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Δ^9-Tetrahydrocannabinol Inhibits Arachidonic Acid Acylation of Phospholipids and Triacylglycerols in Guinea Pig Cerebral Cortex Slices

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SUMMARY

We reported earlier that Δ^9-tetrahydrocannabinol (THC), the main psychoactive ingredient in marihuana, increased markedly the level of unesterified arachidonic acid (AA) in guinea pig cerebral cortex slices prelabeled with [^{14}C]AA. The purpose of the present study was to clarify the mechanism underlying THC-enhanced mobilization of AA. We could find little data to support an involvement for phospholipase A_2 in this response. For example, the levels of lysophosphatidylcholine or lysophosphatidylethanolamine were not elevated after incubation with THC. A role for phosphoinositidase C-initiated lipolytic pathways was excluded, because neither basal nor acetylcholine-stimulated inositol phosphate formation was altered by THC. When we prelabeled slices with [^{14}C]stearate or [^{3}H]glycerol, THC did not elevate levels of unesterified [^{14}C]stearate, nor did we observe significant changes in the phospholipids that were labeled with either precursor. These findings were in marked contrast to the previously reported reductions in [^{14}C]AA-labeled phosphatidylinositol after exposure of prelabeled brain slices to THC; moreover, they suggested that the THC-induced effects on brain lipid metabolism *in vitro* were rather specific for AA. We show here that, when the acylation of brain lipids with AA was measured by addition of [^{3}H]AA in the presence and absence of THC at zero time and incubation for 1 hr at 37°, THC elicited marked, dose-dependent, and saturable reductions in esterified [^{3}H]AA levels. The reductions in incorporation were balanced by increases in unesterified [^{3}H]AA. The IC_{50} for the effect was on the order of 8 μM, and a maximal response occurred at 32 μM. We observed that the THC-induced suppression in acylation of the phospholipids by radiolabeled AA was up to 5-fold greater than the THC-elicited loss of AA from slices prelabeled before exposure to THC. The largest inhibitions of acylation were in phosphatidylinositol; the suppression of radioactivity in this phospholipid accounted for over 50% of the rise in unesterified [^{3}H]AA. The radioactivity incorporated in triacylglycerols were also reduced markedly by THC. In contrast, the incorporation of radioactivity in phosphatidylcholine remained unaffected by THC. Taken together, these findings suggest that THC mobilizes AA by inhibiting acylation of certain lipids with AA, particularly phosphatidylinositol and triacylglycerol, rather than by liberating fatty acids by lipolysis. Comparison of the effects of several primary cannabinoids on lipid acylation with [^{3}H]AA revealed that there was no relationship between the potencies of cannabinoids in inhibiting the incorporation of [^{3}H]AA into membrane lipids and their psychoactive potencies *in vivo*; moreover, the stereoisomers of THC were equipotent. The results indicate that the cannabinoid-elicited suppression of acylation is not a primary mechanism underlying the unique psychopharmacology of cannabinoids.

Considerable effort has been directed towards elucidation of the mechanisms by which THC, the major active ingredient in marihuana, exerts its effects (see Refs. 1–4 for recent reviews). Studies by different groups with a variety of extraneural cells have demonstrated clearly that cannabinoids increase cellular levels of unesterified AA *in vitro* (5–8). An important question arises from this work; namely, is the cannabinoid-induced rise in free AA levels involved in the unique response constellation elicited by cannabinoids, particularly psychoactivity? Part of the problem in interpreting the literature on the role of AA in the neuropharmacology of the cannabinoids is that nonneural tissues or cells in culture have been used almost exclusively to study the effect of THC on AA mobilization. We have been investigating the structure-activity relationships between the effects of cannabinoids *in vitro* on brain lipid and on AA metabolism. For this work, we use brain slices incubated *in*

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ABBREVIATIONS: THC, (−)-*trans*-Δ^9-tetrahydrocannabinol; AA, arachidonic acid; PI, phosphatidylinositol; CBN, cannabinol; CBD, cannabidiol; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; KHBS, Krebs-Henseleit bicarbonate saline; DG, diacylglycerol; TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; PLA_2, phospholipase(s) A_2; ACh, acetylcholine; PA; phosphatidic acid; PS, phosphatidylserine; MG, monoacylglycerol.

vitro, a relevant, functional, neural preparation with intact synapses (9). We reported previously that cannabinoids elevate unesterified [^{14}C]AA levels in slices from guinea pig brain cortex prelabeled with [^{14}C]AA and that the major portion of the AA is liberated from PI (10). The purpose of the present study was to determine the mechanism of the AA response and its involvement in psychoactivity. Our results strongly suggest that cannabinoids mobilize AA mainly by inhibiting acylation mechanisms, rather than by activation of lipolytic enzymes; moreover, the structure-activity relationship data presented herein argue against the proposal that cannabinoid-induced mobilization of AA is involved directly in the mechanisms that mediate psychoactivity.

Experimental Procedures

Materials. The cannabinoids used in this study, which include THC, its inactive stereoisomer (+)-THC, which was 95% stereochemically pure, Δ^8-THC, CBN, and CBD, were obtained through the National Institute on Drug Abuse (Rockville, MD) from the Research Triangle Institute (Research Triangle Park, NC). They were stored in absolute ethanol at −20° under N_2. The purity of the drugs was 98% or greater, as measured by gas chromatography or high pressure liquid chromatography. Just before their use, the ethanol was removed under a stream of N_2, followed by resuspension of the residue in DMSO. [^{14}C]AA (65–80 mCi/mmol) was purchased from NEN Research Products (Boston, MA), [^{3}H]glycerol (1 Ci/mmol) from Amersham (Arlington Height, IL), and [^{3}H]inositol (15–20 Ci/mmol) from ARC products (St. Louis, MO). All glassware in contact with the cannabinoids or [^{14}C]AA was treated with Surfa-Sil (Pierce Chemical Co., Rockford, IL). BSA (fatty acid depleted), phospholipid standards, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). The thin layer chromatography plates used were 0.25-mm-thick silica gel 60 (E. M. Merck, Darmstadt, West Germany), and the solvents were all analytical reagent grade or better.

Tissue preparation. Adult male guinea pigs (400–500 g) were stunned and then decapitated. Their brains were rapidly removed and rinsed in ice-cold KHBS (saturated with 5% CO_2 in O_2, to a final pH value of 7.4) containing 10 mM glucose. A modified Stadie-Riggs tissue slicer was used to remove 500-μm-thick slices bilaterally from the cerebral cortex of two guinea pigs. These brain cortex slices were then cross-chopped in a cold room into 350- × 350-μm sections, using a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY). The slices were placed in a 25-ml Ehrlenmeyer flask containing 5 ml of 0.2% BSA in KHBS. The vessels were then flushed with 5% CO_2 in O_2 and tightly stoppered.

