De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis

Teresa GÓMEZ DEL PULGAR, Guillermo VELASCO, Cristina SÁNCHEZ, Amador HARO and Manuel GUZMÁN[1]

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

Δ^9-Tetrahydrocannabinol (THC) and other cannabinoids have been shown to induce apoptosis of glioma cells via ceramide generation. In the present study, we investigated the metabolic origin of the ceramide responsible for this cannabinoid-induced apoptosis by using two subclones of C6 glioma cells: C6.9, which is sensitive to THC-induced apoptosis; and C6.4, which is resistant to THC-induced apoptosis. Pharmacological inhibition of ceramide synthesis *de novo*, but not of neutral and acid sphingomyelinases, prevented THC-induced apoptosis in C6.9 cells. The activity of serine palmitoyltransferase (SPT), which catalyses the rate-limiting step of ceramide synthesis *de novo*, was remarkably enhanced by THC in C6.9 cells, but not in C6.4 cells. However, no major changes in SPT mRNA and protein levels were evident. Changes in SPT activity paralleled changes in ceramide levels. Pharmacological inhibition of ceramide synthesis *de novo* also prevented the stimulation of extracellular-signal-regulated kinase and the inhibition of protein kinase B triggered by cannabinoids. These findings show that *de novo*-synthesized ceramide is involved in cannabinoid-induced apoptosis of glioma cells.

Key words: extracellular-signal-regulated kinase, G-protein-coupled receptor, glioma, protein kinase B, serine palmitoyltransferase.

INTRODUCTION

Δ^9-Tetrahydrocannabinol (THC), the main active component of marijuana (*Cannabis sativa*), and other cannabinoids exert a wide spectrum of central and peripheral effects, such as alterations in cognition and memory, analgesia, anti-convulsion, anti-inflammation and alleviation of both intraocular pressure and emesis [1,2]. Cannabinoids produce their effects by binding to specific plasma membrane G-protein-coupled receptors [1,2]. To date, two different cannabinoid receptors have been cloned and characterized from mammalian tissues, CB_1 [3] and CB_2 [4]. Activation of these receptors has been shown to trigger several $G_{i/o}$-protein-mediated signalling pathways. For example, both CB_1 and CB_2 receptors induce the inhibition of adenylate cyclase and stimulation of extracellular-signal-regulated kinase (ERK), and the CB_1 receptor is also coupled to modulation of Ca^{2+} and K^+ channels [1,2]. The recent discovery of a family of endogenous ligands of cannabinoid receptors [5,6] has focused much attention on cannabinoids over the last few years. In addition, the renaissance in the study of the therapeutic effects of cannabinoids constitutes a widely debated issue with ample scientific and social relevance. Ongoing research is determining whether cannabinoid ligands may be effective agents in the treatment of pain, glaucoma, multiple sclerosis and the wasting and emesis associated with AIDS and cancer chemotherapy [7,8]. Cannabinoids might also be potential antitumoral agents, owing to their ability to inhibit the growth of various types of cancer cells in culture [9–12]. Moreover, in laboratory animals, cannabinoids induce the regression of gliomas, one of the most malignant forms of cancer [13]. This growth-inhibiting effect was exerted by THC and WIN-55,212-2, a synthetic cannabinoid agonist, and was mediated by cannabinoid receptor activation and ceramide generation [13].

Ceramide is a sphingosine-based lipid that regulates a variety of cellular processes, including differentiation, proliferation and apoptosis [14–16]. Accumulation of ceramide has been usually considered to occur via receptor-mediated activation of neutral or acid sphingomyelinases. However, over the last few years, the *de novo* synthesis pathway has been gaining appreciation as an alternative means of generating a signalling pool of ceramide [15,16]. We have shown previously [13] that exposure of glioma cells to cannabinoids triggers the generation of two peaks of ceramide. The first peak, occurring within minutes of the cannabinoid treatment, may involve neutral sphingomyelinase stimulation via the adaptor protein FAN [17,18], and does not play a significant role in the induction of apoptosis [13]. The second peak of ceramide starts at day 3 of cannabinoid treatment, reaching a maximum by day 5, and has been closely related to the apoptotic death of glioma cells [13]. However, the mechanism by which cannabinoids generate this sustained peak of ceramide is, as yet, unknown. The present study was therefore undertaken to investigate how this pro-apoptotic second peak of ceramide is generated in glioma cells, and whether this ceramide pool modulates ERK and protein kinase B (PKB).

EXPERIMENTAL

Glioma cell culture and viability

The rat glioma C6 line (10–20 passages) was cultured as described previously [10]. Two subclones of C6 glioma cells, which have been previously characterized in terms of cannabinoid-induced apoptosis, were used in this study: the C6.9 subclone, which is sensitive to THC-induced apoptosis, and the C6.4 subclone, which is resistant to THC-induced apoptosis [13]. Cell viability was determined by Trypan Blue exclusion. THC stock solutions

Abbreviations used: ERK, extracellular-signal-regulated kinase; LCB, long-chain base subunit; PKB, protein kinase B; SPT, serine palmitoyltransferase; TBS, Tris-buffered saline; THC, Δ^9-tetrahydrocannabinol; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling.

