review

Biochemistry and pharmacology of arachidonylethanolamide, a putative endogenous cannabinoid

Cecilia J. Hillard[1] and William B. Campbell

Department of Pharmacology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

Abstract This review presents and explores the hypothesis that *N*-arachidonylethanolamine (AEA, also called anandamide) is synthesized in the brain and functions as an endogenous ligand of the cannabinoid receptor. Support for this hypothesis comes from in vitro experiments demonstrating that AEA binds and activates signaling through the cannabinoid receptor. In addition, in vivo AEA produces effects very similar to those of the classical agonists of the cannabinoid receptor. Evidence for the cellular synthesis and release of AEA is not as clear. Data are presented that suggest that AEA is synthesized via a two enzyme process. First, a novel phospholipid (*N*-arachidonylphosphatidylethanolamine) is formed by a calcium-dependent transacylase. This lipid is a substrate for a phosphodiesterase of the phospholipase D type which releases AEA. Although there is some evidence to support this hypothesis, it is clear that AEA is a very minor product of this enzymatic cascade. Several important questions remain to be answered, including whether the concentrations of AEA synthesized by cells are sufficient to support a signaling role in the brain.—**Hillard, C. J., and W. B. Campbell.** Biochemistry and pharmacology of arachidonylethanolamide, a putative endogenous cannabinoid. *J. Lipid Res.* 1997. **38:** 2383–2398.

Supplementary key words Δ^9-tetrahydrocannabinol • *N*-acylethanolamines • amidohydrolase • fatty-acyl amide hydrolase • marijuana

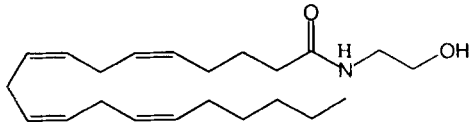
INTRODUCTION

In 1992, Devane and coworkers (1) made the remarkable observation that *N*-arachidonylethanolamine (alternatively called arachidonylethanolamide, anandamide, or AEA, **Fig. 1**), isolated from porcine brain, competed for binding to brain membranes with the cannabinoid receptor ligand [^{3}H]HU-210. In the same report, these investigators presented data that AEA inhibited the electrically evoked twitch response of the mouse vas deferens, a characteristic effect of cannabinoid receptor agonists. Other biochemical, behavioral and physiological studies from a number of laboratories (outlined in more detail below) support the original observation that AEA is an agonist of the cannabinoid receptor. However, far less evidence is available to support or refute the hypothesis that AEA is produced in brain and functions as the endogenous ligand for the cannabinoid receptor. It is our intent in this review to discuss data that shed light on the hypothesis that AEA is formed in the brain and functions as an endogenous ligand of the cannabinoid receptor.

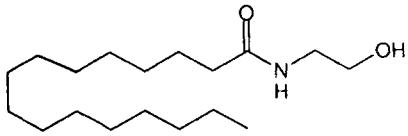
Although the report from Devane and coworkers (1) was the first demonstration of the existence of arachidonylethanolamide, mammalian tissues were shown to contain *N*-acylethanolamine (NAE) derivatives of other fatty acids 30 years earlier by Bachur and coworkers (2). In particular, these investigators found that palmitoylethanolamide (PEA, Fig. 1) is a constituent of lipid extracts from rat brain, liver, and skeletal muscle. *N*-Acylethanolamines, particularly PEA and stearoylethanolamide, are also found in canine heart extracts, accounting for 0.05 mol% of the total lipid (3). Schmid, Schmid, and Natarajan (4) have suggested and provided evidence to support the concept that long chain, saturated and mono-unsaturated NAEs are a family of molecules that share a common mechanism of cellular synthesis and common mechanisms of cellular action (see 4 for a review of this topic).

Abbreviations: AEA, arachidonylethanolamide; CB1, brain cannabinoid receptor; CB2, peripheral cannabinoid receptor; MS, mass spectrometry; NAE, *N*-acylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamine; PMSF, phenylmethylsulfonyl fluoride; PLD, phosphodiesterase of the phospholipase D type; THC, Δ^9-tetrahydrocannabinol.

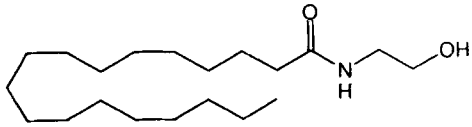
[1]To whom correspondence should be addressed.



N-arachidonoyl ethanolamine
(Anandamide)



N-palmitoyl ethanolamine



N-stearoyl ethanolamine

Fig. 1. Structures of *N*-acylethanolamines.

AEA CONCENTRATIONS IN BRAIN TISSUE

The concentration of AEA in brain tissue is very low and assays with sufficient sensitivity and selectivity to detect AEA have been developed only recently. The primary method used to quantify endogenous AEA in brain tissues is mass spectrometric (MS) analysis with isotopic dilution (5–8). The specifics of the MS methods that have been applied to the analysis of brain tissue differ; the most sensitive of these methods utilizes selective ion monitoring in the negative ion chemical ionization (NICI) mode after derivatization of AEA to the bis-pentafluorobenzoyl (PFB) derivative and separation by gas chromatography (8). The reported lower limit of detection of AEA using this method is 6 pmol/ml. Another group has used liquid chromatography of nonderivatized AEA to separate the protonated molecular ion of AEA which is used as the precursor ion for MS/MS analysis (7). This method has a detection limit of 25 nmol/ml.

Several assays for AEA utilizing the highly sensitive method of high pressure liquid chromatography after fluorescence derivatization have been reported (9, 10). In each of these procedures, the primary hydroxyl group of the ethanolamine is the site of derivatization. As very few lipid species containing primary hydroxyls exist in mammalian tissues, one advantage of this approach is a low number of interfering compounds. A second advantage of HPLC with fluorescence detection is that the concentrations of AEA as well as other NAEs can be determined simultaneously. HPLC analysis of anthroyl derivatives of tissue-derived NAEs, including AEA, have been reported (10). Unfortunately, the sensitivity of this approach was rather low (60 pmol/ml), possibly due to the low fluorescence yield of the anthroyl group. Another derivatization protocol has been reported utilizing a highly fluorescent benzoxadiazole as a derivatizing agent (9). We have successfully derivatized AEA and other NAEs with 7-diethylaminocoumarin-3-carbonyl azide although detection of small concentrations in biological samples has proven to be difficult (S. Muthian, K. Nithipatikom, W. B. Campbell, and C. J. Hillard, unpublished observations). Both of these derivatization procedures coupled with HPLC and fluorescence detection result in sensitive assays; 4–5 fmol/ml of AEA standard derivatized with either of these compounds was detected at a signal to noise ratio of 2. The use of these or similar derivatizations should greatly enhance the sensitivity of AEA measurement in tissues and cells.

The original report from Devane and coworkers (1) estimated the concentration of AEA in porcine brain to be 384 pmol/g of brain tissue. A second group has confirmed the observation that freshly isolated porcine brain contains AEA (17 pmol/g) and demonstrated that cow brain contains 10 pmol/g but AEA concentrations are below the limits of detection in sheep brain (5, 6). Data obtained from rat brain are somewhat conflicting; one group has reported 4 pmol AEA/g tissue (10), another has reported 20–30 pmol/g tissue (7) and a third group reported that AEA was not detectable in freshly isolated rat brain (8). Human brain obtained within 2 h of death contains AEA and differences in the concentration of AEA were noted in some brain regions (7). The highest concentration of AEA was found in hippocampus (110 pmol/g tissue), with lesser amounts in the parahippocampal cortex (75 pmol/g tissue), cerebellum (30 pmol/g tissue), striatum (55 pmol/g tissue), and thalamus (75 pmol/g tissue). In toto, these studies suggest that the amount of AEA in brain is very low. This finding is not at all surprising when AEA is considered in the context of other biologically active arachidonic acid metabolites. Like other eicosanoids, it is probable that AEA and other NAEs are synthesized on an "as needed" basis and are not stored in cells in their free form. It is possible that the concentrations of AEA in brain tissues are dependent upon methods used in the preparation of the tissue rather than the amounts of AEA that are released by "anandamergic" neurons.

One variable of tissue preparation that is an important determinant of tissue AEA concentration is the time between the death of the animal and the extrac-

tion of lipids from the tissue. The concentration of AEA in pig brain increases 100-fold when the brain is kept at ambient temperature for 22 h (5). After 28 h at room temperature, AEA concentrations increase by more than 125-fold in rat brain (8) and by approximately 14-fold in human cerebellum (7). The mechanism by which removal of the brain from its nutrient supply results in an increase in AEA concentration is not known. These studies raise the question whether the concentration of AEA is regulated by more subtle changes in tissue and cellular nutrition, such as ischemia or hypoxia. While the postmortem increase in brain AEA may be an artifact of tissue damage, it is also possible that similar mechanisms may regulate AEA concentrations in living tissue.

In support of this concept, we have recently found that cerebellar granule cells synthesize AEA when they are rendered chemically ischemic by incubation in an N_2 atmosphere for 3 h without serum or glucose. Cerebellar granule cells were isolated, grown in vitro for 7 days then preloaded with [^{3}H]arachidonic acid (100,000 cpm/ml) for 4 h. Twenty hours later, the cells were either washed and incubated in media in a normal O_2 environment or were incubated in media lacking glucose and serum in an N_2 environment. After 3 h, cellular lipids were extracted and resuspended in 100 μl acetonitrile. The samples were separated by reverse-phase HPLC using a gradient from 50% acetonitrile in 0.1% acetic acid to 100% acetonitrile on a C18 column. The ischemic cells exhibited a 193% increase in the amount of tritiated metabolite that comigrated with [^{3}H]AEA standards. These data demonstrate that cells made ischemic for a relatively short period of time synthesize AEA and suggest that AEA synthesis may be regulated by similar events in the intact brain.

