

Isotope Dilution Mass Spectrometric Measurements Indicate That Arachidonylethanolamide, the Proposed Endogenous Ligand of the Cannabinoid Receptor, Accumulates in Rat Brain Tissue Post Mortem but Is Contained at Low Levels in or Is Absent from Fresh Tissue*

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Arachidonylethanolamide (AEA) isolated from porcine brain binds to cannabinoid receptors, mimics cannabinoid pharmacologic effects, and has been proposed as an endogenous cannabinoid receptor ligand. Demonstration of co-distribution of AEA and cannabinoid receptors in various brain regions could provide supportive evidence for this role. We have performed isotope dilution mass spectrometric measurements of AEA and have demonstrated AEA production by rat tissue homogenates *in vitro* from exogenous arachidonate and ethanolamine. No detectable endogenous AEA (<3.5 pmol/g of tissue) was observed in fresh rat brain, whether or not inhibitors of AEA hydrolysis were present during tissue processing. AEA (>1 nmol/g) was produced during saponification of brain phospholipid extracts. This appears not to reflect hydrolysis of *N*-arachidonylethanolamine phospholipid precursors of AEA, because *Streptomyces chromofuscus* phospholipase D, which is active against NAPE, failed to generate AEA from brain phospholipids despite substantial conversion of phospholipids to phosphatidic acid. Such experiments suggested that the abundance of *N*-arachidonylethanolamine phospholipid in fresh rat brain may be less than 1 in 10⁶ phospholipid molecules. AEA generated during saponification of tissue phospholipids appears to arise from base-catalyzed aminolysis of arachidonate-containing glycerolipids, because AEA was produced from synthetic (1-stearoyl, 2-arachidonoyl)-phosphatidylethanolamine under saponification conditions, and the amount produced increased 300-fold when free ethanolamine was included in the hydrolysis solution. Although AEA was not detectable (<0.17 pmol/mg of protein) in fresh rat brain, AEA accumulated post mortem to levels of 126 pmol/mg of brain protein. These findings do not exclude the possibility that AEA is rapidly synthesized and degraded locally *in vivo*, but they indicate that the AEA content of fresh rat brain and of NAPE precursors from which AEA might be derived are exceedingly low and that AEA can be produced artifactually from biological materials.

Psychoactive properties of *Cannabis sativa* (marijuana) (1) are attributable to Δ^9 -tetrahydrocannabinol (THC)¹ (2). Binding sites for THC exist in central nervous system (3), and a receptor for cannabinoid agonists has been identified by cloning of a cannabinoid receptor gene from rat and human brain (4, 5). This receptor is a member of the G-protein-coupled family, and cannabinoid receptors are predominantly distributed in brain tissue, including substantia nigra, pars reticulata, globus pallidus, and cerebellum, which are areas involved in regulation of behavior, emotion, and coordination (6–8). Effects of THC include analgesia, antiemesis, euphoria, and alterations in cognition, memory, and motor performance (9–14).

The existence of cannabinoid receptors in brain and the corresponding functional effects of THC have suggested that an endogenous ligand for cannabinoid receptors may exist by analogy with endogenous ligands for opiate receptors. The search for such ligands has been pursued by chromatographic analysis of lipid extracts of brain and competitive binding assays with the cloned cannabinoid receptor and radiolabeled THC analogs with affinity for the receptor (15). This has resulted in identification of arachidonylethanolamide (AEA) as a candidate for an endogenous cannabinoid receptor ligand (1, 15, 17). AEA exhibits high affinity for cannabinoid receptors, inhibits cannabinoid binding to synaptosomal membranes, and mimics the pharmacologic effects of THC (15). Synthetic AEA also mimics THC biochemical effects, including inhibition of adenylyl cyclase (16, 18, 19), and AEA administered to animals mimics the effects of THC, including induction of analgesia (20). Another group searching for endogenous modulators of Ca²⁺ channels isolated a lipid from lyophilized calf brain powder that inhibited binding of Ca²⁺ channel antagonists to cerebral cortical membranes (21), and this lipid was also demonstrated to be AEA (21). Synthetic AEA inhibits voltage-operated Ca²⁺ currents in cells expressing cannabinoid receptors (22).

No rigorously quantitative measurements of the amounts of AEA in mammalian tissues have been reported. Quantitation of AEA in tissues could provide important evidence for its proposed role as an endogenous cannabinoid receptor ligand, for example, by demonstrating co-distribution of AEA and can-

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¹ The abbreviations used are: THC, Δ^9 -tetrahydrocannabinol; AEA, arachidonylethanolamide; ATFMK, arachidonyltrifluoromethyl ketone; C18:0/C20:4-PE, (1-stearoyl, 2-arachidonoyl)-phosphatidylethanolamine; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NAPE, *N*-arachidonylethanolamine phospholipids; NICL, negative ion chemical ionization; PFB₂, bis-pentafluorobenzoyl; PFP₂, bis-pentafluoropropionyl; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; RP, reverse phase; Mes, 4-morpholineethanesulfonic acid.

nabinoid receptors in various brain regions with differing receptor densities. We have developed an isotope dilution mass spectrometric assay for AEA and have used it in attempts to measure the endogenous AEA content of rat tissues, including cerebrum and cerebellum.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats were obtained from Sasco (O'Fallon, Mo); arachidonic acid from NuChek (Elysian, MN); [$^3\text{H}_8$]arachidonic acid (100 Ci/mmol) from DuPont NEN; and synthetic (1-stearoyl, 2-arachidonoyl)-phosphatidylethanolamine (C18:0/C20:4-PE) from Avanti (Birmingham, AL). [$^2\text{H}_8$]Arachidonic acid was prepared by catalytic reduction of eicosa-(5,8,11,14)-tetraynoic acid with $^2\text{H}_2$ gas and purified by silicic acid chromatography (25).

Preparation of $^2\text{H}_8$ - and $^3\text{H}_8$ -Labeled Arachidonylethanolamide—[$^2\text{H}_8$]/[$^3\text{H}_8$]Arachidonyl chloride was prepared from [$^2\text{H}_8$]/[$^3\text{H}_8$]arachidonic acid and oxalyl chloride (26). [$^2\text{H}_8$]Arachidonic acid (1.32 μmol) and [$^3\text{H}_8$]arachidonic acid (80 μCi) in benzene were mixed with 2 equivalents of oxalyl chloride and 1 equivalent of dimethylformamide and converted to [$^2\text{H}_8$]/[$^3\text{H}_8$]AEA (specific activity, 60.6 $\mu\text{Ci}/\mu\text{mol}$) in a reaction performed at 0 °C for 15 min with a 10-fold molar excess of ethanolamine in CH_2Cl_2 . The reaction mixture was concentrated to dryness under N_2 , and the oily product was dissolved in 2% CH_3OH in CHCl_3 and purified on a silica gel column (Sep-Pak, Waters, Milford, MA). The column was washed with 2% CH_3OH in CHCl_3 (3 ml), and [$^2\text{H}_8$]/[$^3\text{H}_8$]AEA (light yellow oil) was eluted with CH_3OH (3 ml). The product was characterized by GC-MS analyses of the trimethylsilyl ether derivative in electron impact and positive ion chemical ionization modes. The electron impact mass spectrum of native AEA-trimethylsilyl contained a prominent molecular cation (m/z 419) and an ion reflecting loss of CH_3 (m/z 404). Other ions in the spectrum included m/z 328, 258, 230, and 218. Analogous ions in the spectrum of [$^2\text{H}_8$]AEA were m/z 427 (M), m/z 412 (M - CH_3), m/z 336, 264, 238, and 226. The CH_4 -positive ion chemical ionization mass spectrum of AEA-trimethylsilyl included ions at m/z 420 (MH), m/z 418 (M - H), m/z 448 (M + C_2H_5), m/z 460 (M + C_3H_7), and m/z 404 (M - CH_3). Corresponding ions in the spectrum of [$^2\text{H}_8$]AEA were m/z 428, 426, 456, 468, and 412.

Standard Curve for Isotope Dilution Gas Chromatographic-Mass Spectrometric Quantitation of Arachidonylethanolamide—Internal standard [$^3\text{H}_8$]/[$^2\text{H}_8$]AEA was added in a constant amount (30 nCi, 500 pmol) to a series of tubes containing varied amounts (0, 43, 150, 430, 1500, or 4300 pmol) of unlabeled AEA. Samples were concentrated to dryness, reconstituted in HPLC solvent, analyzed by HPLC, extracted, concentrated to dryness, converted to the bis-pentafluoropropionyl or bis-pentafluorobenzoyl derivatives, and analyzed by GC-MS, as described below.

