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Mead Ethanolamide, a Novel Eicosanoid, Is an Agonist for the Central (CB1) and Peripheral (CB2) Cannabinoid Receptors

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SUMMARY

The recently discovered endogenous agonist for the cannabinoid receptor, anandamide (arachidonylethanolamide), can be formed enzymatically by the condensation of arachidonic acid with ethanolamine. 5*Z*,8*Z*,11*Z*-Eicosatrienoic acid (mead acid) has been found to substitute for arachidonic acid in the *sn*-2 position of phospholipids and accumulate during periods of dietary fatty acid deprivation in rats. In the present study, the chemically synthesized ethanolamide of mead acid was evaluated as a potential agonist at the two known subtypes of cannabinoid receptor: CB1 (central) and CB2 (peripheral). This compound was equipotent to anandamide in competing with [^{3}H]CP55,940 binding to plasma membranes prepared from L cells expressing the human CB1 receptor and from ATt-20 cells expressing the human CB2 receptor. Mead ethanolamide was also equipotent to anandamide in inhibiting forskolin-stimulated cAMP accumulation in cells expressing the CB1 receptor. It inhibited N-type calcium currents with a lower potency than anandamide. Mead and arachidonic acid were equally efficacious as substrates for the enzymatic synthesis of their respective ethanolamides in rat and adult human hippocampal P_2 membranes. Palmitic acid was not an effective substrate for the enzymatic synthesis of palmitoyl ethanolamide. Mead ethanolamide exhibits several characteristics of a novel agonist to CB1 and CB2 receptors and may represent another candidate endogenous ligand for the CB1 receptor. Due to the anticonvulsant properties of GABA and the positional similarity of L-serine to ethanolamine in membrane phospholipids, these compounds were synthetically coupled to arachidonic acid, and their resulting arachidonamides were tested as potential cannabinoid agonists. The arachidonamides of GABA and L-serine were inactive in both binding and functional assays at the CB1 receptor.

Until relatively recently, it was not known how Δ^9-THC, the active principle in marijuana, and other cannabinoid agonists exerted their biological effects. In the past several years, Δ^9-THC has been found to bind to two subtypes of cannabinoid receptors: CB1 and CB2. The CB1 (1) and CB2 (2) receptors have been cloned and are members of the superfamily of G protein-coupled receptors. The CB1 receptor transduces its biological signals through the inhibition of adenylate cyclase (1, 3), N-type calcium channels (4, 5), and potassium channels (6). The highest concentrations of these receptors are found in the cerebellum, hippocampus, and striatum (7). Their localization on synaptic terminals of projecting neurons in the striatum suggests a role for the CB1 receptor in presynaptic regulation (8). The CB2 receptor is found on immune cells such as macrophages and B cells in the marginal zone of the spleen (9). Additional pharmacological and signal transduction studies of the CB2 receptor are under way.

The recent discovery of anandamide, a natural brain eicosanoid agonist for the cannabinoid receptor, suggests that lipids can act as neurotransmitters (10). Anandamide is composed of an arachidonic acid molecule coupled through an amide linkage to ethanolamine, and it represents a new eicosanoid structure. Although anandamide is structurally dissimilar to Δ^9-THC, as well as other cannabinoid agonists, both compounds are functional agonists at the cloned human CB1 receptor (3, 11). Anandamide exhibits physiological and behavioral properties similar to those of Δ^9-THC, including analgesia (12), hypothermia, ataxia, and decreased locomotor

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ABBREVIATIONS: CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; GABA, γ-amino-*n*-butyric acid; Δ^9-THC, (−)-*trans*-δ^9-tetrahydrocannabinol; anandamide, arachidonylethanolamide; dihomo-γ-linolenoyl ethanolamide, 8*Z*,11*Z*,14*Z*-eicosatrienoyl ethanolamide; adrenoyl ethanolamide, 7*Z*,10*Z*,13*Z*,16*Z*-docosatetraenoyl ethanolamide; mead acid, 5*Z*,8*Z*,11*Z*-eicosatrienoic acid; arachidonic acid, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl acid; CHO, Chinese hamster ovary; FAB-MS, fast atom bombardment mass spectrometry; BSA, bovine serum albumin; TLC, thin-layer chromatography; BCA, bicichoninic acid; LTB_4, leukotriene B_4.

activity (13). The existence and physiological role of anandamide in the nervous system have yet to be elucidated. A recent structure-activity study of anandamide suggested that related fatty acid ethanolamides may exist in the brain and interact with the cannabinoid receptor (11). Dihomo-γ-linolenoyl ethanolamide and adrenoyl ethanolamide were shown to be agonists at the CB1 receptor and later confirmed to be present in mammalian brain (14).

