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# ENZYMATIC SYNTHESIS AND DEGRADATION OF ANANDAMIDE, A CANNABINOID RECEPTOR AGONIST

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**Abstract** – Enzymatic activities have been identified which catalyze both the hydrolysis and synthesis of arachidonylethanolamide (anandamide). Anandamide was taken up by neuroblastoma and glioma cells in culture, but it did not accumulate since it was rapidly degraded by an amidase activity that resided mainly in the membrane fractions. This amidase activity was expressed in brain and the majority of cells and tissues tested. Phenylmethylsulfonyl fluoride (PMSF) was found to be a potent inhibitor of this amidase. A catalytic activity for the biosynthesis of anandamide from ethanolamine and arachidonic acid was readily apparent in incubations of rat brain homogenates. The stability of anandamide in serum and its rapid breakdown in cells and tissues are consistent with the observation that it is active when administered systemically, and its duration of action will be regulated by its rate of degradation in cells.

The psychoactive marijuana plant-derived cannabinoid, $\Delta^9$-tetrahydrocannabinol, and numerous synthetic derivatives have been shown to bind to a specific brain receptor [1,2,3,4]. Arachidonylethanolamide has been identified recently as a naturally occurring brain constituent that binds to this cannabinoid receptor [5]. Furthermore, using a series of behavioral tests to evaluate cannabinoid analogs [6], anandamide has been shown to be a cannabinoid receptor agonist exhibiting pharmacological activity in mice parallel to that of other psychotropic cannabinoids [7]. In this study, the uptake and breakdown of anandamide in neuroblastoma and glioma cell cultures were characterized. Subcellular fractions of these cultured cells and crude homogenates of other cultured cells and of rat tissues were assayed for enzymatic breakdown of arachidonylethanolamide to arachidonic acid. It was observed that phenylmethylsulfonyl flouride (PMSF) was a potent inhibitor of the breakdown activity (anandamide amidase). Employing ethanolamine and arachidonic acid as substrates, a synthetic activity (anandamide synthase) was readily demonstrated in crude homogenates of rat brain.

## MATERIALS AND METHODS

All cells were grown at 37° in a humidified atmosphere containing 5% $CO_2$ in air. Neuroblastoma cells (N18TG2) were grown in Ham's F12/DMEM (GIBCO, Grand Island, NY) with penicillin, streptomycin and gentamicin plus 10% bovine calf serum (HyClone, Logan, UT). Rat glioma (C6), human epithelioid carcinoma cells (HeLa), bronchioalveolar non-small cell lung carcinoma (H358), human larynx epidermoid carcinoma (Hep2), and human hepatocellular carcinoma (HepG2) all were grown in DMEM containing 10% bovine calf serum with penicillin and streptomycin.

Uptake and degradation of anandamide were studied in neuroblastoma and glioma cell cultures by adding 12 $\mu$Ci of [$^3$H]-anandamide (NEN, NET-1073, 210 Ci/mmol) to 5 mL of cell culture medium in 6 cm dishes containing approximately 6 x $10^6$ cells. At the end of the incubation, the medium was removed and the cells were

washed twice with phosphate-buffered saline (PBS). The cells were removed from the plates after a brief incubation with 2 mL of a 0.05% trypsin in 0.53 mM EDTA solution at 37°. The medium and cells were extracted with 2 vol. of chloroform:methanol (1:1). The organic extract was evaporated under nitrogen and redissolved in 40 $\mu$L of chloroform:methanol (1:1).

Crude homogenates of cultured cells were prepared with approximately 6 x $10^6$ cells in 3 mL of ice-cold TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) with a Dounce glass homogenizer. Subcellular fractions of cultured neuroblastoma and glioma cells were prepared by centrifuging the crude homogenate at 800 *g* for 10 min to obtain the sediment consisting of nuclei, whole cells and cellular debris. The supernatant was centrifuged at 12,000 *g* for 30 min to prepare the mitochondrial fraction and the supernatant was centrifuged at 100,000 *g* for 60 min to prepare the microsomal and soluble fractions.

