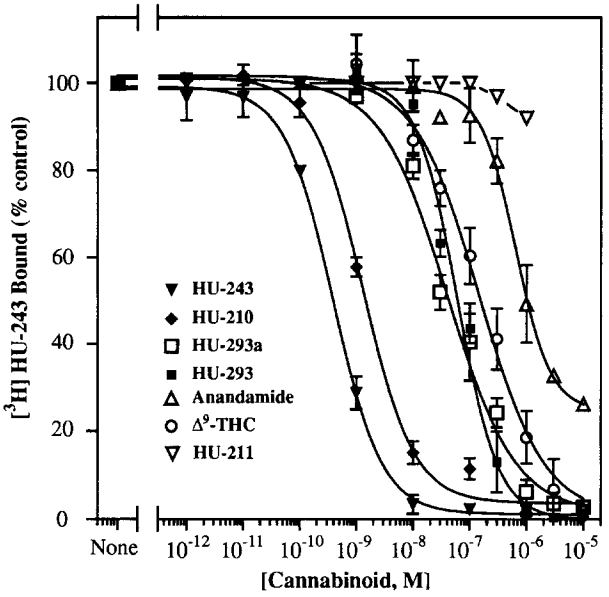


Fig. 2. Tricyclic cannabinoids and anandamide compete with binding of [^{3}H]HU-243 to CB2 receptor in CHO-CB2 cells. Binding of [^{3}H]HU-243 (0.3 nM final) was assayed in the presence of the indicated concentrations of cannabinoids. 100% specific binding was obtained by subtracting the background in the presence of 10 μM HU-210 (ca. 400 cpm, less than 35% of total binding). Phenylmethylsulfonyl fluoride (PMSF) at 0.15 mM was added to the binding assay with anandamide to protect it from degradation [19]. PMSF did not affect binding of the tricyclic cannabinoids. Data are the mean ± S.E.M. of three independent experiments (performed in duplicate).

In the above-described experiment, we have used forskolin to stimulate AC activity. Similar observations were also obtained when AC activation was performed following a 2 h preincubation with cholera toxin (CTX). In the experiment described in Fig. 4, we show that HU-210 at 0.1 μM and HU-293a at 1 μM inhibited CTX stimulated AC activity by 61% and 48%, respectively. These levels of inhibition are similar to those observed when AC was stimulated with forskolin. On the other hand as described above, Δ^9-THC was much less potent in inhibiting AC activity. 1 μM of Δ^9-THC inhibited AC by only 18%. This level of inhibition is much lower than the one obtained with the CB1 receptor with equivalent concentrations of Δ^9-THC [5]. The nature of the weak inhibition of AC by Δ^9-THC and anandamide via the CB2 receptor is under investigation.

To determine the class of the G proteins involved in the inhibition of AC by the CB2 receptor activation, we have treated CHO-CB2 cells with 100 ng/ml of PTX for 20 h prior to AC assay. As shown in Fig. 5, the inhibition of AC by 0.1 μM HU-210 or by 1 μM HU-293a was completely abolished by PTX pretreatment, indicating involvement of the G_i/G_o G proteins in the CB2 coupled inhibition of AC activity.

4. Discussion

We have prepared a cell line that stably expresses the CB2 cannabinoid receptor. The expressed CB2 receptor has the ability to bind Δ^9-THC and a number of synthetic tricyclic cannabinoids as well as anandamide. The binding potencies of HU-243, HU-210, Δ^9-THC, and anandamide were found to be similiar to values reported for the CB1 receptor [5,16,17]. In addition, we have shown that tricyclic cannabinoids were able to mediate inhibition of forskolin stimulated AC activity in these cells. This inhibition is PTX sensitive demonstrating that the peripheral cannabinoid receptor, CB2, is similar to the CB1 receptor in its ability to couple to the G_i/G_o G protein effector-signaling system. Tricyclic cannabinoids did not bind and had no effect on AC activity in parental CHO cells [5]. We therefore conclude that the cannabinoid effect on AC activity is being mediated by the expressed CB2 receptor in the transfected cells. These results are consistent with a recent report indicating that AC inhibition (via CB1) is indeed coupled to cannabinoid receptors as opposed to alternate signal transduction pathways that appear to be stimulated via nonreceptor-mediated cannabinoid-induced effects [3].

