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PHOSPHOLIPASE PARTICIPATION IN CANNABINOID-INDUCED RELEASE OF FREE ARACHIDONIC ACID

SUMNER BURSTEIN,* JOHN BUDROW, MICHELLE DEBATIS, SHEILA A. HUNTER and ASHA SUBRAMANIAN

Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01655, U.S.A.

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Abstract—The exposure of cells in culture to cannabinoids results in a rapid and significant mobilization of phospholipid bound arachidonic acid. *In vivo*, this effect has been observed as a rise in eicosanoid tissue levels that may account for some of the pharmacological actions of Δ^9-tetrahydrocannabinol (THC), the major psychoactive cannabinoid. Fluoroaluminate pretreatment of mouse peritoneal cells potently reduced the cannabinoid response, while promoting arachidonate release on its own, consistent with earlier observations that this effect may be a receptor/G-protein-mediated process. Further support for receptor mediation was the demonstration of saturable, high-affinity cannabinoid binding in these cells. THC potency was reduced in the presence of ethanol, and was accompanied by significant increases in phosphatidylethanol (PdEt) levels, a unique product of phospholipase D (PLD) activity. THC-dependent arachidonate release was reduced partially in similar amounts by either propranolol or wortmannin, further implicating PLD as a mediator of THC action. A central role for diacylglyceride (DAG), a secondary product of PLD metabolism, in this THC-induced process, both as a source of arachidonate and as a stimulator of protein kinase C (PKC), is supported by the data in this report. Cells exposed to phorbol ester for 18 hr prior to THC challenge became less responsive, indicating a possible role for PKC. The involvement of PKC further suggests participation by phospholipase A_2 (PLA_2) whose activity may be regulated by the former. Treatment of cells with interleukin-1α, an agent known to elevate PLA_2 levels, caused an increase in the THC response, supporting a role for this enzyme in the release reaction. Direct evidence, by immunoblotting, for the activation and phosphorylation of PLA_2 by THC was also obtained. In summary, the evidence presented in this report indicates that THC-induced arachidonic acid release occurs through a series of events that are consistent with a receptor-mediated process involving the stimulation of one or more phospholipases.

Key words: cannabinoid; arachidonic acid; mouse peritoneal cells; phospholipase D; phospholipase A_2; diacylglyceride

A number of reports, using diverse experimental approaches, have supported the hypothesis that arachidonic acid and its metabolites are important mediators in the actions of cannabinoids [1]. Interest in this hypothesis has intensified recently with the discovery that the ethanolamide derivative of arachidonic acid is a putative endogenous ligand for the brain cannabinoid receptor [2]. Although a direct connection between this endogenous ligand and the hypothesis is not apparent, it seems unlikely that the two are unrelated. For example, it may be that anandamide itself is capable of releasing cellular arachidonic acid that results in an elevation of eicosanoid levels, as has been shown for other cannabinoids. Thus, a better understanding of the mechanism underlying cannabinoid-induced arachidonate release is a subject of considerable interest.

The exposure of cells in culture to THC†, the principal psychoactive cannabinoid, results in a rapid release of free arachidonic acid into the medium. In humans, as well as in experimental animals, the response is manifested by a rise in plasma and brain levels of eicosanoids [1]. Experiments using mouse peritoneal cells, as well as S49 mouse lymphoma cells, suggest that this effect is mediated by a heterotrimeric GTP-binding complex [3] and, thus, may be receptor initiated. A cannabinoid binding protein, different from the brain receptor [4], that occurs mainly in peripheral tissues has been cloned [5]; however, its functional role has not been reported. Nevertheless, it now seems likely that there is more than one cannabinoid receptor raising the possibility of specificity among the family of cannabinoids and also the likelihood of more than

* Corresponding author: Sumner Burstein, Ph.D., Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. Tel. (508) 755-1866; FAX (508) 856-6231.

† Abbreviations: THC, Δ^9-tetrahydrocannabinol; G-protein, guanine nucleotide-binding protein; PLD, phospholipase D; PKC, protein kinase C; PdEt, phosphatidylethanol; PA, phosphatidic acid; PLC, phospholipase C; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; PAF, platelet activation factor; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; IL, interleukin; MEM, minimal essential medium; MAPK, mitogen-activated protein kinase; DAG, diacylglyceride.

one mechanism of action. The presence of low levels of the brain receptor has also been detected in human leukocytes [6].

The particular signal transduction events involved in this cannabinoid-mediated process, as well as the nature of the effector molecules, are largely unknown. By analogy with other arachidonate mobilizing agonists, it seems likely that one or more phospholipases are responsible for the release effect. In fact, earlier studies support a role for phospholipases in this cellular response to THC [7, 8]; however, neither the specific lipases nor the factors regulating their activities were identified. Two recent publications [9, 10] have reported an apparent inhibition of arachidonic acid uptake by cannabinoids, and the authors stated that this may be the basis for our reported observations on cannabinoid-induced arachidonic acid release [3, 7, 8]. Direct measurements of cannabinoid inhibition of the isolated enzymes involved in arachidonic acid uptake were not reported by those authors, making their conclusions on mechanism somewhat doubtful. Moreover, we have shown previously that cannabinoid-induced eicosanoid synthesis from endogenous arachidonic acid correlates well with the release of radiolabeled arachidonic acid promoted by cannabinoids [11]. Such a finding is difficult to explain by a mechanism based on inhibition of uptake of exogenously added arachidonic acid as has been claimed [9, 10]. The present report gives further evidence on the nature of THC-induced arachidonic acid release and supports our hypothesis that it is caused by a receptor-mediated stimulation of phospholipase activities.

MATERIALS AND METHODS

Materials. The THC was supplied by the National Institute on Drug Abuse, and its purity was monitored by thin-layer chromatography. Phosphatidylethanol, RHC-80267 and sphingosine were purchased from Biomol, Plymouth Meeting, PA. Wortmannin, TPA and staurosporine were obtained from the Sigma Chemical Co., St Louis, MO. Carbon-14 labeled arachidonic acid was purchased from ARC, St Louis, MO, and had a specific activity of 40–60 Ci/mol. The cells were obtained from female, CD-1 mice (20–25 g) by peritoneal lavage as previously described [3]. The ligand used in the binding experiment, 2-iodo-5′-azido-Δ^8-THC, was prepared as described in an earlier report [12]. The antiserum to PLA_2 was obtained through the generosity of Dr Christina C. Leslie, National Jewish Center, Denver, CO.