CELLULAR SYNTHESIS OF AEA

Evidence for the transacylase/PLD pathway

As it is unlikely that AEA is stored in its free form in tissues, elucidation of the mechanisms of AEA synthesis is of utmost importance to understand the role of AEA and the cannabinoid receptor in brain function. Striatal neurons in primary culture preloaded with radiolabeled ethanolamine produce small amounts of radiolabeled AEA along with greater amounts of the ethanolamides of other free fatty acids including 16:0, 18:0, 18:1, 18:2, and 18:3 (11). N18 neuroblastoma cells also make small amounts of radiolabeled AEA when cells are prelabeled with either radiolabeled ethanolamine or arachidonic acid (12). AEA synthesis also occurs in several nonneuronal cell types; including J774 cells, a cell line derived from mouse macrophages (12) and possibly endothelial cells from rabbit mesenteric arteries (13). Cells devoid of AEA synthetic activity include astrocytes (11) and bovine coronary artery endothelial cells although they may make other NAEs (P. F. Pratt, C. J. Hillard, and W. B. Campbell, unpublished results). The calcium ionophore, ionomycin, increases the production of AEA in striatal neurons (11) and J774 cells (12). However, the details of the relationship between an increase in the intracellular calcium concentration and AEA production are not known. High concentrations of THC increase AEA synthesis in N18 neuroblastoma cells preloaded with either [^{3}H]arachidonic acid or [^{14}C]ethanolamine; however, the mechanism for this effect is not clear (14).

A plausible mechanism for the cellular synthesis of AEA is based on the scheme originally suggested by Schmid and coworkers for the synthesis of saturated and mono-unsaturated NAEs (reviewed in refs. 4, 15; outlined in **Fig. 2**). According to this synthetic scheme, a minor phospholipid *N*-acyl phosphatidylethanolamine (*N*-acyl-PE) is found in tissues that synthesize NAEs. This phospholipid is a substrate for a calcium-independent phosphodiesterase of the phospholipase D type which releases NAE and phosphatidic acid. The rate limiting and calcium-dependent step in this cascade of events is the synthesis of *N*-acyl-PE which occurs through the action of an *N*-acyl transferase that catalyzes an exchange reaction between the *sn*-1 position of donor phospholipids and the primary amine of PE. While there is considerable support for this mechanism of synthesis for saturated and mono-unsaturated NAEs, it has been more difficult to demonstrate that AEA is synthesized in this manner. A theoretical problem with the application of this scheme to the production of AEA in brain is that very low amounts of arachidonic acid are found in the *sn*-1 position of phospholipids. It is almost exclusively in the *sn*-2 position of brain phospholipids. However, AEA synthesis does appear to occur by this pathway in rat testis (16) where significant amounts of arachidonate are incorporated into the *sn*-1 position of phosphatidylcholine (17). We will summarize the data that support each step of this mechanism as it relates to AEA below. The reader is also referred to the recent review by Schmid, Schmid, and Natarajan (15) for data that support this mechanism for the production of other NAEs.

The final step of this cascade of events is the release of AEA from *N*-arachidonyl-PE through the action of a phosphodiesterase of the phospholipase D type (referred to herein as PLD) which hydrolyzes the bond between phosphate and ethanolamine resulting in the formation of AEA and phosphatidic acid. This particu-

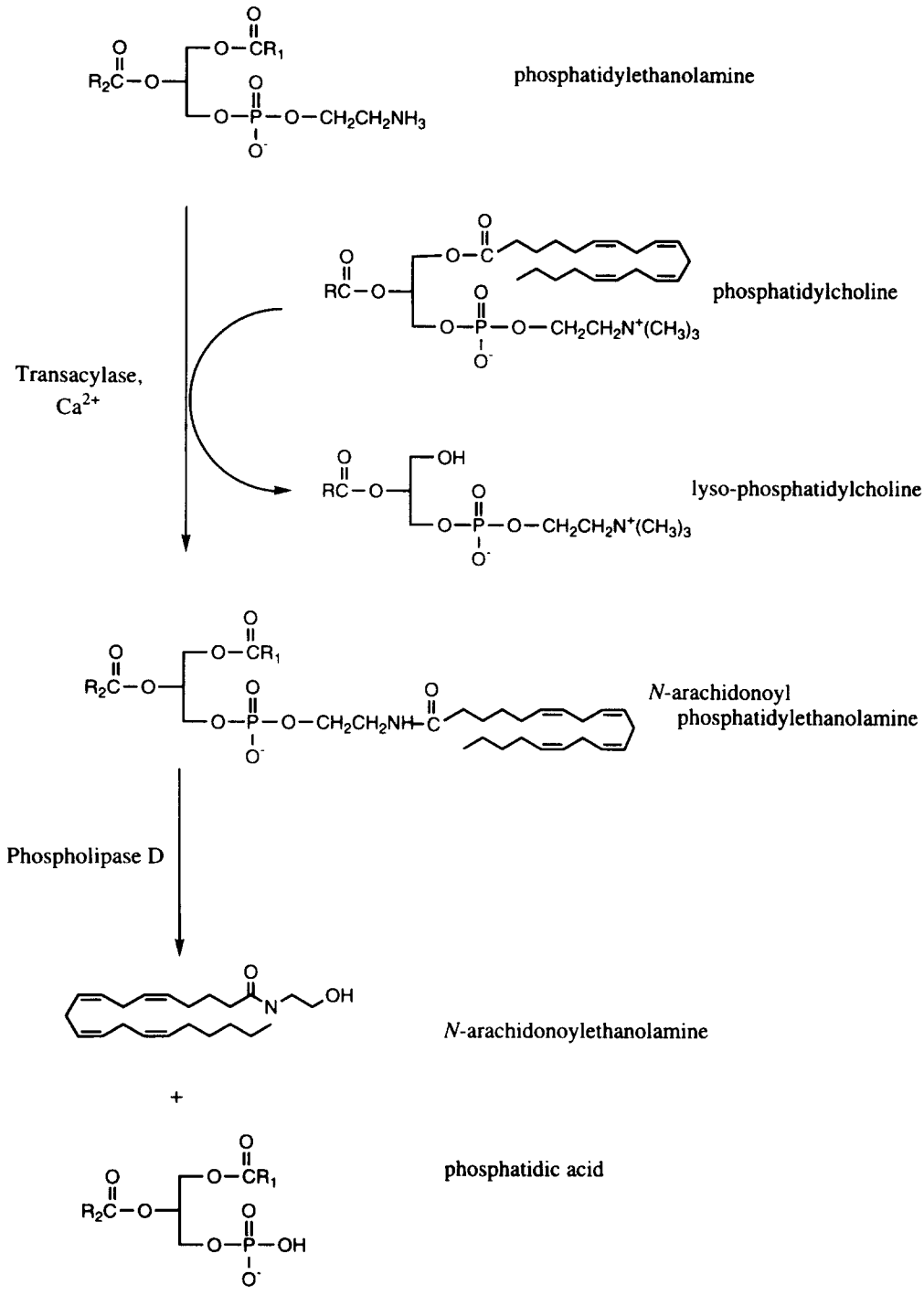


Fig. 2. Proposed mechanism for cellular synthesis of AEA from PE via transacylase and phospholipase D.

lar phosphodiesterase is also a lysophospholipase D and can also hydrolyze glycerophospho-*N*-acylethanolamine (18). Rat brain microsomes convert *N*-[^{3}H]arachidonyl-PE to [^{3}H]AEA in the presence and absence of calcium (10). Similar results have been reported for homogenates from neurons in primary culture (11). There has been only one study of this process in intact cells using the macrophage-like J774 cells prelabeled with [^{3}H]ethanolamine (12). In this experiment, a high concentration (5 μM) of ionomycin was found to increase NAE formation and decrease *N*-acyl-PE content (12). The authors concluded that the release of NAE from *N*-

acyl-PE in these cells is a calcium-dependent process and the rate limiting step in the production of NAE. This conclusion is at odds with the data from brain homogenates cited above and with the hypothesis of Schmid (4, 15) that the formation of *N*-acyl-PE is the rate limiting step in NAE synthesis. Whether the discrepancy is the result of differences between cell homogenates and whole cells or is unique to lymphoid cells is not known.

The next question is whether *N*-arachidonyl-PE is present in brain or in neurons. One group has reported that rat brain contains very low amounts of *N*-arachidonyl-PE (26 pmol/g tissue) accounting for only 0.4% of the total *N*-acyl-PE (10). Earlier studies using less sensitive methods of quantification failed to find *N*-arachidonyl-PE in brain extracts (19). One can make the argument that the synthetic scheme of Schmid outlined above predicts that the concentration of *N*-arachidonyl-PE is low because it is the substrate of an unregulated enzyme. In other words, any *N*-arachidonyl-PE formed should be rapidly converted to AEA. However, significant amounts of other *N*-acyl-PE species are found in brain extracts (19) which suggests that either *N*-arachidonyl-PE is a preferred substrate for PLD or its synthesis occurs at a lower rate than other *N*-acyl-PEs.

Several studies have been carried out to identify *N*-arachidonyl-PE in cells at rest and after stimulation. N18 neuroblastoma cells incorporate [^{14}C]ethanolamine into a lipid component that comigrates on TLC with *N*-acyl-PE (12). When the newly synthesized *N*-acyl-PE was digested with PLD from *S. chromofuscus* and the *N*-acyl moieties were separated using HPLC, a single peak that comigrated with both *N*-linoleoylethanolamine and AEA standards was seen. The absolute identity of these lipids was not reported. Similarly, Cadas and coworkers (20) demonstrated that *N*-acyl-PE content was increased when rat cortical neurons in primary culture were stimulated by a variety of agents that increase intracellular calcium. The contribution of *N*-arachidonyl-PE to the total *N*-acyl-PE pool was not determined. Another group reported that glutamate stimulation of cortical neurons in primary culture resulted in the incorporation of [^{3}H]stearic acid but not [^{3}H]arachidonic acid into the *N*-acyl position of *N*-acyl-PE (21). In summary, *N*-arachidonyl-PE accounts for a very small fraction of the *N*-acyl-PE present in brain or unstimulated neurons. It is not clear whether *N*-arachidonyl-PE concentrations in brain and/or neurons are elevated by increased intracellular calcium concentrations although it is clear that the total amount of *N*-acyl-PE is increased.

