The CB_1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt

Teresa GÓMEZ DEL PULGAR, Guillermo VELASCO and Manuel GUZMÁN[1]

Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, 28040-Madrid, Spain

Cannabinoids exert most of their effects in the central nervous system through the CB_1 cannabinoid receptor. This G-protein-coupled receptor has been shown to be functionally coupled to inhibition of adenylate cyclase, modulation of ion channels and activation of extracellular-signal-regulated kinase. Using Chinese hamster ovary cells stably transfected with the CB_1 receptor cDNA we show here that Δ^9-tetrahydrocannabinol (THC), the major active component of marijuana, induces the activation of protein kinase B/Akt (PKB). This effect of THC was also exerted by the endogenous cannabinoid anandamide and the synthetic cannabinoids CP-55940 and HU-210, and was prevented by the selective CB_1 antagonist SR141716. Pertussis toxin and wortmannin blocked the CB_1 receptor-evoked activation of PKB, pointing to the sequential involvement of a G_i/G_o protein and phosphoinositide 3′-kinase. The functionality of the cannabinoid-induced stimulation of PKB was proved by the increased phosphorylation of glycogen synthase kinase-3 serine 21 observed in cannabinoid-treated cells and its prevention by SR141716 and wortmannin. Cannabinoids activated PKB in the human astrocytoma cell line U373 MG, which expresses the CB_1 receptor, but not in the human promyelocytic cell line HL-60, which expresses the CB_2 receptor. Data indicate that activation of PKB may be responsible for some of the effects of cannabinoids in cells expressing the CB_1 receptor.

Key words: G-protein-coupled receptor, phosphoinositide 3′-kinase, protein phosphorylation.

INTRODUCTION

Cannabinoids, the active components of *Cannabis sativa* (marijuana), exert most of their central and peripheral effects by binding to specific G-protein-coupled receptors [1]. To date, two different cannabinoid receptors have been characterized and cloned from mammalian tissues: CB_1 [2] and CB_2 [3]. The CB_1 receptor is distributed mainly in the central nervous system, but is also present in peripheral nerve terminals as well as in extraneural organs such as testis, uterus, spleen and tonsils. By contrast, the expression of the CB_2 receptor is almost exclusively restricted to cells and organs of the immune system [1–3]. Several signalling pathways have been shown to be regulated by these receptors. Thus both the CB_1- and CB_2-receptor signal inhibition of adenylate cyclase and activation of extracellular-signal-regulated kinase (ERK). In addition, the CB_1 receptor is coupled to modulation of Ca^{2+} and K^+ channels [1]. The recent discovery of a family of endogenous ligands of cannabinoid receptors [4,5] and the potential therapeutic applications of cannabinoids [6] have focused a lot of attention on cannabinoids during the last few years.

One of the most common mechanisms of signal transduction is the activation of the serine/threonine protein kinase B (PKB), also known as Akt. This kinase plays a pivotal role in the regulation of basic cell functions such as energy metabolism and proliferation. Thus in response to insulin and growth factors PKB stimulates glucose uptake through translocation of GLUT-4 to the plasma membrane, glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3), glycolysis through activation of 6-phosphofructo-2-kinase and protein synthesis by de-inhibition of initiation and elongation factors [7–9]. In addition, PKB has been identified as a very important component of pro-survival signalling pathways [8,9]. The anti-apoptotic action of PKB seems to be mediated by phosphorylation and inactivation of Bad, caspase-9 and Forkhead transcription factors, and perhaps by promoting the degradation of $I_\kappa B$, thereby increasing the activity of nuclear factor κB [8–10]. PKB contains a pleckstrin homology domain, and hence a major pathway of PKB activation is that mediated by the lipid products of phosphoinositide 3′-kinase (PI3K) [11–13]. It has been shown that wortmannin, a PI3K inhibitor, blocks the cannabinoid-induced stimulation of ERK [14] and glucose metabolism [15] in cells expressing the CB_1 receptor. However, no direct proof of the coupling of this receptor to PKB activation has been reported to date. The present work was therefore undertaken to test whether PKB may become activated by the CB_1 cannabinoid receptor.