Arachidonic acid labeling and release conditions. The peritoneal cells, approximately 0.5×10^6/18 mm culture well, were incubated at 37° for 2 hr with [1-^{14}C]arachidonic acid (0.5 to 1×10^5 dpm/mL MEM). The medium was removed, and the cells were washed twice with MEM (1.0 mL) containing 0.1% bovine serum albumin to remove unincorporated fatty acid. The cells in fresh MEM (1.0 mL) containing 0.1% BSA were given appropriate treatments and incubated for 30 min at 37°. For arachidonic acid release determinations, the medium was collected and centrifuged at 3000 *g* for 15 min; duplicate aliquots of the supernatant were assayed for radioactivity by liquid scintillation counting. TLC analysis of the medium confirmed that more than 90% of the radioactivity consisted of arachidonic acid.

Measurement of PdEt, PA and DAG synthesis. Phospholipase D activity was measured by the formation of [^{14}C]phosphatidylethanol as reported elsewhere [13]. Briefly, following a 30-min incubation of [^{14}C]arachidonic acid-labeled peritoneal cells with THC in either DMSO or ethanol, as vehicle, the medium was aspirated and 0.5 mL of ice-cold methanol was added to the cells. Unlabeled PdEt (10 μg) was added as a carrier, and the cells were scraped from the dishes using 3×0.5 mL methanol rinses and extracted with 2 mL water and 8 mL chloroform. The aqueous phase was extracted again with 8 mL chloroform, and the combined organic phases were evaporated with a stream of nitrogen. Dried extracts, along with lipid standards, were applied to silica gel plates and eluted with chloroform:methanol:acetic acid (81:19:2.5, by vol.). Lipids were detected by exposure to iodine vapor, and the relevant zones were scraped, added to 10 mL Liquiflor and 1 mL methanol, and then assayed for radioactivity. Radioactive zones of PdEt, PA and DAG were identified by comparison of their mobilities with those of authentic standards (Sigma).

Binding and photolabeling procedure. Peritoneal cells were collected from female CD-1 mice and cultured as previously described [3]. Monolayers containing approximately $4–5 \times 10^5$ cells under 1 mL of medium were equilibrated with ligand (2×10^4–20×10^4 dpm) in 25 μL of ethanol for 30 min at room temperature with gentle agitation by which time earlier studies showed that saturation is reached [12]. The medium was then collected and centrifuged to sediment unattached cells and debris; the radioactive content was measured on duplicate aliquots of the supernatant. The average of these values was taken as a measure of the free ligand concentration. The remaining monolayers were covered with 0.25 mL of Tris (0.05 M, pH 7.4) saline (0.15 M) and irradiated in a Rayonet Photochemical Mini-Reactor (RMR-600) at room temperature with ultraviolet light (254 nm) for 20 min. Two 8 W bulbs at a distance of 10 cm from the cells were used. Microscopic examination of the cells following irradiation showed no changes in morphology or monolayer density. The buffer was removed and the cells were treated briefly with 2×1 mL volumes of ice-cold 10% trichloroacetic acid. The cells were then rinsed with 1 mL of potassium acetate in ethanol followed in 10 min by 1 mL of absolute ethanol to remove lipids and residual unreacted ligand. The ethanol extraction was allowed to proceed for 10 min at 50°. The remaining material on the culture plate, which contained the protein bound ligand, was extracted with a 0.25 mL mixture of 5% SDS and 0.05% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in 0.05 M Tris (pH 7.4). The extraction was performed at 50° for 5 min followed by continuous agitation for 30 min at room temperature after which the solutions were collected. The dishes were rinsed with 0.5 mL of

buffer, combined with the detergent extracts, and radioactivity levels measured to give the values of bound ligand. Three monolayers were used to measure binding at each ligand concentration and the values averaged for binding analysis. In experiments where competition with unlabeled cannabinoids was measured, i.e. THC, (+)Δ^8-THC and Δ^8-THC-11-oic acid, the competing cannabinoid was added to the cells simultaneously with the radiolabeled ligand and the system allowed to equilibrate for 30 min prior to the removal of the medium and photoactivation.

Immunodetection of cPLA$_2$ activation. Cells were prepared as previously reported [3] and treated with THC in DMSO–water (1:1, v/v) for 30 min. A vehicle control in which the final concentration of DMSO was 0.5% was also run in parallel. Then the medium was removed, the cells were washed three times with fresh medium, and a detergent extract of the monolayer was prepared using 20 mM Tris (pH 8.0), 137 mM NaCl, 2 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1% (v/v) Trition X-100, 10% (v/v) glycerol, 25 mM β-glycerophosphate. The extracts were subjected to SDS–PAGE (7.5%), and western blot analyses were performed after electrophoretic transfer onto an Immobilon-P membrane. The membranes were probed with a rabbit polyclonal anti PLA$_2$ described by Qiu *et al.* [14] and the immune complexes detected by enhanced chemiluminescence (Amersham International PLC). The antiserum is able to detect phosphorylated as well as nonphosphorylated PLA$_2$, which can be differentiated by the slightly greater mobility of the latter in the gel system used.

RESULTS

Evidence for receptor involvement in THC-induced arachidonic acid release. In an earlier publication, we described the preparation and use of a high specific activity ligand for the detection of cannabinoid binding sites in intact cells [12]. This molecule, 2-iodo-5′-azido-Δ^8-THC, has now been used to demonstrate the presence of high-affinity, saturable cannabinoid binding in mouse peritoneal cells (Fig. 1). The ligand was intended initially for experiments aimed at photolabeling and isolation of the binding site(s); however, those findings will be presented elsewhere. Advantage was taken of the irreversible nature of the photolabeling process to generate binding data with greatly reduced nonspecific binding. Our previous study [12] showed that by 30 min full equilibrium is reached, so that the level of photobound ligand represents a close estimate of the steady-state value. As can be seen in Fig. 1, the non-specific bound ligand was less than 20% of total binding. Specificity of binding was found when tested with (+)-Δ^8-THC (25%) and Δ^8-THC-11-oic acid (<8%), both of which showed reduced affinities for this site relative to (−)-Δ^8-THC (100%). It is also significant that these binding affinities were in the same rank order as the abilities of these cannabinoids to induce arachidonic acid release in macrophages (data not shown).