Effects of THC on the disposition of radioactivity in prelabeled brain cortex slices. After a preincubation period of 15 min in 5 ml of 0.2% BSA in KHBS, the medium was replaced with 5 ml of the same buffer containing 5 μCi of [^{14}C]AA or [^{14}C]stearate or 50 mCi of [^{3}H]glycerol. The slices were further incubated for 1 hr. We have found that carrying out the incorporation of radiolabeled AA in the presence of 0.2% BSA aids in reducing the basal levels of unesterified radioactivity in the washed slices, thereby reducing the noise in the system, as reported by others (11). Apparently, the BSA acts to buffer the free [^{14}C]AA during the prelabeling and aids in the subsequent removal of unreacted radioactivity. Following this protocol, we find that the radioactivity esterified in the total lipid fraction, as well as in each individual lipid, reaches apparent equilibrium after an incubation period of 1 hr with radiolabeled precursor. After the 60-min incubation, excess label was removed by rinsing three times with 15 ml of KHBS with 0.2% BSA, followed by incubation of the slices twice with 5 ml of the same buffer for 10 min. The BSA was removed by rinsing three times with 15 ml of KHBS, and the slices were suspended in KHBS (without BSA) to a final protein concentration of around 1.0 mg/ml. Aliquots (2-ml) of the suspension were pipetted into Ehrlenmeyer flasks containing the appropriate amounts of THC or the vehicle alone (which was 0.25% DMSO, a concentration that was without effect in our studies), and the vessels were then incubated for 1 hr. The reaction was terminated by placing the vessels in a slurry of dry ice in ethanol, and total lipid extracts were usually prepared immediately afterwards.

Measurement of the effects of THC on lipid acylation. For these studies, the effects of THC and other congeners on the incorporation of [^{3}H]AA into brain lipids were measured. Slices were preincubated in the presence of 5 ml of KHBS, containing 0.2% BSA, for 1 hr, during which time the buffer was changed three times. At the end of the preincubation, the slices were rinsed three times in KHBS (without BSA) and suspended in the appropriate volume of the same buffer. One-milliliter aliquots of the slices, containing around 2 mg of protein, were pipetted into vessels containing the appropriate amounts of cannabinoids or vehicle alone (0.2% final concentration of DMSO) and 0.5–1 mCi of [^{3}H]AA in 1 ml of buffer. The slices were incubated, and the reaction was terminated by the addition of an equal volume of ice-cold buffer, followed by centrifugation for 1 min at 2000 × *g* at 4°. The medium was aspirated and discarded, and the pellet was placed in an acetone/dry ice mixture. In those cases where unesterified AA was not measured, the incubation mixture was frozen over dry ice/acetone, followed by extraction. Pellets were usually extracted either the same day or the next day. Both of the procedures described above yielded equivalent results on the effects of THC on the acylation of neutral lipids and phospholipids.

Determination of radioactivity in individual lipids. The frozen brain slices were extracted by using a modified Bligh and Dyer method (12), in which the aqueous phase was acidified to pH 3.5. The total lipid extract was fractionated using two different chromatographic systems. The neutral lipids, comprising DG, TG, and AA, were separated using a one-dimensional system on activated silica gel G plates in hexane/diethyl ether/acetic acid (65:35:4). In this system, the total phospholipids that remained at the origin were also removed for scintillation counting. In other experiments, the individual phospholipids were separated by thin layer chromatography using a two-dimensional system. Samples and carrier lipids were applied as 2-cm × 2-mm bands and developed in two dimensions (first dimension, tetrahydrofuran/acetone/methanol/water, 50:20:40:8; second dimension, chloroform/methanol/acetic acid/water, 75:45:12:3). The lipid bands were visualized by brief exposure to I_2 and were then scraped from the plate and transferred into scintillation vials. One milliliter of hexane/isopropanol/water (3:20.8) was added to the silica, followed by 10 ml of scintillation fluid. After vigorous mixing, the radioactivity in the lipids was measured with a Packard 2000CA scintillation counter, at 67% efficiency for tritium and 97% for carbon.

Measurement of inositol phosphate formation. Brain cortex slices were washed twice by incubation for 15 min in 4 ml of KHBS buffer, followed by incubation for 60 min in the presence of 200 μCi of [^{3}H]inositol (specific activity, 15 Ci/mmol) in 4 ml of KHBS. The label was removed by rinsing of the slices four times with 10 volumes of KHBS. Aliquots (1-ml) of suspended slices were pipetted into siliconized vessels containing the drugs, and the slices were incubated at 37° in the presence of 10 mM LiCl. The reaction was terminated by transfer of the slices to tubes containing 4 volumes of boiling 2 mM EDTA. After cooling on ice, the slices were homogenized and then centrifuged at 3000 × *g* for 30 min. In some of the experiments, the reaction was terminated by the addition of 1 volume of 10% trichloroacetic acid containing 200 mg of phytic acid, to aid in recovery of inositol polyphosphates, followed by homogenization and centrifugation as described above. The acid was removed by washing of the aqueous extracts four times with 3 volumes of water-saturated diethyl ether. The relative effects of the drugs were similar using either the boiling or trichloroacetic acid method for termination of the incubations. The aqueous extracts were filtered with the aid of Centricon-10 centrifugal microconcentrators (Amicon). Inositol phosphates in the filtrates were separated by high pressure liquid chromatography, by a modified method of Dean and Moyer (13), on a Whatman Partisil SAX column (25 cm

× 0.46 cm) with a precolumn containing Whatman pellicular anion exchange resin, with ammonium phosphate, pH 3.8, at a flow rate of 1 ml/min. The inositol phosphates were eluted with the following program: water for 30 min to remove free [^{3}H]inositol, 0.01 to 0.08 M ammonium phosphate over 30 min, 0.2 to 0.28 ammonium phosphate over 30 min, and 0.5 to 0.52 ammonium phosphate over 30 min. The appropriate fractions were collected with the aid of a Radiomatic Beta-One radioactive flow detector, together with a Gilson model 202 fraction collector. The radioactivity in the fractions was measured with a Packard 2000CA liquid scintillation counter.

Data analysis. In lipids prelabeled with radiolabeled stearate, AA, or glycerol, >95% of the total radioactivity contained in the tissue homogenate was recovered in the lipid extract, with the remainder of the radioactivity being distributed in the (upper) aqueous phase. The radioactivity in the aqueous phase from treated sample extracts did not differ significantly from controls, unless otherwise stated in the text (as in the case of [^{3}H]glycerol-prelabeled lipids). Essentially all (99% ± 3%) of the radioactivity applied to the plate was recovered in the individual bands taken for scintillation counting. There were no significant differences between the total radioactivity contained in the control and experimental groups, a result that indicates that there is a net balance in the disposition of the radiolabeled precursors. Therefore, the dpm values in the total lipid extracts were, in some cases, normalized to the average of the applied radioactivity, in order to correct for differences in tissue content. All data points are averages of quadruplicate (or triplicate) incubations. Experiments were carried out at least three times on different days. Statistical significance was determined using Student's *t* test, and the *p* values are for a two-tailed distribution.