Some of the properties of the *N*-acyl transferase are known. The *N*-acyl transferase activity is membrane associated, with microsomal membranes having the highest activity (22). *N*-Acyl transferase activity is energy independent; neither free fatty acids nor acyl-CoA derivatives serve as the acyl donor. The *N*-acyl moieties in *N*-acyl-PE can be transferred from the O-acyl groups of PE (22), from the *sn*-1 position of phosphatidylcholine (23) and from cardiolipin (24). The *N*-acyl transferase activity in brain is calcium-dependent at concentrations between 0.5 and 2 mM $CaCl_2$ (19). These results suggest that the *N*-acyl transferase is quiescent at resting concentrations of calcium in the cell and is only activated when cellular calcium concentrations rise to very high values.

Two recent studies of the production of *N*-acyl-PE in cells support the conclusions reached from studies using brain microsomal membranes. Glutamate, which causes large increases in neuronal intracellular calcium concentrations (25), increases *N*-acyl-PE formation in cortical neurons (21). Similarly, rat cortical neurons synthesize *N*-acyl-PE in response to ionomycin, veratridine, 4-aminopyrine, and kainate, all of which increase intracellular calcium concentrations (20). *N*-Acyl-PE synthesis is inhibited by the calcium chelator EGTA but not by inhibitors of calcium/calmodulin-dependent protein kinase or protein kinase C (20). Activators of adenylyl cyclase, such as forskolin and the neurotransmitter VIP, have no effect alone but enhance ionomycin-induced *N*-acyl-PE synthesis (20). While these studies shed light on the properties of *N*-acyl transferase in intact cells, neither of these studies identified *N*-arachidonyl-PE as a product of the enzyme.

As indicated above, a major problem with the application of the *N*-acyltransferase/PLD pathway to the synthesis of brain AEA is that only acyl groups in the *sn*-1 position are transferred to the amine group of PE yet very little arachidonic acid is found in the *sn*-1 position. Sugiura and colleagues (10) have confirmed that brain microsomes slowly transfer arachidonic acid from exogenous diarachidonyl-PC to PE resulting in the formation of *N*-arachidonyl-PE by an enzyme-mediated process. Only arachidonate in the *sn*-1 position is transferred, neither arachidonate in the *sn*-2 position nor free arachidonic acid serve as acyl donors. The same authors also examined the distribution of fatty acids at the *sn*-1 position of brain PC and found that arachidonic acid accounts for 0.3% of the total fatty acids. These results offer a compelling explanation for the finding that both *N*-arachidonyl-PE and AEA account for only a small fraction of the *N*-acyl-PE and NAE, respectively, in brain. The data are consistent with the hypothesis that AEA is synthesized via the transacylase/PLD pathway. Future studies will need to address the question of whether this pathway synthesizes AEA in large enough quantities to support its role as a transmitter in brain.

Evidence for the synthetase pathway

An alternative mechanism for the synthesis of AEA has been demonstrated in tissue homogenates but is

not supported by data from whole cells. AEA and other NAEs are synthesized by homogenates and membrane preparations from liver, brain, and kidney by the enzymatic condensation of ethanolamine and the fatty acid (26–30). This pathway utilizes free fatty acid and not the fatty acyl CoA derivatives (29). The physiological importance of this pathway is questionable, largely because the K_m of the enzyme for arachidonic acid is 7–153 μM (10, 29, 30) and for ethanolamine is 27–135 mM (10, 29, 30). The K_m values are much greater than the normal cellular concentrations of free arachidonic acid and ethanolamine. In addition, there is no evidence that AEA is synthesized in intact cells by this mechanism. In fact, DiMarzo and coworkers (11) demonstrated that AEA synthesis by neurons in primary culture in response to agents that increase intracellular calcium concentration is independent of free arachidonic acid concentrations within the neuronal cells. These findings suggest that the synthetase pathway does not occur in cells without the addition of exogenous ethanolamine and/or arachidonic acid.

There are several lines of evidence that the NAE synthetase described by these groups and an amidohydrolase that utilizes AEA as a substrate (described in greater detail below) are the same protein. First, several characteristics of the two enzymes are very similar, including organ and subcellular distribution, pH optima, and inhibition by phenylmethylsulfonyl fluoride (PMSF). Solubilized enzyme preparations from liver mitochondria exhibit both synthetase and hydrolase activities (31). Second, both the amidohydrolase and synthetase activities of a partially purified enzyme from porcine brain coelute from hydrophobic and ion exchange columns, and show identical sensitivities to heat, pH dependence and inhibition by difluorophosphate (DFP), arachidonyl trifluoromethyl ketone and *p*-chloromercuribenzoic acid (32).

ENZYMATIC HYDROLYSIS OF AEA

NAEs are hydrolyzed to free fatty acid and ethanolamine by an amidohydrolase (also called fatty-acid amide hydrolase). This enzyme was first identified in liver (31) and brain (18) by Schmid and coworkers. Amidohydrolase is membrane associated, with the highest specific activity in brain microsomal/myelin preparations (33, 34), liver microsomes, and liver mitochondria (31). Amidohydrolase activity has also been reported in membranes from lung, gastrointestinal tract, and kidney (29, 33). Based on the ability of the amidohydrolase inhibitor PMSF to increase the potency of AEA, there is evidence for the presence of amidohydrolase in guinea pig intestine but not mouse vas deferens (35). We have recently found that bovine coronary vessels and endothelial cells from coronary vessels have amidohydrolase activity (P. F. Pratt, C. J. Hillard, and W. B. Campbell, unpublished observations).

Amidohydrolase activity has been measured in intact cells and in membrane preparations from cells in culture. Neuroblastoma (N18TG2) and C6 glioma cells in culture take up AEA from the media and convert it to arachidonic acid and ethanolamine (28). Cerebellar granule cells in primary culture also take up AEA from the media, but the subsequent hydrolysis to arachidonic acid is very slow (36). It is possible that the low rate of cellular hydrolysis is due to a low amount of amidohydrolase in cerebellar granule cells; cerebellar granule cells exhibit amidohydrolase activity that is only 20% of the activity in membranes prepared from adult rat cerebellum (C. J. Hillard and M. J. Greenberg, unpublished results). We have found that membranes from cerebellar astrocytes and microglial cells in primary culture also exhibit amidohydrolase activity but it is lower than in membranes from granule cells.

In addition to AEA, substrates of the brain-derived amidohydrolase include several other long chain, unsaturated NAEs such as the ethanolamides of 18:3, 20:3, and 20:2 fatty acids (33). AEA is the best substrate for the brain amidohydrolase among this series of NAEs (33); K_m values of 3 μM to 12 μM have been reported for AEA hydrolysis in brain membranes (33, 34). Monounsaturated and saturated NAEs are not good substrates for the brain amidohydrolase although PEA and SEA are hydrolyzed by the liver enzyme (31). Primary amides of fatty acids, particularly *cis*-9,10-octadecenoamide, are also hydrolyzed by brain membranes to free fatty acid (37). These brain-derived lipids have been isolated from the cerebral spinal fluid of sleep-deprived mammals and induce physiological sleep when injected into rats (37). There is convincing evidence that the hydrolysis of *cis*-9,10-octadecenoamide and other unsaturated fatty acid amides occurs via the same amidohydrolase that catabolyzes AEA (38, 39).

Brain-derived amidohydrolase is inhibited by serine protease inhibitors such as PMSF (28, 33, 34) and DFP (34) with IC_{50} values in the micromolar range (34). A highly lipophilic derivative of PMSF, palmitylsulfonylfluoride, is a very potent inhibitor with an IC_{50} value of 50 nM (40) while the hydrophilic serine protease inhibitor AEBSF is not an effective inhibitor of amidohydrolase (34) suggesting that the active site of the enzyme is hydrophobic. The histidine alkylating agent, *p*-bromophenacyl bromide inhibits brain amidohydrolase activity (33) as do sulfhydryl-reactive agents *p*-chloromercuribenzoic acid (31, 32) and thimerosal

(34). Amidohydrolase activity is potentiated in the presence of dithiothreitol (38).

Several AEA-derived inhibitors of the amidase have been identified. One series of inhibitors is based on the hypothesis that polarized carbonyls mimic the intermediates formed during AEA hydrolysis (41). The best amidohydrolase inhibitors among the compounds synthesized and evaluated by these authors are trifluoromethyl ketone arachidonic acid and ethyl-2-oxostearate. We have recently developed an irreversible inhibitor of amidohydrolase, 1-diazo-6Z,9Z,12Z,15Z-heneicosatetra-2-one that covalently binds and inhibits amidase in membranes, cells and in vivo (W. S. Edgemond, C. J. Hillard, S. Muthian, and W. B. Campbell, unpublished results).

The amidohydrolase from liver was recently purified, microsequenced and cloned (39). The sequence information reveals a putative transmembrane domain at the N terminal that is consistent with the presence of enzymatic activity in membrane fractions and its absence from cytosolic fractions. A second interesting feature of the protein is the presence of a polyproline segment that contains a class II SH-3 binding domain, which suggests that the activity or subcellular localization of amidohydrolase may be regulated by other proteins. The expressed enzyme hydrolyzes both AEA and oleamide and the tissue distribution of its mRNA matches the distribution of enzyme activity described above. All of the available data support the contention that the cloned protein is the amidohydrolase. The identification of the protein and mRNA encoding the protein are exciting advances in the field that provide important tools that can be used to study the physiological roles of its substrates in the brain.

TRANSPORT OF AEA ACROSS CELLULAR MEMBRANES

A second possible mechanism for the inactivation of AEA in the brain is cellular reuptake. Primary neuronal cultures of cortical neurons (11) and cerebellar granule cells (36) accumulate AEA from the incubation media by a saturable and temperature-sensitive process. AEA accumulation occurs by facilitated diffusion and is not dependent upon sodium gradients or cellular ATP (36). The uptake process is inhibited by phloretin, a nonselective inhibitor of several membrane transport processes, including carrier-mediated glucose influx into erythrocytes (42). When cells are preloaded with [^{3}H]AEA, temperature-sensitive efflux of [^{3}H]AEA occurs which suggests that the carrier may mediate both AEA release and reaccumulation. It is possible that the carrier and amidohydrolase function in series to catabolyze AEA in a manner analogous to catecholamine inactivation by uptake followed by catabolism by monoamine oxidase. These findings are significant because they suggest that a selective extracellular mechanism exists for the removal of AEA from extraneuronal space and is, therefore, indirect support for the role of AEA as a neurotransmitter. In addition, inhibition of the carrier should enhance the availability of AEA for its receptor and prolong its action.