Preparation of Tissue Homogenates and Extraction of Lipids—Preparation of tissue homogenates was performed as described previously (27). To examine AEA content of fresh tissues, samples of rat cerebrum, cerebellum, heart, or liver were obtained immediately after euthanasia and processed. In experiments to examine post-mortem accumulation of AEA, samples of rat brain were allowed to remain at room temperature (3–48 h) before processing. Tissue processing involved placing specimens in 10-ml conical screw-cap tubes containing 5 volumes of ice-cold buffer (20 mM Tris-HCl, 1 mM EGTA, pH 7.6) and homogenization (Polytron, Brinkman). In some cases, homogenization was performed in the presence of phenylmethylsulfonyl fluoride (1.5 mM) or arachidonyl-trifluoromethyl ketone (7.5 μM). Aliquots of homogenates were removed for protein determination (Bio-Rad assay kit using bovine serum albumin as the standard). Other aliquots were used as enzyme sources for AEA production from exogenous arachidonate and ethanolamine, as described below. Lipids were extracted from the remaining homogenate by adding CH_3OH (2 ml) and CHCl_3 (1 ml) to the homogenization tube, followed by vortexing and sonication (on ice, 30 s, setting 3, power 30%, Branson sonifier). Phosphate-buffered saline (0.8 ml) and CHCl_3 (1 ml) were then added, and tubes were vortex-mixed and centrifuged (2000 rpm, 5 min). The organic layer was transferred to a silanized conical 10-ml screw-cap tube, and extracts were concentrated to dryness. Lipid extracts were then processed for determination of AEA content, used as substrate in phospholipase D digestions, or saponified to determine fatty acyl content.

Alkaline Hydrolysis of Tissue Extracts and of Standard (1-Stearoyl, 2-Arachidonoyl)-phosphatidylethanolamine—In some experiments, lipid extracts from tissues were subjected to alkaline hydrolysis. In others, synthetic C18:0/C20:4-PE with or without free ethanolamine (1 mM) was subjected to alkaline hydrolysis. Phospholipid solutions were concentrated to dryness in 10 ml of silanized, conical, screw-cap glass tubes, to which dimethoxyethane (0.5 ml), 3 N LiOH (0.1 ml), and a magnetic stirring vane were added. Contents were then stirred in

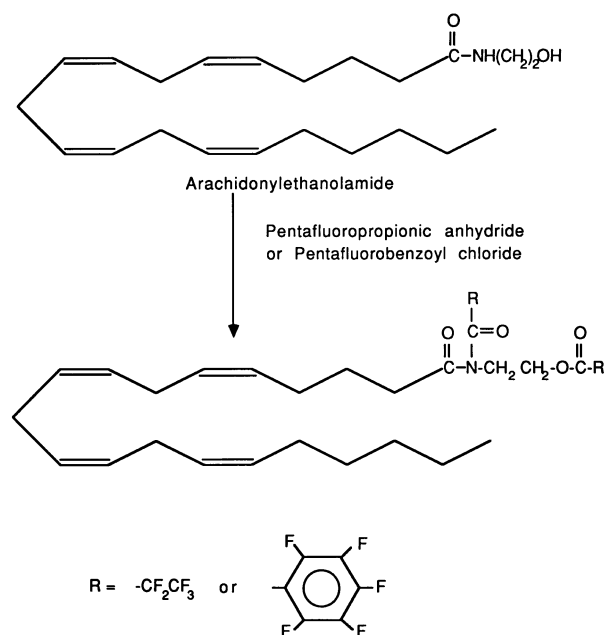


FIG. 1. Structure and derivatization scheme for arachidonylethanolamide. Arachidonylethanolamide was treated with pentafluoropropionic anhydride or with pentafluorobenzoyl chloride to form the bis-pentafluoropropionyl or bis-pentafluorobenzoyl derivative, respectively.

sealed vessels (90 min, room temperature, under N_2), and then water (3.5 ml) was added. After vortex-mixing, CHCl_3 (1 ml) was added, vortex-mixing was repeated, and the CHCl_3 (lower) phase was aspirated into a silanized 5-ml conical screw-cap glass tube. CHCl_3 extraction was repeated on residual aqueous phase, and the second extract was combined with the first and concentrated to dryness. The content of AEA or of free fatty acids in the extracts was then determined.

Phospholipase D Digestion of Tissue Phospholipids—Phospholipase D (PLD) digestion of tissue phospholipids was performed as described elsewhere (28, 29). Phospholipids from tissue homogenates were extracted by the method of Folch *et al.* (30) and concentrated to dryness. To the reaction vessel containing the phospholipid extract were then added diethyl ether (2 ml), buffer (360 μl of 40 mM Mes, pH 5.5), PLD (40 μl of a solution of 125 units/ml) from *Streptomyces chromofuscus* (phospholipase D, type VI, Sigma), and 100 mM CaCl_2 (30 μl). Reaction vessels were then shaken (high speed, 32 °C, 90 min). The diethyl ether was then evaporated under N_2 , and products were extracted by adding 2 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2/1, v/v) and H_2O (0.3 ml) and vortex-mixing. After centrifugation, the CHCl_3 -containing (lower) phase was placed in a separate tube, and the residual aqueous phase was extracted with 1.8 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (86/14/1, v/v/v). The organic phase was combined with the first extract and concentrated to dryness under N_2 . An aliquot of the extract was analyzed by TLC to verify digestion of phospholipids to phosphatidic acid. TLC analyses were performed on scored, channelled Silica Gel G plates (Analtech, Newark, DE) in the solvent system $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{NH}_4\text{OH}$ (67.5/52.5/11/4, v/v/v/v). After development, plates were allowed to dry and exposed to iodine vapor. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid exhibited R_f values of about 0.71, 0.84, and 0.30, respectively. Under the digestion conditions, essentially complete conversion of phosphatidylcholine and phosphatidylethanolamine to phosphatidic acid occurred. The remainder of the lipid extract from the digestion was spiked with [$^3\text{H}_8$]/[$^2\text{H}_8$]AEA internal standard and analyzed in a separate TLC system to isolate AEA, as described below.

Synthesis of Arachidonylethanolamide by Tissue Homogenates—Incubations to examine AEA production from exogenous substrates by tissue homogenates were performed as described previously (27, 32). Aliquots of tissue homogenates (1 mg of protein) were incubated (60 min, 37 °C) in 1 ml of buffer (0.1 M Tris-HCl, pH 7.0–9.0) with or without exogenous arachidonic acid (100 μM) and ethanolamine (7 mM) (27). For incubations at pH 5.0–6.0, citrate/ Na_2HPO_4 buffer was used, and for incubations at pH 10.0, $\text{NaHCO}_3/\text{NaOH}$ buffer was used (32). Incubations were terminated by addition of 2 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1/1, v/v), vortex-mixing, and centrifugation. The organic phase was harvested, mixed with a [$^3\text{H}_8$]/[$^2\text{H}_8$]AEA internal standard, concentrated to

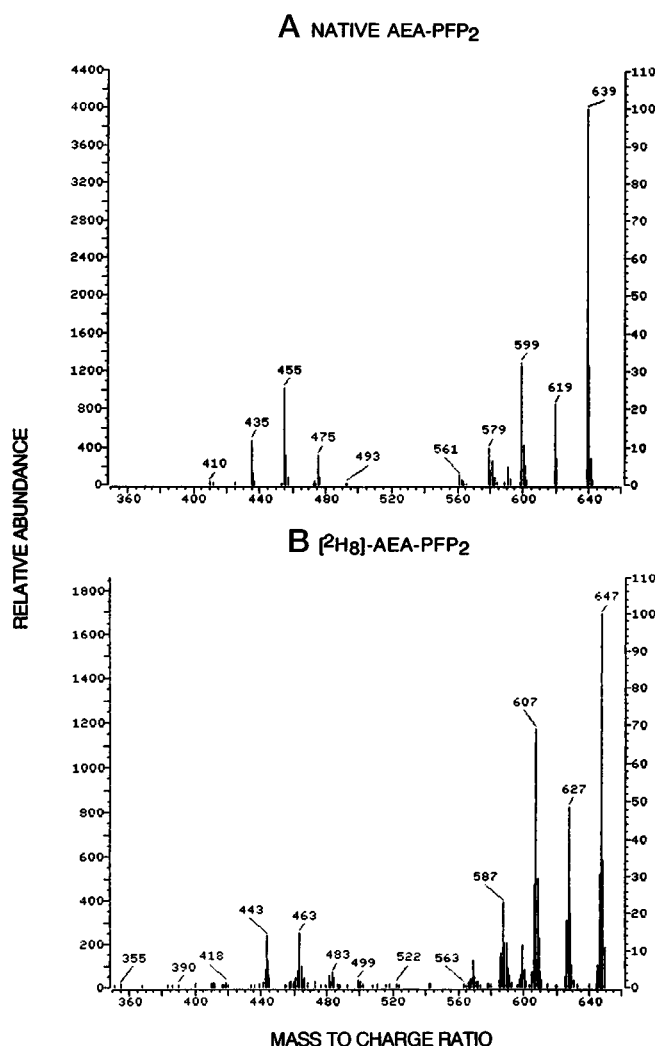


FIG. 2. Negative ion chemical ionization mass spectra of the bis-pentafluoropropionyl derivatives of arachidonylethanolamide and of [²H₈]arachidonylethanolamide. Standard AEA and [²H₈]AEA were converted to bis-pentafluoropropionyl derivatives and analyzed by GC-NICI-MS with CH₄ as moderating gas as described under "Experimental Procedures." *Panel A* is the NICI mass spectrum of the derivative of native AEA and *panel B* the NICI mass spectrum of the derivative of [²H₈]AEA.