Results

Effects of THC on A-type phospholipase activity. We measured the amounts of LPC, LPE, and LPI formed in the presence and absence of THC in brain slices prelabeled with either [^{14}C]AA or [^{3}H]glycerol, to determine whether PLA_2 was involved in the liberation of esterified AA. In the presence of 8 μM THC, which was the EC_{50} for the THC-elicited rise in unesterified AA (>2.5-fold over controls) (10), the levels of LPC, LPE, and LPI were similar to control levels when [^{14}C]AA was used as label (Table 1). When the disposition of [^{3}H]glycerol was monitored, there was a modest but significant increase in LPI, on the order of 20% above control levels. However, the amount of [^{3}H]LPI was quite small, compared with total [^{3}H]PI. We observed no significant changes in LPC and LPE. The latter observation argues against the notion that activation of phospholipases of the A type played a major role in mediating the marked mobilization of AA in response to THC. On the other hand, 5 μg/ml melittin, a low molecular weight peptide in bee venom that activates endogenous PLA_2, elicited marked increases in all of the lysophospholipids, on the order of 2- to >10-fold above control levels (Table 2). This latter observation indicates that, when increases in lysolipids are expected to occur, as with treatment with melittin, we are able to detect these with our methodologies. This observation lends support to our interpretation that the relatively large rises in unesterified AA in brain elicited by THC occur, for the most part, by mechanisms other than activation of A_2-type phospholipases.

Effect of THC on [^{14}C]stearate levels in prelabeled brain slices. To determine whether THC increased levels of free fatty acids nonspecifically or whether the mobilization was specific for AA, we examined whether THC increased levels of [^{14}C]stearate (18:0), the major (saturated) fatty acid in membrane stores, in prelabeled slices. In contrast to its effects on AA disposition, THC did not significantly affect unesterified 18:0 or the levels of this fatty acid esterified in the phospholipids (Table 3). Thus, the THC-induced increases in unesterified fatty acids appear to be specific for AA. We did, however, observe a small, although statistically nonsignificant, rise in unesterified 18:0, which appeared to derive from TG. We have found previously that the turnover of prelabeled TG is very

TABLE 1

Effect of (−)-THC on the levels of lysophospholipids in brain cortex slices prelabeled with [^{14}C]AA and [^{3}H]glycerol

Prelabeled brain cortex slices were incubated with or without 8.0 μM THC for 1 hr at 37°. The indicated lysophospholipids were separated by thin layer chromatography, and their radioactivity was determined. The results are means ± standard deviations of quadruplicate measurements from a single experiment, which was repeated several times.

	Lipid	
	Control	THC
	dpm	
[^{14}C]AA		
LPC	272 ± 8	260 ± 28
LPE	117 ± 16	120 ± 13
LPI	26 ± 3	32 ± 32
[^{3}H]Glycerol		
LPC	772 ± 19	20 ± 53
LPE	505 ± 50	496 ± 49
LPI	186 ± 21	231 ± 6[a]

[a] $p < 0.05$.

TABLE 2

Effect of 5 μg/ml melittin on the levels of lysophospholipids in brain cortex slices prelabeled with [^{14}C]AA and [^{3}H]glycerol

See Table 1 for description of methods.

	Lipid	
	Control	Melittin
	dpm	
[^{14}C]AA		
LPC	253 ± 32	600 ± 32[a]
LPE	80 ± 7	177 ± 40[a]
LPI	60 ± 4	1132 ± 147[a]
[^{3}H]Glycerol		
LPC	671 ± 34	796 ± 90[b]
LPE	278 ± 12	588 ± 44[a]
LPI	104 ± 15	1063 ± 157[a]

[a] $p < 0.005$.
[b] $p < 0.05$.

TABLE 3

Effect of (−)-THC on the radioactivity in lipids prelabeled with [^{14}C] stearate

Prelabeled brain cortex slices were incubated with or without 8.0 μM THC for 1 hr at 37°. The phospholipids and neutral lipids were separated by thin layer chromatography, and their radioactivity was determined. The results were normalized to 100,000 dpm and are means ± standard errors of quadruplicate measurements from two individual experiments.

	Lipid		Change
	Control	THC	
	dpm		%
DG	3,099 ± 257	2,878 ± 171	−2
18:0	14,533 ± 1,256	15,438 ± 1,137	+7
TG	5,730 ± 203	4,661 ± 100	−19
PC	18,778 ± 298	19,590 ± 408	+4
PI	20,938 ± 256	20,666 ± 256	−1
PS	8,642 ± 166	8,611 ± 142	0
PE	24,238 ± 199	24,622 ± 155	+2
PA	1,655 ± 54	1,596 ± 28	−4

sensitive to exposure to THC, in comparison with other lipid species (10).

It is unlikely that the hydrolysis of phospholipids is balanced by re-esterification, resulting in no net change in stearate labeling. We feel that this could only occur if the lipids prelabeled with [^{14}C]stearate are equilibrated isotopically with free [^{14}C]stearate, the same amount of the same specific activity [^{14}C]stearate leaves the lipids as enters the lipids, and there is no mixing with nonradioactive pools in either direction. It is clear that in the case of [^{14}C]AA this is not the case.

Effects of THC on C-type phospholipase activity. Incubation of [^{14}C]AA-prelabeled cerebral cortex slices with ACh resulted in significant changes in DG, PI, and PA levels (Fig. 1). As expected, the level of PI was significantly reduced, and the levels of DG and PA rose concomitantly. The changes indicate a phosphoinositidase-mediated breakdown of PI to yield DG. The latter, in turn, is converted to PA by the action of DG kinase (see Refs. 14–16 for reviews). In marked contrast to the changes elicited by ACh, the apparent loss of PI elicited by THC was not accompanied by elevated levels of DG or PA (Fig. 1). This observation indicates that THC-induced losses in PI do not involve C-type phospholipases. Furthermore, the losses in PI elicited by combining THC and ACh were significantly greater than when either agent was added alone, even at a maximally effective concentration of ACh, a finding that supports the interpretation that THC and ACh elicit PI loss via different mechanisms.