AEA HYDROXYLATION

Free arachidonic acid serves as a precursor for the formation of many biologically active lipids including prostaglandins, leukotrienes, and epoxyeicosatrienoic acid epoxides through the action of cyclooxygenases, lipoxygenases, and cytochrome P450 epoxygenases, respectively. It is of interest to determine whether AEA is a substrate for any of these enzymes and thereby serves as a precursor for other bioactive molecules.

The lipoxygenase family of enzymes incorporates molecular oxygen regiospecifically into arachidonic acid at the 5, 12, or 15 position. AEA is converted to the 12- and 15-hydroperoxy-5,8,10,14-eicosatetraenoylethanolamides by leukocyte (43) and pineal 12-lipoxygenase (44) and soybean 15-lipoxygenase (43), respectively, at rates comparable to oxygenation of free arachidonic acid. Lipoxygenase derivatives of AEA are also made by intact cells. Incubation of human neutrophils with AEA results in the formation of both 12(S)-hydroxy-AEA and 15(S)-hydroxy-AEA (W. S. Edgemond, C. J. Hillard, and W. B. Campbell, unpublished results). 12(S)-Hydroxy-AEA binds to the CB1 receptor with approximately the same affinity as AEA (43, 45) while 15(S)-hydroxy-AEA has 5- to 6-fold lower affinity for the CB1 receptor than AEA (43, 44). Interestingly, 12-hydroxy-AEA has been reported to be either an ineffective (43) or poorly effective (44) inhibitor of electrically evoked contractions of mouse vas deferens, raising the possibility that it is a partial agonist or antagonist of the CB1 receptor. We have examined the affinities of the hydroxy derivatives for the CB2 receptor. The CB2 receptor is expressed primarily in cells of the immune system, including B cells and macrophages (46) and so is more likely target for neutrophil-derived, hydroxylated AEA than the brain receptor. We find that 12(S)-hydroxy-AEA binds to the CB2 receptor with a K_i of 131 nM compared to AEA which has a K_i of 94 nM. Neither 12(R)-hydroxy-AEA nor 15(S)-hydroxy-AEA competed for binding at a concentration of 1 μM.

AEA is a substrate for mouse hepatic microsomal P450s, at least 20 different metabolites are produced (45, 47). Based on studies with inducers and blockade of enzyme activity with antibodies, cytochrome P450 3A is a major contributor to the metabolism. However, none of the metabolites have been identified and the physiological role(s) of these metabolites is completely unknown.

AEA IS A LIGAND OF THE CANNABINOID RECEPTOR

Several laboratories have confirmed the original observation that AEA competes for binding to the brain cannabinoid receptor using the four radiolabeled ligands of the cannabinoid receptor that are currently available; the agonists HU210, CP55940, and Win 55212-2, and the antagonist SR141716A (48–53). There is fairly good agreement among the K_i values reported for AEA binding to the CB1 receptor of rat brain membranes regardless of the radiolabeled ligand used when the binding assays are carried out in the presence of PMSF or another amidohydrolase inhibitor. With the exception of the original study of Devane (1), K_i values for AEA are in the range of 1–10 μM in the absence of amidohydrolase inhibitors when rat brain membrane preparations are used (49–52). The original study of Devane et al. (1) avoided the problem of amidohydrolase-mediated degradation by using purified synaptosomal membranes, which are essentially devoid of amidohydrolase activity (34). AEA has a lower affinity for the CB1 receptor in fibroblast L cells expressing the human CB1 receptor than in brain membranes; however, [^{3}H]CP55940 also has a lower affinity for the human CB1 receptor in L cells than in brain membranes which

TABLE 1. Structure activity relationships among AEA analogs for binding to the CB1 receptor

Compound	Relative affinity[a]	Citation
Ethanolamides of:		
20:3 (5Z,8Z,11Z)	0.78	54
22:4 (n–6)	0.65–1.6	53,55
20:3 (n–6)	1.0–1.6	53,55
20:4 (5Z,7E,9E,14Z)	6.3	56
20:3 (5Z,8Z,14Z), 12(R)hydroxy	13	[b]
20:5 (n–3)	17	51
22:6 (n–3)	22.5	53
cis-9,10-Octadecanoic acid	33	57
18:3 (n–6)	>75	53
20:1 (n–9)	>111	51
16:0	>1000	58
20-Hydroxy-AA	0.6	[b]
12(S)-Hydroxy AA	0.4–1.4	44,[b]
12(R)-Hydroxy AA	2.8	[b]
15(S)-Hydroxy AA	5–6	44,[b]
20:3 (5Z,8Z,14Z), 12(R)hydroxy	13	[b]
11-Hydroxy AA	15	44
PGE_2	>5000	50
PGA_2	>5000	50
PGB_1	>5000	50
PGB_2	>5000	50
Amides of arachidonic acid		
2′-Chloroethylamide	0.003	[b]
Cyclopropylamide	0.01	[b]
Cyclobutylamide	0.04	[b]
2-Methylarachidonyl-(2′-fluoroethyl)amide	0.06	51
2′-Fluoroethylamide	0.1	51
(R)1′-Hydroxy-2′-Propylamide	0.25	59,60
Propylamide	0.33	50
(R)2′-Hydroxy-1′-propylamide	0.33	59
3′-Hydroxy-1′-propylamide	0.6–0.7	50,53
Sulfonamide	1.3	51
Butylamide	1.4	50
1′-Hydroxy-2′-propylamide (racemic)	1.5	61
(S)2′-Hydroxy-1′-propylamide	1.5	59
1′-Methyl-2′-hydroxylethylamide	1.5	51
2′-(4-Benzenesulfonamide)ethylamide	1.8	51
(S)1′-Hydroxy-2′-propylamide	2.2	59

(*continued*)

suggests that characteristics of transfected receptor are responsible for the lower affinity (53). As one would expect for an agonist, K_i of AEA is increased when the CB1 receptor is uncoupled from G protein effectors as occurs in the presence of the GTP analog, GppNHp (C. S. Kearn and C. J. Hillard, unpublished observations).

Several laboratories have synthesized AEA analogs and determined their affinity for the CB1 receptor relative to AEA (**Table 1**). These studies give interesting insights into the nature of the binding interactions between AEA and the CB1 receptor. Few of the alterations in the arachidonate backbone of AEA enhance binding affinity, suggesting that a chain length of 20–22 carbons containing 3 or 4 double bonds is optimal. Several modifications in this region do not significantly affect binding, including removal of the double bond at the 14 position and the addition of hydroxyl groups at the 12 position. Confining the molecule to a hairpin configuration, as occurs with the ethanolamides of the prostaglandins, results in complete loss of activity.

Addition of methyl groups at C2 enhances the binding affinity relative to AEA. Both the monomethyl and dimethyl derivatives are twice as potent as AEA itself. These methylated analogs are not substrates for the amidohydrolase and, therefore, are useful AEA analogs. Binding affinity can also be enhanced when the ethanolamine portion of the molecule is modified. The primary hydroxyl group is not necessary for activity and its replacement with a methyl group results in increased affinity (e.g., arachidonyl propylamide). Replacement of the primary hydroxyl with methyl, chlorine, or fluorine greatly enhances binding affinity. These results suggest that the hydroxyl does not participate in hydrogen bonding and that hydrophobic interactions between AEA and the receptor in this region of the molecule are important. Binding affinity is dimin-

TABLE 1 (*Continued*). Structure activity relationships among AEA analogs for binding to the CB1 receptor

Compound	Relative affinity[a]	Citation
N,N-bis(2-Hydroxyethyl)arachidonylamide	2.2	60
N-(2-Hydroxy-2-ethylethyl)arachidonylamide	2.6	60
4′-Hydroxybutylamide	2–4.5	50,51
4′-Hydroxybenzylamide	2.8	60
2′-(4-Hydroxyphenyl)ethylamide	4.2	62
2′-Phenoxyethylamide	4.5	51
N-[2-Hydroxy-1-(hydroxymethyl)ethyl-arachidonylamide	4.6	60
2′-Hydroxy-1′-propylamide (racemic)	7.4	53
Benzylamide	8	50
Arachidonylmorpholine	13	51
5′-Hydroxypentylamide	10–21	50,51
2′-Hydroxyphenylamide	15–21	62,60
Arachidonamide	18	53
2′-(N-Formyl)aminoethylamide	18	50
1′-Hydroxyethylthioamide	18	60
3′-Hydroxyphenylamide	20	60
1-(Arachidonylcarbonyl)-3-hydroxypiperidine	22	60
4′-Hydroxyphenylamide	23	60
2′-Methoxyethylamide	20–30	50,51
1′-Hydroxy-2′-propylthioamide	28	60
N-Methyl-1′-hydroxyethylamide	32	60
N-(*trans*-2-Hydroxycyclohexyl)-arachidonylamide	>38	60
(R)2′-(1′-Hydroxy-4′-methyl)pentylamide	61	61
(S)2′-(1′-Hydroxy-4′-methyl)pentylamide	>95	61
4′-Benzenesulfonamide	>95	51
2′-Aminoethylamide	>450	50
Methylations of AEA		
2,2-Dimethyl AEA	0.5	51,61
2-Methyl AEA	0.6	51,61
2-Ethyl AEA	5.2	61
2,N-Dimethyl AEA	26	61
2-Isopropyl AEA	45	51,61
N-Methyl-AEA	56	61
Other related compounds		
2-Arachidonylglycerol	1.9–24	58,63
cis-9,10-Octadecanoamide	550	57
Arachidonic acid	>1000	58

[a]The relative affinity was calculated as the ratio of the K_i for the compound of interest to the K_i for AEA reported in the same study.