It has been known for some time that essential fatty acid deficiency in rats leads to the accumulation of mead acid (15). Furthermore, the increase of mead acid is paralleled by a concomitant decrease in the overall level of arachidonate, although preferential conservation of arachidonate in specific phospholipids in the heart and renal cortex has been observed in fat-deficient rats (16). In the present study, we coupled mead acid to ethanolamine to investigate the potential of the resulting amide to act as an agonist for the CB1 and CB2 receptors. Mead ethanolamide displayed binding and functional characteristics equipotent to those of anandamide at the CB1 and CB2 receptors. In addition, mead ethanolamide was enzymatically synthesized from mead acid in rat and human brain plasma membrane preparations.

A number of bioactive compounds exist in the brain that contain a free amine capable of forming an amide bond with arachidonic acid and thus may be potential ligands for the CB1 receptor. The neurotransmitter GABA is abundant in brain and, like Δ^9-THC, has been implicated in mediating anticonvulsant effects (17). Therefore, GABA was synthetically coupled to arachidonic acid for evaluation as a potential CB1 agonist. L-Serine is relatively abundant in plasma membranes, contains a free amine, and was also selected for synthesis of its corresponding arachidonamide due to its analogous role with ethanolamine in functioning as a polar head moiety in phospholipids. The arachidonamides of both GABA and L-serine were inactive at the CB1 receptor in binding and functional assays.

Experimental Procedures

Materials. Arachidonic acid and mead acid were obtained from Biomol (Plymouth Meeting, PA), and [1,2-^{14}C] ethanolamine hydrochloride was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Preparative and analytical TLC plates were obtained from Merck (Darmstadt, Germany). CHO and L cells were obtained from the American Type Culture Collection (Rockville, MD). Membranes from AtT-20 cells transfected with the human cannabinoid CB2 receptor (AtT-20-hCB2) were kindly provided by Dr. Yvonne Lai (PanLabs, Seattle, WA). Human hippocampus was a gift from Dr. Miklos Palkovits (National Institute of Mental Health, Bethesda, MD).

Chemical synthesis. Anandamide and mead ethanolamide were synthesized via formation of the fatty acid chlorides as previously reported (11). The arachidonamide derivatives of GABA and L-serine were synthesized through the reaction of arachidonic acid with 1.5 M equivalents of iso-butylchloroformate in the presence of 2.25 M equivalents of *N*-methylmorpholine at −20° for 30 min. Then, 3 M equivalents of GABA or L-serine were dissolved in water and 3 M equivalents of *N*-methylmorpholine and added to the reaction mixture containing the arachidonic acid anhydride. After 2 hr, the reaction products were extracted in 10% HCl/ethyl acetate and washed with 10% HCl and then water. The products were separated by silica column chromatography (Biosil A, 200–400 mesh; Bio-Rad, Richmond, CA) and eluted with chloroform/hexane/methanol, 4:3:0.5 (v/v/v). In the case of the arachidonamide of L-serine, further purification on a preparative silica TLC plate was necessary; however, only 60% final purity was achieved. Structural confirmation of the synthesized compounds was performed with 300-MHz proton nuclear magnetic resonance analysis (in $CDCl_3$) and FAB-MS (meta-nitrobenzyl-alcohol derivatization for the ethanolamide of mead acid and monothioglycerol derivatization for the arachidonamides of GABA and L-serine).

Cell culture and stable expression of the human cannabinoid receptor. CHO and L cells were transfected with the human cannabinoid CB1 receptor (CHO-hCB1 and L-hCB1, respectively) as previously described (11). CHO cells stably expressing the muscarinic acetylcholine m5 receptor (CHOm5) were used as a control cell because they were subjected to similar transfection and selection procedures.

cAMP accumulation and radioligand binding assay. The accumulation of cAMP was measured over 5 min in CHO cells expressing the human cannabinoid receptor and in CHOm5 cells in suspension as previously described (3). Plasma membranes were prepared from L-hCB1 cells and were used in radioligand binding experiments as previously reported (11). The compounds synthesized were competed against 500 pM [^{3}H]CP55,940, and the K_i values were determined by nonlinear regression analysis with GraphPad computer software (GraphPad Software, San Diego, CA).