The relationship between binding and functionality, i.e. arachidonic acid release, is further demonstrated by the data in Fig. 2. There appears to be a close correspondence between the binding of THC and its ability to promote the mobilization of arachidonic acid, suggesting that the latter may

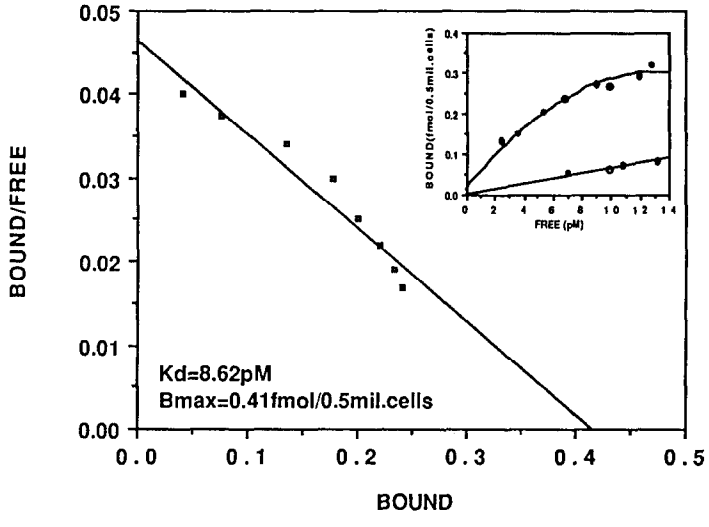

Fig. 1. Saturable, high-affinity cannabinoid binding to intact mouse peritoneal cells. The ligand 2-iodo-5′-azido-Δ^8-THC was synthesized from 5′-azido-Δ^8-THC as previously reported [12] and had a specific activity of 2200 Ci/mmol. The binding protocol is described under Materials and Methods. The inset shows total binding (solid circles) and nonspecific binding (open circles) in the presence of 50 μM THC. The data shown are representative of three separate experiments in which each point was measured in triplicate.

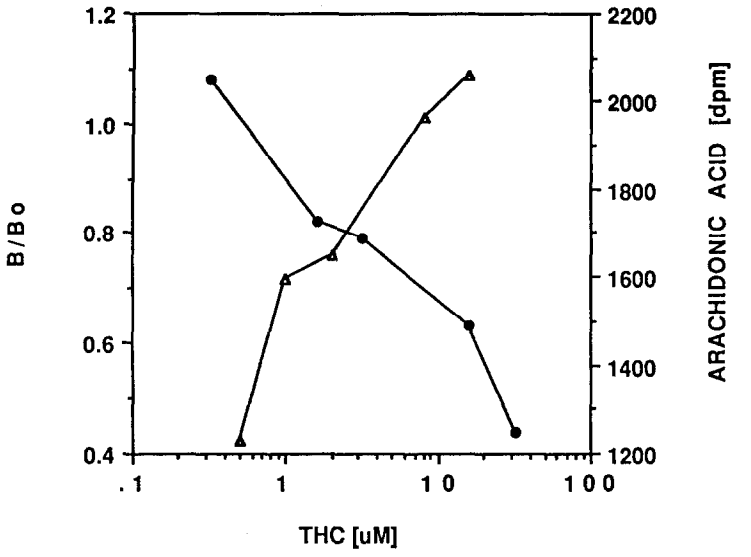

Fig. 2. Concentration–response relationships for inhibition of binding and stimulation of arachidonate release by THC. The ligand binding and arachidonic acid release studies were done as described in Materials and Methods with increasing concentrations of THC in DMSO, as indicated. Circles indicate the inhibition of ligand binding by THC. Triangles represent [^{14}C]arachidonic acid released into the medium. All data points were done in triplicate; the vehicle control value for the release experiment was 960 ± 70 dpm.

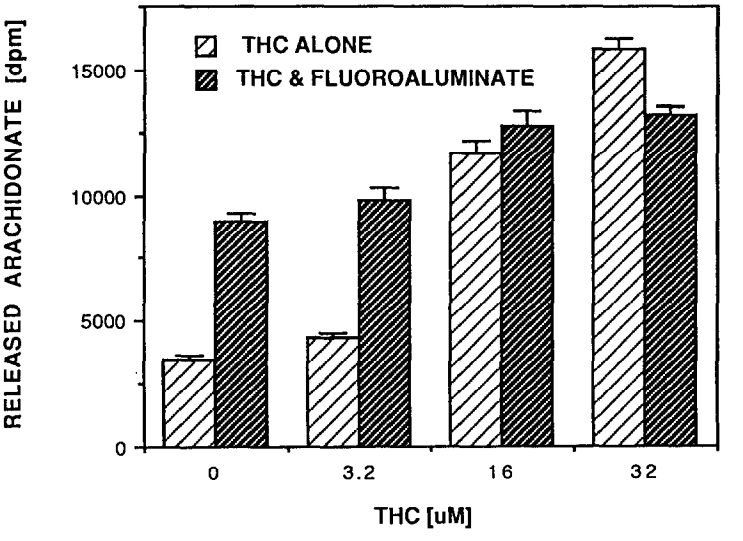

Fig. 3. Decreased THC response in the presence of $NaAlF_4$. Cells were labeled and treated as described in Materials and Methods. Pretreatment for 5 min with $NaAlF_4$ (50 μM) was followed by exposure to THC in ethanol at the indicated concentrations for 30 min. Values are the means ± SD of four determinations and are based on monolayers containing 0.5×10^6 cells.

be a receptor-mediated process. The onset of binding occurred at slightly lower concentrations of THC than the release effect; however, this may be due to the differences in experimental conditions imposed by each of the procedures, or possibly it could reflect the complex nature of the release response. Although the added amounts of THC needed to bring about displacement were somewhat higher than the K_d value, they are in close agreement with concentrations reported by Munro *et al.* [5] in a similar system. Moreover, at least part of the difference could be due to the structural modifications present in the ligand, which may increase its affinity for the receptor over that shown by THC itself.

Effect of AlF_4 on the release response. G-protein-mediated signaling pathways have been shown to be

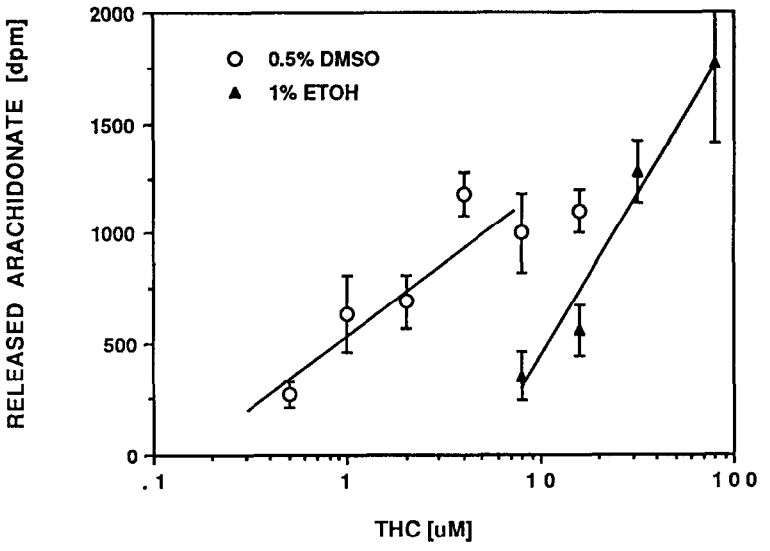

Fig. 4. Effect of vehicle on THC-induced arachidonate release. Cells were prepared and labeled as described in Materials and Methods. They were then exposed to THC in either DMSO or ethanol as the vehicle for 30 min in fresh MEM containing 0.1% BSA. The final concentrations of vehicle were 0.5% for DMSO and 1% for ethanol. Each data point is the mean ± SD of dpm ^{14}C in the medium from four individual monolayers minus the vehicle control values (DMSO, 960 ± 70; EtOH, 980 ± 40). Monolayers containing 0.5×10^6 cells were used for each data point.