[1] To whom correspondence should be addressed (e-mail mgp@bbm1.ucm.es).

were prepared in DMSO and control incubations contained the same amount of DMSO. No significant effect of DMSO was observed in any of the parameters determined throughout this study at the final concentration used (0.1–0.2 %, v/v).

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) staining

C6 glioma cells were washed with PBS, fixed for 20 min at 20 °C in PBS/4 % paraformaldehyde/5 % (w/v) sucrose, permeabilized with 0.05 % Triton-X-100/PBS for 5 min, and blocked with 0.5 % BSA/PBS for 30 min. DNA ends were subsequently labelled for 2 h at 37 °C in Tris-buffered saline [TBS: 50 mM Tris/HCl (pH 7.2) and 100 mM NaCl] containing 2 mM $CoCl_2$, 0.1 unit/μl terminal deoxynucleotidyl transferase (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) and 2.5 pmol/μl biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany). Finally, cells were incubated for an additional 2 h with 3.5 μg/ml streptavidin–Alexa®Fluor 488 (Molecular Probes, Leiden, The Netherlands) in 0.1 % BSA/PBS.

Ceramide levels

Ceramide levels were determined as described previously [19]. Briefly, after incubation of the cells in the different conditions, lipids were extracted, saponified, and incubated with *Escherichia coli* diacylglycerol kinase in the presence of [γ-^{32}P]ATP. Finally, ceramide 1-phosphate was resolved by TLC.

ERK activity and levels

Cells were washed, lysed, supernatants were obtained, and ERK activity was determined via the incorporation of [γ-^{32}P]ATP into APRTPGGRR (where single-letter amino-acid notation has been used), a specific peptide substrate, as described previously [20]. ERK protein levels were determined by Western-blot analysis with an antibody that recognizes both the phosphorylated and non-phosphorylated form of ERK (New England Biolabs, Beverly, MA, U.S.A.).

PKB activity and levels

PKB activity was determined as described previously [21]. Briefly, PKB was immunoprecipitated from cell lysates with 2 μg of anti-PKBα antibody bound to Protein G–Sepharose. Kinase activity was determined via the incorporation of [γ-^{32}P]ATP into GRPRTSSFAEG (where single-letter amino-acid notation has been used), a specific peptide substrate. PKB protein levels were determined by Western-blot analysis with an antibody that recognizes both the phosphorylated and non-phosphorylated form of PKB.

Serine palmitoyltransferase (SPT) assay

SPT activity was determined in digitonin-permeabilized C6 glioma cells via the incorporation of radiolabelled L-serine into ketosphinganine by using a new procedure [22] with minor modifications. Briefly, the medium was aspirated and cells were washed twice with PBS. Reactions were started by the addition of 100 mM Hepes (pH 8.3)/200 mM sucrose/2.5 mM EDTA/5 mM dithioerythritol/50 μM pyridoxal phosphate/ 1.0 mg/ml BSA/8.75 μg/ml digitonin/0.3 mM palmitoyl-CoA/0.25 mM L-[U-^{14}C]serine (3 μCi/assay). After 30 min, reactions were stopped with 0.5 M NH_4OH, the [^{14}C]ketosphinganine product was extracted with chloroform/methanol (2:1, v/v), and the organic phases were subsequently washed with 1 % (w/v) NaCl. Preliminary experiments defined the optimal concentration of digitonin and other reagents in the assay, as well as its linearity with time.

Western-blot analysis of SPT

Western-blot analysis was carried out with polyclonal antibodies raised against hamster SPT long-chain base subunit (LCB) 1 and LCB2 [22,23]. Briefly, cells were lysed, and samples were subjected to centrifugation at 12000 ***g*** for 1 h at 4 °C. Pellets were resuspended in lysis buffer [21], 50 μg of total protein from each sample was subjected to SDS/PAGE on 10 % (w/v) gels, and proteins were transferred on to nitrocellulose membranes [21]. The blots were blocked with TBS/0.1 % Tween-20/5 % (w/v) non-fat dried milk and incubated with anti-(LCB1) or anti-(LCB2) antibodies [1:150 dilution in TBS/0.1 % Tween-20/5 % (w/v) non-fat dried milk]. Finally, membranes were subjected to luminography with an enhanced chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Northern-blot analysis of SPT

For RNA preparation, cells were washed with ice-cold PBS and total RNA was extracted using the RNAgents total RNA extraction kit (Promega, Madison, U.S.A.). Briefly, 30 μg of total cellular RNA was separated on 37 % formaldehyde-containing 1.2 % agarose gels and transferred on to nylon membranes (Roche, Basel, Switzerland). After prehybridization for 8 h at 42 °C in 5 × SSC (1 × SSC is 0.15 M NaCl/15 mM sodium citrate)/50 % formamide/0.1 % laurosyl sarcosine/0.2 % SDS/2 % blocking reagent (Roche, Basel, Switzerland), membranes were incubated with cDNA fragments labelled by random primers in the presence of [α-^{32}P]dATP. The probes were an 867 bp *Sma*I fragment of human LCB1 and a 747 bp *Hin*dIII/*Sca*I fragment of human LCB2, obtained from digestion of plasmids encoding LCB1 and LCB2 of SPT [24]. Membranes were subsequently washed twice with 2 × SSC/0.1 % SDS (5 min at 20 °C); twice with 0.2 × SSC/0.1 % SDS (15 min at 42 °C); and once with 0.2 × SSC/0.1 % SDS (15 min at 55 °C). Blots were exposed finally to Kodak X-AR films with intensifier screens at −70 °C.