Electrophysiological recording. Calcium currents were recorded from N18 neuroblastoma cells differentiated in 2% DMSO with the use of techniques and solutions previously described (18), except 10 mM $BaCl_2$ replaced the $CaCl_2$ and 2 μM nifedipine was added to inhibit L-type calcium currents. Mead ethanolamide was dissolved as a 10-mM stock solution in ethanol and diluted into external solution containing 3 μM BSA. The concentration of BSA in all recording solutions was 3 μM.

Enzymatic synthesis of ethanolamides. Enzymatic formation of the ethanolamide of mead acid was measured in rat whole brain and adult human hippocampal P_2 membranes as previously described (19). Briefly, P_2 membranes were prepared and diluted in 125 mM Tris-HCl/1 mM EDTA (pH 9). Each assay was performed in duplicate with each tube containing approximately 100 μg of protein, ethanolamine hydrochloride [1,2-^{14}C] (550 nCi), 20 mM ethanolamine, and 1 μl of fatty acid of a specified concentration ranging from 10^{-5} to 10^{-3} M in a total volume of 200 μl. Lipid products were extracted in 15 vol of toluene, dried, and spotted on a silica-gel TLC plate for migration in a solvent system containing dichloromethane/methanol/ammonium hydroxide, 70:5:0.5 (v/v/v). Products were identified by comparison with authentic standards prepared in our laboratory (11). TLC plates were developed overnight, and quantification of ethanolamides was performed with the FUJIX BAS 2000 (FUJIX, Stamford, CT) phosphor-imaging analyzer. Protein measurements were made with the Pierce BCA kit (Pierce, Rockford, IL).

Results and Discussion

The binding of mead ethanolamide was compared with that of anandamide using membranes of L cells and AtT-20 cells transfected with cloned CB1 and CB2 receptors, respectively. Mead ethanolamide was equipotent to anandamide in displacing the binding of the synthetic cannabinoid agonist [^{3}H]CP55,940 in membranes prepared from L-hCB1 cells (Fig. 1). Mead ethanolamide and anandamide exhibited similar K_i values for binding to the CB2 receptor in membranes prepared from AtT-20-hCB2 cells. However, the binding affinities of both mead ethanolamide and anandamide were slightly lower at the CB2 receptor. Mead ethanolamide was equally effective at inhibiting cAMP accumulation as anandamide in CHO-hCB1 cells (Fig. 2). No inhibition of cAMP accumulation by anandamide or mead ethanolamide was

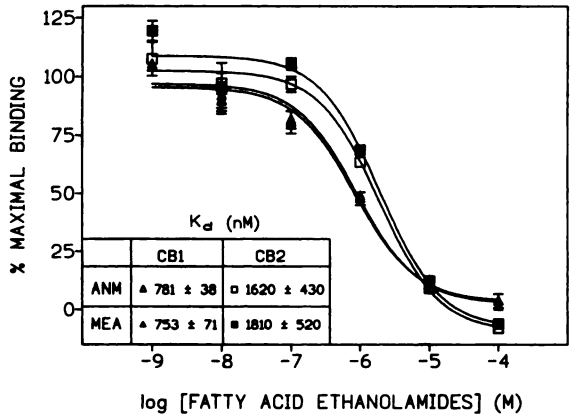

Fig. 1. Mead ethanolamide (*MEA*) and anandamide (*ANM*) inhibit binding of [^{3}H]CP55,940 (500 pM) to L cell and AtT-20 cell membranes expressing the CB1 and CB2 receptors, respectively. Data are the mean ± standard deviation of at least two representative experiments, each performed in triplicate.

