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SPECIFIC INHIBITION OF AN ACYLTRANSFERASE BY Δ^9-TETRAHYDROCANNABINOL

JEFFREY H. GREENBERG and ALAN MELLORS

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Abstract—Increased phospholipid turnover in mouse lymphocytes during mitogen-induced blastogenesis can be prevented by low levels of Δ^9-tetrahydrocannabinol (Δ^9-THC) *in vitro*. Acyl CoA: lysophosphatidylcholine acyltransferase, a lymphocyte plasma membrane enzyme, is inhibited by Δ^9-THC with a K_i of 0.35 μM. Other cannabinoids with much lower psychoactivity inhibit the enzyme similarly only if present at more than 100 times the concentration needed for Δ^9-THC inhibition. For cannabinoids other than Δ^9-THC and for a variety of membrane-active lipids, the enzyme inhibition at higher concentrations of lipid correlates well with their anesthetic activity as measured by their ability to protect erythrocytes against hemolysis. However, the inhibition by Δ^9-THC is much greater than that predicted by its anti-hemolytic activity, which indicates stereospecific requirements for inhibition of the enzyme. The inhibition of acyltransferase by Δ^9-THC is not restricted to the lymphocyte enzyme and can also be demonstrated in mouse brain synaptosomes, suggesting a possible mechanism for psychoactive specificity.

Cannabinoids have been shown to inhibit cell division [1], and the impairment of T-lymphocyte transformation *in vitro* has been of particular interest [2]. Mitosis and differentiation of lymphocytes to form lymphoblasts are major events in the cell-mediated immune response. The inhibition of blastogenesis by μM levels of cannabinoids [3] may be related to the lowered cellular immune responses seen in regular cannabis users [4] though other workers have disputed this observation [5, 6]. It is clear that lymphocyte transformation as monitored by the lymphokine, migration inhibition factor (MIF), can be suppressed by the administration *in vivo* of delta-9-tetrahydrocannabinol (Δ^9-THC) to rats, mice and guinea pigs [7]. An early event in lymphocyte transformation is the increased turnover of lipids in the lymphocyte plasma membrane that is seen within 2 hr of the initial stimulus by mitogen [8, 9]. This early event is also inhibited by μM concentrations of Δ^9-THC [7]. In the present study we describe the inhibition of an enzyme responsible for membrane lipid turnover by Δ^9-THC at low levels *in vitro* and *in vivo*. The enzyme is acyl CoA:lysophosphatidylcholine acyltransferase (EC 2.3.1.23), a key enzyme in the turnover of phospholipids. It has been suggested [10] that the increased activity of this enzyme in lymphocyte plasma membranes shortly after stimulation by mitogen may be an early event in the development of transformation, leading via membrane changes to the later increases in DNA, RNA and protein synthesis. The latter changes, usually monitored by thymidine uptake studies, occur about 20 hr after the stimulation by mitogen, whereas the lipid changes are apparent within 2–4 hr. Since LPC acyltransferase has a greater specificity for unsaturated fatty acyl CoA than for saturated acyl groups, an increase in this enzyme may lead to increased membrane fluidity. The fluidity changes may in turn be responsible for increased Ca^{2+} ion uptake and cyclic GMP production, both of which have been observed during lymphocyte transformation [11, 12]. In the present study we describe the inhibition of LPC acyltransferase by Δ^9-THC, and the high specificity of this effect, in that it is restricted to psychoactive cannabinoids and is not given by similar levels of non-psychoactive but membrane-active lipids.

METHODS

Materials. [^{14}C]acetate (53 mCi/m-mole) and [^{32}P]phosphoric acid (carrier free) were obtained from New England Nuclear Inc., Boston, MA. [^{32}P]lysophosphatidylcholine was prepared from [^{32}P]phosphatidylcholine using *Crotalus adamanteus* venom [13, 14]. [^{32}P]phosphatidylcholine was obtained from rats injected with [^{32}P]phosphate [15]. Acyl coenzyme A (CoA) derivatives and other lipids were purchased from Sigma Chemical Co., St. Louis, MO. Cannabinoids were kindly provided by Health and Welfare Canada. For the acetate incorporation studies, cannabinoids were dispersed in 10% (w/v) bovine serum albumin (BSA) solution, while for LPC acyltransferase experiments, cannabinoids and other lipids were administered in dimethylsulfoxide (DMSO) such that 50 μl solution contained the required amount of lipid. Appropriate controls were performed containing BSA and DMSO solutions respectively. Media, biological extracts and salt solutions were obtained from Grand Island Biological Co., NY.