Further evidence that the THC- and ACh-elicited losses of esterified AA are mediated by different mechanisms is derived from the results of studies that used slices prelabeled with [^{3}H]glycerol rather than [^{14}C]AA (Fig. 2). The former is used to monitor "turnover" of the phosphoglyceride backbone and better represents changes in mass, compared with radiolabeled

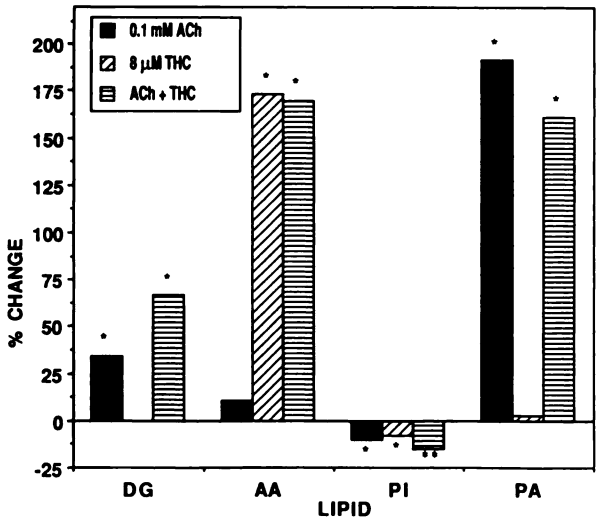

Fig. 1. Effect of THC and ACh on [^{14}C]AA levels in prelabeled brain lipids. Brain cortex slices were prelabeled with [^{14}C]AA and then incubated with either 8 μM THC, 0.1 mM ACh, or both drugs, for 1 hr at 37°. The lipids were separated by thin layer chromatography, and their radioactivity was measured. The results are expressed as the percentage increase relative to controls and are based on the means of quadruplicate determinations from a single representative experiment, which was replicated three times. The control dpm (mean ± standard deviation) values were DG = 1,691 ± 146; AA = 4,370 ± 710; PI = 52,138 ± 104; PA = 1,215 ± 21. *, $p < 0.05$, relative to controls; **, $p < 0.05$, relative to ACh or THC alone.

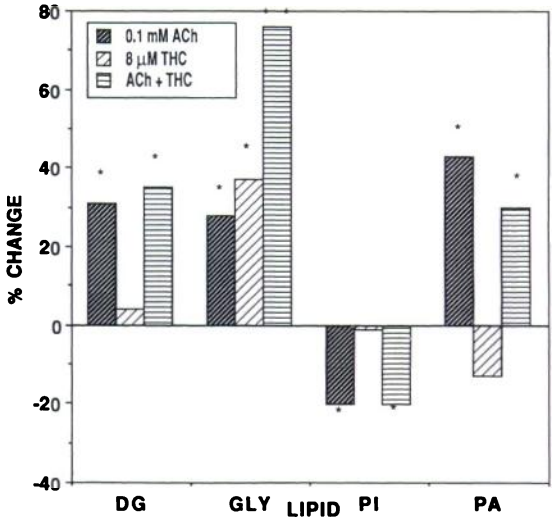

Fig. 2. Effect of THC and ACh on [^{3}H]glycerol levels in prelabeled brain lipids. Brain cortex slices were prelabeled with [^{3}H]glycerol (*GLY*) and then incubated with either 8 μM THC, 0.1 mM ACh, or both drugs for 1 hr at 37°. The lipids were separated by thin layer chromatography, and their radioactivity was measured. The results are expressed as the percentage increase relative to controls and are based on the means of quadruplicate determinations from a single representative experiment, which was replicated several times. The control dpm (mean ± standard deviation) values were DG = 2,656 ± 207; free [^{3}H]glycerol (*GLY*) = 8,015 ± 492; PI = 36,147 ± 508; PA = 3,450 ± 87. *, $p < 0.05$; **, $p < 0.05$, relative to ACh or THC alone.

AA, which is used to estimate turnover of the 2′-acyl group of phospholipids. The ACh-induced changes in the radioactivity in [^{3}H]glycerol-prelabeled slices were very similar to the results obtained when [^{14}C]AA was used as label; the radioactivity in PI decreased, whereas the radioactivity in DG and PA increased, as did the radioactivity in the aqueous medium, most of which was free [^{3}H]glycerol. We have reported that ACh increases free [^{3}H]glycerol levels in the aqueous medium in pancreatic acinar cells as a result of DG lipase activity on the elevated DG levels (17). Incubation with THC also increased free [^{3}H]glycerol in the medium, although it had no significant effect on [^{3}H]glycerol levels in PI (compare Figs. 1 and 2). Although the level of free [^{3}H]glycerol in the medium was elevated in response to THC, we could observe no rise in either DG or PA. The latter observation, together with the finding that the increases in [^{3}H]glycerol elicited by THC and ACh were, in general, additive when these agents were combined, provides further support for our interpretation that THC does not affect phosphoinositidase C action in brain.

The formation of water-soluble inositol phosphates, i.e., inositol monophosphates, bisphosphates, and trisphosphates, provides a reliable and sensitive measure of phosphoinositidase C action in phosphoinositides, including the polyphosphoinositides (14, 16). As shown in Fig. 3, in [^{3}H]inositol-prelabeled brain cortex slices, the ACh-elicited rises in the various inositol phosphates were on the order of 35–80-fold over controls (note logarithmic scale on ordinate). In contrast, inositol phosphate levels in slices treated with 8.0 μM THC alone were not significantly different from controls. Moreover, ACh-enhanced inositol phosphate formation remained unaffected by pretreatment with 8.0 μM THC. Thus, THC affected neither basal nor ACh-stimulated inositol phosphate and polyphosphate formation, an observation that buttresses the interpretation that THC