MATERIALS AND METHODS

Reagents

The following materials were kindly donated: Chinese hamster ovary (CHO) cells stably transfected with the rat CB_1 cannabinoid receptor cDNA by Dr T. I. Bonner (Natonal Institutes of Health, Bethesda, MD, U.S.A.) and Dr Z. Vogel (The Weizmann Institute, Rehovot, Israel); the anti-PKBα antibody, the anti-phospho-Ser-21-GSK-3α antibody and the PKB substrate peptide (GRPRTSSFAEG) by Dr D. R. Alessi (Dundee University, Dundee, U.K.); SR141716 and SR144528 by Sanofi Recherche (Montpellier, France); HU-210 by Professor R. Mechoulam (Hebrew University, Jerusalem, Israel); and CP-55940 by Dr J.

Abbreviations used: CHO, Chinese hamster ovary; ERK, extracellular-signal-regulated kinase; GSK-3, glycogen synthase kinase-3; PI3K, phosphoinositide 3′-kinase; PKB, protein kinase B/Akt; THC, Δ^9-tetrahydrocannabinol.

[1] To whom correspondence should be addressed (e-mail mgp@solea.quim.ucm.es).

A. Ramos and Dr J. J. Fernández-Ruiz (Complutense University, Madrid, Spain). Δ^9-Tetrahydrocannabinol (THC) and anandamide were from Sigma (St. Louis, MO, U.S.A.). The protein kinase A inhibitor peptide (TTYADFIASGRTGRRNAIHD) was from Calbiochem (La Jolla, CA, U.S.A.).

Cell culture

Wild-type CHO cells were maintained in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum and non-essential amino acids. CHO cells transfected with the CB_1 receptor cDNA were grown in the same medium supplemented with 0.5 mg/ml geneticin. Wild-type and transfected CHO cells are referred to as CHO-wt and CHO-CB_1 cells, respectively. The human astrocytoma cell line U373 MG [16] and the human promyelocytic cell line HL-60 [17] were cultured as described previously. Before the experiment (24 h), cells were transferred to their respective serum-free media. Stock solutions of cannabinoid agonists and antagonists were prepared in DMSO. Control incubations had corresponding DMSO contents. No significant influence of DMSO on PKB activity was observed at the final concentration used (0.1%, v/v).

PKB activity

PKB activity was determined as described by Alessi et al. [18] with some modifications. Cells were scraped in lysis buffer consisting of buffer A [50 mM Tris/HCl, pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol] supplemented with 0.5 μM microcystin-LR, 17.5 μg/ml PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 20 μg/ml soya bean trypsin inhibitor and 5 μg/ml benzamidine. Lysates were centrifuged for 5 min at 13000 ***g***, and PKB was immunoprecipitated from $\approx$ 100 μg of cell-lysate protein with 2 μg of anti-PKBα antibody bound to Protein G–Sepharose. The immune complexes were washed extensively with buffer A containing 0.5 M NaCl and then with buffer B [50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol] supplemented with 0.03% (w/v) Brij-35. Assay of PKB activity was performed in buffer B containing 50 μM PKB substrate peptide, 15 μM protein kinase A inhibitor peptide, 100 μM [γ-^{32}P]ATP (1 μCi/assay) and 15 mM magnesium acetate. After 10 min at 30 °C, reactions were stopped and PKB activity was determined as described in [18].

GSK-3 phosphorylation

Cells lysates were obtained as described above for PKB. Samples were subjected to SDS/PAGE in 10% gels and proteins were transferred from SDS gels on to nitrocellulose membranes. The blots were then blocked with 10% fat-free dried milk in 50 mM Tris/HCl, pH 7.8, 100 mM NaCl and 0.1% Tween 20 (TBST). They were incubated subsequently for 2 h at 4 °C with the anti-phospho-Ser-21-GSK-3α antibody (2 μg/ml) in TBST supplemented with 3% fat-free dried milk, and washed thoroughly. The blots were then incubated with anti-(sheep IgG) peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature, and finally subjected to luminography with an electrochemiluminescence detection kit.