Analysis of the structure–activity relationship of the various tricyclic cannabinoids tested could demonstrate which structural elements of the tricyclic ligand can influence binding. Indeed, small modifications based on the basic structure of Δ^9-THC can increase or decrease binding to the cannabinoid receptors by almost 1000-fold and markedly change the capacity of the cannabinoids to inhibit AC. For example, the addition of a hydroxyl group at the C-11 position in Δ^8-THC (which parallels Δ^9-THC activity) and the conversion of the hydrophobic pentyl carbon chain into a 1,1-dimethylheptyl chain (i.e. converting Δ^8-THC to HU-210) led to high affinity ligand–receptor interaction. These modifications of Δ^9-THC have also greatly increased ligand affinity toward the cannabinoid receptor of brain [18].

In agreement with the binding studies, HU-210 and HU-293a were effective agonists and gave similar inhibition of AC; their EC_{50} values being in the concetration range of their K_i values. On the other hand, Δ^9-THC and anandamide demonstrated a much lower ability to inhibit AC activity even though they were

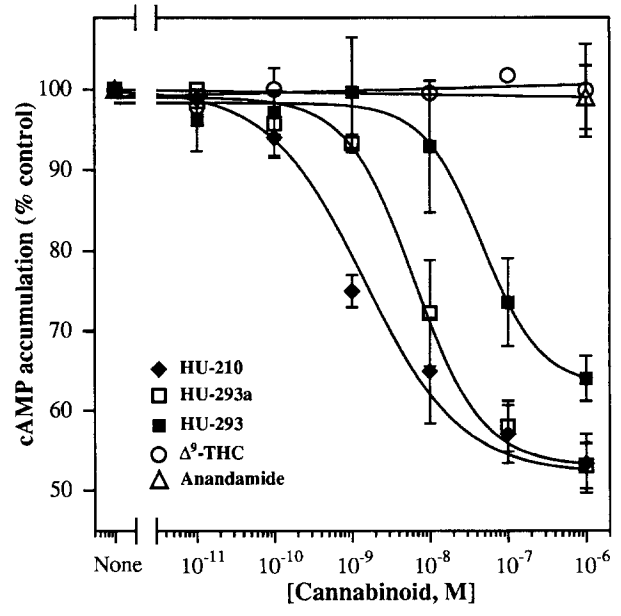


Fig. 3. Tricyclic cannabinoids inhibit the forskolin-stimulated AC activity in CHO-CB2. Cannabinoids were added at the indicated concentrations. PMSF at 0.15 mM was present in the anandamide assay and did not have any effect by itself on AC activity. The amount of [^{3}H]cAMP in the absence of forskolin was subtracted. Data are the mean ± S.E.M. of three to four independent experiments (performed in duplicate).

able to bind to the CB2 receptor. For example, the affinity of Δ^9-THC (K_i of 39 nM) to this receptor was similar to some of the active tricyclic cannabinoids (e.g. HU-293a with a K_i of 20 nM). This raises the possibility that Δ^9-THC and anandamide serve as weak partial agonists of the CB2 receptor (i.e. ligands which bind but do not effectively activate the receptor) indicating a difference in the signal transduction compared with the previously reported inhibition of AC by Δ^9-THC and anandamide for the CB1 receptor [5,8,9]. Indeed, preliminary experiments have shown that Δ^9-THC can antagonize the AC inhibition induced by HU-293 or HU-293a through the CB2 receptor (data not shown). This result suggests that use of marijuana (in which the active component is mainly Δ^9-THC) could have different effects on AC activity in the nervous and peripheral systems due to the differential ability of Δ^9-THC to mediate its effects depending on the receptor with which it interacts. It is interesting to note that anandamide, as well as its two endogenous analogues docosotetraenylethanolamide and homo-γ-linoenylethanolamide, have been shown to act as partial agonists at the CB1 receptor in cultured $N_{18}TG_2$ neuroblastoma cells [4,9,20] and that anandamide and docosotetraenylethanolamide were shown to have partial agonistic effects in vivo as well [20].