[b]S. Manna, W. S. Edgemond, M. J. Greenberg, W. B. Campbell, and C .J. Hillard, unpublished results.

ished if this region of the molecule either becomes too bulky (e.g., phenyl or pentyl derivatives) or is removed (e.g., arachidonamide). The high affinity of the 1′-chloroethyl, cyclopropyl, and cyclobutyl arachidonoylamides suggests that the region of the receptor that binds the amide is a hydrophobic pocket that is not very large.

There is considerably less information regarding the binding of AEA and structural analogs to the peripheral cannabinoid receptor, CB2. The CB2 receptor is located predominantly in cells of the peripheral immune system such as macrophages in the marginal zone of the spleen (64), the tonsils, and B cells (65). A wide range of K_i values for AEA binding to the CB2 receptor have been reported, ranging from 30 to 2000 nM (66–70). One reason for the wide array of results is that most of the assays have used cell lines transfected with the CB2 receptor and it is possible that post-translational modification of the receptor may be necessary for AEA binding. In this regard, AEA competes for [^{3}H]Win 55212-2 binding to CB2 receptors in membranes from a rat basophilic leukemia cell line with an IC_{50} value of 33 nM (67). Only a few studies comparing the CB2 binding affinities of AEA and structural analogs are published. Facci and coworkers (67) have reported that PEA is a high affinity ligand for the CB2 receptor in leukemia cells although a second group has reported that PEA does not bind to the CB2 receptor expressed in CHO cells (70). As discussed above, we have found that 12(S)-hydroxy-AEA binds to the CB2 receptor with a K_i of 131 nM compared to AEA which has a K_i of 94 nM while neither 12-(R)-hydroxy-AEA nor 15(S)-hydroxy-AEA competed for binding at a concentration of 1 μM.

AEA IS AN AGONIST OF THE CANNABINOID RECEPTOR: BIOCHEMICAL EVIDENCE

The brain cannabinoid receptor (CB1) has the biochemical and structural characteristics of a G-protein coupled receptor: agonist binding is decreased in the presence of GTP analogs (71, 72); CB1 agonists increase [^{35}S]GTPγS binding (73) and stimulate a low K_m GTPase activity (74), and the amino acid sequence of the receptor is structurally related to other members of the G-protein-coupled receptor superfamily (75). Activation of the CB1 receptor results in decreased adenylyl cyclase activity (76, 77), decreased opening of voltage-operated calcium channels (78, 79), and activation of several types of potassium channels, including A-type channels in hippocampal neurons (80) and inwardly rectifying (GIRK1) channels (81). The effects of CB1 agonists on adenylyl cyclase and voltage-operated calcium channel inhibition are reversed by incubation of the cells with pertussis toxin (79, 82), indicating that these effects are mediated by CB1 coupling to a G protein of the Gi or Go subtype.

There is substantial biochemical evidence that AEA is an agonist of the CB1 receptor. AEA inhibits forskolin-stimulated adenylyl cyclase activity in N18TG2 cells (IC_{50} 540 nM; 48), in CHO cells expressing the human CB1 receptor (IC_{50} values in the range of 160–322 nM; 48, 53, 66) and in cerebellar membranes (IC_{50} 1.9 μM; 49). However, in several of these studies, AEA had lower efficacy (i.e., a lower maximal effect) than the high affinity cannabimimetics Win 55212-2 and CP55940 (48, 49). AEA inhibits calcium currents in N18 neuroblastoma cells (53, 83) and in AtT20 cells expressing the human CB1 receptor (66). However, AEA has the properties of a partial agonist; it inhibits a smaller fraction of the total calcium current (83, 84) and decreases the inhibition produced by the agonist Win 55212-2 (83).

There is some controversy in the literature regarding the efficacy of AEA at the CB2 receptor. In one study, AEA was reported to have agonist activity at the CB2 receptor as it inhibited adenylyl cyclase activity with a K_i of 900 nM (66). However, several other studies report that AEA has no CB2 agonist activity (67, 69). In fact, in mast cells, AEA decreases the inhibitory effect of synthetic cannabinoids on adenylyl cyclase (67) and may function as an antagonist of the CB2 receptor. Further studies are essential to clarify the efficacy of AEA at the CB2 receptor.

AEA IS A CANNABINOID RECEPTOR AGONIST: PHYSIOLOGICAL EVIDENCE

AEA mimics many of the physiological effects of the cannabinoids when administered in vivo. The cannabinoids produce a unique spectrum of behavioral effects in animals, including catalepsy (85), decreased spontaneous activity in an open field (86), antinociception (87), and hypothermia (88) that are not unique to the cannabinoids individually but when taken together are highly predictive of cannabinoid activity (86). AEA mimics the cannabinoids in the production of these four behavioral effects in mice (89–91). AEA is generally less potent than THC and the behavioral changes have a shorter duration of action. It is likely that rapid breakdown by amidohydrolase, particularly in the liver, is responsible for the short duration of action. In support of this hypothesis, 2-methyl AEA, an active analog of AEA that is resistant to hydrolysis, produces more

long-lasting effects than AEA on motor behavior (92). Although high doses of AEA produce THC-like effects, very low doses of AEA (less than 0.1 mg/kg) have been shown to antagonize some effects of THC (93, 94). One explanation for this finding is that AEA is a partial agonist of the CB1 receptor, a concept that is supported by data that AEA has lower efficacy than other cannabinoids in vitro (48, 49, 84).

AEA and THC have similar effects on a variety of non-behavioral physiological systems. AEA mimics the ability of the cannabinoids to inhibit electrically induced contractions of the mouse vas deferens (1) and the guinea pig ileum (35). AEA decreases intraocular pressure in rabbits (95) as does THC (96). Like THC (97), AEA reduces sperm fertilizing capacity in sea urchins by inhibition of the acrosome reaction (98). AEA has other effects on reproductive function that are mediated by the CB1 receptor, including inhibition of the development of preimplantation embryos in the mouse (99). AEA administration to anesthetized rats produces a spectrum of changes in blood pressure depending upon the dose and time after administration (100, 101). The CB1 antagonist SR141716A inhibits the depressor response of AEA, suggesting that this component is mediated by the CB1 receptor. Both AEA and THC produce dilation of cerebral arterioles at very low concentrations likely by stimulation of the release of arachidonic acid from astrocytes (102).

One method that is commonly used to demonstrate that drugs share a binding site is to induce tolerance to one of the drugs and determine whether the animal is also tolerant to the other agent. In vivo pretreatment of mice with THC produces tolerance to the in vitro inhibitory effects of both THC and AEA on electrically evoked contractions of the isolated vas deferens (103). However, pretreatment with THC produces tolerance to THC-induced hypothermia but not to AEA-induced hypothermia (103). In contrast, chronic exposure of mice to high doses of AEA produces both tolerance to AEA and cross-tolerance to THC when motor activity, catalepsy, hypothermia, and analgesia are used as indices of cannabinoid action (104). Similarly, mice tolerant to the antinociceptive effects of THC exhibit cross-tolerance to AEA and 3′-fluoro-AEA (93). These studies support the concept that THC and AEA interact with the same receptor.

Drug discrimination studies have been used to determine whether AEA substitutes for THC in rats that have been trained to bar-press when treated with THC (105). This method provides a very sensitive approach to the question of whether two drugs "feel the same" to an animal. Rats responded to AEA, but only at one dose and the effect was accompanied by severe decreases in bar pressing overall. These results are consistent with the tolerance studies and suggest that the behavioral effects of AEA and THC are similar but do not completely overlap.

EFFECTS OF AEA NOT MEDIATED BY THE CB1 RECEPTOR

A possible explanation for the findings discussed above is that AEA produces effects that are not mediated by CB1 receptors. One very interesting effect of AEA is that it blocks gap junctions and astrocyte junctional conductance (106). Gap junctions in striatal astrocytes conduct calcium between adjacent astrocytes and their function can be measured using either double whole-cell recording or intercellular dye diffusion. The calcium waves that propagate through the astrocyte network are thought to be part of a long-range signalling loop between neurons and astrocytes (107). This effect of AEA requires fairly high concentrations (5 μM) and is neither mimicked by the cannabinoid agonist CP55940 nor inhibited by the CB1 receptor antagonist SR141716A, leading to the conclusion that the effect is not mediated by cannabinoid receptors. Interestingly, however, the effect is sensitive to pertussis toxin, which suggests the involvement of a G protein and possibly a non-cannabinoid, G protein-coupled receptor. Arachidonic acid also inhibited gap junctional conductance; however, the concentrations required were 10-fold higher than AEA and pertussis toxin did not reverse its effect. These results are very interesting and could be the first clue that a non-cannabinoid receptor binds AEA.

There are several reports that NAEs inhibit cation permeability of membranes, although none of these studies have been extended to AEA as yet. *N*-Oleoyl ethanolamine inhibits oxalacetate-induced release of calcium and magnesium from heart and liver mitochondrion at concentrations between 10 and 95 μM (108). The mechanism of NAE action is not clear but seems to be consistent with decreased opening of the permeability transition pore, a voltage-dependent channel of the inner mitochondrial membrane that is responsible for the sudden permeability increase to solutes that occurs following calcium accumulation, thiol reduction or metabolic uncoupling (109). In another study, long chain, saturated NAEs, including PEA and *N*-stearoyl ethanolamine, have been shown to prevent veratridine-induced increases in rubidium efflux from neuroblastoma cells in culture at sub-micromolar concentrations (110). The NAEs also inhibited veratridine-induced increases in lyso-PC content of neuroblastoma cell lipid extracts. The authors put forward

the hypothesis that NAEs are "membranotropic" agents that can prevent cellular damage resulting from neurotoxin treatment and possibly from other injury. This idea is interesting (although far from substantiated) and, when combined with the observation that NAE concentrations increase during ischemia (3, 5–8), raises the possibility that increased production of NAEs is a protective mechanism used by cells in an attempt to prevent ionic damage.