Statistics

Results shown represent means ± S.D. Statistical analysis was performed by ANOVA with a *post hoc* analysis by the Student–Neuman–Keuls test.

RESULTS

Inhibition of ceramide synthesis *de novo* prevents THC-induced ceramide accumulation and apoptosis

Treatment of C6 glioma cells with THC and other cannabinoids has been shown to induce apoptosis via ceramide generation [13]. However, the metabolic origin of the ceramide pool responsible for cannabinoid-induced apoptosis remains unknown. Two major pathways may contribute to intracellular ceramide accumulation: sphingomyelin hydrolysis and ceramide synthesis *de novo* [14,15]. Hence we tested whether selective pharmacological inhibitors of those two pathways [16,25–28] were able to prevent cannabinoid-induced apoptosis of C6 glioma cells. As shown in Figure 1, none of desipramine (an inhibitor of acid sphingomyelinase), scyphostatin (an inhibitor of neutral sphingomyelinase) or PMA (which inhibits neutral sphingomyelinase via protein kinase C activation) prevented THC-induced death of C6.9 glioma cells. In contrast, blockade of ceramide synthesis *de novo* with L-cycloserine (an inhibitor of SPT) or fumonisin B_1

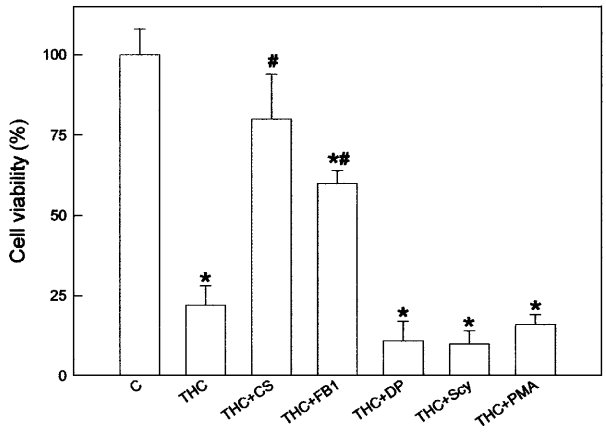

Figure 1 Inhibitors of ceramide synthesis *de novo* prevent THC-induced C6.9 cell death

Cells were incubated in the absence (C) or presence of 0.5 μM THC, with the following additions being made to cells treated with THC: 0.5 mM L-cycloserine (THC + CS), 10 μM fumonisin B_1 (THC + FB1), 10 μM desipramine (THC + DP), 0.2 μM scyphostatin (THC + Scy) and 50 nM PMA (THC + PMA). Cell viability was determined by Trypan Blue exclusion after 5 days of treatment. Results are expressed as a percentage of incubations with no additions and were obtained from six different experiments. $^{*}P < 0.01$ compared with incubations with no additions; $^{\#}P < 0.01$ compared with incubations with THC.

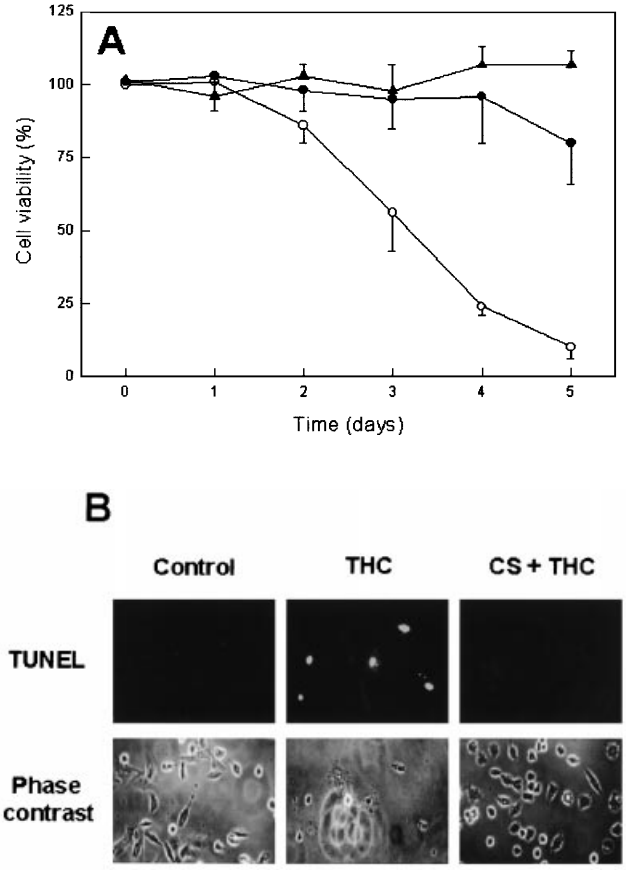

Figure 2 L-Cycloserine prevents THC-induced apoptosis in C6.9 cells

(**A**) C6.9 cells were incubated in the presence of 0.5 μM THC with (●) or without (○) 0.5 mM L-cycloserine (CS). C6.4 cells were also incubated in the presence of 0.5 μM THC (▲). Results are expressed as a percentage of incubations with no additions and were obtained from six different experiments. (**B**) C6.9 cells were treated as in (**A**) for 5 days and TUNEL staining was performed. Representative micrographs (phase contrast and TUNEL-stained cells) from one experiment are shown. Similar data were obtained in three additional experiments. Control, untreated cells.