The presence of multiple cannabinoid receptors and the elucidation of their signal transduction pathways should allow for the identification of cannabinoid effects according to which cannabinoid receptor is being activated. Because CB2 has been found in spleen and has been implicated to be present in cells of the immune system, the immune modulatory effects of cannabinoids are possibly mediated through this receptor. Our work demonstrates that activation of the CB2 receptor can mediate inhibition of AC activity. Recent work [13,22] indeed demonstrates that cannabinoids inhibit cAMP formation in spleenocytes and that this effect is associated with inhibition of the humoral immune response in spleen cells. The development of selective agonists and antagonists should allow for the specific elucidation of the effects mediated by each cannabinoid receptor.

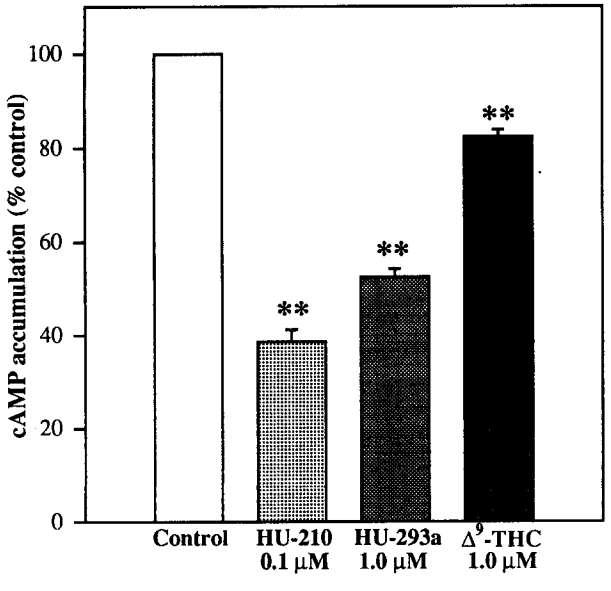


Fig. 4. Cannabinoids inhibit the CTX-stimulated AC activity. CHO-CB2 cells were preincubated for 2 h in the presence of 100 ng/ml of CTX. This was followed by the addition of phosphodiesterase inhibitors and cannabinoids. The background, defined as the level of cAMP in cells prior to the addition of phosphodiesterase inhibitors (ca. 1500 cpm), was subtracted. Control, represented as 100%, was equivalent to ca. 5500 cpm. Data are the mean ± S.E.M. of three independent experiments (performed in duplicate). $^{**}P < 0.001$, significantly different from control according to Student's t-test.

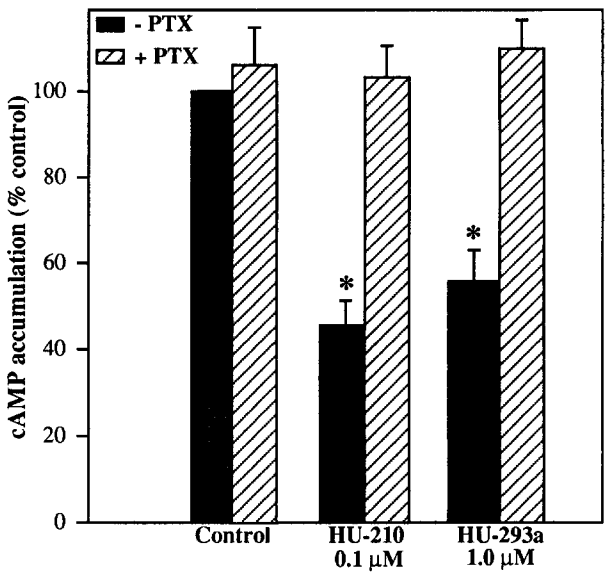


Fig. 5. Pretreatment of CHO-CB2 cells with PTX blocks the inhibition of AC activity by cannabinoids. CHO-CB2 cells were preincubated for 20 h with 100 ng/ml of PTX (fresh PTX was supplemented upon the addition of [^{3}H]adenine). The forskolin-stimulated AC activity in the absence of PTX and cannabinoids is represented as 100% and was equivalent ca. 2150 cpm. The basal level was ca. 300 cpm. Data are the mean ± S.E.M. of three independent experiments (performed in duplicate). $^{*}P < 0.005$, significantly different from control according to Student's t-test.

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