dryness under N₂, and analyzed by HPLC or TLC to isolate AEA, as described below.

Liquid Chromatographic Analyses—For HPLC analyses, samples of tissue extracts, saponification products, phospholipase D digestion products, or standard curve solutions were spiked with internal standard [³H₈]/[²H₈]AEA (30 nCi, 500 pmol), concentrated to dryness under N₂, reconstituted in HPLC solvent, and analyzed by RP-HPLC on an octadecylsilicic acid column (Ultrasphere ODS 4.6 × 250 mm, Alltech, Deerfield, IL) with the solvent system (flow rate, 2 ml/min) CH₃CN/H₂O/CH₃CO₂H (57/43/0.01). Aliquots (50 μl) of each fraction (2 ml) were analyzed by liquid scintillation spectrometry to determine ³H content. The [³H₈]AEA standard exhibited a retention time of about 21.9 min under these conditions. TLC analyses were performed as described elsewhere (27). AEA-containing solutions were concentrated to dryness under N₂, dissolved in CHCl₃/CH₃OH (1/1, v/v), and applied to alternate lanes of channeled, scored Silica Gel G plates (Analtech, Newark, DE). A blank lane was interposed between each lane containing sample. Standard AEA (2.3 nmol) was applied to outer lanes of the plates. Plates were then developed in the solvent system ethyl acetate/hexane/CH₃CO₂H/H₂O (100/50/20/100, v/v/v/v). After the developed plates were allowed to dry, the outer portions containing AEA standard were broken from the remainder of the plate and the AEA migration position identified with iodine vapor (*R_F* ~ 0.41). Silicic acid from appropriate regions of lanes from the remainder of the plate was then scraped into conical glass tubes and extracted three times with aliquots (1 ml) of

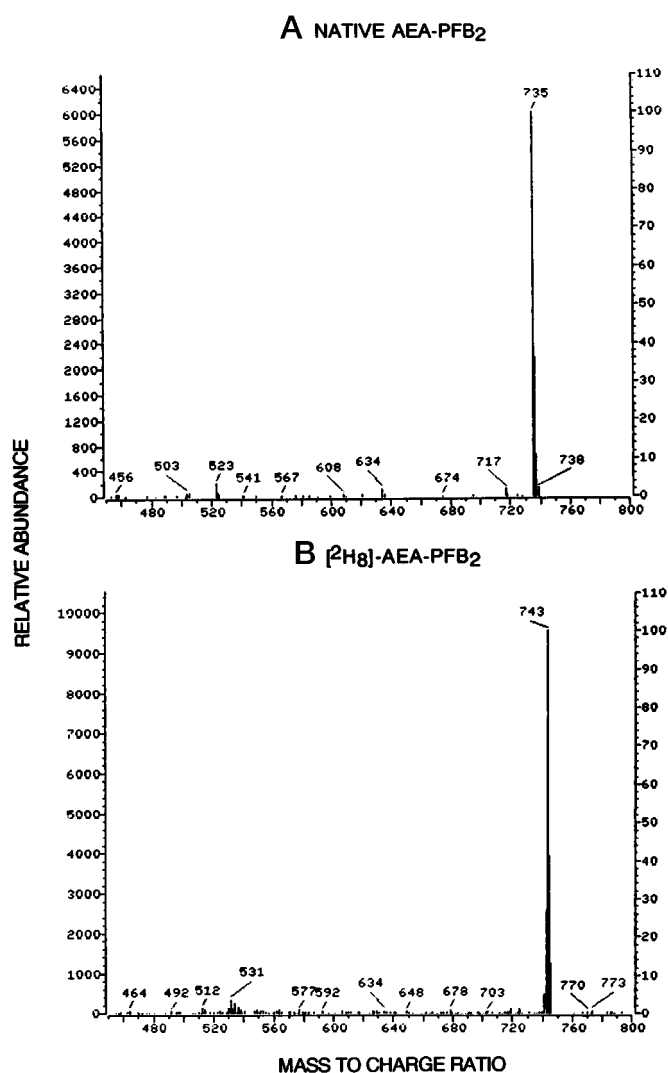


FIG. 3. Negative ion chemical ionization mass spectra of the bis-pentafluorobenzoyl derivatives of arachidonylethanolamide and of [²H₈]arachidonylethanolamide. Standard AEA and [²H₈]AEA were converted to the bis-pentafluorobenzoyl derivatives and analyzed by GC-NICI-MS as in Fig. 2. *Panel A* is the NICI mass spectrum of the derivative of native AEA and *panel B* the NICI mass spectrum of the derivative of [²H₈]AEA.

CHCl₃/CH₃OH (1/1, v/v). AEA-containing solutions from either HPLC or TLC analyses were concentrated to dryness, derivatized, and analyzed by GC-MS.

Derivatization of Arachidonylethanolamide—The trimethylsilyl ether derivative of AEA was prepared with pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide, as described previously (33–35). The AEA-bis-pentafluoropropionyl (PFP₂) derivative was prepared by mixing AEA with pentafluoropropionic anhydride/ethyl acetate (1/5, v/v) and heating (60 °C, 30 min). The AEA-bis-pentafluorobenzoyl (PFB₂) derivative was prepared by concentrating AEA-containing solutions to dryness in a conical reaction tube, adding 4% pyridine in toluene (50 μl), vortex-mixing, adding pentafluorobenzoyl chloride (10 μl, Aldrich), and vortex-mixing. This solution was incubated (90 min, 80 °C) and then concentrated to dryness under N₂. Water (0.5 ml) was then added, and vortex-mixing was performed. Hexane (0.8 ml) was then added, and vortex-mixing was repeated. The mixture was then centrifuged, and the aqueous phase was discarded. The residual hexane phase was then washed twice with H₂O (0.5 ml). After preparation of all of the above derivatives, solutions containing them were concentrated to dryness under N₂, and derivatives were dissolved in heptane (20 μl) for GC-MS analysis.

Gas Chromatography—Derivatized samples of AEA were introduced into a Hewlett-Packard 5890 gas chromatograph via a Grob-type injector (splitless mode, temperature 225 °C) and analyzed on an HP Ultra-performance capillary column (8-m length, cross-linked methyl-silicone, inside diameter 0.31 mm, film thickness 0.17 μm) interfaced with a

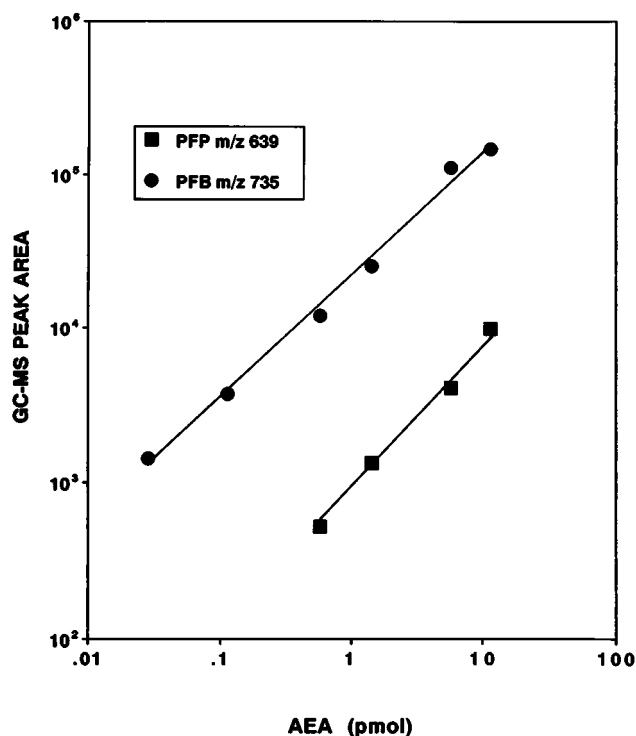


FIG. 4. Comparison of GC-MS signal intensity from the bis-pentafluoropropionyl and bis-pentafluorobenzoyl derivatives of arachidonylethanolamide. Standard arachidonylethanolamide was converted to the bis-pentafluoropropionyl (squares) or the bis-pentafluorobenzoyl derivative (circles), and varied amounts of the derivatives were analyzed by GC-NICI-MS, as described under "Experimental Procedures," with selected monitoring of the molecular anions.