(an inhibitor of ceramide synthase) prevented THC-induced death of C6.9 glioma cells (Figures 1 and 2).

To confirm that ceramide synthesis *de novo* is involved in THC-induced ceramide accumulation and cell death, we determined ceramide levels and cell viability in parallel. L-Cycloserine prevented not only THC-induced death of C6.9 glioma cells (Figure 2A), but also THC-induced long-term ceramide accumulation in these cells (Figure 3). A good relationship was observed between the kinetics of cell death and that of ceramide levels, although at day 3 of treatment there was significant cell death (Figure 2A), but only slight ceramide accumulation (Figure 3).

TUNEL-staining analysis was performed to test whether THC-induced apoptosis of C6.9 glioma cells occurs by apoptosis. As shown in Figure 2(B), TUNEL-positive nuclei were evident upon THC treatment, and this effect was efficiently prevented by L-cycloserine.

THC enhances SPT activity without major changes in its mRNA and protein levels

Because SPT catalyses the rate-limiting step of ceramide synthesis *de novo* [28], we determined whether ceramide accumulation correlated with SPT activity during the treatment with THC. As shown in Figure 4(A), incubation of C6.9 glioma cells with THC enhanced SPT activity in parallel with ceramide accumulation (Figure 3). A good relationship was also observed between the kinetics of cell death and that of SPT activity, although, as mentioned above for ceramide levels, at day 3 of treatment there was significant cell death (Figure 2A), but only slight SPT activation (Figure 4A). Of interest, SPT activation was not observed in C6.4 glioma cells (Figure 4A), a subclone of C6 glioma cells in which THC does not induce apoptosis (Figure 2A) and ceramide accumulation (Figure 3) [13].

To test whether THC enhances SPT activity by inducing SPT expression, enzyme mRNA and protein levels were monitored by Northern and Western blot respectively. As shown in Figure 4(B), the levels of the regulatory LCB1 were only slightly enhanced in C6.9 glioma cells after THC challenge (1.8 ± 0.4 fold increase, $n = 3$; $P < 0.05$ compared with incubations with no additions). In addition, no significant changes in the levels of LCB1 in C6.4 cells and the catalytic LCB2 in the two subclones were evident upon THC treatment (Figure 4B and results not shown). Likewise, THC did not significantly increase the mRNA levels of either SPT subunits in C6.9 cells (1.4 ± 0.4- and 1.1 ± 0.3-fold increase for LCB1 and LCB2 respectively, $n = 3$; not significantly different than incubations with no additions) (Figure 4C).

De novo-synthesized ceramide activates ERK and inhibits PKB

Ceramide-induced apoptosis may be mediated, at least in part, via modulation of the ERK [14] and PKB [29] signalling pathways. In particular, we have shown previously that sustained ERK activation may contribute to ceramide-induced apoptosis in C6 glioma cells [13] and primary astrocytes [30]. Therefore we studied the contribution of *de novo*-synthesized ceramide in the regulation of ERK and PKB in C6.9 and C6.4 glioma cells. Blockade of ceramide synthesis *de novo* with L-cycloserine

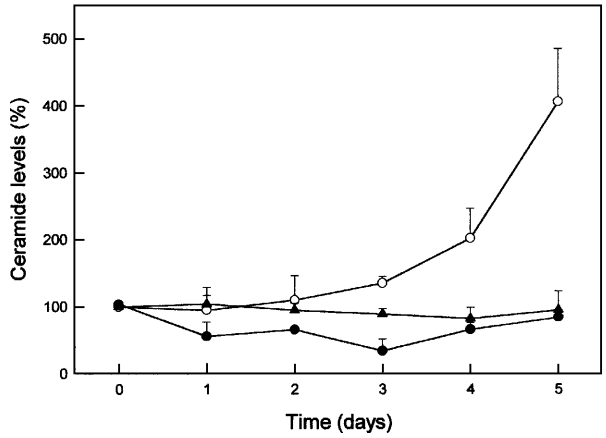

Figure 3 L-Cycloserine prevents THC-induced ceramide accumulation in C6.9 cells

C6.9 cells were incubated in the presence of 0.5 μM THC with (●) or without (○) 0.5 mM L-cycloserine. C6.4 cells were incubated in the presence of 0.5 μM THC (▲). Results are expressed as a percentage of incubations with no additions and were obtained from four different experiments.