Female adult Sprague-Dawley rats were decapitated and the desired tissues were dissected and homogenized in 5 vol. of ice-cold TE with a Polytron homogenizer (Brinkmann). These crude homogenates were stored at -80°. All incubations were conducted at 37° in a water bath with shaking. The reactions were terminated by the addition of 2 vol. of chloroform:methanol (1:1). The organic extract was evaporated under nitrogen and redissolved in 40 $\mu$L of chloroform:methanol (1:1). It was found, by liquid scintillation counting of the aqueous and organic phases, that arachidonic acid and anandamide distributed in the organic phase, whereas ethanolamine was virtually all in the aqueous phase.

Thin-layer chromatography (TLC) was performed on silica gel coated plates with a solvent system consisting of the organic layer of an ethyl acetate:hexane:acetic acid:water (100:50:20:100) mixture. The plates were sprayed with a surface autoradiography enhancer (EN$^3$HANCE, Dupont) and exposed to X-ray film (Kodak X-OMAT AR) at -80°. For quantitation, the plates were scanned on a Bioscan System 200 Imaging Scanner (Washington, DC). [$^3$H]-Arachidonate (NET-298Z, 210 Ci/mmol) was obtained from New England Nuclear and [2-$^{14}$C]-ethanolamine (54 mCi/mmol) was from Amersham. Anandamide was purchased from Cayman Chemicals (Ann Arbor, MI). Arachidonate, *Crotalus adamanteus* phospholipase $A_2$ ($PLA_2$), PMSF, and emetine were obtained from the Sigma Chemical Company (St. Louis, MO).

## RESULTS

When anandamide was incubated with N18TG2 or C6 cells, there was a time-dependent decrease of its levels in the media (Fig. 1). Anandamide was taken up by the cells immediately, but it did not accumulate since it was converted to arachidonate and other lipids containing arachidonate which migrate on TLC near phospholipids, triglycerides, and cholesterol esters. Control experiments indicated that anandamide was stable in medium, with or without serum, for up to 2.5 hr. Anandamide levels were greater in the cells when emetine, an inhibitor of protein synthesis [8], was included.

When the neuroblastoma and glioma cells were fractionated into membrane and soluble fractions, the enzymatic activity for the degradation of anandamide resided mainly in the membrane fractions. Under these conditions, the sole degradation product detected was arachidonate. It was found that 1.5 mM PMSF completely abolished the amidase activity (Fig. 2) . In a separate set of experiments, other inhibitors were tested (aprotinin, benzamidine, leupeptin, chymostatin, and pepstatin) and found to have no effect on this activity. Crude homogenates of HeLa cells were inactive (Fig. 2). No activity was detected in human larynx epidermoid carcinoma (Hep2) or human hepatocellular carcinoma cells (HepG2), whereas a crude homogenate of cultured bronchioalveolar non-small cell lung carcinoma (H358) did convert anandamide to arachidonate (data not shown).

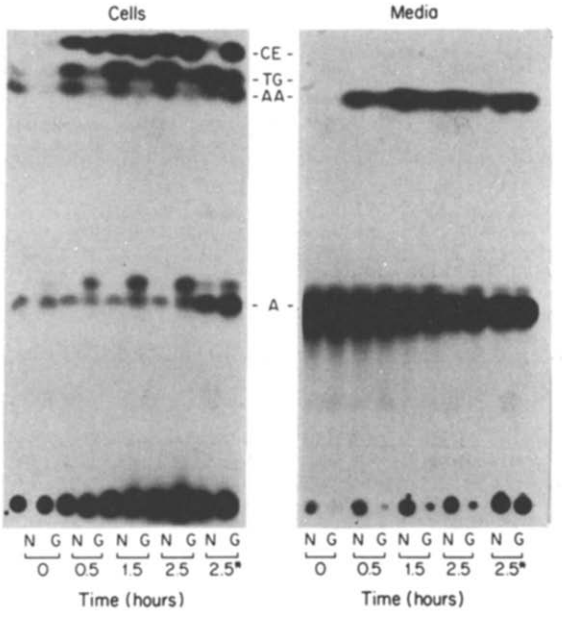


Fig. 1. Uptake and degradation of anandamide by neuroblastoma (N) and glioma (G) cells as a function of time. [$^3$H]-Anandamide (12 $\mu$Ci) was added to the cell culture media. The radioactive products from the media and cells were analyzed by TLC and autoradiography to detect tritiated anandamide (A) and its putative metabolites: arachidonic acid (AA), triglyceride (TG), and cholesterol ester (CE). Phospholipids remained at the origin. The breakdown product with a slightly faster mobility than anandamide was not identified. In one set of experiments, $10^{-3}$ M emetine (*) was included in a 2.5-hr incubation.