Lymphocyte extraction. Spleens were removed from 25 g male inbred white mice and gently homogenized in 20 ml Hank's balanced salt solution (BSS). The homogenate was passed through a short column of cotton which was washed with a further 10 ml BSS. The eluted cells were centrifuged in a clinical centrifuge (5000 $g \cdot$ min) and the pellet was cleared of intact erythrocytes by the method of Boyle [16]. The resultant pellet was resuspended, either in medium RPMI

1640 or in Hank's minimal essential medium (MEM), the latter being a more suitable agent for the acyltransferase assay. Cell concentration was adjusted to 10^7/ml. based on cell counts and viabilities measured by the Trypan blue exclusion technique. For incubations of lymphocytes in excess of 3 hr, the medium was supplemented with 10% fetal calf serum.

[^{14}C]acetate incorporation. To 10^7 lymphocytes in 1 ml BSS was added 50 μl BSS containing 10 μg concanavalin A (ConA), and the mixture was incubated for 30 min at 37°. Controls received no ConA. After this preincubation, 50 μl of an aqueous solution containing 25 nmoles and 1.4 μCi [^{14}C]acetate was added, together with 50 μl of 10 μM Δ^9-THC in 10% BSA. Another group of controls received [^{14}C]acetate and 50 μl of 10% BSA. All groups were incubated at 37° for 1, 2, 8 or 22 hr; and cells were killed at the end of this period by addition of 2 Ml of cold 5% trichloracetic acid. After clinical centrifugation (10.000 $g \cdot$ min), lipids were extracted [17] and separated by thin-layer chromatography on Silica gel 7 G (Baker Chemical Co., Philipsburg, NJ.) developed with chloroform–methanol–water (65:25:4). Radioactivity was determined by liquid scintillation counting, after comparison of separated lipids with known standards.

AH_{50} determination. The concentration of a drug that will give 50 per cent protection of erythrocytes against hemolysis (AH_{50}) was obtained from the literature (18) or was determined on equine erythrocytes according to the method of Machleidt *et al.* (19). Equine values were corrected by the experimentally determined factor of 0.36 in order to correspond with the literature data on human erythrocytes. The AH_{50} values for the lipid-soluble cannabinoids were obtained as follows. The cannabinoids (100 mg/ml of ethanol) were dried to an oily film under nitrogen in silanized glass test tubes. One ml of an erythrocyte suspension which had been prepared according to Machleidt *et al.* (19) and at a hematocrit of 85–95 was added to each tube and vortexed 2 min. The cells were further incubated in a shaking water bath 1.5 hr at 37°. The erythrocyte suspension was diluted to a hematocrit of 5–7 with 0.9% saline in 15 mM Tris–HCl, pH 7.0, and 0.1 ml added to 2.5 ml of 0.45% saline in 15 mM Tris–HCl, pH 7.0. After swirling on a vortex shaker for 2 min, the cells were sedimented in a clinical centrifuge (1500 $g \cdot$ min), and the absorbance of the supernatant was determined using a Beckman DB-GT spectrophotometer at 540 nm.

Determination of LPC acyltransferase activity. All incubation mixtures were preincubated 30 min at 37° with the test lipid prior to enzyme assay. After the preincubation, 25 nmoles oleoyl CoA and 20–30 nmoles [^{32}P]lysophosphatidylcholine in a volume of 0.1 ml were added directly to the 1-ml reaction mixtures and allowed to incubate a further 15 min at 37°. Lymphocyte assays were stopped and lipids analyzed as described above. Synaptosome assays were stopped by the addition of 3.75 ml chloroform–methanol (1:2) followed by 1.25 ml chloroform prior to lipid analysis.

The percentage of total ^{32}P radioactivity which appeared in the phosphatidylcholine band was compared with controls containing DMSO to determine enzyme activity.

Synaptosome preparation. Synaptosomes were prepared from mice according to scheme 1 of Cotman (20). Preparations were stored at ten times the desired final concentration in 0.1 M NaH_2PO_4–Na_2HPO_4 buffer, pH 7.4, at −7°. Lower storage temperatures substantially decreased enzyme activity. Before assaying, the preparations were diluted to 1–1.5 mg protein/ml with Krebs–Ringer's phosphate solution (21).