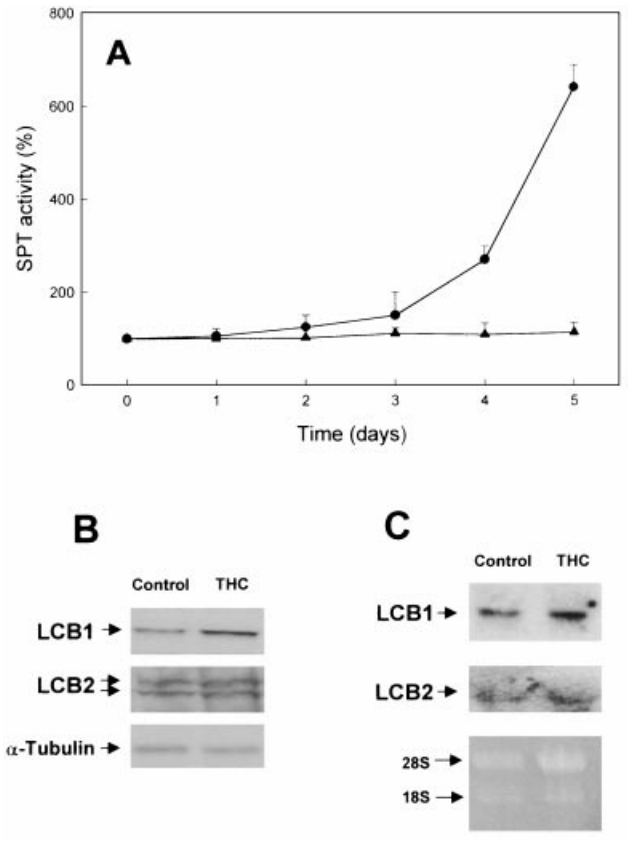

Figure 4 THC enhances SPT activity in C6.9 cells without major changes in its mRNA and protein levels

(**A**) C6.9 (●) or C6.4 (▲) cells were incubated with 0.5 μM THC for up to 5 days. Results are expressed as the percentage of incubations with no additions and were obtained from six different experiments. (**B** and **C**) C6.9 cells were treated as in (**A**) for 5 days and Western- (**B**) and Northern- (**C**) blot analysis of SPT subunits was performed. Protein and mRNA loadings were determined using α-tubulin (**B**) and 18S and 28S (**C**) respectively. Representative blots from one experiment are shown. Similar data were obtained in two additional experiments.

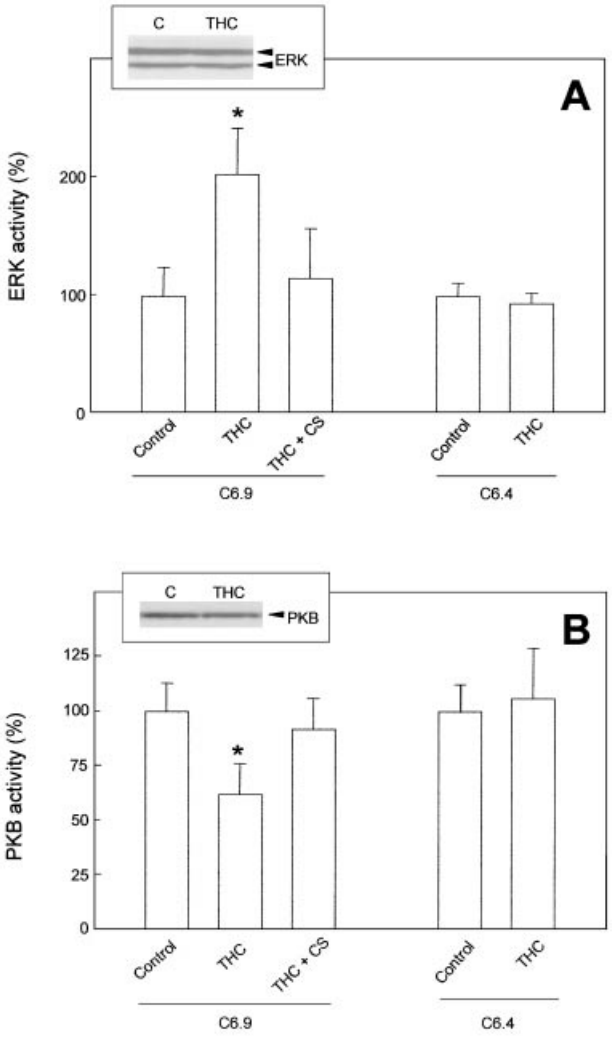

Figure 5 L-Cycloserine prevents THC-induced ERK activation and PKB inhibition in C6.9 cells

C6.9 cells were incubated with 0.5 μM THC in the absence or presence of 0.5 mM L-cycloserine (THC + CS). C6.4 cells were incubated in the presence of 0.5 μM THC. Cells treated in the absence of THC were used as controls. After 5 days, ERK (**A**) and PKB (**B**) activity was determined. Results are expressed as a percentage of incubations with no additions and were obtained from four (**A**) or six (**B**) different experiments. $^*P < 0.01$ compared with incubations with no additions. Insets. C6.9 cells were incubated in the absence (C) or presence of 0.5 μM THC. After 5 days, ERK (**A**) and PKB (**B**) levels were evaluated by Western blot. Representative blots from one experiment are shown (inset). Similar data were obtained in two additional experiments.

abolished the THC-induced stimulation of ERK (Figure 5A) and THC-induced inhibition of PKB (Figure 5B) observed in C6.9 cells. In contrast, ERK (Figure 5A) and PKB (Figure 5B) activities remained unchanged in C6.4 cells after challenge with THC. The modulation of ERK (Figure 5A, inset) and PKB (Figure 5B, inset) activities induced by THC treatment in C6.9 cells was not due to changes in total enzyme levels.