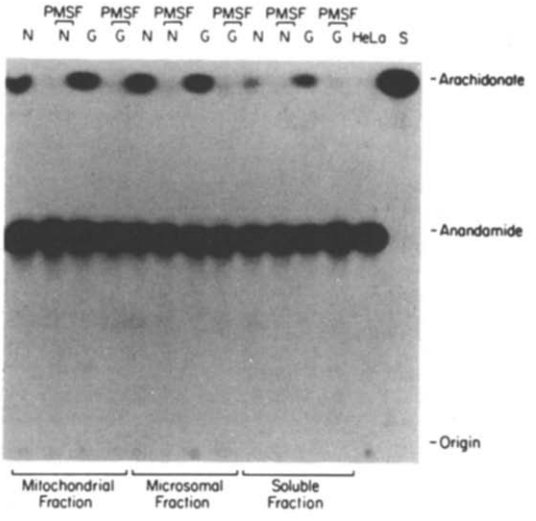


Fig. 2. Amidase activity in subcellular fractions of neuroblastoma (N) and glioma (G) cells. Incubations of whole fractions were for 45 min with 0.4 $\mu$Ci [$^3$H]-anandamide in PBS at 37°. Where indicated, the fractions were incubated with 1.5 mM PMSF. The HeLa cells were assayed as a crude homogenate. The last sample on the plate is a [$^3$H]-arachidonic acid standard (0.16 $\mu$Ci).

To characterize the distribution of amidase activity in animals, tissues from the rat were studied (Fig. 3). Enzymatic activity was present in homogenates of tissues from brain, liver, kidney and lung. In addition to the arachidonic acid degradation product, lung and liver produced an unknown second catabolite. No activity was present in homogenates of rat heart and skeletal muscle.

A study was undertaken to detect the biosynthesis of arachidonylethanolamide in a rat brain tissue homogenate. Using [$^{14}$C]-ethanolamine as the label, no fatty acid amide synthesis (0 cpm) was detected on the TLC plates unless PMSF (680 cpm), arachidonate (120 cpm), PMSF + arachidonate (790 cpm), $PLA_2$ (70 cpm) or $PLA_2$ + PMSF (740 cpm) was added to the incubation (Fig. 4).

To observe anandamide synthesis, when [$^3$H]-arachidonate was employed as the labeled compound (data not shown), ethanolamine had to be added to the rat brain homogenate incubation mixture. For example, using [$^3$H]-arachidonic acid (1 $\mu$Ci), in the presence of 1.5 mM PMSF and 1.6 mM unlabeled ethanolamine, anandamide synthesis was clearly demonstrated with more than 7% of the total counts on the TLC plate from anandamide (50 cpm anandamide, 620 cpm arachidonate). Anandamide synthesis increased with increasing amounts of ethanolamine so that 35% of the [$^3$H]-arachidonate counts were incorporated into anandamide with 7 mM unlabeled ethanolamine.

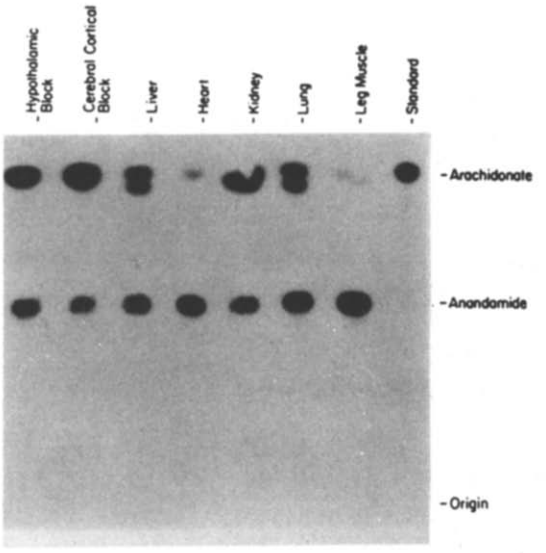


Fig. 3. Degradation of anandamide in homogenates of rat tissue. Incubations were at 37° for 45 min with 10 mg of tissue homogenate protein and 0.4 $\mu$Ci [$^3$H]-anandamide in PBS.