Hewlett-Packard 5988 mass spectrometer. Helium was the carrier gas (total flow, 10 ml/min; head pressure, 4 p.s.i.). The initial oven temperature was 85 °C. The injector and interface temperatures were 225 °C. At 0.8 min after injection, the oven temperature was increased (30 °C/min) to a final temperature of 280 °C.

Mass Spectrometry—The GC was interfaced with a Hewlett-Packard 5988 mass spectrometer controlled via a Hewlett-Packard RTE-A data system (36, 37). For chemical ionization analyses, CH₄ was the reagent gas (source pressure of 1.5 torr). Source temperature was 140 °C for negative ion chemical ionization (NICI) analyses and 200 °C for positive ion analyses. For analyses of tissue content of AEA, selected ion monitoring of fluorinated derivatives of native AEA and of the [²H₈]AEA internal standard was performed in an NICI mode. For the bis-pentafluoropropionyl derivative, ions monitored were *m/z* 647 (M⁻ for [²H₈]AEA-PFP₂) and *m/z* 639 (M⁻ for native AEA-PFP₂). For the bis-pentafluorobenzoyl derivative, ions monitored were *m/z* 743 (M⁻ for [²H₈]AEA-PFB₂) and *m/z* 735 (M⁻ for native AEA-PFB₂).

Quantitation of the Fatty Acyl Content of Phospholipid Saponification Products—Fatty acids liberated from phospholipids by alkaline hydrolysis were extracted, converted to pentafluorobenzyl ester derivatives, and quantitated by GC-NICI-MS, as described previously (36, 37). Extracts of alkaline hydrolysates were spiked with [²H₈]arachidonic acid (800 pmol) and concentrated to dryness. To the dry sample, 20 μl of a solution of *N,N*-dimethylacetamide/tetramethylammonium hydroxide/methanol (1/0.5/1.5) and 20 μl of a solution of pentafluorobenzyl bromide/*N,N*-dimethylacetamide (1/3) were then added, and the tubes were vortex-mixed and incubated (room temperature, 15 min). After concentration to dryness, H₂O (50 μl) was added, vortex-mixing performed, CH₂Cl₂ (200 μl) added, vortex-mixing repeated, and the aqueous phase discarded. The CH₂Cl₂ phase was then concentrated to dryness. Samples were then dissolved in heptane (0.5 ml) and analyzed by capillary column (Hewlett-Packard Ultraperformance, 8 m, 0.17 μm dimethyl-silicone film, inside diameter 0.31 mm) GC (initial temperature 85 °C for 0.5 min, followed by a 20 °C/min increasing ramp to 240 °C) mass spectrometry (Hewlett Packard 5988) in the CH₄ (source pressure 1 torr) NICI mode. Fatty acid-pentafluorobenzyl esters were detected by selected monitoring of their M-PFB (M-181) ions at characteristic retention times and were quantitated relative to the [²H₈]arachidonate internal standard (36, 37).

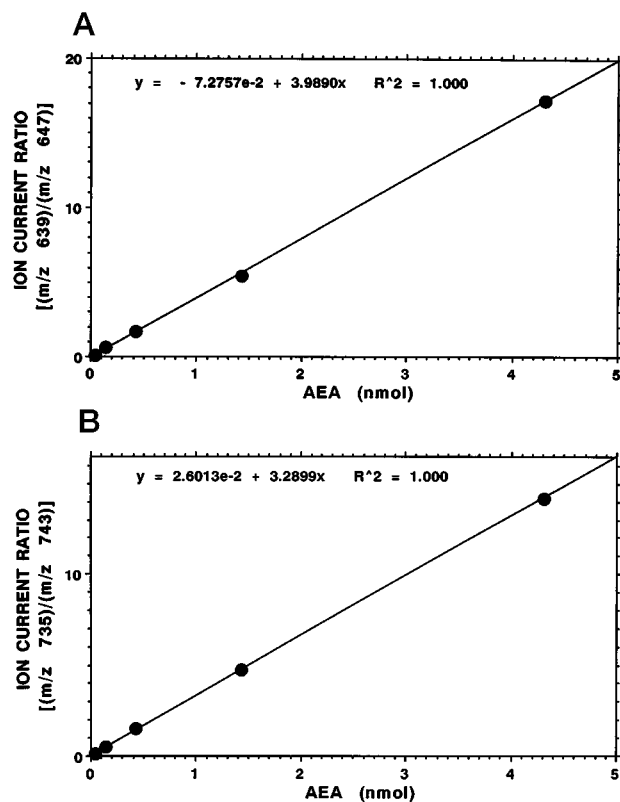


FIG. 5. Standard curves for stable isotope dilution gas chromatographic negative ion chemical ionization mass spectrometric measurement of arachidonylethanolamide. To each sample in the standard curve set, a fixed amount of [²H₈]/[³H₈]AEA internal standard (30 nCi, 500 pmol) was added, and varied amounts (0, 43, 150, 430, 1500, or 4300 pmol) of native AEA were added to individual samples. Each sample was then analyzed by RP-HPLC, and the AEA peak was located by liquid scintillation counting of aliquots of column eluant. AEA was then extracted, converted to the bis-pentafluoropropionyl (panel A) or bis-pentafluorobenzoyl (panel B) derivative and analyzed by GC-NICI-MS with selected monitoring of molecular anions for PFP₂ derivatives (*m/z* 647 for [²H₈]AEA-PFP₂ and *m/z* 639 for native AEA-PFP₂) or for PFB₂ derivatives (*m/z* 743 for [²H₈]AEA-PFB₂ and *m/z* 735 for native AEA-PFB₂), respectively. Abscissa values represent the amount of native AEA in each sample, and ordinate values represent the ratio of the ion current peak areas for the molecular anions of the unlabeled derivative divided by that for the ²H₈-labeled derivative for the peak at the appropriate GC retention time for AEA-PFP₂ (upper panel) or for AEA-PFB₂ (lower panel).

RESULTS

To exploit the great sensitivity of NICI, mass spectrometry for measurement of AEA, fluorinated, electron-capturing derivatives of AEA were prepared as in Fig. 1. NICI mass spectra of the PFP₂ derivatives of native AEA and of [²H₈]AEA are illustrated in Fig. 2. The AEA-PFP₂ spectrum contains the molecular anion (M) at *m/z* 639. Consecutive losses of HF yield ions at *m/z* 619, 599, and 579. Loss of CF₃CF₂CO₂H from M yields an ion at *m/z* 475, and consecutive losses of HF yield ions at *m/z* 455 and 435. Corresponding ions in the [²H₈]AEA-PFP₂ spectrum are *m/z* 647, 627, 607, 483, 463, and 443. NICI mass spectra of the PFB₂ derivative of native AEA and of [²H₈]AEA are illustrated in Fig. 3; they exhibit little fragmentation and are dominated by the molecular anions (*m/z* 735 for AEA-PFB₂ and *m/z* 743 for [²H₈]AEA-PFB₂). Comparison of GC-MS peak areas from selected monitoring of molecular anions of AEA-PFP₂ and of AEA-PFB₂ revealed that greater signal was obtained from the latter derivative for a given amount injected on-column (Fig. 4). A signal to noise ratio of 2/1 was obtained with about 30 fmol of AEA-PFB₂ and with about 600 fmol of AEA-PFP₂. Either derivative could be employed in isotope di-