DISCUSSION

Origin of pro-apoptotic ceramide

One of the areas of current cannabinoid research is focused on the ability of these compounds to control the cell survival/death

decision. Thus cannabinoids may induce proliferation, growth arrest or apoptosis in a number of cells, including neurons, lymphocytes and various transformed neural and non-neural cells [31]. Upon THC challenge, two peaks of ceramide are generated in C6 glioma cells [13]. In the present study, we show that the pro-apoptotic sustained peak of ceramide is generated by the stimulation of SPT and the *de novo* synthesis pathway. Although it has been usually considered that ceramide generation through sphingomyelin hydrolysis is the norm in ceramide signalling pathways [14,15], over the last few years long-term ceramide accumulation through enhanced *de novo* synthesis has been gaining appreciation as an alternative means of generating a pro-apoptotic pool of this compound in neural [22,30,32,33] and non-neural cells [25,34–37]. However, the mechanisms that regulate SPT activity are still a matter of controversy. For example, Weiss and Stoffel [38] showed that overexpression of the catalytic LCB2 is sufficient to enhance the activity of the enzyme, whereas Hanada et al. [39] observed that LCB1 is essential for the activity of the enzyme. Likewise, Farrell et al. [40] observed that LCB2 protein and mRNA levels are up-regulated in human keratinocytes in response to UV-B, whereas other reports have shown that the stimulation of SPT activity in response to different pro-apoptotic stimuli occurs without changes in the levels of SPT protein [33,37]. Our present data clearly support the notion that, besides changes in enzyme levels, post-translational mechanisms may be involved in the control of SPT activity. We are currently studying the molecular basis of these mechanisms.

Ceramide targets

The precise mechanism by which ceramide controls cell fate is still a matter of debate. Ceramide has been implicated in stimulation of, among other targets, ERK, c-Jun N-terminal kinase ('JNK') and p38 mitogen-activated protein kinase cascades, several protein kinase C isoforms, kinase suppressor of Ras, and a ceramide-activated protein phosphatase [14–16]. In addition, ceramide inhibits PKB [29]. Our present results, together with previous observations [13], indicate that long-term ceramide accumulation may be involved in apoptosis of C6.9 glioma cells by activating ERK and inhibiting PKB. A large body of evidence supports the implication of both signalling pathways in the control of neural-cell fate. Thus inhibition/lack of stimulation of the phosphoinositide 3-kinase/PKB route in neural cells may lead to apoptosis [41]. In addition, although the ERK cascade has been traditionally considered as mediating cell proliferation rather than apoptosis [41], it has been reported recently that sustained ERK activation may mediate cell-cycle arrest and apoptosis in neural cells [30,42–44]. Nevertheless, it should be noted that in C6.9 glioma cells, cannabinoids, besides activating ERK and inhibiting PKB, also mediate c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation [13]. In addition, we are aware that the time of glioma cell death observed in the present study is somewhat earlier than the time of SPT activation and ceramide generation. Hence, cannabinoid-induced apoptosis of glioma cells may depend on the cross-talk among the different pathways that would be affected by cannabinoid challenge, rather than the modulation of a single signalling route.

Therapeutic potential

The antitumoral action of cannabinoids on gliomas is mediated by cannabinoid receptors [13]. Of importance, recent data from our laboratory have shown that selective CB_2 agonists, which are devoid of typical CB_1 receptor-mediated psychotropic side-effects, produce the same antitumoral actions as their non-selective counterparts through a mechanism that is also dependent on ceramide accumulation (C. Sánchez, T. Gómez del Pulgar, G. Velasco and M. Guzmán, unpublished work). In agreement with a pathophysiological role for *de novo*-synthesized ceramide in the control of neural cell fate, hereditary sensory neuropathy I, the most common hereditary disorder of peripheral sensory neurons, occurs via activating mutations in the gene that encodes LCB1 of SPT [45,46]. These mutations would lead in turn to enhanced apoptosis of neurons via ceramide synthesis *de novo*. Of interest, cannabinoids, in line with their suggested neuroprotective role [8], do not induce apoptosis or sustained ceramide accumulation in primary neurons and astrocytes [18]. Therefore a potential cannabinoid therapy for the treatment of malignant gliomas could be on the basis of the ability of these compounds to selectively stimulate ceramide synthesis *de novo* and apoptosis in transformed glial cells, but not in non-transformed neural cells.