RESULTS AND DISCUSSION

Concanavalin A and other mitogens induce an early increase in the glycerolipid turnover of lymphocyte membranes within a few hours of the exposure to mitogen *in vitro*. Figure 1 shows that this increase can be abolished by the presence of 10 μM Δ^9-THC. The cannabinoid inhibits the incorporation of [^{14}C]acetate into glycerolipids both after Con A stimulation and in the absence of mitogen. Thus it appears that Δ^9-THC interferes not only with the mitogen-stimulated glycerolipid turnover but also with the basal rate of turnover in unstimulated lymphocytes. While the incorporation of [^{14}C]acetate into phosphatidylinositol shows the most stimulation due to Con A, and the least interference by Δ^9-THC, this is a minor glycerolipid in these lymphocyte cultures comprising only 10 per cent of the total ^{14}C label incorporated. Phosphatidylcholine (44 per cent), phosphatidylethanolamine (11 per cent) and triglyceride (35 per cent) are the major glycerolipids and show similar patterns of decreased [^{14}C]acetate incorporation due to 10 μM Δ^9-THC, in mitogen-treated and unstimulated lymphocyte cultures.

It is not possible to distinguish by precursor incorporation studies whether the interference by Δ^9-THC is due to decreased synthesis or increased degradation of glycerolipids. In an attempt to answer this question, the fate of [^{32}P]phosphatidylcholine and [^{32}P]-lysophosphatidylcholine added *in vitro* to lymphocyte cultures during early blastogenesis was studied. In these preliminary attempts to measure phospholipid turnover rates it became apparent that a major phospholipid interconversion in the lymphocyte preparations was conversion of [^{32}P]lysophosphatidylcholine to [^{32}P]phosphatidylcholine. The rate of this synthetic reaction was such that it masked any degra-

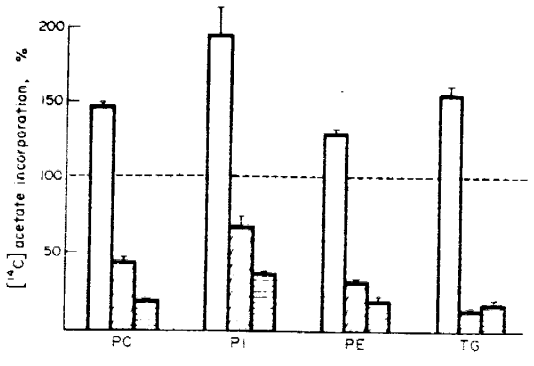

Fig. 1. Incorporation of [^{14}C]acetate (56 μCi/m-mole) into lymphocyte lipids in the presence of 10 μg/ml Con A (□), 10 μg/ml Con A + 10 μM Δ^9-THC (▨), and 10 μM Δ^9-THC (▤). Incubations were for 24 hr at 37° before lipid analysis. Values are expressed as percentages of control values determined for each lipid and represent means ± standard deviation.

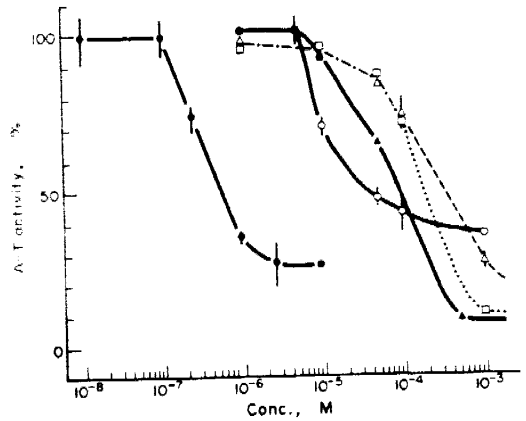

Fig. 2. Inhibition of lymphocyte LPC acyltransferase activity by cannabinoids. Lymphocytes were preincubated 30 min at 37° with Δ^9-THC (●), Δ^8-THC (○), olivetol (▲), cannabigerol (□) and cannabinol (△) before assaying for enzymme activity. Values are expressed as percentages of controls and are given as means ± standard deviations.

dation of phospholipids. Especially interesting was the observation that this conversion was completely abolished by 10 μM Δ^9-THC. This suggested the likely involvement of LPC acyltransferase in a Δ^9-THC-sensitive synthesis of phosphatidylcholine. Figure 2 shows the sensitivity of the lymphocyte LPC acyltransferase enzyme to a variety of cannabinoids over a wide range of concentration. Only Δ^9-THC is substantially inhibitory at concentrations below 10^{-5} M, and its concentration for half-maximal inhibition (K_i) is 0.35 μM. Other cannabinoids inhibited the LPC acyltransferase activity when present in the concentration range 10^{-4} to 10^{-3} M and inhibition ranged from 60 to 80 per cent at the higher concentration. Of the cannabinoids tested, only Δ^9-THC and Δ^8-THC have psychoactivity, the former being the most potent (22). Thus it is interesting that these two compounds showed the most inhibition and that the K_i for Δ^8-THC (13 μM) was intermediate between the values for the more psychoactive Δ^9-THC and the psycho-inactive cannabinoids.

