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Short Communication

STIMULATION OF ANANDAMIDE BIOSYNTHESIS IN N-18TG2 NEUROBLASTOMA CELLS BY Δ^9-TETRAHYDROCANNABINOL (THC)

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Abstract—A concentration-related stimulation of anandamide (arachidonylethanolamide) synthesis by Δ^9-tetrahydrocannabinol (THC) was observed in N-18TG2 neuroblastoma cells. Anandamide was detected and measured using an approach in which [^{3}H]arachidonic acid and [^{14}C]ethanolamine were incorporated into the phospholipids of subconfluent monolayers of cells, and the radiolabeled products were analyzed by TLC following agonist exposure. Both precursors showed similar concentration–response relationships and time dependencies consistent with the production of a product containing both the ethanolamine and arachidonyl moieties. The radiolabeled product also migrated together with authentic anandamide on two-dimensional TLC, confirming its identity as arachidonylethanolamide. Approximately two-thirds of the observed synthesis could be inhibited by 1 μM wortmannin, an agent previously reported to inhibit THC-stimulated arachidonic acid release. These findings are in agreement with reports showing that THC can mobilize phospholipid bound arachidonic acid, leading to the production of other eicosanoids.

Key words: anandamide; THC; N-18TG2 cells; arachidonic acid; ethanolamine; eicosanoids

A putative endogenous ligand for cannabinoid receptors has been isolated recently from brain lipid extracts and discovered to be a member of the eicosanoid family [1]. Its structure was shown to be the ethanolamide derivative of AA† [1], suggesting that its biosynthesis might involve the coupling of some form of AA with EA, presumably in a physiologically regulated process. Anandamide displays many of the biological activities of the exogenous cannabinoids [2–6] and may serve as a neuroregulatory substance analogous to the classical eicosanoids such as the prostaglandins and leukotrienes [7].

Despite its recent discovery [1], several papers have already been published on the enzymic nature of anandamide biosynthesis [8–10]. Deutsch and Chin [8] reported that crude rat brain homogenates have the ability to catalyze the formation of anandamide from free AA and EA. They also demonstrated the presence of hydrolytic activity that could reverse the synthesis; however, it is not clear whether the same site is involved. A second report by Kruska and Gross [9], in which subcellular fractions from rabbit brain were used, gave evidence that anandamide synthesis is, apparently, CoA and ATP independent, is selective for AA, specific for EA, and calcium independent. They suggested that a novel process was involved and that intracellular phospholipases may be important mediators in anandamide synthesis under physiological conditions. Using bovine brain tissues, Devane and Axelrod [10] also found that AA is the preferred substrate for anandamide synthase, although certain other polyunsaturated fatty acids could be coupled to EA in their system. They also reported that membrane preparations from the hippocampus showed the highest specific activity and suggested the existence of anandamide-containing neurons.

Key questions to be addressed at this time are whether there are specific agents that can initiate the biosynthesis of anandamide and what they might be. Clearly, substances that are known to promote the release of free AA from cellular lipid pools would be likely candidates. While the literature is replete with such molecules, an especially interesting possibility is the major psychoactive exogenous cannabinoid, THC. We have demonstrated recently that THC can promote AA release in mouse peritoneal cells through a receptor-mediated process involving phospholipase D [11]. This process would not only provide free AA but could also result in elevated cellular EA levels in the event that phosphatidylethanolamine is the substrate. In this report, we give evidence showing that, indeed, THC can promote anandamide synthesis in intact cells, suggesting a possible mechanism for THC action.

Materials and Methods

N-18TG2 cells [12] were grown at 37° under 95% O_2:5% CO_2 in DMEM, high glucose, 1% penicillin:streptomycin containing 10% FBS to 70% confluence in 12 well plates. The cells were prepared from a stock supply maintained by the University tissue culture facility. Cells were labeled with either 2.5×10^5 dpm [^{14}C]EA (ARC, Inc., St. Louis, MO; sp. act. 55 mCi/mmol) for 18 hr or 2.0×10^5 dpm [^{3}H]AA (NEN, Boston, MA; sp. act. 100 Ci/mmol) for 2 hr in 0.1% FBS:DMEM. Media were monitored for uptake, and it was found that 80% EA and 90% AA were incorporated. The cells were then washed three times with 0.1% BSA:DMEM to remove essentially all of the free AA and EA. Each well containing 1 mL DMEM: 0.1% BSA and either 10 μL vehicle, or drug, was incubated at 37° for 30 min unless otherwise specified. Media were added immediately to the extraction solvents:

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† AA, arachidonic acid; EA, ethanolamine; THC, Δ^9-tetrahydrocannabinol; PLC, phospholipase C; PLD, phospholipase D; DMEM, Dulbecco's modified Eagle's medium; and FBS, fetal bovine serum.