Table 1. Increased cellular levels of phosphatidylethanol following THC treatment compared with arachidonic acid release

Treatment	Phosphatidylethanol* (% ^{14}C)	Released arachidonate† (dpm)
Ethanol (1%)	1.09, 0.967 (1.03)	1420 ± 320
THC (8 μM) in ethanol	1.68	2620 ± 690‡
THC (16 μM) in ethanol	1.92, 3.01 (2.47)	5050 ± 910‡
DMSO (0.5%)	1.01, 0.998 (1.00)	2050 ± 220
THC (8 μM) in DMSO	1.28	4010 ± 400‡
THC (16 μM) in DMSO	1.09, 1.07 (1.08)	5010 ± 600‡

* Cells were prepared and their PdEt levels measured following treatment for 30 min as described in Materials and Methods. The values shown in brackets are the averages of two experiments.

† Cells were labeled and treated as described in Materials and Methods. Values are means ± SD, N = 3.

‡ P = 0.001 (vehicle vs THC-treated cells).

highly responsive to the presence of AlF_4. It has been suggested that AlF_4 mimics the γ-phosphate of GTP and functions as an activator of the trimeric G-protein–GDP complex [15]. In labeled mouse peritoneal cells, we found that AlF_4 elicited a massive release of free arachidonic acid (Fig. 3). The response could not be stimulated further by THC, indicating that the THC-sensitive and the AlF_4-sensitive pools of arachidonate may be derived from the same source. This supports earlier observations that the release reaction is a G-protein-mediated response.

Concentration–response relationship dependence on vehicle. Cells were labeled by equilibration with [^{14}C]arachidonic acid as described in Materials and Methods. Under these conditions, the various phospholipid and neutral lipid pools take up 80–90% of the added radioactivity and provide a suitable system to test for agonist-induced release of arachidonate. THC in two vehicles, 95% ethanol and 50% DMSO, was studied over a range of concentrations for its effect on arachidonate release (Fig. 4). The final concentration for each vehicle was 1% for ethanol and 0.5% for DMSO. We observed that the potency of THC was reduced markedly in the presence of ethanol. A possible explanation for this observation is that ethanol reacts with a phospholipid intermediate, effectively shunting it from the metabolic pathway leading to arachidonic acid mobilization. Alternatively, ethanol might directly interfere with receptor interactions of

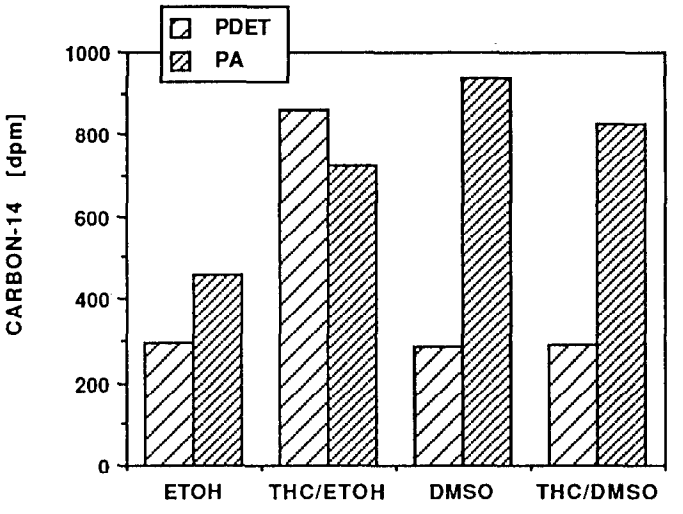

Fig. 5. Phosphatidylethanol (PdEt) and phosphatidic acid (PA) formation following THC treatment. Peritoneal cells (2×10^6/well) were prepared, labeled and analysed by TLC as described in Materials and Methods. Equipotent concentrations of THC (16 μM in ethanol and 4 μM in DMSO) in terms of arachidonate release were used (see Fig. 4). The values shown were obtained by liquid scintillation counting of the appropriate TLC zones and are representative of three separate experiments.

THC; however, we feel that this is a less likely explanation.

Stimulation of PLD activity following THC exposure. To support the contention that the vehicle effect (*vide supra*) is due to a reaction with a phospholipid intermediate, we subjected extracts from labeled cells to chromatographic analysis. Concentration-related increases in free arachidonic acid were seen (Table 1) in agreement with the results shown in Fig. 4. Concomitant concentration-related increases in phosphatidylethanol were observed specifically when ethanol, and not DMSO, was used as the vehicle for THC (Table 1). This lipid is generally considered to be an indicator of agonist-promoted PLD activity when ethanol is present [13]. The apparent formation of PdEt in the DMSO-treated cells is probably due to reactions occurring during the 2-hr labeling period. An analysis of untreated cells, immediately following arachidonate labeling, showed the same levels of PdEt as found in the DMSO-treated cells. We believe that this is due to the presence of small amounts of ethanol during labeling, which could lead to PdEt formation.

Further evidence for the activation of PLD is seen in Fig. 5 where we give data on the formation of PA, the normal product of PLD action on substrates such as phosphatidylcholine. Its formation was measured following treatment of cells with 16 μM THC in ethanol and 4 μM THC in DMSO; these concentrations gave approximately equal amounts of released arachidonate (Fig. 4). In ethanol, we saw an increase in PA with THC that was parallel to that of PdEt; however, in DMSO, neither the level of PA nor that of PdEt changed in the presence of THC. This could possibly be due to downstream effects of THC in DMSO since PA is subject to further metabolism in intact cell systems. The generally elevated levels of PA in the DMSO- versus ethanol-treated cells support the idea of PLD activation by THC and the occurrence of PdEt formation.

Inhibition of PLD and phosphohydrolase activities. In view of the above findings, suggesting a role for PLD, we studied two agents known to affect PLD action by rather different mechanisms. Wortmannin, which is believed to uncouple receptors to PLD [16], and propranolol, which is an inhibitor of phosphohydrolase [17], were examined for their effects on THC-induced arachidonate release. As shown in Fig. 6, both compounds inhibited the release of arachidonic acid. The inability of propranolol to completely inhibit release suggests that other parallel pathways might also comprise part of the signaling cascade mediating this cannabinoid response.