AEA has several biochemical effects that result from its conversion to arachidonic acid. Incubation of hepatocytes with 5 μM AEA results in a variety of effects on hepatic fatty acid metabolism including inhibition of acetyl-CoA carboxylase activity and cholesterol synthesis and increased carnitine palmitoyltransferase I activity and ketogenesis from palmitate (111). These effects of AEA are mimicked by arachidonic acid and not Δ^9-THC and prevented by coincubation with PMSF which inhibits the conversion of AEA to arachidonic acid (28). AEA conversion to arachidonic acid may be responsible for the reported inhibition of 1,4-dihydropyridine binding to calcium channels in rat brain (112), an effect that is mimicked by arachidonic acid (113). Similarly, AEA is converted to arachidonic acid by endothelial cells which may explain its reported activity as an endothelial-derived hyperpolarizing factor (P. F. Pratt, C. J. Hillard, and W. B. Campbell, unpublished observations).

AEA shares several nonreceptor-mediated effects of the cannabinoids, including increased arachidonic acid release (53) possibly due to increased MAP kinase activity (114) and alterations in membrane lipid ordering (115, 116). In addition, high concentrations of AEA stimulate protein kinase C activity (117) as do high concentrations of the plant-derived cannabinoids (118) although most likely the two classes of cannabinoids act through different mechanisms. Most of the effects occur at high AEA concentrations so it is not likely that they play an important role in the response of cells and tissues to AEA.

SUMMARY

AEA is a newly discovered endogenous cannabinoid that shares many of the biochemical and physiological effects of the plant-derived and synthetic cannabinoids. In addition, AEA also produces effects that are not shared by the cannabinoids and suggest that it may have other signaling targets. The most intriguing of these is its effect on astrocytic gap junctional conductance. AEA has the properties one would expect of an endogenous, eicosanoid signaling molecule. It is not stored in final form in cells and its synthesis is regulated by intracellular calcium concentrations. There are at least two mechanisms by which the action of AEA is terminated: cellular reuptake and hydrolysis.

Several very important questions regarding AEA as a signaling molecule remain to be answered. First, the synthetic pathway proposed requires that arachidonic acid be present in *sn*-1 position of PC yet only vanishingly small amounts of this lipid are found in brain. In addition, synthesis requires very high concentrations of intracellular calcium, which may only be achieved when cells are dying. This contention is supported by the finding that the brain concentrations of AEA increase postmortem and that ischemic cells make AEA. These results lead one to ask the most fundamental question, which is what is the role of the AEA/cannabinoid signaling system in the body.

Experiments that were carried out in the authors' laboratories were supported by NIH grants DA09155 and DA08098. The authors thank S. Muthian for assistance in making the figures.

Manuscript received 1 April 1997 and in revised form 28 May 1997.

REFERENCES

1. Devane, W. A., L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* **258:** 1946–1949.
2. Bachur, N. R., K. Masek, K. L. Melmon, and S. Udenfriend. 1965. Fatty acid amides of ethanolamine in mammalian tissues. *J. Biol. Chem.* **240:** 1019–1024.
3. Epps, D. E., P. C. Schmid, V. Natarajan, and H. H. O. Schmid. 1979. *N*-Acylethanolamine accumulation in infarcted myocardium. *Biochem. Biophys. Res. Commun.* **90:** 628–633.
4. Schmid, H. H. O., P. C. Schmid, and V. Natarajan. 1990. *N*-Acylated glycerophospholipids and their derivatives. *Prog. Lipid Res.* **29:** 1–43.
5. Schmid, P. C., R. J. Krebsbach, S. R. Perry, T. M. Dettmer, J. L. Maasson, and H. H. O. Schmid. 1995. Occurrence and postmortem generation of anandamide and other long chain *N*-acylethanolamines in mammalian brain. *FEBS Lett.* **375:** 117–120.
6. Schmid, P. C., R. J. Krebsbach, S. R. Perry, T. M. Dettmer, J. L. Maasson, and H. H. O. Schmid. 1996. Corrigendum to: Occurrence and postmortem generation of anandamide and other long chain *N*-acylethanolamines in mammalian brain. *FEBS Lett.* **382:** 125–126.
7. Felder, C. C., A. Nielsen, E. M. Briley, M. Palkovits, J. Priller, J. Axelrod, D. N. Nguyen, J. M. Richardson, R. M. Riggin, G. A. Koppel, S. M. Paul, and G. W. Becker. 1996. Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS Lett.* **393:** 231–235.

8. Kempe, K., F-F. Hsu, A. Bohrer, and J. Turk. 1996. Isotope dilution mass spectrometric measurements indicate that arachidonylethanolamide, the proposed endogenous ligand of the cannabinoid receptor, accumulates in rat brain tissue post mortem. *J. Biol. Chem.* **271:** 17287–17295.
9. Koga, D., T. Santa, K. Hagiwara, K. Imai, H. Takizawa, T. Nagano, M. Hirobe, M. Ogawa, T. Sato, and K. Inoue. 1995. High-performance liquid chromatography and fluorometric detection of arachidonylethanolamide (anandamide) and its analogues, derivatized with 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-N,N-dimethylaminosulphonyl-2,1,3-benzoxadiazole (DBD-COCl). *Biomed. Chromatogr.* **9:** 56–57.
10. Sugiura, T., S. Kondo, A. Sukagawa, T. Tonegawa, S. Nakane, A. Yamashita, Y. Ishima, and K. Waku. 1996. Transacylase-mediated and phosphodiesterase-mediated synthesis of *N*-arachidonylethanolamine, an endogenous cannabinoid-receptor ligand, in rat brain microsomes. *Eur. J. Biochem.* **240:** 53–62.
11. DiMarzo, V., A. Fontana, H. Cadas, S. Schinelli, G. Cimino, J-C. Schwartz, and D. Piomelli. 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature.* **372:** 686–691.
12. DiMarzo, V., L. De Petrocellis, N. Sepe, and A. Buono. 1996. Biosynthesis of anandamide and related acylethanolamides in mouse J774 macrophages and N18 neuroblastoma cells. *Biochem. J.* **316:** 977–984.
13. Randall, M. D., S. P. H. Alexander, T. Bennett, E. A. Boyd, J. R. Fry, S. M. Gardiner, P. A. Kemp, A. I. McCulloch, and D. A. Kendall. 1996. An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem. Biophys. Res. Commun.* **229:** 114–120.
14. Burstein, S. H., and S. A. Hunter. 1995. Stimulation of anandamide biosynthesis in N-18TG2 neuroblastoma cells by Δ^9-tetrahydrocannabinol (THC). *Biochem. Pharmacol.* **49:** 855–858.
15. Schmid, H. H. O., P. C. Schmid, and V. Natarajan. 1996. The *N*-acylation-phosphodiesterase pathway and cell signaling. *Chem. Phys. Lipids.* **80:** 133–142.
16. Sugiura, T., S. Kondo, A. Sukagawa, T. Tonegawa, S. Nakane, A. Yamashita, and K. Waku. 1996. Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through *N*-acylphosphatidylethanolamine pathway in testis: involvement of Ca^{2+}-dependent transacylase and phosphodiesterase activities. *Biochem. Biophys. Res. Commun.* **218:** 113–117.
17. Blank, M. L., E. A. Cress, M. Robinson, and F. Snyder. 1985. Metabolism of unique diarachidonoyl and linoleoylarachidonoyl species of ethanolamine and choline phosphoglycerides in rat testes. *Biochim. Biophys. Acta.* **833:** 366–371.
18. Natarajan, V., P. C. Schmid, P. V. Reddy, and H. H. O. Schmid. 1984. Catabolism of *N*-acylethanolamine phospholipids by dog brain preparations. *J. Neurochem.* **42:** 1613–1619.
19. Natarajan, V., P. C. Schmid, P. V. Reddy, M. L. Zuzarte-Augustin, and H. H. O. Schmid. 1983. Biosynthesis of *N*-acylethanolamine phospholipids by dog brain preparations. *J. Neurochem.* **41:** 1303–1312.
20. Cadas, H., S. Gaillet, M. Beltramo, L. Venance, and D. Piomelli. 1996. Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J. Neurosci.* **16:** 3934–3942.
21. Hansen, H. S., L. Lauritzen, A. M. Strand, B. Moesgaard, and A. Frandsen. 1995. Glutamate stimulates the formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cortical neurons in culture. *Biochim. Biophys. Acta.* **1258:** 303–308.
22. Natarajan, V., P. V. Reddy, P. C. Schmid, and H. H. O. Schmid. 1982. *N*-Acylation of ethanolamine phospholipids in canine myocardium. *Biochim. Biophys. Acta.* **712:** 342–355.
23. Reddy, P. V., V. Natarajan, P. C. Schmid, and H. H. O. Schmid. 1983. *N*-Acylation of dog heart ethanolamine phospholipids by transacylase activity. *Biochim. Biophys. Acta.* **750:** 472–480.
24. Reddy, P. V., P. C. Schmid, V. Natarajan, and H. H. O. Schmid. 1983. The role of cardiolipin as an acyl donor in dog heart *N*-acylethanolamine phospholipid biosynthesis. *Biochim. Biophys. Acta.* **751:** 241–246.
25. Nakanishi, S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science.* **258:** 597–603.
26. Colodzin, M., N. R. Bachur, H. Weissbach, and S. Udenfriend. 1963. Enzymatic formation of fatty acid amides. *Biochem. Biophys. Res. Commun.* **10:** 165–170.
27. Bachur, N. R., and S. Udenfriend. 1965. Microsomal synthesis of fatty acid amides. *J. Biol. Chem.* **241:** 1308–1313.
28. Deutsch, D. G., and S. A. Chin. 1993. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol.* **46:** 791–796.
29. Kruszka, K. K., and R. W. Gross. 1994. The ATP- and CoA-independent synthesis of arachidonylethanolamide. *J. Biol. Chem.* **269:** 14345–14348.
30. Devane, W. A., and J. Axelrod. 1994. Enzymatic synthesis of anandamide, an endogenous ligand for the cannabinoid receptor, by brain membranes. *Proc. Natl. Acad. Sci. USA.* **91:** 6698–6701.
31. Schmid, P. C., M. L. Zuzarte-Augustin, and H. H. O. Schmid. 1985. Properties of rat liver *N*-acylethanolamine amidohydrolase. *J. Biol. Chem.* **260:** 14145–14149.
32. Ueda, N., Y. Kurahashi, S. Yamamoto, and T. Tokunaga. 1995. Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J. Biol. Chem.* **270:** 23823–23827.
33. Desarnaud, F., H. Cadas, and D. Piomelli. 1995. Anandamide amidohydrolase activity in rat brain microsomes. *J. Biol. Chem.* **270:** 6030–6035.
34. Hillard, C. J., D. M. Wilkison, W. S. Edgemond, and W. B. Campbell. 1995. Characterization of the kinetics and distribution of *N*-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim. Biophys. Acta.* **1257:** 249–256.
35. Pertwee, R. G., S. R. Fernando, G. Griffin, V. Abadji, and A. Makriyannis. 1995. Effect of phenylmethylsulphonyl fluoride on the potency of anandamide as an inhibitor of electrically evoked contractions in two isolated tissue preparations. *Eur. J. Pharmacol.* **272:** 73–78.
36. Hillard, C. J., W. S. Edgemond, A. Jarrahian, and W. B. Campbell. 1997. Accumulation of *N*-arachidonylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J. Neurochem.* **69:** 631–638.
37. Cravatt, B. F., O. Prospero-Garcia, G. Siuzdak, N. B. Gilul, S. J. Henriksen, D. L. Bolger, and R. A. Lerner. 1995. Chemical characterization of a family of brain lipids that induce sleep. *Science.* **268:** 1506–1509.
38. Maurelli, S., T. Bisogno, L. DePetrocellis, A. DiLuccia,