The specificity of the inhibition of acyltransferase by Δ^9-THC is in marked contrast to the reported lack of specificity for the impairment of blastogenesis by cannabinoids (23). Nahas and Desoize have shown that a variety of psychoactive and psycho-inactive cannabinoids and analogs all produce inhibition of thymidine uptake during phytohemagglutinin-induced lymphocyte transformation, in the concentration range 10^{-3} to 10^{-4} M for half-maximal inhibition. This concentration range is of the same order as that seen for inhibition of LPC acyltransferase by psycho-inactive cannabinoids in the present study but 1000-fold greater than that required for Δ^9-THC inhibition. It should be noted that the thymidine-uptake experiments use lymphocytes that have been cultured in the presence of cannabinoids for 3 days, whereas the LPC acyltransferase effects are apparent after a 30-min exposure to cannabinoids.

It has been proposed that the increased LPC acyltransferase activity seen in lymphocyte cultures shortly after stimulation with mitogen is an essential early event in the process of blastogenesis (24). Should this be the case, inhibition of LPC acyltransferase activity may be associated with the inhibition by Δ^9-THC of macromolecular synthesis associated with blastogenesis, which follow acyltransferase activation by 20 hr or more.

Cannabinoids are highly lipophilic molecules with a high affinity for biological membranes. Furthermore the LPC acyltransferase of this study is a plasma membrane-bound enzyme (25) and is likely to be susceptible to the fluidity state of the hydrophobic phospholipid–cholesterol matrix. Electron spin resonance studies on the fluidity of this matrix have indicated that the psychoactive cannabinoids have a "partial anesthetic" effect (26), that is, they are membrane fluidizers which expand the membrane, as do other anesthetics, but that they do not exert the maximum effect consistent with their size. Their anesthetic potency is poor compared to specific psycho-active effects. It appeared to be important to compare the inhibition of acyltransferase activity for cannabinoids with their ability to fluidize membranes. It has been shown by Seeman that all lipid-soluble anesthetics can protect erythrocytes against hemolysis to an extent which is consistent with their membrane-expanding properties (27), and with the critical volume hypothesis for anesthetic action (28). We have measured the ability of some cannabinoids to protect erythrocytes against hemolysis (AH_{50}) and in Fig. 3 we plot the AH_{50} for cannabinoids and some other compounds against their ability to inhibit LPC acyltransferase in lymphocytes. For the anesthetics and the psycho-inactive cannabinoids there is a clear positive correlation between the AH_{50} and the K_i for LPC acyltransferase inhibition. A linear regression analysis of the correlation between AH_{50} and K_i for LPC acyltransferase inhibition was carried out for the non-cannabinoids of Fig. 3, namely octanol, nonanol, decanol, retinol, 2-chlorophenol, chlorpromazine, 4-chlorophenol and barbital. The correlation as indicated in Fig. 3 is high, with a correlation coefficient r of 0.99, and is consistent with the critical volume hypothesis for anesthesia. It indicates that the inhibition of the enzyme is

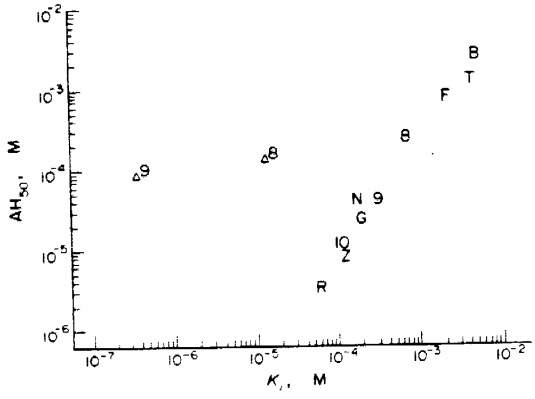

Fig. 3. LPC acyltransferase inhibition as a function of antihemolytic potency. AH_{50} values for Δ^9-THC (Δ^9), Δ^8-THC (Δ^8), cannabinol (N), cannabigerol (G) and retinol (R) were determined as described in the text. Values for barbital (B), 2-chlorophenol (T), 4-chlorophenol (F), octanol (8), nonanol (9), decanol (10) and chlorpromazine (Z) were obtained from the literature [18]).