Diacylglyceride involvement as a source of arachidonic acid. In addition to PA, we were able to monitor DAG levels in which radiolabeled arachidonate was shown to be present by chromatographic analysis of cell extracts. Figure 7 shows the time dependence of DAG levels as well as that of PA and released arachidonic acid. As expected, arachidonic acid in the medium increased steadily over the 30-min duration of the study. Since the medium was changed at time zero, the initial levels were quite low; however, at that point, levels of labeled cellular PA and DAG were relatively high as a result of the 2-hr labeling period. After the addition of THC, there was a rapid small decrease in PA levels followed by a period of no measurable change. This suggests that because of its intermediate position, the rate of synthesis of PA is the same as its rate of conversion to DAG and other products. Arachidonyl-containing DAG levels, on the other hand, showed a steady decline following THC

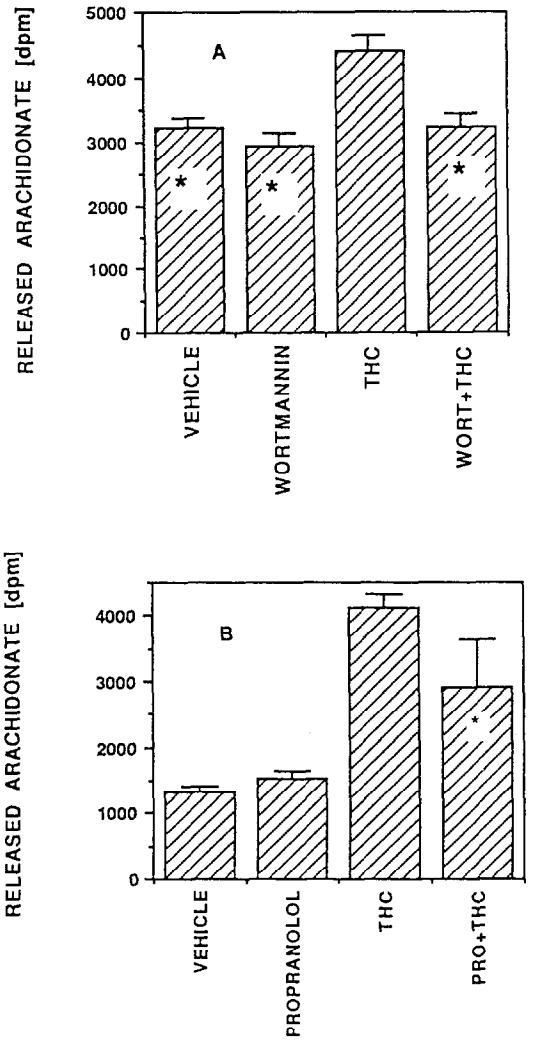

Fig. 6. Inhibition of phospholipase D and phosphohydrolase activities. Cells were treated and labeled as described in Materials and Methods. (A) Wortmannin (1.0 μM) in 50% DMSO was added to the cells 15 min prior to the THC (2.0 μM) in 50% DMSO. Values are the means ± SD of four determinations. Key: (*) Indicates 95% confidence level by ANOVA vs THC. (B) Propranolol (100 μM) in water was added to the cells 15 min prior to the THC (8 μM) in 50% DMSO. Values are the means ± SD of four determinations. Key: (*) Indicates 95% confidence level by ANOVA vs THC.

exposure. This observation is consistent with the suggestion that, at least part of the released radiolabeled arachidonic acid, arises directly from DAG.

Further evidence for a precursor role for DAG comes from the use of a diglyceride lipase inhibitor, RHC-80267 [18], which significantly inhibited THC-induced arachidonic acid release at low, but not high concentrations of THC (Fig. 8). The inhibitor was present at a concentration of 15 μM and at this level had no effect on the basal release of arachidonic acid (data not shown). When challenged with THC at 3.2 and 16 μM concentrations, inhibitor-treated cells showed about a 50% reduction in the release response while a concentration of 32 μM THC resulted in a much smaller inhibition (Fig. 8). It appears from these findings that DAG is the immediate source for about half of the arachidonate acid released through THC action.

Evidence for PLA_2 participation. Lin *et al.* [19] have reported that the exposure of WI-38 human lung fibroblasts to the cytokine IL-1α for periods of greater than 5 hr causes increased $cPLA_2$ expression levels in these cells. We performed a similar experiment on mouse peritoneal cells and, following 18 hr of IL-1α treatment, the cells were labeled with [^{14}C]arachidonic acid as described in Materials and Methods, and then challenged with 1.3 μM THC in DMSO for 30 min. A small increase in the vehicle-treated cells was observed; however, the THC challenged cells showed a 45% increase in arachidonic acid release for the cytokine-treated cells versus control cells (Table 2). Prolonged exposure of macrophages to phorbol ester (TPA) has been shown to decrease agonist-induced arachidonate mobilization probably by down-regulation of PKC [20]. We observed a similar effect with THC as seen by the data shown in Fig. 9 where as much as 70% of the release could be blocked by an 18-hr pretreatment of the cells with TPA. These results again suggest involvement of PLA_2 at some stage of the response.

Confirmation of the stimulatory action of THC on PLA_2, as well as its induction in peritoneal cells by IL-1α, was obtained by SDS–PAGE immunoblot analysis of cell extracts. A polyclonal antiserum raised against purified, cytosolic, 85 kDa PLA_2 [14] was used to probe the samples obtained from western blotting and revealed that THC did cause a concentration-dependent increase in phosphorylated $cPLA_2$ (Fig. 10), as evidenced by the appearance of a slightly slower moving band that has been shown to be indicative of such a process [19]. The levels of unphosphorylated $cPLA_2$ were decreased only slightly by THC; however, IL-1α treatment did increase $cPLA_2$ levels in agreement with the findings of Lin *et al.* [19].

DISCUSSION

The question we sought to address in the present study is, what signal transduction events lead from THC binding to the eventual release of free arachidonic acid and its ultimate conversion to various eicosanoid mediators? A recent report by Munro *et al.* [5] gave evidence for a cannabinoid receptor, different from the brain receptor, that is found at peripheral sites such as splenic macrophages. In this study, we have presented evidence (Fig. 1) that suggests the existence of a cannabinoid receptor in a very similar cell type, the mouse peritoneal macrophage. We have further shown (Fig. 2) that a potential functional consequence of cannabinoid binding to this site is the mobilization of phospholipid bound arachidonic acid. Unfortunately, Munro *et al.* [5] did not report any data suggesting a functional relationship for their cloned receptor. By contrast,