G. Marino, and V. DiMarzo. 1995. Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma "anandamide amidohydrolase." *FEBS Lett.* **377:** 82–86.
39. Cravatt, B. F., D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner, and N. B. Guila. 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature.* **384:** 84–87.
40. Lang, W., C. Qin, W. A. G. Hill, S. Lin, A. D. Khanolkar, and A. Makriyannis. 1996. High-performance liquid chromatographic determination of anandamide amidase activity in rat brain microsomes. *Anal. Biochem.* **238:** 40–45.
41. Koutek, B., G. D. Prestwich, A. C. Howlett, S. A. Chin, D. Salehani, N. Akhavan, and D. G. Deutsch. 1994. Inhibitors of arachidonoyl ethanolamide hydrolysis. *J. Biol. Chem.* **37:** 22937–22940.
42. Krupka, R. M., and R. Deves. 1981. An experimental test for cyclic versus linear transport models. *J. Biol. Chem.* **256:** 5410–5419.
43. Ueda, N., K. Yamamoto, S. Yamamoto, T. Tokunaga, E. Shirakawa, H. Shinkai, M. Ogawa, T. Sato, I. Kudo, K. Inoue, H. Takizawa, T. Nagano, M. Hirobe, N. Matsuki, and H. Saito. 1995. Lipoxygenase-catalyzed oxygenation of arachidonylethanolamide, a cannabinoid receptor agonist. *Biochim. Biophys. Acta.* **1254:** 127–134.
44. Hampson, A. J., W. A. G. Hill, M. Zan-Phillips, A. Makriyannis, E. Leung, R. M. Eglen, and L. M. Bornheim. 1995. Anandamide hydroxylation by brain lipoxygenase: metabolite structures and potencies at the cannabinoid receptor. *Biochim. Biophys. Acta.* **1259:** 173–179.
45. Bornheim, L. M., K. Y. Kim, B. Chen, and M. A. Correia. 1993. The effect of cannabinol on mouse hepatic microsomal cytochrome P450-dependent anandamide metabolism. *Biochem. Biophys. Res. Commun.* **197:** 740–746.
46. Bouaboula, M., M. Rinaldi, R. Carayon, C. Carillon, B. Delpech, D. Shire, G. Le Fur, and P. Casellas. 1993. Cannabinoid-receptor expression in human leukocytes. *Eur. J. Biochem.* **214:** 173–180.
47. Bornheim, L. M., K. Y. Kim, B. Chen, and M. A. Correia. 1995. Microsomal cytochrome P450-mediated liver and brain anandamide metabolism. *Biochem. Pharmacol.* **50:** 677–686.
48. Vogel, Z., J. Barg, R. Levy, D. Saya, E. Heldman, and R. Mechoulam. 1993. Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J. Neurochem.* **61:** 352–355.
49. Childers, S. R., T. Sexton, and M. B. Roy. 1994. Effects of anandamide on cannabinoid receptors in rat brain membranes. *Biochem. Pharmacol.* **47:** 711–715.
50. Pinto, J. C., F. Potie, K. C. Rice, D. Boring, M. R. Jonson, D. M. Evans, G. H. Wilken, C. H. Cantrell, and A. C. Howlett. 1994. Cannabinoid receptor binding and agonist activity of amides and esters of arachidonic acid. *Mol. Pharmacol.* **46:** 516–522.
51. Adams, I. B., W. Ryan, M. Singer, B. F. Thomas, D. R. Compton, R. K. Razdan, and B. R. Martin. 1995. Evaluation of cannabinoid receptor binding and in vivo activities for anandamide analogs. *J. Pharmacol. Exp. Ther.* **273:** 1172–1181.
52. Hillard, C. J., W. S. Edgemond, and W. B. Campbell. 1995. Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: application to anandamide. *J. Neurochem.* **64:** 677–683.
53. Felder, C. C., E. M. Briley, J. Axelrod, J. T. Simpson, K. Mackie, and W. A. Devane. 1993. Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA.* **90:** 7656–7660.
54. Priller, J., E. M. Briley, J. Mansouri, W. A. Devane, K. Mackie, and C. C. Felder. 1995. Mead ethanolamide, a novel eicosanoid, is an agonist for the central (CB1) and peripheral (CB2) cannabinoid receptors. *Mol. Pharmacol.* **48:** 288–292.
55. Hanus, L., A. Gopher, S. Almog, and R. Mechoulam. 1993. Two new unsaturated fatty acid ethanolamides that bind to the cannabinoid receptor. *J. Med. Chem.* **36:** 3032–3034.
56. Wise, M. L., K. Soderstrom, T. F. Murray, and W. H. Gerwick. 1996. Synthesis and cannabinoid receptor binding activity of conjugated triene anandamide, a novel eicosanoid. *Experientia.* **52:** 88–92.
57. Boring, D. L., B. A. Berglund, and A. C. Howlett. 1996. Cerebrodiene, arachidonyl ethanolamide and hybrid structures: potential for interaction with brain cannabinoid receptors. *Prostaglandins Leukot. Essent. Fatty Acids.* **55:** 207–210.
58. Sugiura, T., S. Kondo, A. Sukagawa, S. Nakane, A. Shinoda, K. Itoh, A. Yamashita, and K. Waku. 1995. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* **215:** 89–92.
59. Abadji, V., S. Lin, G. Taha, G. Griffin, L. A. Stevenson, R. G. Pertwee, and A. Makriyannis. 1994. (R)-Methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. *J. Med. Chem.* **37:** 1889–1893.
60. Khanolkar, A. D., V. Abadji, S. Lin, W. A. G. Hill, G. Taha, K. Abouzid, Z. Meng, P. Fan, and A. Makriyannis. 1996. Head group analogs of arachidonylethanolamide, the endogenous cannabinoid ligand. *J. Med. Chem.* **39:** 4515–4519.
61. Adams, I. B., W. Ryan, M. Singer, R. K. Razdan, D. R. Compton, and B. R. Martin. 1995. Pharmacological and behavioral evaluation of alkylated anandamide analogs. *Life Sci.* **56:** 2041–2048.
62. Edgemond, W. S., W. B. Campbell, and C. J. Hillard. 1995. The binding of novel phenolic derivatives of anandamide to brain cannabinoid receptors. *Prostaglandins Leukot. Essent. Fatty Acids.* **52:** 84–86.
63. Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, R. G. Pertwee, G. Griffin, M. Bayewitch, J. Barg, and Z. Vogel. 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50:** 84–90.
64. Munro, S., K. L. Thomas, and M. Abu-Shaar. 1993. Molecular characterization of a peripheral receptor for cannabinoids. *Nature.* **365:** 61–65.
65. Galiegue, S., S. Mary, J. Marchand, D. Dussossoy, D. Carriere, P. Carayon, M. Bouaboula, D. Shire, G. Le Fur, and P. Casellas. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* **232:** 54–61.
66. Felder, C. C., K. E. Joyce, E. M. Briley, J. Mansouri, K.