Statistical analysis

Results shown represent the means $\pm$ S.D. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance (ANOVA). A *post hoc* analysis was made using the Student–Neuman–Keuls test.

RESULTS AND DISCUSSION

The CB_1 cannabinoid receptor is coupled to PKB activation

CHO cells stably transfected with the CB_1 receptor cDNA constitute a well-characterized model to study the signal-transduction pathways modulated by that receptor (see [1]). Here we used those cells to test the possible coupling of the CB_1 receptor to PKB activation. CHO cells were treated for different times with THC, the major active component of marijuana; PKB was then immunoprecipitated and enzyme activity was determined. As shown in Figure 1(a), THC induced a time-dependent stimulation of PKB in CHO-CB_1 cells. The effect of THC was transient, reaching a maximal 8-fold stimulation at 10 min. Several experiments were performed subsequently to demonstrate the involvement of the CB_1 receptor in the THC-induced stimulation of PKB in CHO-CB_1 cells.

(i) Half-maximal stimulation of PKB by THC occurred at a concentration of $\approx$ 15 nM (Figure 1b), i.e. in the range of the K_d value of THC for the CB_1 receptor [1]. (ii) Like THC, the

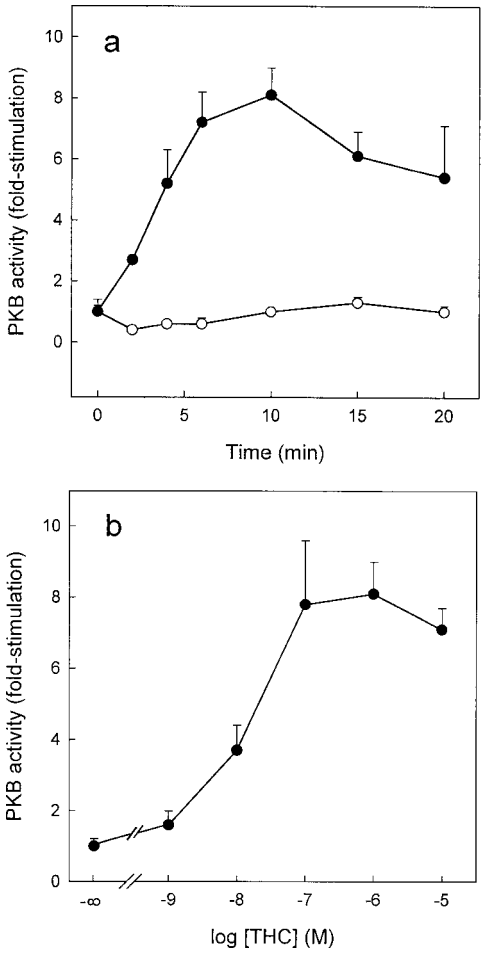

Figure 1 THC induces PKB activation

(a) Time course. CHO-CB_1 (●) and CHO-wt cells (○) were incubated with 1 μM THC for different periods of time. (b) Dose–response. CHO-CB_1 cells were incubated with different THC concentrations for 10 min. In both panels, results are expressed as fold-stimulation over incubations with no additions and correspond to four different experiments.

Table 1 The CB_1 cannabinoid receptor is coupled to PKB activation

CHO cells were incubated with or without 1 μM SR141716 for 20 min, and then with or without the cannabinoids (1 μM THC, 25 nM CP-55940, 25 nM HU-210 or 15 μM anandamide) for an additional 10 min. Results are expressed as fold-stimulation over incubations with no additions and correspond to four different experiments. Significant differences from incubations with no additions are indicated by * ($P < 0.01$) and ** ($P < 0.05$).