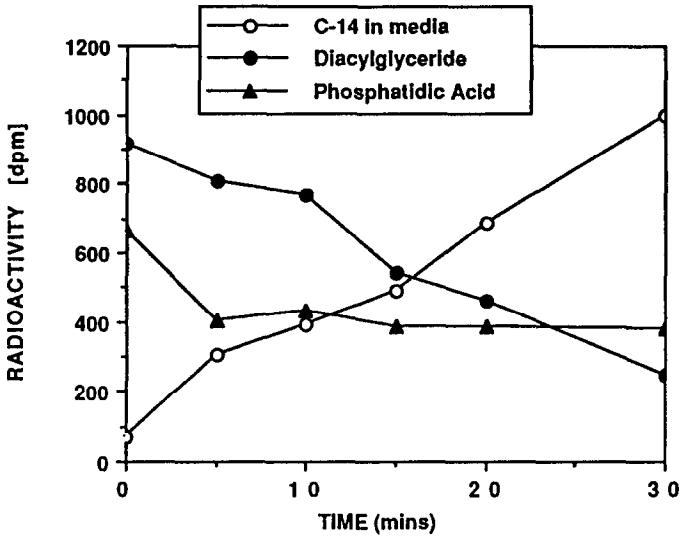

Fig. 7. Time dependence of diacylglyceride (DAG), phosphatidic acid (PA) and media C-14 levels. Cells (2×10^6/well) were prepared and labeled for 2 hr as described in Materials and Methods. Following thorough washing, the cells were challenged with 8 μM THC in DMSO, and the results were monitored at the indicated times. DAG and PA were measured by TLC analysis as described in Materials and Methods. The media content of C-14 was measured by liquid scintillation counting of duplicate aliquots and consists of greater than 90% free arachidonic acid.

a number of studies, as described in Ref. 21, have demonstrated that the brain receptor, cloned by Matsuda *et al.* [4], is negatively coupled to adenylate cyclase; however, the *in vivo* significance of this process still lacks direct experimental evidence. In the case of arachidonic acid release, we have shown that one of the sequelae is eicosanoid synthesis, both *in vitro* and *in vivo*, and that certain cannabimimetic responses, such as catalepsy in the mouse, are best understood by this mechanism [1]. These observations raised the obvious question concerning the nature of the molecular events that follow THC binding to the macrophage receptor leading to the release of eicosanoids.

The possible participation of a G-protein in the THC-induced release of arachidonate from mouse peritoneal cells was suggested in an earlier report from our laboratory [3]. The data given on the effects of pertussis toxin, cholera toxin, GDP-β-s and GTP-γ-s were all consistent with the idea that this cannabinoid action is initiated by a receptor–G-protein complex. In the present report we have

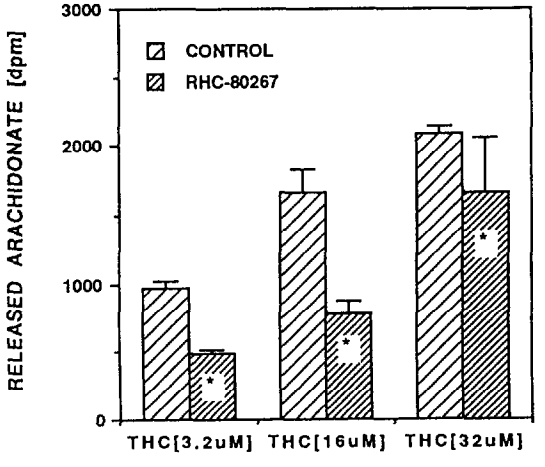

Fig. 8. Decreased arachidonate release following treatment with the diacylglyceride lipase inhibitor, RHC-80267. Cells were labeled and treated as described in Materials and Methods. The inhibitor (15 μM) in ethanol was added 15 min prior to the THC (also in ethanol). RHC-80267 at a concentration of 15 μM had no measurable effect on basal release values. Data are the means ± SD of four values minus vehicle challenged cells (1010 ± 110 dpm). Key: (*) Indicates 95% confidence levels vs control (no RHC-80267).

Table 2. Increased THC-induced arachidonic acid release following interleukin-1α treatment

Treatment*	Released arachidonic† (dpm)	Increase (dpm)
DMSO	1460 ± 270	
THC (1.3 μM)	2780 ± 580	1320
IL-1α + DMSO	1680 ± 50	
IL-1α + THC (1.3 μM)	3600 ± 440	1920

* Cells were treated with IL-1α (0.2 nM) for 18 hr after which they were labeled with [^{14}C]arachidonic acid and challenged with THC (1.3 μM) for 30 min; the extent of release was measured as described in Materials and Methods.

† Values are means ± SD, N = 3. ANOVA of the data resulted in P = 0.0001.

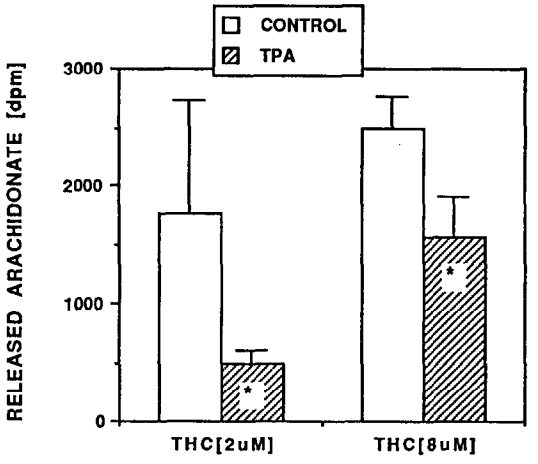

Fig. 9. Effect of phorbol ester pre-exposure on arachidonic acid release. Data designated as "TPA" were obtained from cells in which phorbol ester (1.0 μM) was added 18 hr prior to the release procedure. Control cells were exposed to vehicle for the same period. At the end of the pretreatment, the cells were labeled and challenged with THC in DMSO as in Fig. 4. The values shown are the means ± SD of four monolayers minus vehicle-challenged values. Key: (*) Indicates 95% confidence levels vs control. Released arachidonate from the control was 2050 ± 220 dpm.

data supporting the concept that THC-induced arachidonate release is a heterotrimeric-G-protein-mediated process. Figure 3 gives the results of an experiment in which fluoroaluminate pretreatment of the cells, prior to a THC challenge, causes a profound dampening of the drug-induced response. Fluoroaluminate is believed to hold certain G-protein α-subunits in their active state by substituting for the γ-phosphate of GTP, thereby inhibiting further activation by agonist–receptor complexes [15]. Such effects are not seen with other GTP-binding proteins like the members of the RAS superfamily.

A comparison of vehicles for THC used in measuring the concentration–response relationships shown in Fig. 4 hinted at the possibility that phospholipase D may be involved in THC-induced arachidonate release in peritoneal cells. The significant shift to higher concentrations in ethanol compared with DMSO could be explained by the formation of phosphatidylethanol following receptor activation by THC. It is well known that the presence of ethanol during PLD action on phosphatidylcholine can reduce the formation of PA, the normal product of the process. Along with PA, PdEt is formed and, since this molecule is unable to give rise to PA, or DAG, a net reduction in arachidonate release is observed. In fact, over the concentration range of 0.5 to 8 μM where THC in DMSO showed a significant concentration-related response, THC in ethanol caused virtually no release of arachidonic acid (Fig. 4).