Mackie, O. Blond, Y. Lai, A. L. Ma, and R. L. Mitchell. 1995. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol. Pharmacol.* **48:** 443–450.
67. Facci, L., R. Dal Toso, S. Romanello, A. Buriani, S. D. Skaper, and A. Leon. 1995. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl. Acad. Sci. USA.* **92:** 3376–3380.
68. Slipetz, D. M., G. P. O'Neill, L. Favreau, C. Dufresne, M. Gallant, Y. Gareau, D. Guay, M. LaBelle, and K. M. Metters. 1995. Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol. Pharmacol.* **48:** 352–362.
69. Bayewitch, M., T. Avidor-Reiss, R. Levy, J. Barg, R. Mechoulam, and Z. Vogel. 1995. The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett.* **375:** 143–147.
70. Showalter, V. M., D. R. Compton, B. R. Martin, and M. E. Abood. 1996. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *J. Pharmacol. Exp. Ther.* **278:** 989–999.
71. Devane, W. A., F. A. Dysarz, M. R. Johnson, L. S. Melvin, and A. C. Howlett. 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* **34:** 606–613.
72. Houston, D. B., and A. C. Howlett. 1993. Solubilization of the cannabinoid receptor from rat brain and its functional interaction with guanine nucleotide-binding proteins. *Mol. Pharmacol.* **43:** 17–22.
73. Sim, L. J., D. E. Selley, and S. R. Childers. 1995. In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[gamma-[^{35}S] thio]-triphosphate binding. *Proc. Natl. Acad. Sci. USA.* **92:** 7242–7246.
74. Pacheco, M. A., S. J. Ward, and S. R. Childers. 1993. Identification of cannabinoid receptors in cultures of rat cerebellar granule cells. *Brain Res.* **603:** 102–110.
75. Matsuda, L. A., S. J. Lolait, M. J. Brownstein, A. C. Young, and T. I. Bonner. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature.* **346:** 561–564.
76. Howlett, A. C. 1985. Cannabinoid inhibition of adenylate cyclase. Biochemistry of the response in neuroblastoma cell membranes. *Mol. Pharmacol.* **27:** 429–436.
77. Felder, C. C., J. S. Veluz, H. L. Williams, E. M. Briley, and L. A. Matsuda. 1992. Cannabinoid agonists stimulate both receptor and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol. Pharmacol.* **42:** 838–845.
78. Caulfield, M. P., and D. A. Brown. 1992. Cannabinoid receptor agonists inhibit Ca current in NG108-15 neuroblastoma cells via a pertussis toxin-sensitive mechanism. *Br. J. Pharmacol.* **106:** 231–232.
79. Mackie, K., and B. Hille. 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc. Natl. Acad. Sci. USA.* **89:** 3825–3829.
80. Deadwyler, S. A., R. E. Hampson, J. Mu, A. Whyte, and S. Childers. 1995. Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. *J. Pharmacol. Exp. Ther.* **273:** 734–743.
81. Henry, D. J., and C. Chavkin. 1995. Activation of inwardly rectifying potassium channels (GIRK1) by coexpressed rat brain cannabinoid receptors in *Xenopus* oocytes. *Neurosci. Lett.* **186:** 91–94.
82. Howlett, A. C., J. M. Qualy, and L. L. Khachatrian. 1986. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol. Pharmacol.* **29:** 307–313.
83. Mackie, K., W. A. Devane, and B. Hille. 1993. Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol. Pharmacol.* **44:** 498–503.
84. Gebremedhin, D., W. B. Campbell, C. J. Hillard, and D. R. Harder. 1996. Inhibitory effects of anandamide on Ca^{2+} currents in cat cerebral arterial smooth muscle cells. *FASEB J.* **10:** A302.
85. Pertwee, R. G. 1972. The ring test: a quantitative method for assessing the cataleptic effect of cannabis in mice. *Br. J. Pharmacol.* **46:** 753–763.
86. Martin, B. R., D. R. Compton, B. F. Thomas, W. R. Prescott, P. J. Little, R. K. Razdan, M. R. Johnson, L. S. Melvin, R. Mechoulam, and S. J. Ward. 1991. Behavioral, biochemical and molecular modeling evaluations of cannabinoid analogs in mice. *Pharmacol. Biochem. Behav.* **40:** 471–478.
87. Buxbaum, D. M. 1972. Analgesic activity of Δ^9-tetrahydrocannabinol in the rat and mouse. *Psychopharmacology.* **25:** 275–280.
88. Bloom, A. S., and C. J. Kiernan. 1980. Interaction of ambient temperature with the effects of Δ^9-tetrahydrocannabinol on brain catecholamine synthesis and plasma corticosterone levels. *Psychopharmacology.* **67:** 215–219.
89. Fride, E., and R. Mechoulam. 1993. Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. *Eur. J. Pharmacol.* **231:** 313–314.
90. Crawley, J. N., R. L. Corwin, J. K. Robinson, C. C. Felder, W. A. Devane, and J. Axelrod. 1993. Anandamide, an endogenous ligand of the cannabinoid receptor, induces hypomotility and hypothermia in vivo in rats. *Pharmacol. Biochem. Behav.* **46:** 967–972.
91. Smith, P. B., D. R. Compton, S. P. Welch, R. K. Razdan, R. Mechoulam, and B. R. Martin. 1994. The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J. Pharmacol. Exp. Ther.* **270:** 219–227.
92. Romero, J., E. Garcia-Palermo, S. Y. Lin, J. A. Ramos, A. Makriyannis, and J. J. Fernandez-Ruiz. 1996. Extrapyramidal effects of methanandamide, an analog of anandamide the endogenous CB1 receptor ligand. *Life Sci.* **58:** 1249–1257.
93. Welch, S. P., L. D. Dunlow, G. S. Patrick, and R. K. Razdan. 1995. Characterization of anandamide- and fluoroanandamide-induced antinociception and cross-tolerance to delta-9-THC after intrathecal administration to mice: blockade of delta-9-THC-induced analgesia. *J. Pharmacol. Exp. Ther.* **273:** 1235–1244.
94. Fride, E., J. Barg, R. Levy, D. Saya, E. Heldman, R. Mechoulam, and Z. Vogel. 1995. Low doses of anandamides inhibit pharmacological effects of Δ^9-tetrahydrocannabinol. *J. Pharmacol. Exp. Ther.* **272:** 699–707.
95. Pate, D. W., K. Jarvinen, A. Urti, P. Jarho, and T. Jarvinen. 1995. Ophthalmic arachidonylethanolamide decreases intraocular pressure in normotensive rabbits. *Curr. Eye Res.* **14:** 791–797.
96. Merritt, J. C., S. McKinnon, J. R. Armstrong, G. Hatem,

and L. A. Reid. 1980. Oral delta-9-tetrahydrocannabinol in heterogeneous glaucomas. *Ann. Ophthalmol.* **12:** 947–950.
97. Schuel, H., R. Schuel, A. M. Zimmerman, and S. Zimmerman. 1987. Cannabinoids reduce fertility of sea urchin sperm. *Biochem. Cell Biol.* **65:** 130–136.
98. Schuel, H., E. Goldstein, R. Mechoulam, A. M. Zimmerman, and S. Zimmerman. 1994. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc. Natl. Acad. Sci. USA.* **91:** 7678–7682.
99. Paria, B. C., S. K. Das, and S. K. Dey. 1995. The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. *Proc. Natl. Acad. Sci. USA.* **92:** 9460–9464.
100. Varga, K., K. Lake, B. R. Martin, and G. Kunos. 1995. Novel antagonist implicates the CB1 receptor in the hypotensive action of anandamide. *Eur. J. Pharmacol.* **278:** 279–283.
101. Stein, E. A., S. A. Fuller, W. S. Edgemond, and W. B. Campbell. 1996. Physiological and behavioural effects of the endogenous cannabinoid, arachidonylethanolamide (anandamide), in the rat. *Br. J. Pharmacol.* **119:** 107–114.
102. Ellis, E. F., S. F. Moore, and K. A. Willoughby. 1995. Anandamide and Δ^9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am. J. Physiol.* **269:** H1859–H1864.
103. Pertwee, R. G., L. A. Stevenson, and G. Griffin. 1993. Cross-tolerance between delta-9-tetrahydrocannabinol and cannabimimetic agents, CP 55,940, Win 55,212-2 and anandamide. *Br. J. Pharmacol.* **110:** 1483–1490.
104. Fride, E. 1995. Anandamides: tolerance and cross-tolerance to Δ^9-tetrahydrocannabinol. *Brain Res.* **697:** 84–90.
105. Wiley, J., R. Balster, and B. R. Martin. 1995. Discriminative stimulus effects of anandamide in rats. *Eur. J. Pharmacol.* **276:** 49–54.
106. Venance, L., D. Piomelli, J. Glowinski, and C. Glaume. 1995. Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. *Nature.* **376:** 590–594.
107. Nedergaard, M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science.* **263:** 1768–1771.
108. Epps, D. E., J. W. Palmer, H. H. O. Schmid, and D. R. Pfeiffer. 1982. Inhibition of permeability-dependent Ca^{2+} release from mitochondria by *N*-acylethanolamines, a class of lipids synthesized in ischemic heart tissue. *J. Biol. Chem.* **257:** 1383–1391.
109. Petronelli, V., A. Nicolli, P. Costantini, R. Colonna, and P. Bernardi. 1994. Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporin-A. *Biochim. Biophys. Acta.* **1187:** 255–259.
110. Gulaya, N. M., A. A. Melnik, D. I. Balkov, G. L. Volkov, M. V. Vysotsky, and V. E. Vaskovsky. 1993. The effect of long-chain *N*-acylethanolamines on some membrane-associated functions of neuroblastoma C1300 N18 cells. *Biochim. Biophys. Acta.* **1152:** 280–288.
111. Guzman, M., J. J. Fernandez-Ruiz, C. Sanchez, G. Velasco, and J. A. Ramos. 1995. Effects of anandamide on hepatic fatty acid metabolism. *Biochem. Pharmacol.* **50:** 885–888.
112. Johnson, D. E., S. L. Heald, R. D. Dally, and R. A. Janis. 1993. Isolation, identification and synthesis of an endogenous arachidonic amide that inhibits calcium channel antagonist 1,4-dihydropyridine binding. *Prostaglandins Leukot. Essent. Fatty Acids.* **48:** 429–437.
113. Shimasue, K., T. Urushidani, M. Hagiwara, and T. Nagao. 1996. Effects of anandamide and arachidonic acid on specific binding of (+)-PN200-110, diltiazem and (−)-desmethoxyverapamil to L-type Ca^{2+} channel. *Eur. J. Pharmacol.* **296:** 347–350.
114. Wartmann, M., D. Campbell, A. Subramanian, S. H. Burstein, and R. J. Davis. 1995. The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett.* **359:** 133–136.
115. Hillard, C. J., R. A. Harris, and A. S. Bloom. 1985. Effects of the cannabinoids on physical properties of brain membranes and phospholipid vesicles: fluorescence studies. *J. Pharmacol. Exp. Ther.* **232:** 579–588.
116. Bloom, A. S., W. S. Edgemond, and J. C. Moldvan. 1997. Nonclassical and endogenous cannabinoids: effects on the ordering of brain membranes. *Neurochem. Res.* **22:** 563–568.
117. DePetrocellis, L., P. Orlando, and V. DiMarzo. 1995. Anandamide, an endogenous cannabinomimetic substance, modulates rat brain protein kinase C in vitro. *Biochem. Mol. Biol. Int.* **36:** 1127–1133.
118. Hillard, C. J., and J. A. Auchampach. 1994. In vitro activation of brain protein kinase C by the cannabinoids. *Biochim. Biophys. Acta.* **1220:** 163–170.