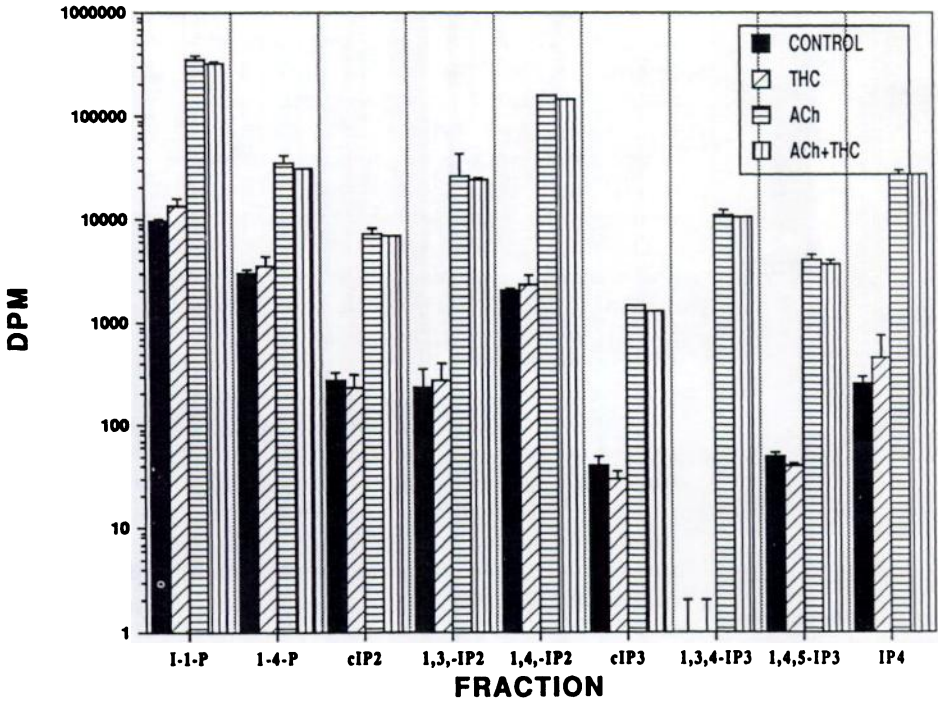

Fig. 3. Effect of THC and ACh on [^{3}H]inositol phosphate formation. Cortical slices that were prelabeled with [^{3}H]inositol were incubated for 5 min with 8.0 μM THC, followed by an additional incubation of 10 min in the presence and absence of 0.1 mM ACh. The inositol phosphates were measured as described in Experimental Procedures. The results are means of quadruplicate determinations from a representative experiment, which was repeated at least twice. cIP2, cyclic inositol triphosphate.

affects neither basal nor receptor-coupled phosphoinositidase activity.

Effects of THC on lipid acylation. A pathway that regulates cellular levels of unesterified AA is lipid acylation via the acyl-CoA synthetase and transferase complexes. If acylation were blocked, the levels of free AA could rise as the arachidonyl residues in the lipids decreased consequent to basal turnover. We, therefore, examined whether THC interfered with the acylation of lipids with AA, by measuring the uptake (esterification) of radiolabeled AA into brain lipids. Incubation with 8.0 μM THC resulted in selective inhibition of incorporation of radioactivity in neutral lipids and phospholipids. The incorporation of [^{3}H]AA in TG, PI, and PE was significantly reduced, compared with controls, whereas the radioactivity in PC was not altered significantly (Fig. 4). The reductions in esterified radioactivity were balanced by concomitant increases in unesterified [^{3}H]AA levels. The concentration of THC eliciting one half of the maximal response was 8 μM, and the maximally effective concentration was 32 μM (see below). These same values for the potency of THC were observed when we measured the release of esterified AA from prelabeled slices in our earlier study (10). However, an important observation in the acylation studies is that the percentage decreases in radioactivity in the phospholipids were far greater when THC-induced suppression of acylation was measured, compared with the THC-induced reductions in phospholipids prelabeled before exposure to THC. For example, at a maximally effective concentration of THC (32 μM), the percentage decrease in PI radioactivity was almost 5-fold greater when uptake of radiolabeled AA was measured, compared with the THC-induced reduction of esterified radioactivity from prelabeled slices (Fig. 5). Similar results were obtained when the incorporation of [^{3}H]AA into PE and PS was monitored. The relative decrease of radioactivity in PE and PS due to suppressed acylation with AA was on the order of 3-fold and 5-fold greater, respectively, than the apparent THC-induced loss of AA from these phospholipids in slices

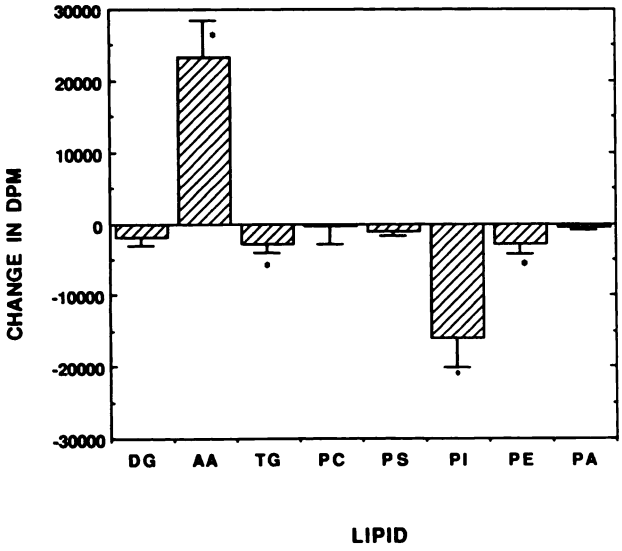

Fig. 4. Effect of THC on AA disposition in brain slices. Slices were incubated with [^{3}H]AA in the presence of 8.0 μM THC for 1 hr, in order to measure the effect of THC on the incorporation of label into esterified stores. The results are depicted as a "balance sheet" expressing the absolute magnitude of the rise in unesterified [^{3}H]AA and the contribution from each of the lipid species to this rise. The results are means of quadruplicate determinations and are representative of those obtained in several similar experiments. Control dpm (mean ± standard deviation) values were as follows: DG = 9,465 ± 592; AA = 39,311 ± 3,439; TG = 6,917 ± 501; PC = 16,020 ± 725; PS = 3,242 ± 252; PI = 37,294 ± 1,821; PE = 8,844 ± 587; PA = 2,382 ± 124. *, $p < 0.05$.

prelabeled before incubation with THC. In none of our studies did we detect significant changes in PC.

Structure-activity relationships for cannabinoid-induced inhibition of acylation by AA. All the results thus far presented are consistent with the interpretation that THC-induced mobilization of AA in brain occurs via interference with lipid acylation by AA, rather than by the liberation of AA from esterified stores by activation of lipolytic enzymes. We

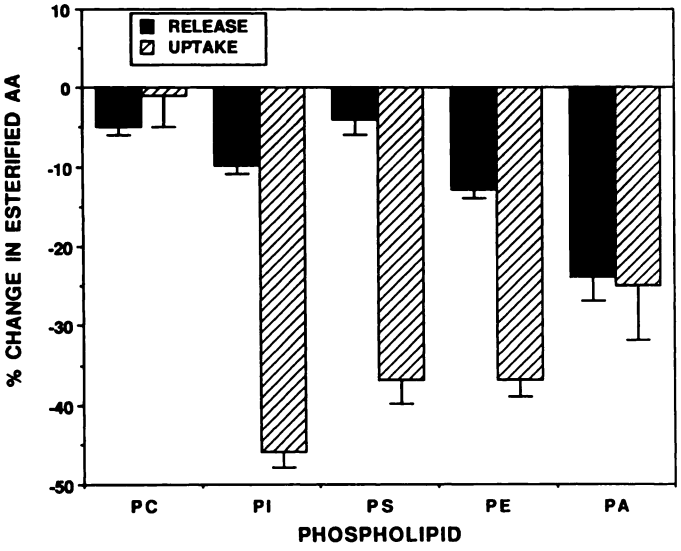

Fig. 5. Comparison of THC-elicited decreases in esterified AA in prelabeled and unlabeled slices. Brain cortex slices were either prelabeled with [^{14}C]AA before incubation with THC for 1 hr or co-incubated with [^{3}H]AA together with THC for 1 hr at 37°. The esterified radioactivity in the phospholipids was measured after separation by thin layer chromatography. The results are means ± standard errors from three separate experiments, each carried out in triplicate or quadruplicate.