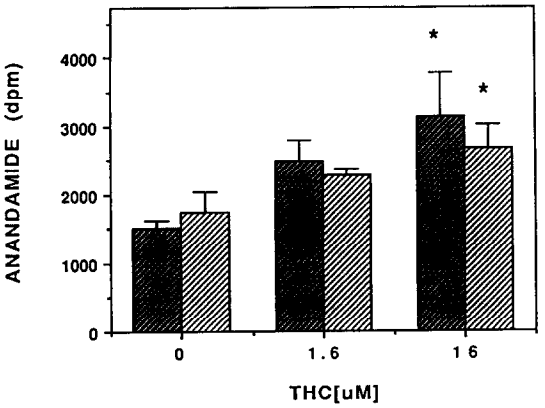

Fig. 1. Stimulation of anandamide synthesis by THC. Cells were grown and labeled with [^{3}H]AA or [^{14}C]EA, as described in Materials and Methods. THC in 95% ethanol (10 μL) was added, and after 30 min the cells were extracted and analyzed by TLC. Controls were vehicle-treated cells and gave values for anandamide of 430 dpm for [^{3}H]AA precursor and 1500 dpm [^{14}C]EA precursor. Dark bars are EA and light bars are 4 × AA responses. Values shown are the means of triplicate determinations for a typical experiment. Key: * indicates 95% significance level when compared with controls.

Table 1. THC-induced arachidonic acid release in N-18TG2 cells

Treatment*	Released arachidonic acid† (dpm)
Vehicle	520 ± 70
THC (1.6 μM)	580 ± 10
THC (16 μM)	1200 ± 140

* Cells were grown and labeled with [^{3}H]AA, as described in the legend of Fig. 1. Following a 30-min exposure to THC, the cells and media were extracted and analyzed by radio-TLC.

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

chloroform:methanol:0.05 M KCl:water (12:6:1:5, by vol.) including 2 μg unlabeled anandamide (Biomol, Plymouth Meeting, PA). The cells were extracted twice with 1 mL methanol, which was then added to the extraction mixture. The combined extracts were evaporated to dryness under nitrogen, applied in 2 × 40 μL $CHCl_3$:MeOH (2:1, v/v) to silica gel TLC plates and chromatographed in chloroform:methanol:glacial acetic acid (90:6:6, by vol.) with appropriate standards; R_f values for authentic anandamide and AA were 0.56 and 0.73, respectively. Two-dimensional TLC was done using the above system in the first direction and the system published by Deutsch and Chin [8] in the second direction where we found that anandamide showed an R_f of 0.25. Plates were sprayed with phosphomolybdic acid, and the anandamide and AA zones were assayed for radioactivity by liquid scintillation counting. Alternatively, the plates were scanned using a Molecular Dynamics phosphorimager. Data were analyzed using a Statview 512+ statistics program (Abacus Concepts, Inc.), and curve fitting was done with Kaleidagraph 2.1 (Abelbeck Software).

Results and Discussion

Treatment of subconfluent monolayer cultures of N-18TG2 cells with THC resulted in correspondingly greater amounts of radioactivity in the area of the TLC plate where unlabeled, authentic anandamide appeared when compared with extracts from vehicle-treated cells (Fig. 1). The TLC systems used in this report were similar to those used for anandamide analysis by others [8–10], and the identity of the product was further established by a two-dimensional TLC analysis. For this purpose, an extract obtained following exposure to 16 μM THC was applied to a silica gel TLC plate and eluted, as described in Materials and Methods. Duplicate experiments were performed in which radioactivity was found in the anandamide zone when both EA and AA were used as precursors. A slightly slower moving product was also observed throughout these experiments; however, its

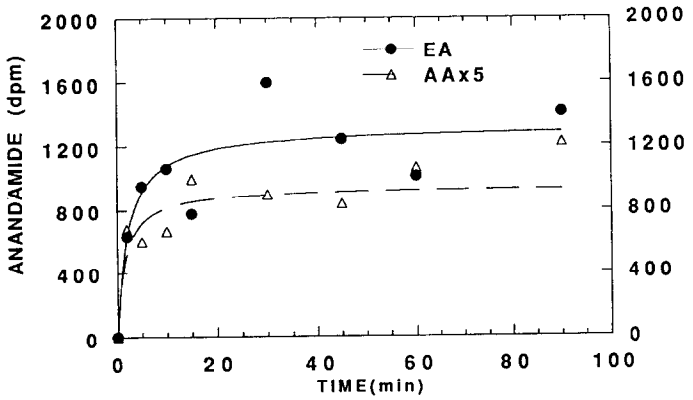

Fig. 2. Time course for the stimulation of anandamide synthesis by THC. Cells were grown, labeled and treated as described in the legend of Fig. 1 except that the exposure times were varied as shown. The THC concentration was 16 μM, and all points were measured in triplicate. Curve fitting was done by non-linear regression analysis, as described in Materials and Methods. Correlation coefficients were 0.87 for each of the two plots.