We are indebted to the following for kindly providing reagents: Dr K. Hanada (National Institute for Infectious Diseases, Tokyo, Japan) for antibodies against SPT subunits and the plasmids coding the SPT subunits; Dr D. Alessi (Dundee University, U.K.) for antibodies and peptides for the PKB assay; and Dr T. Ogita (Samkyo Co., Tokyo, Japan) for the scyphostatin. We are also indebted to Dr M. J. H. Geelen (Utrecht University, The Netherlands) for his contribution in setting up the SPT assay in permeabilized cells. We also thank Dr I. Galve-Roperh for his critical comments and general contribution to this work, and Dr D. Rueda, Dr C. Blázquez, Dr S. Alonso and Dr E. Martínez for their assistance in cell culture and Northern-blot experiments. This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (PM 98/0079), Comunidad Autónoma de Madrid (CAM 08.1/0079/2000), Universidad Complutense de Madrid (64/99-8532) and Fundación Ramón Areces.

REFERENCES

1 Felder, C. C. and Glass, M. (1998) Cannabinoid receptors and their endogenous agonists. Annu. Rev. Pharmacol. Toxicol. **38**, 179–200
2 Pertwee, R. G. (2000) Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development. Expert Opin. Invest. Drugs **9**, 1553–1571
3 Matsuda, L. A., Lolait, S. J., Brownstein, M., Young, A. and Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature (London) **346**, 561–564
4 Munro, S., Thomas, K. L. and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature (London) **365**, 61–65
5 Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (Washington, D.C.) **258**, 1946–1949
6 Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R. et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. **50**, 83–80
7 Voth, E. and Schwartz, R. (1997) Medicinal applications of Δ^9-tetrahydrocannabinol and marijuana. Ann. Intern. Med. **126**, 791–798
8 Piomelli, D., Giuffrida, A., Calignano, A. and Rodríguez de Fonseca, F. (2000) The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol. Sci. **21**, 218–224
9 De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (1998) The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **95**, 8375–8380
10 Sánchez, C., Galve-Roperh, I., Canova, C., Brachet, P. and Guzmán, M. (1998) Δ^9-Tetrahydrocannabinol induces apoptosis in C6 glioma cells. FEBS Lett. **436**, 6–10
11 Ruiz, L., Miguel, A. and Díaz-Laviada, I. (1999) Δ^9-Tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. FEBS Lett. **458**, 400–404
12 Maccarrone, M., Lorenzon, T., Bari, M., Melino, G. and Finazzi-Agrò, A. (2000) Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. J. Biol. Chem. **275**, 31938–31945