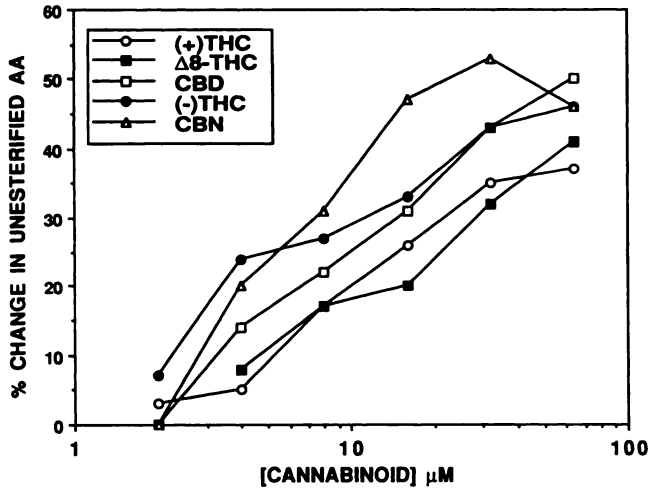

Fig. 6. Cannabinoid-elicited elevation in unesterified [^{3}H]AA. Brain cortex slices were incubated with [^{3}H]AA, together with increasing concentrations of cannabinoids, for 1 hr at 37°. The radioactivity in the free fatty acid band was measured after separation by thin layer chromatography. The results are from at least three experiments and are expressed as the mean percentage changes in the dpm values relative to control samples. The average ± standard error for the control AA values was 49,577 ± 2,659.

next addressed whether this response was involved in the neurochemistry underlying psychoactivity, by comparing the potencies of several well characterized primary cannabinoids in suppressing AA incorporation.

Although the cannabinoids we examined exhibited modest structural specificity in elevating [^{3}H]AA, there was little correlation between this neurochemical response and the psychoactive potencies *in vivo* (Fig. 6). For example, CBN, which is relatively inert pharmacologically, was 2- to 3-fold more potent than Δ^{8}-THC. The latter is a naturally occurring, acid-stable cannabinoid that is almost equipotent to THC in humans and animals. Furthermore, the potencies of THC and CBD in elevating [^{3}H]AA levels were similar, in spite of the relative lack of psychoactivity residing in the latter. Moreover, the potency of the inactive stereoisomer of THC, (+)-THC, was similar to that of THC; *in vivo*, there is up to a 100-fold difference in psychoactive potencies between the stereoisomers of THC (18). Thus, the effects of THC in suppressing lipid acylation with AA are probably not receptor mediated.

The uptake of [^{3}H]AA by TG was very sensitive to cannabinoid treatment; cannabinoids reduced by up to 80% the formation of labeled TG (Fig. 7). However, the effect again appears to be unrelated to psychoactivity, because the relative rank order in the potencies of the congeners was CBD > CBN > (−)-THC = Δ^{8}-THC = (+)-THC. Also, all the cannabinoids decreased the acylation of phospholipids with [^{3}H]AA similarly, by 35–45%, compared with control samples (Fig. 8). In summary, the structure-activity relationships for the cannabinoids in inhibiting lipid acylation, as measured by several different parameters, do not correlate well with the structure-activity relationships of the cannabinoids as psychoactive agents in humans and animals.

Discussion

We reported earlier that THC, over a range of 2–32 μM, increased markedly the levels of unesterified [^{14}C]AA in prelabeled brain cortex slices incubated for 1 hr (10). The EC_{50} for the response was 8 μM, with a maximal stimulation of >5-fold over control levels occurring at 32 μM. We observed that most of the AA was derived from the phospholipids, particularly PI. The present work had two major aims. First, we sought to determine the mechanism underlying the THC-induced mobilization of AA in a neuropharmacologically relevant preparation with functioning synapses. Second, we sought to test the proposal that THC-elicited mobilization of AA is a factor that underlies the psychoactivity of cannabinoids.

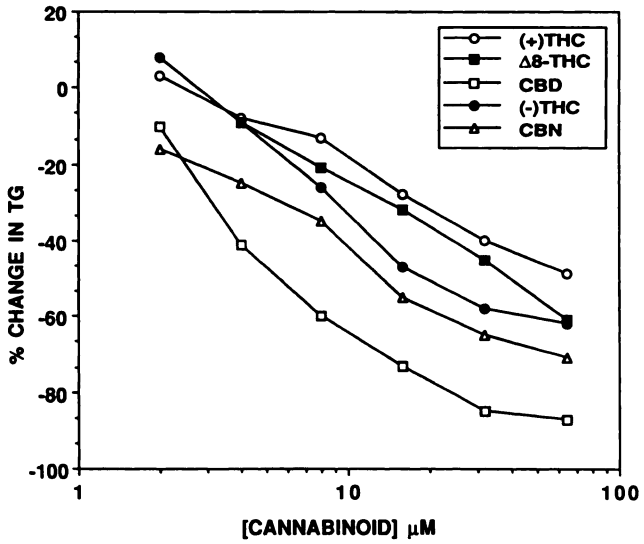

Fig. 7. Cannabinoid-elicited inhibition of incorporation of radioactivity into TG. Brain cortex slices were incubated with [^{3}H]AA, together with increasing concentrations of cannabinoids, for 1 hr at 37°. The radioactivity in the TG band was measured after separation by thin layer chromatography. The results are from at least three experiments and are expressed as the mean percentage changes in the dpm values, relative to control samples. The average ± standard error for the control TG values was 3,991 ± 209.

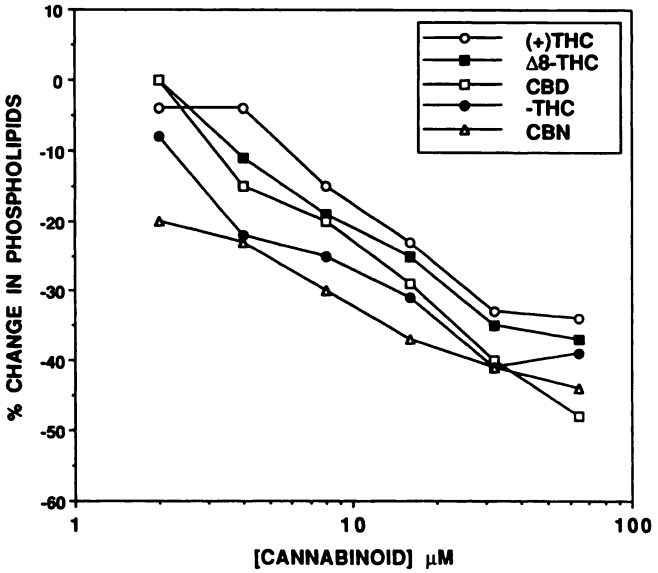

Fig. 8. Cannabinoid-elicited inhibition of incorporation of radioactivity into the phospholipid fraction. Brain cortex slices were incubated with [^{3}H] AA, together with increasing concentrations of cannabinoids, for 1 hr at 37°. The radioactivity in the total phospholipid band was measured after separation by thin layer chromatography. The results are from at least three experiments and are expressed as the mean percentage changes in the dpm values, relative to control samples. The average ± standard error for the control phospholipid values was 41,601 ± 2,576.