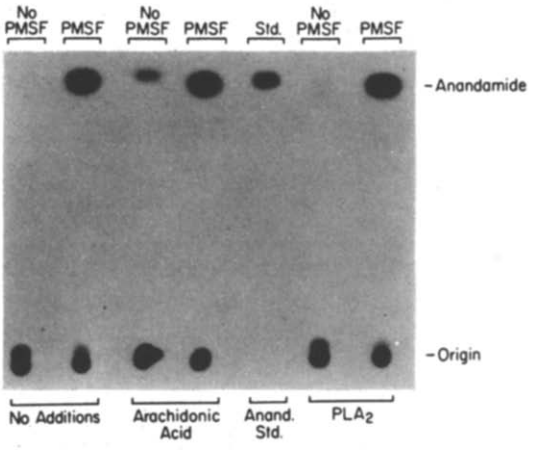


Fig. 4. Synthesis of anandamide in homogenates of rat brain tissue. [ 2-$^{14}$C]-Ethanolamine hydrochloride (3 $\mu$Ci) and 1 mg of brain homogenate protein were incubated in 1 mL of 0.1 M Tris-HCl, pH 9.0, for 60 min at 37°. PMSF (1.5 mM), sodium arachidonate (1 mM), $PLA_2$ (37 $\mu$g/mL) and the anandamide standard (0.04 $\mu$Ci [$^3$H]-anandamide plus 0.5 $\mu$g anandamide) were added as indicated.

## DISCUSSION

Recently, anandamide was identified as an endogenous agonist for the $\Delta^9$-tetrahydrocannabinol receptor. The levels of anandamide are regulated by enzymes responsible for its synthesis and degradation. In this report, evidence is presented for both of these enzymatic activities. Anandamide was readily taken up by neuroblastoma and glioma cells, but it did not accumulate to a great extent because it was degraded to arachidonic acid (which was subsequently incorporated into other lipids) and possibly to other as of yet uncharacterized catabolites. Emetine decreased the breakdown of anandamide, most likely by inhibiting the synthesis of the enzyme(s) responsible for catabolism.

Arachidonylethanolamide amidase was not expressed in all tissues. Activity was found in neuroblastoma, glioma, and non-small cell lung carcinoma cells, but not in HeLa cells, larynx epidermoid carcinoma and hepatocellular carcinoma. Amidase activity was detected in brain, liver, kidney and lung, but not in heart or skeletal muscle. In the two types of cells that were subfractionated (glioma and neuroblastoma), this activity resided mainly in the membrane fractions. PMSF (which was originally added to prevent proteolytic degradation of the enzymes in the homogenates) completely abolished the amidase activity. The mechanism by which PMSF inhibited this activity remains to be elucidated. PMSF is known to inhibit serine proteases, some thiol proteases, and non-protease enzymes such as erythrocyte acetylcholinesterease.

An enzymatic activity (or activities) was detected which catalyzes the formation of anandamide in crude homogenates of brain. This activity was also detected in liver and heart (to be published). When [$^3$H]-arachidonic

acid was employed as the label, addition of exogenous ethanolamine was necessary to observe anandamide synthesis, indicating that ethanolamine is limiting in the brain homogenate. When [$^{14}$C]-ethanolamine was employed as the label, fatty acid ethanolamides other than anandamide may have been formed which were not resolved by TLC. However, arachidonylethanolamide was resolved from palmitoylethanolamide with the TLC system employed in this study (to be published). Palmitoylethanolamide has been reported to constitute 0.1% of the total brain lipid [9].

The relationship of the enzymatic activities found here and those reported in the literature remains to be investigated. Bachur and co-workers [10,11,12] described a rat liver microsomal system utilizing aliphatic fatty acids and several amines as substrates for the synthesis of fatty acid amides. Schmid and co-workers [13,14] have demonstrated that mammalian tissue can N-acylate ethanolamine phospholipids which release long chain amides of ethanolamine as a result of phosphodiesterase activity. Activities for the hydrolysis and synthesis of fatty acid amides via reversible amidases have also been reported [15,16,17]. Our studies, which showed inhibition of the amidase but not the synthase by PMSF, strongly suggest that separate enzymatic activities mediate the breakdown and synthesis of anandamide. The role that these enzymes play under physiological conditions is presently under investigation.

The stability of anandamide in serum and its rapid breakdown in cells and tissues are consistent with the observation that it may be administered systemically, but is not long acting. The amidase and synthase activities described in this report should be taken into consideration when anandamide is employed in receptor binding studies.

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