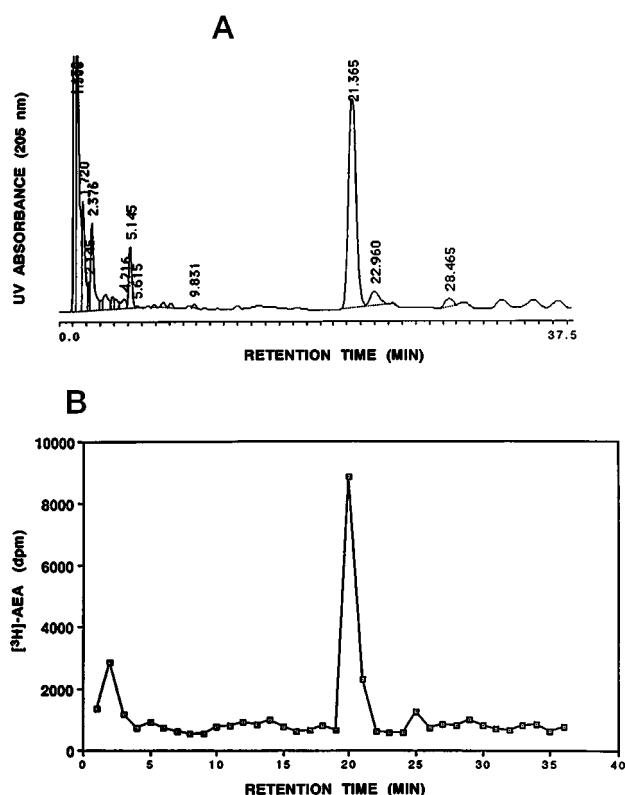


FIG. 6. Reverse phase HPLC analysis of unlabeled arachidonylethanolamide and recovery of $[^3\text{H}_8]/[^2\text{H}_8]$ arachidonylethanolamide internal standard from extracts of rat cerebellum. In *panel A*, standard AEA was analyzed by RP-HPLC, as described under "Experimental Procedures," with flow-through UV monitoring (205 nm). In *panel B*, samples of rat cerebellum were homogenized, mixed with the $[^2\text{H}_8]/[^3\text{H}_8]$ AEA internal standard (30 nCi, 500 pmol), and extracted. The extract was analyzed by RP-HPLC, and aliquots (50 μl) of each fraction were subjected to liquid scintillation spectrometry to measure ^3H content.

lution measurements of AEA (Fig. 5), which yielded linear standard curves with both derivatives (43 pmol to 4.3 nmol).

To isolate AEA from biological sources, an RP-HPLC method was developed (Fig. 6). *Panel A* illustrates analysis of standard AEA (6.6 nmol) with flow-through UV monitoring (205 nm) to detect absorbance from the double-bonds of the arachidonyl moiety. To detect the sub-nanomolar amounts of the $[^2\text{H}_8]/[^3\text{H}_8]$ AEA internal standard used in analysis of biological samples, the ^3H content of aliquots of the RP-HPLC eluant was determined by liquid scintillation spectrometry, as illustrated in *panel B*, which represents isolation of $[^3\text{H}_8]/[^2\text{H}_8]$ AEA that had been spiked into a lipid extract from rat cerebellum. Isolated AEA was then derivatized and analyzed by GC-NICI-MS. A described TLC method (27) was also employed for AEA isolation in some cases.

These methods were then used to measure AEA produced in a biological matrix. AEA production by tissue homogenates has been demonstrated radiochemically using $[^3\text{H}]$ arachidonic acid or $[^{14}\text{C}]$ ethanolamine as substrates (27, 31). Tissue homogenates were incubated with exogenous arachidonate and ethanolamine under previously described conditions (27). Internal standard $[^3\text{H}_8]/[^2\text{H}_8]$ AEA was then added, lipid extraction was performed, and AEA was isolated by liquid chromatography, derivatized, and analyzed by GC-NICI-MS with selected monitoring of molecular anions. Liver homogenates incubated in this way yielded a robust signal for AEA when analyzed as the PFB₂ derivative (Fig. 7, *panel B*). Little AEA production occurred with liver homogenates incubated without exogenous arachidonate and ethanolamine (Fig. 7, *panel A*). AEA produc-

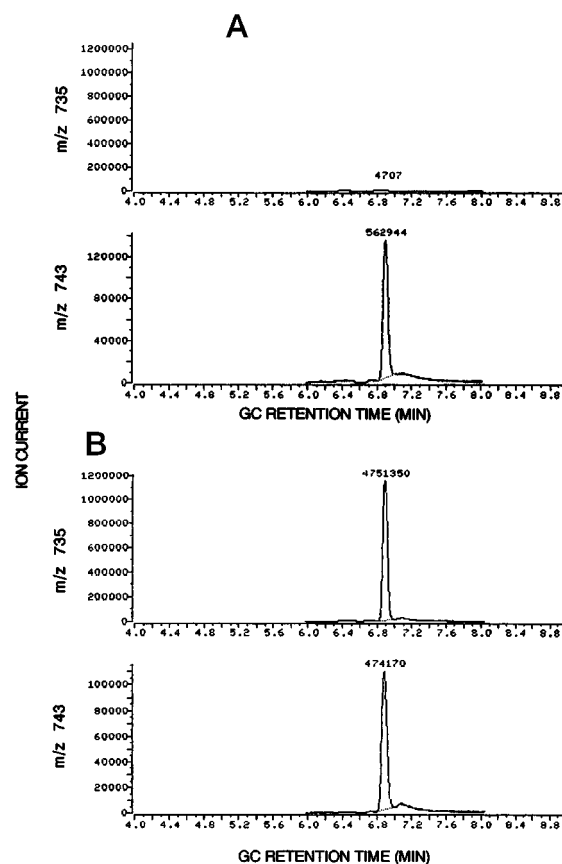


FIG. 7. Demonstration of arachidonylethanolamide production by rat liver homogenates supplemented with arachidonic acid and ethanolamine by stable isotope dilution gas chromatographic mass spectrometry. An aliquot of rat liver homogenate (1 mg of protein) was incubated (60 min, 37 $^{\circ}\text{C}$) in 1 ml of 0.1 M Tris-HCl, pH 9.0, without (*panel A*) or with (*panel B*) exogenous arachidonate (100 μM) and ethanolamine (7 mM). Incubations were terminated by addition of 2 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1) containing $[^2\text{H}_8]/[^3\text{H}_8]$ AEA internal standard (30 nCi, 500 pmol), vortexing, and centrifugation. The organic phase was harvested, concentrated, and analyzed by HPLC. The AEA peak was identified by liquid scintillation spectrometry for ^3H content, extracted, converted to the bis-pentafluorobenzoyl derivative, and analyzed by GC-NICI-MS with selected monitoring of the molecular anions for native AEA-PFB₂ (m/z 735) and for $[^2\text{H}_8]$ AEA-PFB₂ (m/z 743).

tion was ablated by boiling the homogenate before incubation with substrates, by omission of homogenate from the incubation, or by omission of either arachidonate or ethanolamine (Table I). Arachidonyltrifluoromethyl ketone (ATFMK) reduced the amount of AEA produced. This compound (32), like phenylmethylsulfonyl fluoride (PMSF) (27, 32, 38), has been reported to inhibit enzymatic hydrolysis of AEA, and such inhibition has been reported to increase AEA yield from radiolabeled substrates incubated with tissue homogenates under some conditions (27). Both ATFMK and PMSF also inhibit AEA synthesis (32, 39), however, and this effect appeared to dominate in our incubations, as in similar systems (39).

Comparison of abilities of homogenates of various tissues to generate AEA indicated that rat liver homogenate yielded more AEA per unit protein mass than did brain homogenates, although demonstrable AEA production occurred with both cerebellar and cerebral homogenates (Fig. 8). Little AEA was produced by heart homogenate. The relative amounts of AEA mass produced by these tissue homogenates correspond well to the relative abundance of $[^3\text{H}]$ AEA produced from radiolabeled substrate by homogenates of these tissues (31). With liver homogenate, AEA production exhibited an alkaline pH optimum (Fig. 9), as reported for AEA production from radiolabeled

TABLE I

Arachidonylethanolamide synthesis by liver homogenate requires arachidonate and ethanolamine and is ablated by boiling the homogenate

Aliquots of rat liver homogenate (1 mg of protein) were incubated in 1 ml of 0.1 M Tris-HCl, pH 9.0, for 60 min at 37 °C with or without exogenous arachidonic acid (100 μ M), ethanolamine (7 mM), and arachidonyltrifluoromethyl ketone (7.5 μ M). One of the aliquots of liver homogenate was boiled for 10 min before the incubation, and the remaining aliquots were not boiled. Incubations were terminated by addition of 2 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1) containing [$^2\text{H}_8$]/[$^3\text{H}_8$]AEA internal standard (30 nCi, 500 pmol), vortexing, and centrifugation. The organic phase was harvested, concentrated to dryness, and analyzed by HPLC. The AEA peak was identified by liquid scintillation spectrometry for ^3H content, extracted, converted to the bis-pentafluorobenzoyl derivative, and analyzed by GC-NICI-MS.