Table 2. Inhibition of THC-stimulated anandamide synthesis by wortmannin

Treatment*	Control† (dpm)	THC† (dpm)	% Change (THC vs Control)
Vehicle	320 ± 130	480 ± 110	+49.8
Wortmannin (1 μM)	540 ± 120	370 ± 40‡	−32.5
Wortmannin (10 μM)	470 ± 220	350 ± 90	−24.9

* Cells were grown and labeled with [^{3}H]AA as described in the legend of Fig. 1. Wortmannin in 50% DMSO (10 μL) was added to the cells 15 min prior to 16 μM THC in 50% DMSO. After 30 min, the cells and media were extracted and analyzed by radio-TLC.

† Values are means ± SD, N = 3. ANOVA of the data resulted in P = 0.03.

‡ 95% significance, control vs THC.

identity is unknown at this time. It is unlikely to be an acid analog of the type reported by Devane and Axelrod [10] and probably contains EA since it also appeared when [^{14}C]EA was a precursor. Possibly, it is a hydroxylated metabolite of anandamide of the type seen by Bornheim *et al.* [13]. The time dependency of anandamide synthesis is given in Fig. 2 and shows a rapid synthesis of anandamide with either precursor reaching a steady state in about 20 min (k = 0.30 and 0.29/min, R = 0.87, for EA and AA, respectively).

The close similarities in both concentration and time dependencies for THC-stimulated anandamide synthesis when the labeled precursor used was either AA or EA strongly support the conclusion that the labeled product seen on TLC is, in fact, anandamide. There are no known substances that contain both AA and EA moieties that also show the same TLC mobility as anandamide. Moreover, these similarities suggest the possibility that both precursors could arise from the same cellular storage pool. A likely candidate for such a common precursor would be 2-arachidonyl-phosphatidylethanolamine, which has been reported to be a major constituent in N-18TG2 cells [12].

Several reports have shown that THC is able to mobilize AA in a variety of experimental models including humans [14]. Recently, it has been further demonstrated that, in mouse peritoneal cells, this involves a receptor-mediated activation of phospholipases, including PLD [11]. Table 1 gives data showing that the system utilized for this report, the N-18TG2 cell, also responds to THC exposure by releasing AA. It has also been observed that part of the released AA in most cell types is subsequently converted to one or more eicosanoids depending on the nature of the particular system. In N-18TG2 cells we have also found that THC-stimulated levels of prostaglandin E_2 were comparable to those seen for anandamide (data not shown).

Evidence suggesting some similarity of these findings to the effects of THC reported for macrophages [11] is provided in Table 2. The data shown indicate that about two-thirds of the stimulatory effect of THC on anandamide synthesis can be inhibited by prior treatment of the cells with wortmannin. Wortmannin, at the concentrations used by us, has also been reported to inhibit receptor-stimulated PLC as well as PLD [15], so that only limited conclusions can be drawn from its effects. In any case, the actions of both PLC and PLD would ultimately lead to the release of free AA via diacylglyceride intermediates. Moreover, both pathways could also result in increased cellular EA levels, either directly by PLD-mediated hydrolysis of the EA phosphate bond in phosphatidylethanolamine, or by cleavage of the glycerol–phosphate bond by PLC on the same substrate to yield phosphoryl EA that could then be hydrolyzed to give EA itself. Thus, the separate or combined actions of PLD and PLC could effectively provide precursors for cellular anandamide synthesis. The suggested mechanism for the THC-induced anandamide synthesis reported here is only a matter for speculation at this time; however, it may be similar to that involved in THC-stimulated eicosanoid synthesis, in general [11, 14].

The broader implications of the observations from this study could be quite significant. For example, a possible mechanism for the actions of *exogenous* cannabinoids such as THC is suggested whereby they would promote the synthesis of putative *endogenous* cannabinoids with an eicosanoid-like character, such as anandamide. This would be in general agreement with an earlier hypothesis on the relationship between cannabinoids and eicosanoids [14] since anandamide can certainly be considered as a novel eicosanoid. If this putative mechanism is valid, it is further suggested that the cellular responses to both exogenous and endogenous cannabinoids, may involve a positive feedback loop. This would require that anandamide itself show stimulatory effects on AA release and, in fact, such effects have been seen to occur in other systems.* A limit control for such a positive feedback loop could be the level of free AA, which Devane and Axelrod [10] have shown becomes inhibitory for anandamide synthesis at high concentrations.

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