	PKB activity (fold-stimulation)	
Additions	CHO-CB_1	CHO-wt
None	1.0 ± 0.2	1.0 ± 0.4
THC	8.1 ± 0.9*	1.0 ± 0.1
CP-55940	11.6 ± 0.4*	1.3 ± 0.1
HU-210	10.0 ± 0.8*	1.0 ± 0.2
SR141716	1.0 ± 0.2	0.9 ± 0.1
THC + SR141716	1.0 ± 0.5	0.8 ± 0.2
Anandamide	14.9 ± 3.3*	3.2 ± 1.3*
Anandamide + SR141716	2.1 ± 0.8**	2.7 ± 0.3*

Table 2 The CB_1 cannabinoid receptor induces PKB activation via a G_i/G_o-protein- and PI3K-dependent pathway

CHO cells were incubated with 0.2 μM wortmannin (20 min), 100 ng/ml pertussis toxin (14 h) or 1 μM SR141716 (20 min), and then with or without 1 μM THC or 100 nM insulin for an additional 10 min. Results are expressed as fold-stimulation over incubations with no additions and correspond to four different experiments. Significant differences from incubations with no additions are indicated by *$P < 0.01$.

	PKB activity (fold-stimulation)	
Additions	CHO-CB_1	CHO-wt
None	1.0 ± 0.2	1.0 ± 0.4
THC	8.1 ± 0.9*	1.0 ± 0.1
Insulin	23.8 ± 7.0*	23.6 ± 2.5*
Wortmannin	0.2 ± 0.1*	0.4 ± 0.1*
THC + wortmannin	0.1 ± 0.1*	0.2 ± 0.2*
Insulin + wortmannin	0.1 ± 0.1*	0.1 ± 0.1*
Pertussis toxin	0.9 ± 0.3	0.8 ± 0.1
THC + pertussis toxin	1.2 ± 0.1	0.8 ± 0.1
Insulin + pertussis toxin	24.0 ± 1.2*	23.6 ± 0.2*
Insulin + SR141716	29.1 ± 2.6*	24.0 ± 3.2*

synthetic cannabinoids CP-55940 and HU-210 were able to stimulate PKB (Table 1). These cannabinoid agonists elicited PKB activation at lower doses than THC, in line with their higher affinity for the CB_1 receptor [1]. Doses of CP-55940 and HU-210 exerting maximal activation of PKB were used in the experiments shown in Table 1. (iii) The stimulatory effect of THC on PKB was abolished by treatment of cells with SR141716, a selective CB_1 receptor antagonist (Table 1). (iv) The stimulation of PKB induced by THC, CP-55940 and HU-210 in CHO-CB_1 cells was not evident in CHO-wt cells (Figure 1a and Table 1).

The endogenous cannabinoid anandamide stimulated PKB to a higher extent than other cannabinoid agonists (Table 1). Anandamide is known to be actively degraded by cellular fatty acid amide hydrolase to arachidonic acid and ethanolamine [5]. As shown in Table 1, in CHO-CB_1 cells the effect of anandamide on PKB was not blocked completely by SR141716. In addition, in CHO-wt cells anandamide induced a slight though reproducible activation of PKB that was insensitive to SR141716 (Table 1) and to the selective CB_2 antagonist SR144528 at 1 μM (results not shown). Moreover, arachidonic acid stimulated PKB in CHO-wt cells (5.0 ± 1.2-fold stimulation at 15 μM arachidonic acid, $n = 3$, $P < 0.01$ compared with incubations with no additions). These observations indicate that anandamide stimulates PKB activity mostly by a CB_1-receptor-dependent mechanism, with a minor contribution from a cannabinoid-receptor-independent mechanism.