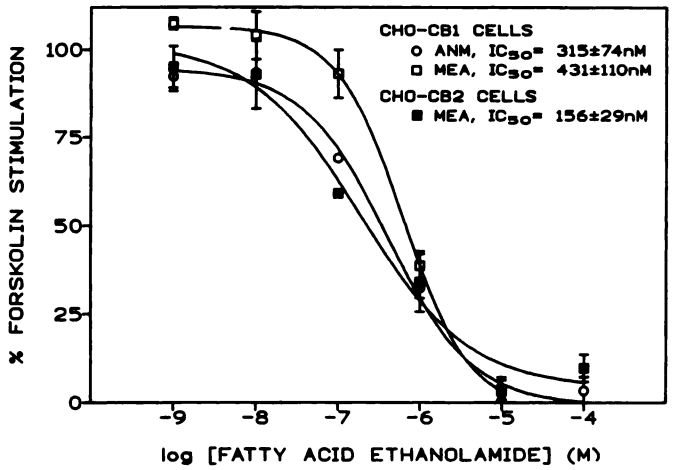

Fig. 2. Inhibition of forskolin-stimulated (500 nM) cAMP accumulation in CHO cells expressing the human CB1 cannabinoid receptor (CHO-CB1) or the human CB2 cannabinoid receptor (CHO-CB2) by mead ethanolamide (*MEA*). The effect of anandamide (*ANM*) on CHO-CB1 cells was included as a control. Data are the mean ± standard error of at least three experiments, each performed in triplicate. *BSL*, basal.

detected in control cells expressing the muscarinic m5 receptor (data not shown).

The ability of mead ethanolamide to inhibit N-type calcium currents (I_{Ca}) in N18 neuroblastoma cells endogenously expressing CB1 receptors was also determined. Exposure of cells to increasing concentrations of mead ethanolamide inhibited an increasing proportion of the calcium current elicited by step depolarization to 0 mV from a holding potential of −90 mV. Inhibition was half-maximal at 124 ± 19 nM mead ethanolamide and showed little cooperativity, with a Hill coefficient of 1.14 (Fig. 3). Inhibition of I_{Ca} by mead ethanolamide was poorly reversible under the recording conditions used in the study (n = 5, data not shown). Previously, we found that anandamide inhibits N-type I_{Ca} with an IC_{50} of 20 nM (11). Therefore, mead ethanolamide is approximately 6-fold less potent than anandamide in inhibiting N-type I_{Ca}. Mead ethanolamide satisfies the basic criteria of binding and stimulation of functional coupling to known signal transduction pathways of the human CB1 receptor to be classified as a CB1 receptor agonist.

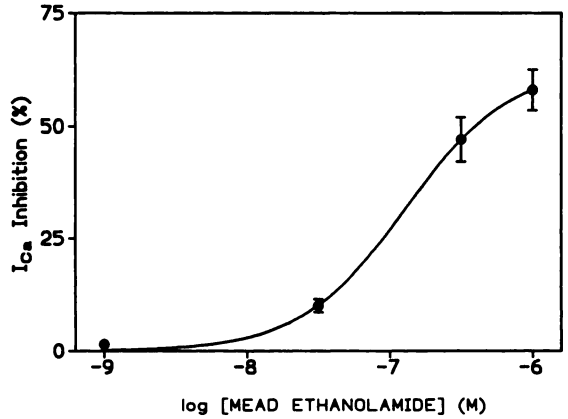

Fig. 3. Mead ethanolamide inhibits I_{Ca} in a concentration-dependent fashion. Mead ethanolamide was applied via bath perfusion, and the calcium current evoked by a step depolarization to 0 mV from a holding potential of −90 mV was recorded every 5 sec. Inhibition was half-maximal at a mead ethanolamide concentration of 124 ± 19 nM. Data are presented as mean ± standard error of n = 3–6 for each cell.

A previous study has demonstrated that arachidonic acid was the preferred substrate for the enzymatic synthesis of fatty acid ethanolamides in bovine P_2 membrane preparations (19). It was also observed that high concentrations of arachidonic acid were inhibitory to anandamide synthesis. In the present study, as a substrate, mead acid was as effective as arachidonic acid in the synthesis of the corresponding ethanolamide in rat whole brain and adult human hippocampal membranes (Fig. 4). However, at high concentrations (1 mM), mead acid did not inhibit synthesis of its ethanolamide, as was observed for arachidonic acid. The high concentrations of fatty acid and ethanolamine substrates required for *in vitro* fatty acid ethanolamide synthesis are consistent with previous reports (19, 20), suggesting that the regulation of the fatty acid ethanolamide synthase may be controlled by the availability of substrates. Therefore, fatty acid ethanolamide biosynthesis may be primarily regulated by phospholipase A_2 and phospholipase D, enzymes considered to regulate the release of fatty acids or ethanolamine, respectively.