If the above speculation is correct, it should be possible to observe a concentration-related increase in PdEt formation following THC exposure. Data to this effect are shown in Table 1 where a comparison of two vehicles revealed that, as expected, THC in DMSO did not give rise to increased levels of PdEt whereas, in ethanol, a THC-induced increase was seen. The formation of PdEt is considered to be highly specific for PLD action so that the results in Table 1 suggest that this lipase is involved in THC-stimulated arachidonic release. The normal product of PLD action is PA whose formation we were also able to observe in our system (Figs. 5 and 7). As would be predicted, the levels of PA were higher in the absence of ethanol consistent with the production of PdEt when this alcohol is present. Since PA is subject to further transformations and may arise from several sources, its levels may not necessarily reflect a specific action on PLD. This may explain why we did not see an increase in PA with time following THC treatment under conditions where arachidonic acid release was observed (Fig. 7).

There are few truly specific inhibitors of PLD so that this type of approach to showing a role for PLD is somewhat limited. One substance claimed to act by reducing the coupling of receptors to PLD is wortmannin [16], although it may also inhibit certain PLC coupled pathways [22]. In our system, 1 μM wortmannin given to the cells 15 min prior to a challenge with THC reduced the formation of PdEt (data not shown), suggesting that, under these conditions, it did exert a major effect on THC-induced PLD activation. The inhibitory action of wortmannin on arachidonate release is shown in Fig. 6A. We were able to demonstrate a complete reduction, in release, consistent with the direct evidence discussed above suggesting that PLD is an important mediator of THC action.

The product of PLD action, PA, is converted to DAG by the action of a phosphatase. The drug propranolol has been reported to inhibit this process [17]; thus, we thought it would be of interest to study its effects in our system. The data in Fig. 6B show that while propanolol (100 μM) had little effect on control values, pretreatment of the cells for 15 min caused a sizeable reduction in the THC response. These observations further support a role for PLD action and suggest that DAG may play an important role in the process, either as a source of arachidonic acid or, possibly, as a messenger molecule. It is interesting to note that propranolol has been reported to antagonize several of the effects of marihuana in humans [23]. Pretreatment of the subjects with propranolol completely abolished the marihuana-induced cardiovascular effects and also prevented the marihuana impairment on a learning task paradigm. Some reduction of the subjective "high" associated with cannabis was also observed.

In some systems, DAG has been shown to be a major source of arachidonic acid for eicosanoid synthesis. This appears to be the case for THC-stimulated release in mouse peritoneal cells, as our data indicate. The fall in DAG levels with time as free acid levels increased (Fig. 7) suggests a precursor–product relationship between the two that would support the idea that not all of the free arachidonic acid comes directly from phospholipid precursors by the action of PLA_2. To further test for the possibility that part of the released

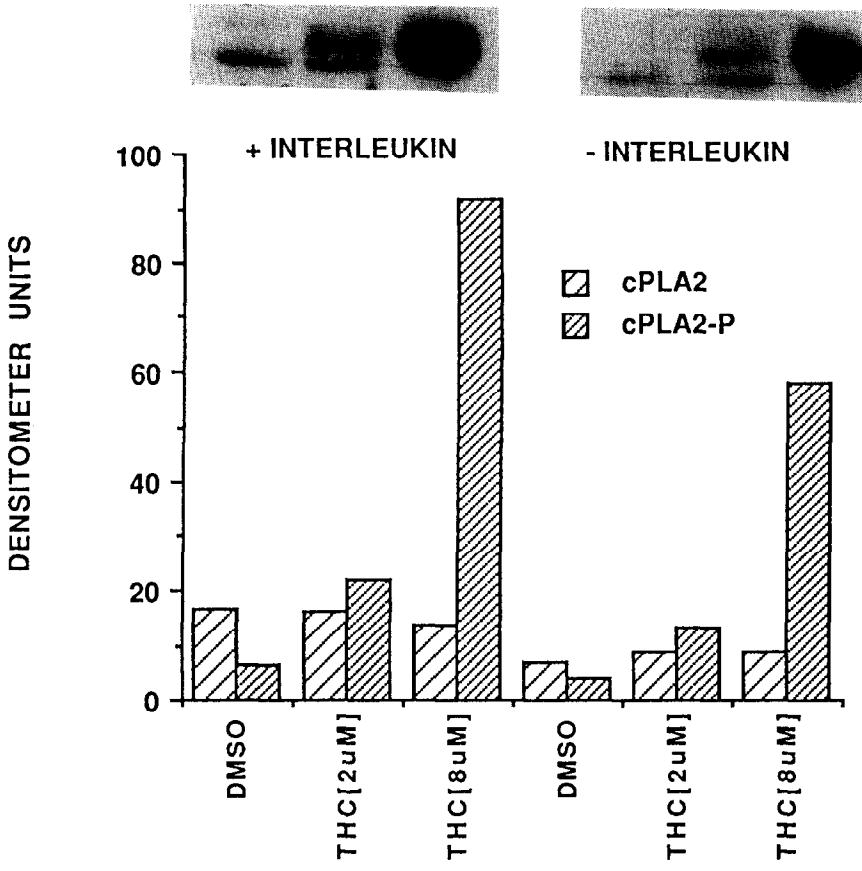

Fig. 10. Western blot analysis with anti-PLA_2 following THC exposure. Cells were prepared as described in Materials and Methods and treated with THC or vehicle (50% DMSO) for 30 min. Detergent extracts were made and subjected to SDS–PAGE (7.5%) followed by transfer to Immobilon-P. The membrane was probed with a polyclonal antiserum raised against purified, cytosolic, 85 kDa PLA_2 [14], and reactive zones were detected by enhanced chemiluminescence. Quantitative estimations of the immune complexes were obtained by laser densitometry. $cPLA_2$-P = phosphorylated $cPLA_2$.

arachidonate arises from DAG, we utilized a selective diglyceride lipase inhibitor, RHC-80267 [18]. This compound is reported to have no effect on platelet PLC, and only a small inhibition of PLA_2 was seen at a concentration of 100 μM. We found that 15 μM RHC-80267 effectively reduced arachidonate release in peritoneal cells exposed to THC (Fig. 8). The extent of the inhibition suggests that up to half of the free arachidonic acid could come from DAG.

The remainder of the released arachidonate not arising from DAG directly could conceivably be accounted for by a signal transmitted through PKC. In fact, there are published data showing that PKC causes activation of phospholipase A_2 via the action of a MAPK [24]. Such a transduction route involving messenger DAG could result in the generation of free arachidonic acid from a source such as phosphatidylcholine. Some of our earlier studies on THC-induced arachidonate release did suggest the involvement of PLA_2 at some stage [7, 8]; however, the potential complexity of the process was not understood at that time.