The CB_1 cannabinoid receptor induces PKB activation via a G_i/G_o-protein- and PI3K-dependent pathway

To further investigate the signal-transduction pathway responsible for PKB activation, the effects of pertussis toxin and wortmannin were examined. Parallel experiments were carried out with insulin, which is known to activate PKB through class-I_A PI3Ks, i.e. PI3K isoenzymes containing Src homology domains and hence activated by binding to phosphotyrosine residues [11,12]. As shown in Table 2, the stimulatory effect of both THC and insulin on PKB was fully prevented by wortmannin. However, pertussis toxin abrogated the activation of PKB induced by THC but not by insulin, in agreement with a selective coupling of the CB_1 receptor to G_i/G_o proteins. Hence, unlike insulin, cannabinoids seem to induce PKB activation through class-I_B PI3Ks, i.e. PI3K isoenzymes devoid of Src homology domains and activated by G-protein $\beta\gamma$ subunits [11,12].

The notion that cannabinoids and insulin activate PKB through different receptor-coupled mechanisms constrasts with the observation that not only wortmannin but also pertussis toxin and SR141716 are able to block the insulin-induced stimulation of ERK [14]. On the basis of these data, Bouaboula et al. suggested that insulin stimulates ERK through class-I_B PI3Ks, and that SR141716 behaves as an inverse agonist that induces the sequestration of G_i proteins by the CB_1 receptor [14]. Although we are aware that SR141716 has been shown to behave as an inverse agonist in a number of experimental models *in vitro* (e.g. [14,19,20]) and *in vivo* (e.g. [1,21]), we have been unable to demonstrate any effect of SR141716 on basal (Table 1) or insulin-stimulated PKB activity (Table 2).

Cannabinoids induce GSK-3 phosphorylation through the CB_1 receptor

The functionality of the cannabinoid-induced stimulation of PKB was tested by examining the phosphorylation of a PKB substrate such as GSK-3α, whose serine 21 residue is selectively phosphorylated by PKB [7,8]. As shown in Figure 2, THC and CP-55940 were able to induce GSK-3 phosphorylation in CHO-CB_1 cells under the same experimental conditions in which they activated PKB. The effect of the two cannabinoids was prevented by SR141716 and wortmannin, and was not evident in CHO-wt cells, which were, however, responsive to insulin. These observations indicate that the cannabinoid-induced activation of PKB is functional.

Cannabinoids stimulate PKB in U373 MG cells but not in HL-60 cells

To test whether the cannabinoid-induced stimulation of PKB observed in CHO cells overexpressing the CB_1 receptor could be extrapolated to cells naturally expressing the receptor, we examined the effect of cannabinoids on the human astrocytoma cell line U373 MG, which expresses the CB_1 receptor mRNA [22]

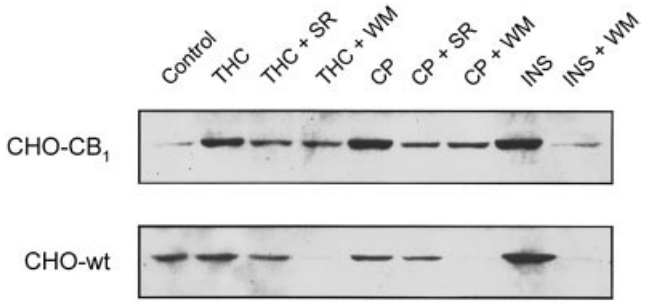

Figure 2 Cannabinoids induce GSK-3 phosphorylation through the CB_1 receptor

CHO cells were incubated with the additions indicated in Tables 1 and 2. Cells were lysed, proteins were subjected to SDS/PAGE, and Western blots were performed with the anti-phospho-Ser-21-GSK-3α antibody. A representative luminogram is shown. Similar results were obtained in two other experiments. CP, CP-55940; INS, insulin; SR, SR141716; WM, wortmannin.

Table 3 Cannabinoids stimulate PKB in U373 MG cells but not in HL-60 cells

U373 MG and HL-60 cells were incubated with the additions indicated in Tables 1 and 2. Results are expressed as fold-stimulation over incubations with no additions and correspond to four different experiments. n.d., not determined. Significant differences from incubations with no additions are indicated by $^*P < 0.01$.