13 Galve-Roperh, I., Sánchez, C., Cortés, M. L., Gómez del Pulgar, T., Izquierdo, M. and Guzmán, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular-signal regulated kinase activation. Nat. Med. **6**, 313–319
14 Kolesnick, R. N. and Krönke, M. (1998) Regulation of ceramide production and apoptosis. Annu. Rev. Physiol. **60**, 643–665
15 Hannun, Y. and Luberto, C. (2000) Ceramide in the eukaryotic stress response. Trends Cell Biol. **10**, 73–80
16 Huwiler, A., Kolter, T., Pfeilschifter, J. and Sandhoff, K. (2000) Physiology and pathophysiology of sphingolipid metabolism and signaling. Biochim. Biophys. Acta **1485**, 63–99
17 Sánchez, C., Rueda, D., Ségui, B., Levade, T., Galve-Roperh, I. and Guzmán, M. (2001) The CB_1 cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein FAN. Mol. Pharmacol. **59**, 955–959
18 Guzmán, M., Galve-Roperh, I. and Sánchez, C. (2001) Ceramide: a new second messanger of cannabinoid action. Trends Pharmacol. Sci. **22**, 19–22
19 Galve-Roperh, I., Sánchez, C., Ségui, B., Haro, A., Díaz-Laviada, I. and Levade, T. (1998) Evidence for the lack of involvement of sphingomyelin hydrolysis in the tumor necrosis factor-induced secretion of nerve growth factor in primary astrocyte cultures. J. Neurochem. **71**, 498–505
20 Sánchez, C., Galve-Roperh, I., Rueda, D. and Guzmán, M. (1998) Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Δ^9-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. Mol. Pharmacol. **54**, 834–843
21 Gómez del Pulgar, T., Velasco, G. and Guzmán, M. (2000) The CB_1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem. J. **347**, 369–373
22 Blázquez, C., Geelen, M. J. H., Velasco, G. and Guzmán, M. (2001) The AMP-activated protein kinase prevents ceramide synthesis *de novo* and apoptosis in astrocytes. FEBS Lett. **489**, 149–153
23 Hanada, K., Hara, T. and Nishijima, M. (2000) Purification of the serine palmitoyltransferase complex responsible for sphingoid base synthesis by using affinity peptide chromatography techniques. J. Biol. Chem. **275**, 8409–8415
24 Hanada, K., Hara, T., Nishijima, M., Kuge, O., Dickson, R. C. and Nagiec, M. (1997) A mammalian homolog of the yeast LCB1 encodes a component of serine palmitoyltransferase, the enzyme catalyzing the first step in sphingolipid synthesis. J. Biol. Chem. **272**, 32108–32114
25 Xu, J., Chen-Hsiung, Y., Chen, S., He, L., Sensi, S. L., Canzoniero, L. M. T., Choi, D. W. and Hsu, C. Y. (1998) Involvement of *de novo* ceramide biosynthesis in tumor necrosis factor-α/cycloheximide-induced cerebral endothelial cell death. J. Biol. Chem. **273**, 16521–16526
26 Brann, A. B., Scott, R., Neuberger, Y., Abulafia, D., Boldin, S., Fainzilber, M. and Futerman, A. H. (1999) Ceramide signaling downstream of the p75 neurotrophin receptor mediates nerve growth factor on outgrowth of cultured hippocampal neurons. J. Neurosci. **19**, 8199–8206
27 Levade, T. and Jaffrézou, J. P. (1999) Signalling sphingomyelinases: which, where, how and why? Biochim. Biophys. Acta **1438**, 1–17
28 Dickson, R. C., Lester, R. L. and Nagiec, M. M. (2000) Serine palmitoyltransferase. Methods Enzymol. **311**, 3–9
29 Schubert, K. M., Scheid, M. P. and Duronio, V. (2000) Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473. J. Biol. Chem. **275**, 13330–13335
30 Blázquez, C., Galve-Roperh, I. and Guzmán, M. (2000) *De novo*-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. FASEB J. **14**, 2315–2322
31 Guzmán, M., Sánchez, C. and Galve-Roperh, I. (2001) Control of the cell survival/death decision by cannabinoids. J. Mol. Med. **78**, 613–625
32 Lehtonen, J. Y. A., Horiuchi, M., Daviet, L., Akishita, M. and Dzau, V. (1999) Activation of *de novo* biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. J. Biol. Chem. **274**, 16901–16906
33 Herget, T., Esdar, C., Oehrlein, S. A., Heinrich, S., Maelicke, A. and van Echten-Deckert, G. (2000) Production of ceramide causes apoptosis during early neural differentiation *in vitro*. J. Biol. Chem. **275**, 30344–30354
34 Paumen, M. B., Ishida, Y., Muramatsu, M., Yamamoto, M. and Honjo, T. (1997) Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. J. Biol. Chem., **272**, 3324–3329
35 Shimabukuro, M., Zhou, Y.-T., Levi, M. and Unger, R. (1998) Fatty acid-induced β cell apoptosis: a link between obesity and diabetes. Proc. Natl. Acad. Sci. U.S.A. **95**, 2498–2502
36 Liao, W.-C., Hainovitz-Friedman, A., Persaud, R. S., McLoughlin, M., Ehleiter, D., Zhang, N., Gatei, M., Lavin, M., Kolesnick, R. and Fuks, Z. (1999) Ataxia telangiectasia-mutated gene product inhibits DNA damage-induced apoptosis via ceramide synthase. J. Biol. Chem. **274**, 17908–17917
37 Perry, D. K., Carton, J., Shah, A. K., Meredith, F., Uhlinger, D. J. and Hannun, Y. A. (2000) Serine palmitoyltransferase regulates *de novo* ceramide generation during etoposide-induced apoptosis. J. Biol. Chem. **275**, 9078–9084
38 Weiss, B. and Stoffel, W. (1997) Human and murine serine palmitoyltransferase. Cloning expression and characterization of the key enzyme on sphingolipid synthesis. Eur. J. Biochem. **249**, 239–247
39 Hanada, K., Hara, T., Fukasawa, M., Yamahi, A., Umeda, M. and Nishihima, M. (1998) Mammalian cell-mutant resistant to a sphingomyelin-directed cytolisin. Kinetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. J. Biol. Chem. **273**, 33787–33794
40 Farrell, A. M., Uchida, Y., Nagiec, M. M., Harris, I. R., Dickson, R. C., Elias, P. M. and Holleran, W. M. (1998) UVB irradiation up-regulates serine palmitoyltransferase in cultured human keratinocytes. J. Lipid Res. **39**, 2031–2038
41 Yuan, J. and Yankner, B. A. (2000) Apoptosis in the nervous system. Nature (London) **407**, 802–809
42 Murray, B., Alessandrini, A., Cole, A. J., Yee, A. G. and Furshpan, E. J. (1998) Inhibition of the p42/p44 MAP kinase pathway protects hippocampal neurons in a cell culture model of seizure activity. Proc. Natl. Acad. Sci. U.S.A. **95**, 11975–11980
43 Runden, E., Seglen, P. O., Haug, F. M., Ottersen, O. P., Wieloch, T., Shamloo, M. and Laake, J. H. (1998) Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. J. Neurosci. **18**, 7296–7305
44 York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W. and Stork, P. J. S. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature (London) **392**, 622–626
45 Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M. and Nicholson, G. A. (2001) Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. Nat. Genet. **27**, 309–312
46 Bejaoui, K., Wu, C., Scheffler, M. D., Haan, G., Ashby, P., Wu, L., de Jong, P. and Brown, Jr, R. H. (2001) SPTLC1 is mutated in hereditary sensory nuropathy, type 1. Nat. Genet. **27**, 261–262

Received 12 July 2001/8 November 2001; accepted 23 January 2002