Data supporting PKC participation in the release effect were obtained from an experiment designed to down-regulate the activity of this kinase in peritoneal cells. In alveolar macrophages, it was reported that exposure to phorbol ester for 5 hr reduces PKC activity by about two-thirds [20]. We found that an 18-hr exposure of our cells to 0.5 μM TPA reduced THC-induced arachidonic release by 72.5% (Fig. 9). A possible explanation for this effect is that down-regulation of PKC had occurred, and, therefore, this enzyme is a part of the signal transduction process involved in cannabinoid-mediated arachidonate release. Further support for the involvement of PKC came from an experiment with sphingosine in which a concentration-related inhibition of release was observed when challenged with 4 μM THC (data not shown). Interestingly, Kadiri *et al.* [20] reported an almost identical sphingosine inhibition of arachidonate release in alveolar macrophages that were challenged with PAF or fMLP. This suggests that there may be a significant degree of commonality in the mechanisms involved in arachidonate release between cannabinoids and agonists such as PAF or fMLP. Possibly the peripheral THC receptor and the

receptors for PAF and fMLP are coupled to similar elements of a G-protein complex.

Finally, we have obtained direct evidence that suggests a role for cytosolic PLA_2 in the release reaction. Lin *et al.* [19] have reported recently that treatment of WI-38 human lung fibroblasts with IL-1α results in the accumulation of $cPLA_2$ by increasing its expression levels. If this effect of IL-1α were to occur in mouse peritoneal cells, a subsequent challenge with THC should result in a significant increase in arachidonate release provided that $cPLA_2$ were an important factor. Interestingly, an 18-hr exposure of the cells to the cytokine resulted in a 44.8% elevation of free arachidonic acid released into the medium following a THC challenge (Table 2), consistent with a postulated increase in $cPLA_2$ expression levels. The results obtained following IL-1α treatment were characterized further by an SDS–PAGE immunoblotting study that is shown in Fig. 10. It has been demonstrated in other cell types that $cPLA_2$ is activated by phosphorylation and that this can be monitored by a western blot analysis where the $cPLA_2$ position is shifted to a slightly slower moving position following kinase action [24]. For this study, we used a polyclonal antiserum obtained by the use of purified macrophage $cPLA_2$ as the antigen [14]. Vehicle-treated cells showed virtually no phosphorylated $cPLA_2$, whereas THC-treated cells exhibited concentration-related increases in phosphorylated enzyme in close agreement with the effects of the drug on arachidonic acid release. The same patterns were observed in cells with or without IL-1α except that the overall effects were greater with the cytokine in agreement with its reported ability to increase the expression of $cPLA_2$ [19]. Thus, our data fully support the suggestion that part of the release effect of THC is due to PLA_2 participation.

Our findings suggest a central role for DAG in the THC-induced release of arachidonic acid and further suggest that part of the DAG originates from a PLD-mediated pathway. The data also indicate that at least one-half of the arachidonic acid arises directly from a DAG precursor by the action of a DAG lipase. An earlier report from this laboratory showed that synaptosomal DAG lipase could be inhibited weakly by THC [8]. This suggests that the dominance of this pathway may very well be a function of the tissue type under consideration. A second role for DAG in THC action is also suggested by our data, namely, the activation of an isoform of PKC. It is now fairly well established that $cPLA_2$ is a major mediator in eicosanoid formation and that secretory PLA_2 has no role in this process [24]. It has been shown that $cPLA_2$ is phosphorylated and translocated to a membrane site where it exerts its effect. A recent report [24] demonstrated that MAPK action is involved in this process and that it can be activated by either a PKC-dependent or a PKC-independent pathway. Our data suggest that PKC may have a role in THC-induced arachidonic acid release in the peritoneal cell and, therefore, by inference that MAPK may also be involved. At present, however, there is no direct experimental evidence for this last point.

By considering all of the above findings, it is possible to propose a signaling pathway that would lead from the binding of THC by a receptor to the eventual release of free arachidonic acid. Thus, following the binding event, PLD would be activated by G-protein mediation, either by an α subunit, or possibly a $\beta\gamma$ subunit. Our data do not clearly distinguish between these two alternatives. PLD would act on one or more of the phospholipid pools to produce PA that would be converted to DAG by a phosphohydrolase. DAG containing arachidonate, at the 2 position, could serve as a substrate for diglyceride lipase giving rise to free arachidonic acid. A second route, in which PKC is activated, would be initiated by DAG acting as a messenger molecule. In this role, the DAG may or may not contain arachidonate. This would result in the activation of PLA_2, possibly involving the action of a MAPK; however, we have no direct experimental evidence for MAPK mediation. The phosphorylation of PLA_2, as evidenced by our western analysis, would result in the translocation of the $cPLA_2$ to the plasma membrane where it would act on one or more of the arachidonate containing phospholipid pools to release free arachidonic acid. Finally, we cannot rule out the simultaneous operation of a PLC-mediated route that could also lead to DAG and arachidonic acid release, based on the available data.

The findings reported here will help to resolve the issues surrounding the cannabinoid-induced mobilization of arachidonic acid and the resulting elevation of eicosanoid levels. Two of the questions over which there is a lack of agreement are (a) is the process initiated by a ligand–receptor binding event, and (b) is the effector molecule a phospholipase or an acyltransferase? The report of a cannabinoid receptor isotype in macrophages [5] and our findings that show a relationship between binding of THC and its ability to induce arachidonic acid release support the idea that this effect of THC is receptor initiated. In addition, the data demonstrating G-protein participation (Fig. 3) are difficult to explain by a mechanism involving membrane perturbation as has been suggested by others [9, 10]. While there are numerous precedents showing that PLD activation is initiated by receptor–ligand interactions, there do not appear to be any documented examples of such a process being initiated by physical changes in cell membranes.

The second question, namely, inhibition of uptake versus stimulation of release as the cause of cannabinoid-induced elevation of arachidonic acid is difficult to answer by studying systems in which the potential for both processes exists, as is the case for most of the reports dealing with this issue [1]. Reichman *et al.* [9] concluded that cannabinoids elevate free arachidonic acid in cortical brain slices by inhibiting acyltransferase; however, their data are also consistent with a PLD-mediated process. In fact, they reported that PA levels were elevated by cannabinoids; this is a direct product of PLD action and can lead to arachidonate release via a DAG intermediate for which we have given evidence (Figs. 7 and 8). Felder *et al.* [10] also favor a non-receptor-mediated inhibition of acyltransferase as the mechanism in their model, which involves a comparison of responses in cells transfected with the

cloned brain cannabinoid receptor versus wild type cells. A potential difficulty with this system is that while the transfected cells contain greater numbers of brain receptors, they may not have correspondingly elevated levels of the particular G-protein required to transduce the signal resulting in arachidonate release. For example, Winitz *et al.* [25] have shown recently that there are strict structural requirements for G-protein participation in $cPLA_2$-mediated arachidonic acid release. Thus, the weight of the evidence at this stage would favor a receptor-mediated stimulation of arachidonic acid release as the primary mechanism responsible for the elevation of eicosanoid levels following cannabinoid exposure.

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