related to the ability of the lipid to cause membrane expansion, an effect which has been shown many times to be a function of the molar volume of the lipid. The two psychoactive cannabinoids tested, however, show clear deviations from this pattern in proportion to their relative psychoactive potency. Δ^9-THC and Δ^8-THC both give much more enzyme inhibition than might be expected from their ability to expand membranes. Such deviations from linear free energy correlations are often interpreted as being due to specific interactions. These anomalies suggest that the psychoactive cannabinoids may have a highly specific interaction with the membrane-bound enzyme. While inhibition of the enzyme may be achieved by anesthetics at relatively high concentrations, acting to modify membrane fluidity and volume, the inhibition seen for the psychoactive Δ^9-THC and the less active Δ^8-THC is at much lower concentrations where anesthetic effects are not seen. Retinol, the most potent membrane fluidizer tested in this series, gave no enzyme inhibition at the concentration for half-maximal inhibition by Δ^9-THC.

While it appears unlikely that the specific LPC acyltransferase inhibition is a direct cause of the unspecific inhibition of blastogenesis in lymphocytes, the participation of LPC acyltransferase appears to be important in other physiological processes. In particular the presence of LPC acyltransferase in synaptosomes of central nervous tissue leads us to speculate that the specific psychoactive effects of cannabinoids may be related to their specific inhibition of synaptosomal LPC acyltransferase. The enzyme is thought to be important in the maintenance of membrane fluidity, and hence in vesicle fusion and rupture, associated with neurotransmitter release (29). Preliminary experiments in progress in our laboratory suggest that mouse brain synaptosomal LPC acyltransferase is inhibited *in vitro* by Δ^9-THC with a K_i of 0.3 μM, very similar to that seen for the lymphocyte inhibition. Further work is necessary to ascertain whether the specificity of the synaptosomal LPC acyltransferase inhibition by cannabinoids resembles that reported in the present study for the lymphocyte enzyme. A specific inhibition of synaptosomal LPC acyltransferase by low levels of Δ^9-THC has some interesting implications for the psychoactive specificity of the drug.

LPC acyltransferase appears to be important in regulating the polyenoic fatty acid content of membranes because of its substrate specificity toward unsaturated acyl CoAs (30). The membrane fluidity of synaptosomes, which is dependent on the polyenoic fatty acid content, has been shown to regulate acetylcholinesterase and Na^+-K^+-adenosine triphosphatase (ATPase) activities (31). Thus any inhibition of LPC acyltransferase in synaptosomes may lead to alterations in the biochemical events of synaptic neurotransmission. In addition, evidence has been presented to indicate that electrical stimulation or neurotransmitter release results in increased phospholipid turnover in synaptosomal preparations (32) though this is an area of some controversy (33). The importance of other acyltransferases in contributing to the steady state level of phosphatidic acid and other glycerolipids is well-established, and any interference with these enzymes might give rise to rapid changes in the levels of such lipids with consequences for th release of neurotransmitter. We have not establishe that lysophosphatidic acid acyltransferases are inh bited by Δ^9-THC in a manner analogous to LP acyltransferases. The inhibition of [^{14}C]acetate incor poration into triglyceride by lymphocytes exposed t Δ^9-THC is similar to that seen for incorporation int phosphatidylcholine. This suggests some inhibition glycerolipid synthesis prior to the metabolism o phosphatidic acid, and LPA acyltransferase is a like site of inhibition. Further work is needed to deter mine if LPC and LPA acyltransferases are separat enzymes in the lymphocyte and synaptosome prep arations used in this study, and if both activities ar susceptible to inhibition by Δ^9-THC.

While other membrane-bound enzymes have bee shown to be inhibited by Δ^9-THC, the concentration required for half-maximal inhibition are severa orders of magnitude greater than that seen in th present study and are consistent with the "partia anesthetic" properties of cannabinoids. Thus erythr cyte Na^+-K^+ ATPase activity is inhibited half-ma mally at between 10^{-5} and 10^{-4} M (34). In tissu cultures of human epidermoid neoplastic KB ce and African green monkey kidney fibroblastic cel the plasma membrane ATPases are inhibited b 0.5 M Δ^9-THC (35). The present study indicates tha such changes at high cannabinoid concentrations ar due to the increased degree of fluidity of the lipi bilayer induced non-specifically by these compounds The specific inhibition at low levels of Δ^9-THC occur at concentrations below those which will induce flu idity changes, and is probably due to a specific inter action with the hydrophobic region of membrane pro teins. Future studies may concentrate on the relativ sensitivities of various membrane enzymes to sub-μM concentrations of cannabinoids, and on the relation ship of the observed biochemical effects to the phar macological effects of cannabinoids *in vivo*.

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