Unesterified AA is maintained at very low levels in brain and other tissues under homeostatic conditions. Almost all of the cellular stores of AA remain esterified in membrane phospholipids, with lesser amounts contained in the neutral lipids. The activation of PLA_2 is one of the more well studied pathways that serve to mobilize esterified AA (19). The enzyme plays a major role in liberating AA in several cellular systems, including stimulated platelets, neutrophils, and mast cells (20, 21). Typically, the enzyme deacylates several phospholipid species, including choline and ethanolamine phosphoglycerides, the predominant phosphoglycerides in biomembranes, thereby generating lysophospholipids and free fatty acids. Formation of the lysophospholipids is generally considered to indicate that the fatty acids arise through enhanced PLA_2 activity. For the most part, we were unable to detect THC-elicited increases in these lysophospholipids, a finding that argues against an involvement of PLA_2. To determine the validity of our methodologies, we incubated brain slices with melittin, which has been shown to activate endogenous PLA_2. Exposure of prelabeled slices to melittin elicited marked rises in lysophospholipids. Thus, our methods could detect increases in lysolipids if they occurred upon exposure to THC. We conclude from these studies that activation of PLA_2 is not the major mechanism whereby THC mobilizes AA in brain slices.

Others have reported that THC increases PLA_2 activity, based on the observation that THC increased the hydrolysis of exogenous PC added to suspensions of membrane fragments (6, 8). Because cannabinoids are membrane perturbants (22), it is conceivable that THC can alter the activity of interfacial enzymes, such as PLA_2, by affecting the physicochemical properties of the phospholipid-enzyme interface. For example, detergents, alcohols, and organic solvents can enhance the lipolytic activity of PLA_2 in suspensions of membrane fragments. In the present study, which utilized intact brain slices and endogenously formed radiolabeled lipids, THC did not appear to affect phospholipase A-type activity.

We examined the effects of 8 μM THC on the disposition of [^{14}C]stearate, which is esterified at the 1′-position of glycerophospholipids, in order to examine whether the THC-elicited changes in prelabeled lipids were due to nonspecific perturbation of lipid metabolism or whether the effects of THC were specific for AA metabolism. Incubation of prelabeled slices with THC had little effect on unesterified [^{14}C]stearate or on the esterified stores in the phospholipids, with the latter comprising as much as 90% of the esterified stores of fatty acids. This finding contrasts markedly with the effects of THC on the disposition of unesterified and esterified [^{14}C]AA in prelabeled slices. For example, we have shown previously that under similar conditions THC increases unesterified [^{14}C]AA up to 2.5-fold over control levels in prelabeled brain cortex slices, with over half of this AA deriving from PI (10). Thus, it appears that the effects of THC are rather specific for AA, compared with its effects on 18:0, the most abundant fatty acid in brain. The small but statistically nonsignificant rise in unesterified 18:0 appeared to be due to a concomitant loss from TG. We have found that, relative to the other lipids, TG turnover appears to be very sensitive to exposure to cannabinoids in prelabeled brain slices. The level of fatty acids esterified in this neutral lipid represents <5% of the total esterified free fatty acid stores.

Another pathway that can serve to liberate AA from esterified stores involves the action of PI-specific phospholipase C (termed phosphoinositidase C to differentiate it from less specific phospholipases C) (23). The primary products of phosphoinositidase C action on phosphatidylinositol 4,5-bisphosphate are DG and inositol 1,4,5-trisphosphate. The latter is an important intracellular second messenger involved in signal transduction, which serves to elevate intracellular Ca^{2+} levels. DG, which remains in the membrane phase, also serves second messenger roles by activating protein kinase C. The metabolism of DG occurs by phosphorylation via DG kinase to yield PA, which ultimately generates PI via the sequential actions of PA-cytidyltransferase and PI synthase (14, 16, 23). Alternatively, DG may be acted upon, in turn, by DG and MG lipases, which ultimately liberate free fatty acids (mainly AA and stearate) and glycerol (17). The DG kinase pathway is believed to preponderate in brain during homeostasis (24), whereas the phosphoinositidase C/DG lipase pathway route occurs during the ischemia that immediately follows neural trauma (25, 26). Determining whether THC affects the activity of phosphoinositidase C is relevant not only in studying mechanisms underlying AA mobilization but also for determining whether THC interacts with the "PI signaling system" in brain. If the latter were true, it would be an important clue to the mechanism of THC action. We addressed this important question by several different methods, all of which yielded results consistent with one another.

As shown in the present study, the ACh-elicited fall in [^{14}C]AA-PI levels is accompanied by a concomitant rise in DG and PA, as expected for receptor-mediated phosphoinositidase C activation. These results provide validation that our preparation is viable, with intact synapses, and that we can monitor changes in lipid turnover that occur in ^{14}C-prelabeled brain slices incubated *in vitro*. In contrast, the THC-elicited fall in [^{14}C]AA-PI levels is not accompanied by rises in labeled DG or

PA. Furthermore, the decreases in [^{14}C]AA-PI elicited by THC and ACh alone are additive when both agents are combined, even if maximally effective concentrations are used (0.1 mM ACh was a maximally effective concentration; results not shown). This finding is one that strongly suggests that ACh and THC reduce PI levels via separate mechanisms. We did note a tendency for THC to increase ACh-stimulated DG levels and a concomitant reduction in the level of PA; however, the effect was not always significant. It may be that THC inhibits DG kinase activity to some extent. Others have proposed that THC inhibits phosphoinositide kinase activity indirectly via mobilization of AA, the latter affecting formation of polyphosphoinositides (27).

Our interpretation that THC does not affect the activity of phosphoinositidase C is supported further by the results from the studies on the effects of both THC and ACh on lipid turnover in slices prelabeled with [^{3}H]glycerol. The turnover of [^{3}H]glycerol has been used as a measure of lipid mass, whereas measurement of radiolabeled AA levels in prelabeled slices provides somewhat more limited information, i.e., it gives information only about the turnover of a specific esterified fatty acyl residue. The effects of ACh on the disposition of [^{3}H]glycerol were very similar to those obtained in slices prelabeled with [^{14}C]AA; the levels of DG and PA rose, whereas the radioactivity contained in the PI fraction fell. This pattern is typical of phosphoinositidase C action, as discussed above. On the other hand, exposure to THC had no effect on the ACh-induced changes in the radioactivity in PI, DG, or PA, i.e., THC did not affect receptor-mediated PI turnover. Furthermore, THC did not elicit any significant changes in prelabeled [^{3}H]glycerol-PI. The latter observation suggested that THC did not affect mass levels of PI, only its 2′ fatty acyl composition (discussed further below).