| Liver homogenate | Boiling | Arachidonate | Ethanolamine | Arachidonyltrifluoromethyl ketone | AEA production |
|------------------|---------|---------------|--------------|-----------------------------------|-------------------|
| mg | | μM | mM | μM | nmol |
| 1 | No | 100 | 7 | 0 | 2.351 \pm 0.460 |
| 1 | Yes | 100 | 7 | 0 | 0.035 \pm 0.007 |
| 0 | No | 100 | 7 | 0 | <0.007 |
| 1 | No | 0 | 0 | 0 | 0.016 |
| 1 | No | 100 | 0 | 0 | 0.023 |
| 1 | No | 0 | 7 | 0 | 0.010 |
| 1 | No | 100 | 7 | 7.5 | 0.734 |

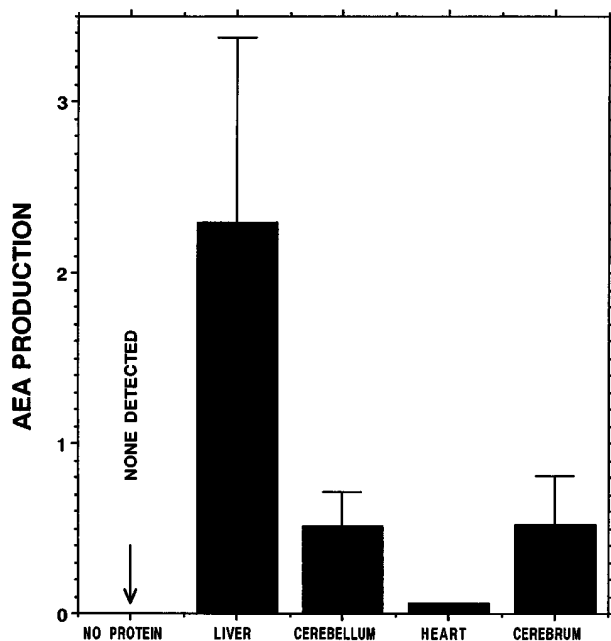


FIG. 8. Arachidonylethanolamide production by homogenates of various rat tissues supplemented with arachidonic acid and ethanolamine. Aliquots (containing 1 mg of protein each) of homogenates from rat liver (*second column*), cerebellum (*third column*), heart (*fourth column*), or cerebrum (*fifth column*) were incubated (60 min, 37 °C) in 1 ml of 0.1 M Tris-HCl, pH 9.0, with exogenous arachidonic acid (100 μ M) and ethanolamine (7 mM). A control incubation was performed without addition of tissue homogenate (*first column*). At the end of the incubations, the AEA content of the incubation medium was then determined by isotope dilution mass spectrometry.

substrates by tissue homogenates (27, 39). These data indicate that our analytical methods can detect AEA from biological matrices.

We next attempted to measure endogenous AEA content in brain regions and other tissues. Isolation of about 380 pmol of AEA per g of tissue from porcine brain preparations has been reported (15), and about 4 g of cerebrum and cerebellum can be obtained from five rats. If the abundances of AEA in rat and porcine brain were similar, this amount of tissue might be expected to contain 1500 pmol of AEA, which should be readily measurable by isotope dilution GC-NICI-MS. Fresh samples of rat cerebrum, cerebellum, liver, and heart were therefore homogenized, mixed with [$^2\text{H}_8$]/[$^3\text{H}_8$]AEA internal standard, subjected to lipid extraction, and concentrated. Some homogenates were prepared and extracted in the presence of PMSF or AT-FMK because inhibition of tissue amidases by these compounds (27, 32) might have suppressed AEA hydrolysis and increased

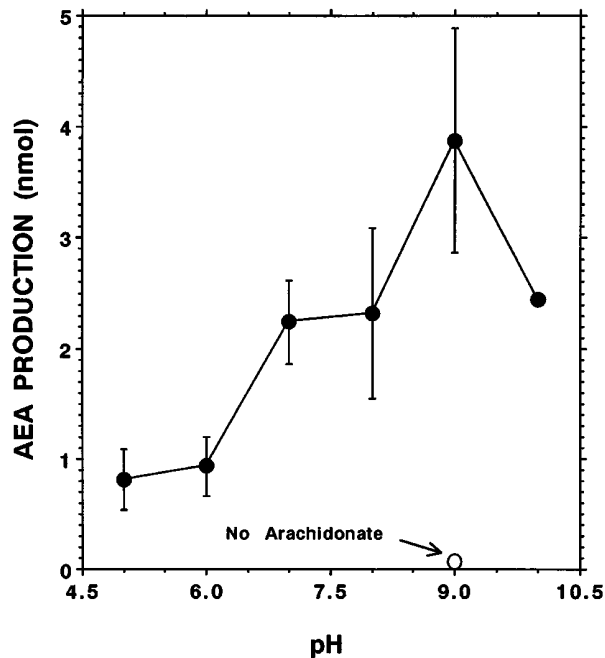


FIG. 9. Influence of pH on arachidonylethanolamide production by rat liver homogenate. Aliquots of rat liver homogenate (1 mg of protein per condition) were incubated (60 min, 37 °C) in buffer with (*closed circles*) or without (*open circle*) exogenous arachidonate (100 μ M) and ethanolamine (7 mM). For incubations from pH 7.0 to 9.0, the buffer was 0.1 M Tris-HCl. For incubations at pH 5.0–6.0, citrate/ Na_2HPO_4 buffer was used, and for incubations at pH 10.0, $\text{NaHCO}_3/\text{NaOH}$ buffer was used (32). At the end of the incubations, the AEA content of the incubation medium was determined by isotope dilution mass spectrometry.

its yield from tissues. Aliquots of lipid extracts were subjected to mild alkaline hydrolysis to hydrolyze glycerolipids to determine if this would facilitate chromatographic analysis. AEA has no base-labile linkages and was not degraded under these conditions. The remainder of each tissue extract was processed similarly but without alkaline hydrolysis.

Fig. 10 illustrates the clear visualization of the internal standard on GC-NICI-MS analyses of extracts from rat cerebrum and cerebellum. Alkaline hydrolysis (saponification) of tissue glycerolipids improved [$^2\text{H}_8$]/[$^3\text{H}_8$]AEA recovery from RP-HPLC slightly (to 60%), but recovery was similar (50%) in nonsaponified samples. *Panel A* illustrates the blank value of the internal standard upon GC-NICI-MS analysis after extraction, RP-HPLC analysis, and conversion to the PFP₂ derivative. Similar tracings were obtained with the PFB₂ derivative (not shown). *Panels B* and *D* illustrate GC-NICI-MS signals