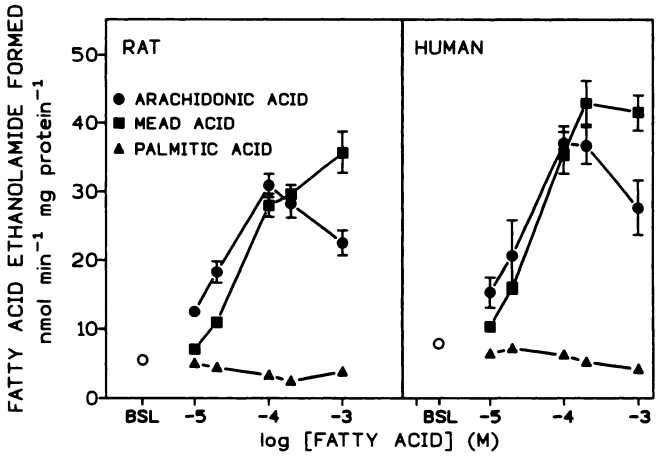

Fig. 4. Mead acid, arachidonic acid, and palmitic acid serve as substrates for the enzymatic synthesis of their respective ethanolamides in rat whole brain and human hippocampal P_2 membrane preparations. Data are the mean ± standard deviation of at least two experiments, each performed in duplicate. *BSL*, basal activity in the absence of added fatty acid.

Palmitic acid was not an effective substrate for enzymatic synthesis of its ethanolamide. Although enzymatic formation of mead ethanolamide has been shown, the isolation and distribution of mead ethanolamide in brain or peripheral tissues have not been demonstrated and are under investigation.

Mead acid substitutes for arachidonic acid in phospholipids during periods of fatty acid deprivation (15). This observation indicates that mead ethanolamide is formed when levels of arachidonic acid are depleted, suggesting the presence of a compensatory mechanism for the formation of anandamide during starvation. The fact that mead ethanolamide was enzymatically synthesized as effectively as anandamide in human brain supports this hypothesis. Although the compensatory substitution of mead acid for arachidonic acid may be relevant in the formation of endogenous cannabinoid agonists, this mechanism does not appear to play a role in eicosanoid production. Dietary fatty acid depletion has been shown to be a useful tool in determining the physiological role of arachidonic acid, particularly in studies of inflammation, since mead acid does not functionally substitute for arachidonic acid or its eicosanoid metabolites (21). For example, the accumulation of mead acid leads to an altered production of eicosanoids in macrophages from mice affected with glomerulonephritis, implicating mead acid as a potential mediator of anti-inflammatory effects (22). In this study, mead acid accumulation during fatty acid deprivation led to a loss of arachidonic acid-derived leukotriene B_4 (LTB_4). LTB_4 plays a role in the migration of polymorphonuclear neutrophils, a vital component of the body's inflammatory response. In addition to its role in disrupting the physiological effects of arachidonic acid and its metabolites, mead acid may mediate distinct physiological functions, independent of those exerted by members of the arachidonic acid cascade. For example, mead acid has been shown to mediate platelet aggregation via its metabolite 12-hydroxy-5,8,10-eicosatrienoate (23, 24). Further biochemical characterization of anandamide and mead ethanolamide metabolism and regulation of synthesis and release will elucidate the physiological function of these cannabinoid agonists.

A number of neurotransmitters and neuromodulators contain active nitrogens that could condense with arachidonic acid or other fatty acids to form novel bioactive molecules. When the arachidonamides of GABA and L-serine were chemically synthesized and evaluated as potential cannabinoid receptor agonists, they were found to be inactive in both binding and functional assays in cells expressing the CB1 receptor (data not shown). Unlike most biogenic amines, L-serine does not contain an aromatic ring, and thus its arachidonamide was likely to display less steric hindrance as a potential CB1 receptor agonist. However, the arachidonamides of both GABA and L-serine appear to have sterically unfavorable conformations for high-affinity binding to the CB1 receptor. Therefore, it is unlikely that GABA or L-serine would substitute for ethanolamine in the formation of high-affinity cannabinoid receptor agonists. Our previous study demonstrated no loss in agonist activity when the ethanolamine moiety of anandamide was increased by one carbon atom in the formation of arachidonyl-3-amino-1-propanolamide, but considerable loss of activity occurred when a branched structure was introduced to form arachidonyl-DL-1-amino-2-propanolamide (11). However, a recent study reported an increased affinity of arachidonyl-3-amino-1-propanolamide for the CB1 receptor compared with anandamide (25). Furthermore, this study revealed that the substitution of the ethyl group in the ethanolamine moiety by a propyl structure resulted in a 2-fold more potent CB1 agonist compared with anandamide, whereas further increase in the alkyl chain length or substitution of a benzyl group decreased binding affinity. Additional structure-activity analysis studies are needed to provide further insight into the binding requirements of cannabinoid agonists.

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