Despite our finding that two different experimental paradigms utilizing different lipid precursors yielded similar results, namely, that THC did not appear to affect phosphoinositidase C activity, it was, nonetheless, possible that THC affected the turnover of polyphosphoinositides without affecting their parent, i.e., PI. We, therefore, examined what effect THC had on basal and receptor-mediated inositol phosphate formation, a sensitive indicator of phosphoinositide and polyphosphoinositide breakdown via phosphoinositidase C activation. Incubation of [^{3}H]inositol-prelabeled cortical slices with THC had no significant effect on basal or receptor-mediated inositol phosphate liberation. These results, together with those discussed above, demonstrate that THC does not affect the PI signaling system in brain. This finding contrasts with the recent report that THC markedly inhibited agonist-stimulated inositol polyphosphate formation in pancreatic acinar cells incubated *in vitro* (27). Apparently, receptor-mediated activation of phosphoinositidase C in brain is less sensitive than acinar cells to THC.

As mentioned above, THC did not elicit any significant changes in prelabeled [^{3}H]glycerol-PI, in contrast to the consistent THC-elicited reductions in [^{14}C]AA-PI in prelabeled slices (10). This observation, together with the THC-mediated modest increase in free [^{3}H]glycerol in the incubation medium from [^{3}H]glycerol-prelabeled slices, suggested that THC might affect lipid acylation with AA. This would also explain the small increase we observed in the level of [^{3}H]glycerol-containing LPI after exposure to THC (Table 1). We, therefore, measured the effects of THC on the incorporation of [^{3}H]AA into brain lipids. The results of these studies, together with all of the results discussed thus far, are consistent with the interpretation that THC mobilizes AA in brain by inhibition of acylation, e.g., the percentage decrease of esterified AA in the phospholipids, which contribute 80% of the THC-induced release of AA, is up to 5-fold greater when the uptake of AA is measured, compared with monitoring of AA release from esterified stores in prelabeled slices (Fig. 5 and Ref. 10). In other words, THC had a much more marked effect on inhibiting the incorporation of radiolabeled AA due to a suppression of acylation mechanisms, compared with its effect on radiolabeled AA in lipid stores prelabeled before exposure to THC. In addition to the well characterized microsomal synthesis of lipids *de novo*, acylation of individual lysophospholipid species occurs as part of deacylation-reacylation cycles, which impart the characteristic "fingerprint profile" to the 1′- and 2′-acyl residues of phospholipids. How the fatty acyl composition of membrane lipids is regulated remains unclear. There is evidence that in brain (28), as well as in other tissues (29), the deacylation-reacylation cycle occurs independently of *de novo* synthesis. It is generally accepted that this is true for most tissues. For example, newly formed PI in platelets and in brain is not enriched in AA in the 2′-position immediately after exposure to agonists (29). Only some time after termination of stimulation does PI assume its characteristic enrichment in AA. The results of earlier studies by others on the mechanisms underlying the normal enrichment of arachidonyl residues in PI in rat brain suggested that this enrichment occurs at a stage beyond *de novo* synthesis, i.e., at the level of the acylation-deacylation cycles (28). We propose that these pathways are more sensitive to THC than *de novo* synthetic mechanisms, which utilize stearate and glycerol.

The inhibition of AA-specific acylation mechanisms has been relatively ignored in the literature as the mechanism underlying THC-induced mobilization of AA. The present results in brain confirm the observation made by others regarding the inhibition by cannabinoids of lipid acylation in lymphocytes (30, 31). In the earlier work in lymphocytes, the effect was specific for THC; however, Δ^{8}-THC, a cannabinoid regarded generally as being roughly equipotent to THC in humans and animals, was much less potent than THC, as were other inactive cannabinoids. Thus, the relationship between the inhibition of acylation in lymphocytes and psychoactivity remains unclear from the earlier work. Regardless of the mechanism underlying the inhibition of acylation in the present study, we observed no correlation between this effect in brain slices and the psychoactive potencies of cannabinoids; this is the other main point of the present work. The cannabinoids we used for this study are primary cannabinoids, i.e., they occur naturally in cannabis and are very well characterized pharmacologically in humans and animals. All of the cannabinoids increased AA levels with similar potencies. Moreover, the stereoisomers of THC were equipotent, an observation that suggests that the increases in AA levels are not receptor mediated.

All the primary cannabinoids we studied inhibited markedly the incorporation of AA into the TG fraction. We observed in our earlier study that the apparent relative percentage disappearance of label from TG in prelabeled slices was much greater than in the other lipids (10). Because the TG pool is one that has a high turnover rate, it might be that TG levels are

particularly sensitive to inhibition of acylation mechanisms. Although the effect in the TG fraction was rather marked, we are unsure of its significance. Although our structure-activity relationship data show that cannabinoid-elicited AA mobilization via inhibition of the acylation of phospholipids and TGs is not related directly to psychoactivity, it may be involved in other, more subtle, effects of cannabis. For example, the only other agents that elevate AA levels *in vivo* involve either intense neural stimulation or neural trauma, e.g., electroconvulsive shock, chemically induced seizures, and ischemia (32). Certain neurotransmitters and KCl-induced depolarization will elevate levels of AA and its metabolites (33, 34); however, in our hands THC is considerably more effective in mobilizing AA than is 45 mM KCl.[2] Thus, the possibility that cannabinoid-induced mobilization of AA through inhibition of acylation is involved in effects of cannabis other than psychoactivity, particularly those responses observed at high doses or with prolonged exposure, cannot be excluded.

Upon reviewing the literature on the biochemical pharmacology of cannabinoids (1–4), one is struck by the lack of consistency in the findings over the years, i.e., cannabinoids either inhibit, stimulate, or do not affect the myriad of biochemical responses measured thus far in many different preparations. Apparently, cannabinoids have different quantitative and qualitative effects in different model systems. The single notable exception to these disparate effects is that, in every model thus far examined, cannabinoids increase the mobilization of AA. Therefore, the proposal that THC-enhanced lipolytic activity is involved in psychoactivity was especially attractive. The results of our work, in summary, demonstrate that cannabinoids increase AA levels in brain by interfering with lipid acylation mechanisms specific for AA, not by activation of lipases. Furthermore, the structure-activity relationships we observed do not appear to relate to psychoactivity.

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