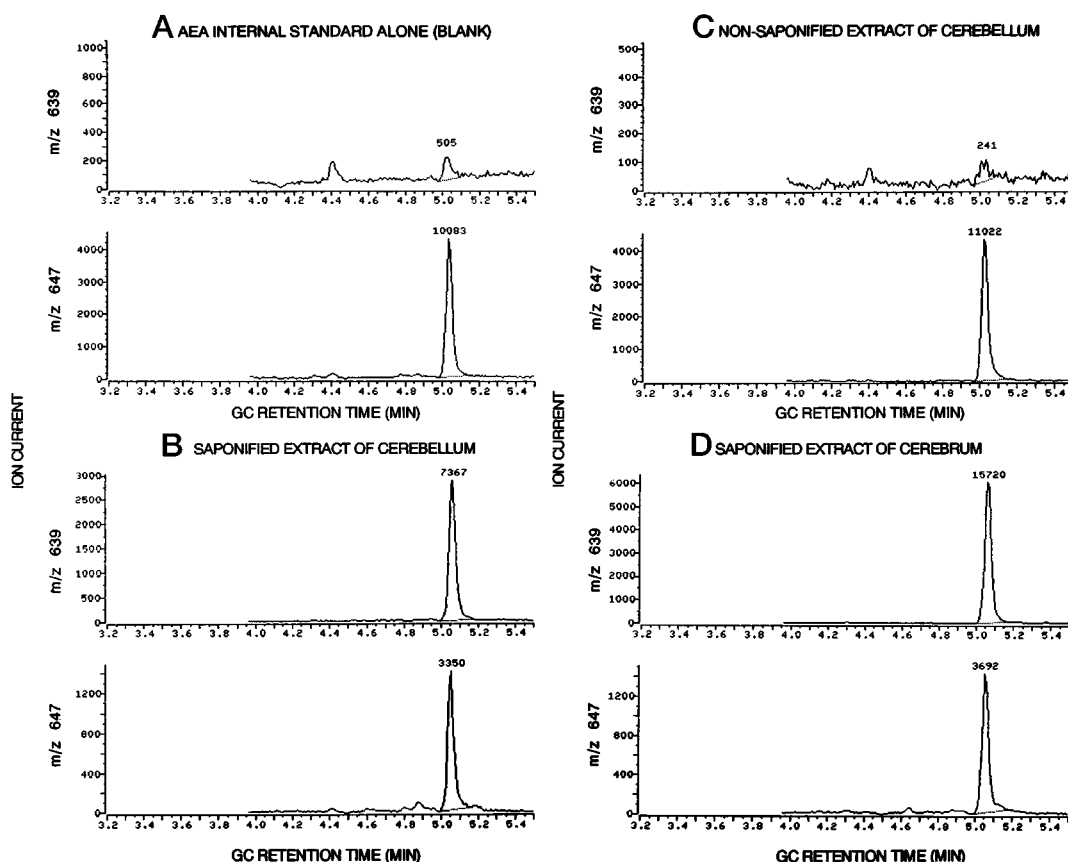


FIG. 10. Analysis of the arachidonylethanolamide content of saponified and nonsaponified lipid extracts from rat brain regions. In panel A, a solution of CHCl_3 (1 ml) and CH_3OH (2 ml) was added to the $[\text{}^2\text{H}_8]/[\text{}^3\text{H}_8]$ AEA internal standard (30 nCi, 500 pmol) and mixed with phosphate-buffered saline (0.8 ml) and then with CHCl_3 (1 ml). The mixture was then vortex-mixed and centrifuged, and the organic phase was concentrated and analyzed by RP-HPLC. The AEA peak was located by liquid scintillation counting for ^3H content, and AEA was extracted, converted to the bis-pentafluoropropionyl derivative, and analyzed by GC-NICI-MS with selected monitoring of the molecular anions for native AEA-PFP₂ (m/z 639) or for $[\text{}^2\text{H}_8]$ AEA-PFP₂ (m/z 647). In panel B, a $\text{CHCl}_3/\text{CH}_3\text{OH}$ solution containing an extract of the saponification products of a lipid extract from rat cerebellum (2 g tissue wet weight) was processed and analyzed as in panel A. In panel C, a $\text{CHCl}_3/\text{CH}_3\text{OH}$ solution containing a lipid extract from rat cerebellum (2 g of tissue, wet weight) that had not been saponified was processed and analyzed as in panel A. In panel D, a $\text{CHCl}_3/\text{CH}_3\text{OH}$ solution containing an extract of the saponification products of a lipid extract from rat cerebrum (2 g of tissue, wet weight) was processed and analyzed as in panel A.

obtained for AEA from lipid extracts of rat cerebellum and cerebrum, respectively, that were subjected to saponification. About 645 pmol of AEA were obtained per g of cerebellum (panel A) and about 1270 pmol/g of cerebrum (panel B) after extraction and saponification. With nonsaponified cerebellar (panel B) or cerebral samples (not shown), no detectable AEA signal was obtained. This was also true for nonsaponified samples from liver and heart, although saponified samples from each tissue yielded substantial signal for AEA (not shown). Several approaches were employed in attempts to identify AEA from nonsaponified tissue extracts, including processing tissues in the presence or absence of ATFMK or PMSF, increasing amounts of tissue processed, comparing analysis by TLC with RP-HPLC, and comparing signal obtained from PFP₂ and PFB₂ derivatives. In no case was a detectable amount of AEA obtained from nonsaponified samples, although in every case, substantial amounts of AEA were obtained from saponified samples.

It was considered possible that saponification released AEA from a phospholipid precursor. *N*-Acylethanolamine phospholipids have been found in vertebrate tissues (28, 29, 40), and such phospholipids have been suggested to serve as precursors to AEA (41), from which AEA may be released by a phospholipase D (PLD) enzyme (41). Although the *N*-acylethanolamine-phosphodiester linkage is stable to conditions of mild alkaline hydrolysis, release of *N*-acylethanolamides from such phospholipids has been observed under harsher conditions of alkaline

hydrolysis at elevated temperatures (40). We therefore attempted to generate AEA by digesting rat tissue phospholipids with a PLD enzyme from *S. chromofucus*, which is active against *N*-acylethanolamine phospholipids and yields *N*-acylethanolamides and phosphatidic acid as products (28, 29, 40). Phospholipid extracts from cerebellum, cerebrum, liver, and heart were digested with this PLD under conditions which yield *N*-acylethanolamides from *N*-acylethanolamine phospholipids of the fish central nervous system and infarcted dog heart (28, 29, 40). TLC analyses of phospholipid extracts before and after PLD digestion indicated that substantially complete conversion of phosphatidylcholine and phosphatidylethanolamine to phosphatidic acid occurred (not shown), indicating that the enzyme was active under the incubation conditions.

Digested samples were then mixed with the $[\text{}^2\text{H}_8]/[\text{}^3\text{H}_8]$ AEA internal standard, and AEA was extracted, isolated by liquid chromatography, converted to the PFB₂ derivative, and analyzed by GC-NICI-MS. In each case, the internal standard was clearly visualized, but in no case was a detectable amount of unlabeled AEA recovered from phospholipid digests from any of the tissues (not shown), suggesting that the content of *N*-arachidonylethanolamine phospholipids in these tissues is low. To place constraints on the upper limit of amounts of such species that might be contained in the extracts, the phospholipid mass extracted from the tissues was determined by measuring the phospholipid fatty acyl content, after alkaline hydroly-

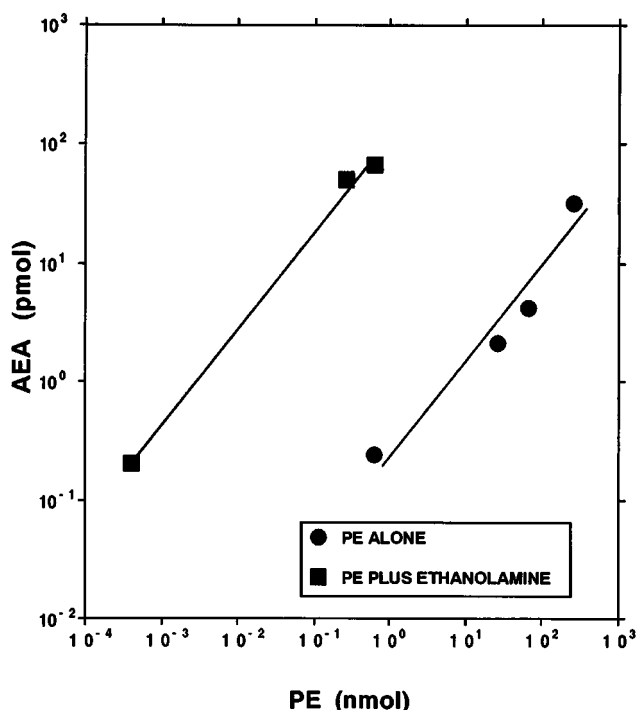


FIG. 11. Production of arachidonylethanolamide by saponification of synthetic standard (1-stearoyl, 2-arachidonoyl)-phosphatidylethanolamine. $\text{CHCl}_3/\text{CH}_3\text{OH}$ solutions containing synthetic C18:0/C20:4-PE standard were concentrated and reconstituted in dimethoxyethane (0.5 ml) and 3 N LiOH (0.1 ml) without (circles) or with (squares) free ethanolamine (1 mM). The contents of the tubes were stirred (90 min, room temperature), and then water was added. Products were extracted (CH_2Cl_2), added to the $[\text{}^2\text{H}_8]/[\text{}^3\text{H}_8]$ AEA internal standard (30 nCi, 500 pmol), concentrated, and analyzed by TLC. AEA was harvested from the TLC plate, converted to the PFB_2 derivative, and quantitated by GC-NICI-MS.

ysis, by GC-NICI-MS with $[\text{}^2\text{H}_8]$ arachidonic acid as the internal standard (36, 37). These measurements indicated that less than 0.6 pmol of AEA per μmol of phospholipid fatty acyl mass was liberated by PLD digestion of tissue extracts, suggesting that the abundance of any *N*-arachidonylethanolamine phospholipids may be less than about one part per million phospholipid molecules in the fresh rat tissues that were examined.