	PKB activity (fold-stimulation)	
Additions	U373 MG	HL-60
None	1.0 ± 0.5	1.0 ± 0.1
Insulin	$2.4 \pm 0.5^*$	$2.6 \pm 0.8^*$
THC	$2.1 \pm 0.2^*$	0.9 ± 0.1
THC + SR141716	1.0 ± 0.1	n.d.
CP-55940	$2.5 \pm 0.7^*$	1.1 ± 0.1
CP-55940 + SR141716	0.9 ± 0.1	n.d.

and protein [23]. As shown in Table 3, THC and CP-55940 induced a significant activation of PKB in U373 MG cells that was prevented by SR141716, therefore pointing to a CB_1-receptor-mediated process. The lower magnitude of the cannabinoid-induced PKB activation in U373 MG cells compared with CHO-CB_1 cells may be explained by the lower levels of CB_1-receptor expression ([16] and this study, results not shown).

The CB_1 receptor is more widely expressed than the CB_2 receptor, and CB_1 signals through a more ample array of transduction systems than CB_2 [1]. Nevertheless, we tested whether cannabinoids stimulate PKB in the human promyelocytic cell line HL-60, a paradigmatic example of cells expressing the CB_2 receptor [1,3,17]. Cannabinoids were unable to stimulate PKB in HL-60 cells, which in contrast were responsive to insulin (Table 3). Although other factors might be involved in the response of PKB to cannabinoids, these results indicate that the CB_2 receptor does not mediate PKB activation. This is not surprising because CB_2 is not coupled to a number of signalling pathways that are regulated by CB_1, e.g. modulation of Ca^{2+} and K^+ channels [1] and NO generation [24,25]. In addition, Bayewitch et al. [26] have shown that the inhibition of adenylate cyclase by the CB_2 receptor is not exerted by naturally occurring cannabinoids such as THC and anandamide, which seem to behave as partial agonists – or even antagonists – of that receptor [26]. The differential signalling properties of the CB_1 and CB_2 receptors may be due to their relatively low sequence similarity, particularly regarding their C-terminal cytoplasmatic domain, supposedly involved in the coupling to signalling effectors [1–3].

Physiological considerations

PKB plays a pivotal role in the regulation of basic cell functions such as energy metabolism, proliferation and migration [7–10]. Regarding energy metabolism, we have shown previously that cannabinoids stimulate glucose oxidation and glycogen synthesis in astroglial cells, and that this effect is prevented by SR141716, pertussis toxin and PI3K inhibitors [15,27]. The present data indicate that the CB_1 receptor may activate cellular glucose utilization by stimulating PKB, which is well known to mediate stimulation of glycolysis through activation of 6-phosphofructo-2-kinase and stimulation of glycogen synthesis through inhibition of GSK-3/de-inhibition of glycogen synthase [7–9]. Regarding cell proliferation, one of the most intriguing and unexplored actions of cannabinoids is their ability to control cell growth. Thus cannabinoids have been shown to induce anti-proliferative [23,28–31] or proliferative effects [32–34] in a number of cultured-cell systems. Data from the present study indicate that modulation of PKB may be one of the signals involved in the control of cell fate by cannabinoids. Moreover, activation of the CB_1 receptor has been shown recently to induce cell migration [35].

Although the actual biological functions of the endogenous cannabinoid system are as yet unknown, it is generally accepted that endogenous cannabinoids might play a role in brain development and function [36,37]. The significance of the endogenous cannabinoid system is supported by the high levels of cannabinoid receptors found in brain [38]; the specific mechanisms of endocannabinoid synthesis, uptake and degradation [5,36]; and the neuromodulatory properties of endogenous cannabinoids [36]. In the context of our findings, one could speculate that by coupling to PKB activation the CB_1 cannabinoid receptor might regulate the utilization of glucose (e.g. for synaptic activity and myelination) and the fate of precursor neural cells (e.g. regarding migration and protection from apoptosis) in the brain. It is clear anyway that further research is required to understand the physiological role of cannabinoids as modulators of brain function.

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