Because of this evidence that AEA generated from saponification of tissue phospholipids did not arise from hydrolysis of *N*-arachidonylethanolamine phospholipid precursors, the possibility was considered that AEA is generated by base-catalyzed aminolysis of arachidonate residues esterified in tissue phospholipids (40, 42). Under alkaline conditions, ethanolamine participates in an aminolysis reaction to yield fatty acylethanolamides from tissue phospholipids artifactually (40, 42). Synthetic C18:0/C20:4-PE was therefore subjected to saponification conditions, and the AEA content of the hydrolysate was determined by isotope dilution GC-NICI-MS. AEA was produced from synthetic C18:0/C20:4-PE under these conditions (Fig. 11), but no AEA was observed in nonsaponified samples of the standard. Inclusion of free ethanolamine (1 mM) in the saponification mixture increased the yield of AEA from C18:0/C20:4-PE by 300-fold, supporting the possibility that AEA generated under these conditions reflects base-catalyzed aminolysis. This phenomenon, rather than hydrolysis of an *N*-acylethanolamine phospholipid precursor, is therefore the likely explanation for generation of AEA from saponification of tissue phospholipids.

It was considered possible that the apparent difference between the reported observations that reasonably large amounts of AEA have been isolated from porcine brain (15) and from

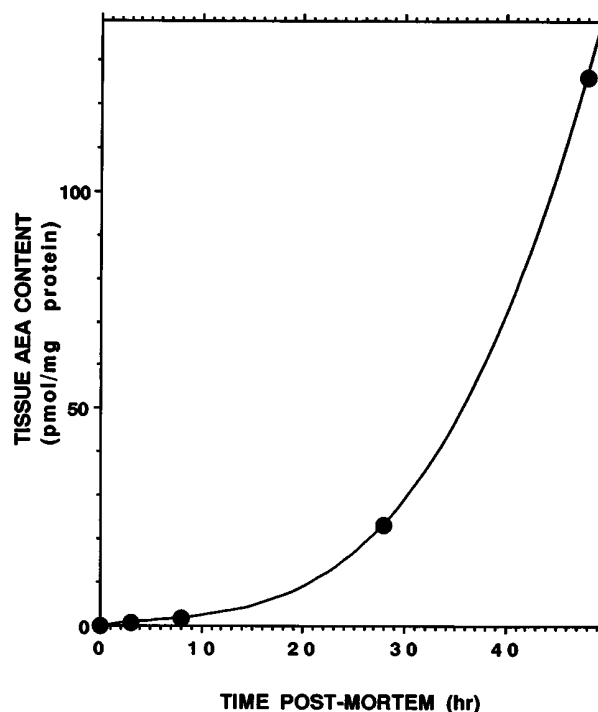


FIG. 12. Accumulation of arachidonylethanolamide in rat brain tissue as a function of time post mortem. Specimens of rat brain were harvested from animals immediately after euthanasia and allowed to remain at room temperature for periods ranging from 0 to 48 h. At the end of the target intervals, specimens were homogenized, mixed with the $[\text{}^2\text{H}_8]/[\text{}^3\text{H}_8]$ AEA internal standard (30 nCi, 500 pmol), and subjected to lipid extraction. AEA was isolated from the extracts by liquid chromatography and quantitated by GC-NICI-MS. Values are means of two to three determinations for each time point.

lyophilized calf brain powder (21) and our findings that no detectable AEA is obtained from nonsaponified fresh rat brain might reflect post-mortem accumulation of AEA in brain. Our studies were performed with freshly isolated rat brain samples that were processed within minutes of euthanasia. *N*-Acylethanolamine phospholipids and free *N*-acylethanolamides have been reported to accumulate in infarcted but not in non-infarcted mammalian myocardium and brain (40). We therefore examined by isotope dilution mass spectrometry the accumulation of AEA in rat brain tissue as a function of time post mortem. As illustrated in Fig. 12, no detectable AEA (<0.17 pmol/mg of protein) was observed in freshly isolated brain tissue. By 3 h post mortem, barely detectable amounts of AEA had accumulated (0.52 ± 0.36 pmol/mg of protein). Thereafter, the amount of AEA continued to rise as a function of time post mortem and achieved levels of 1.87, 23.2 ± 0.56 , and 126.4 pmol/mg of protein at 8, 28, and 48 h post mortem, respectively.

DISCUSSION

This is the first study of which we are aware that examines in a quantitative manner the amounts of endogenous AEA that are contained in fresh mammalian tissues. Our measurements employed $[\text{}^2\text{H}_8]/[\text{}^3\text{H}_8]$ AEA as an internal standard and electron-capturing, fluorinated derivatives, which permit exploitation of the great sensitivity of negative ion chemical ionization mass spectrometry. The sensitivity of these methods should be more than sufficient to permit measurement of the amounts of AEA previously isolated from porcine brain, and it was our intent to determine whether the AEA content of various brain regions corresponds to the differing cannabinoid receptor density among these regions (6–8).

Our measurements failed to reveal detectable levels of AEA in fresh rat brain and indicate that any AEA content of this

tissue is at least two orders of magnitude lower than the amount of AEA isolated per g of tissue weight from porcine brain (15), despite the fact that rat brain homogenates produce AEA from exogenous arachidonate and ethanolamine. Our methods are clearly capable of detecting AEA production by tissue homogenates from exogenous substrates and provide structural evidence that the product previously observed in radiochemical experiments (27, 31, 32, 39) with such homogenates is AEA. Although these measurements unequivocally establish that AEA is produced by tissue homogenates from exogenous substrates *in vitro*, it is uncertain whether formation of AEA in this manner represents a biosynthetic mechanism that is operative *in vivo*. K_m values for arachidonic acid and ethanolamine in such systems have been reported to be 100 μM and 50 mM , respectively (32). Although nonesterified arachidonic acid may accumulate to concentrations approaching 100 μM in biological systems (25), levels of free ethanolamine in mammalian tissues fall in the range of 0.1 to 3.1 $\mu\text{mol/g}$ of fresh tissue weight (40), and these values are lower than those required to support AEA production by tissue homogenates *in vitro*. Such considerations have led to the suggestion that AEA production from exogenous arachidonate and ethanolamine by tissue homogenates reflects the action of an amidohydrolase driven in reverse by mass action effects of concentrations of ethanolamine and arachidonate that are unlikely to accumulate *in vivo* (41). Partial purification of a porcine brain enzyme which catalyzes AEA synthesis from exogenous arachidonate and ethanolamine has revealed that the enzyme does express amidohydrolase activity and catalyzes AEA hydrolysis (32).

An alternative mechanism for AEA biosynthesis is hydrolysis of *N*-arachidonylethanolamine phospholipid precursors by a phospholipase D enzyme (41). Our measurements suggest that, if such phospholipid species are present in fresh rat brain, their abundance is low, perhaps less than one part per million phospholipid molecules. These results are consonant with previous reports that neither *N*-acylethanolamine phospholipids nor free *N*-acylethanolamides are demonstrable in fresh mammalian tissues, although such compounds accumulate in infarcted mammalian heart and brain (40). The fatty amide substituents in these molecules consist predominantly of residues of palmitate, stearate, and linoleate, and *N*-linked residues of arachidonate have not been observed in the *N*-acylethanolamine phospholipids or free *N*-acylethanolamides of infarcted mammalian heart or brain (40).

The previously observed accumulation of *N*-acylethanolamine phospholipids and free *N*-acylethanolamides in infarcted mammalian tissues and their absence in fresh tissues (28, 29, 40) are consonant with our findings that, although AEA is not demonstrable in fresh rat tissues, AEA accumulates post mortem in rat brain tissue. It is appropriate to consider the possibility that some or all of the AEA isolated from brain preparations may be generated post mortem by tissue necrosis. Our studies also indicate that AEA can be generated by base-catalyzed aminolysis of tissue phospholipids, as reported for other acylethanolamides (40, 42), and it is appropriate to consider the possibility that this may occur under milder alkaline conditions and contribute to AEA generation during processing of samples with high content of arachidonate-containing ethanolamine phospholipids, such as the central nervous system.

Our findings do not exclude the possibility that AEA is rapidly synthesized and degraded at local sites *in vivo*, but they indicate that the AEA content of fresh rat brain and of *N*-arachidonylethanolamine phospholipid precursors from which

AEA might be derived are exceedingly low and that AEA can be produced artifactually from biological materials. While there is no question that AEA is a ligand for the cannabinoid receptor and has cannabinomimetic actions, it is perhaps less certain that it is an endogenous ligand formed *in vivo* in living mammalian brain (43, 44). The quantitative methods described here may be useful in the further study of this important question.

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