0022-3565/98/2861-0184$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 286, No. 1
Printed in U.S.A.

JPET 286:184–190, 1998

Synthesis and Characterization of Diazomethylarachidonyl Ketone: An Irreversible Inhibitor of N-Arachidonylethanolamine Amidohydrolase[1]

WILLIAM S. EDGEMOND, MARCIE J. GREENBERG, P. J. MCGINLEY, SHANMUGAM MUTHIAN, WILLIAM B. CAMPBELL and CECILIA J. HILLARD

Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin

Accepted for publication March 31, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

N-Arachidonylethanolamine (AEA), a putative endogenous agonist of neuronal (CB1) cannabinoid receptors, is a substrate for N-arachidonylethanolamine amidohydrolase (AEA amidohydrolase), a serine amidase present in cell membranes. Following a strategy that has been used to develop inhibitors that covalently bind to the active site of serine peptidases, diazomethyl arachidonyl ketone (DAK) was synthesized and its effects on AEA amidohydrolase were determined. DAK inhibits the hydrolysis of AEA by rat brain membranes with an IC_{50} value of 0.5 μM. At low concentrations, DAK reduces the V_{max} and increases the K_m of the enzyme for its substrate AEA, which suggests that it is both a competitive and noncompetitive inhibitor. At higher concentrations, DAK inhibition is completely noncompetitive. DAK inhibition of membrane-associated AEA amidohydrolase is irreversible because hydrolytic activity is not restored with extensive washing or dialysis of the membranes. Furthermore, DAK inhibition is not reversible by anion exchange chromatography of the subsequently solubilized enzyme. In contrast, DAK inhibition of detergent-solubilized enzyme exhibits competitive kinetics and is reversible upon ion exchange chromatography. Exposure of C6 glioma cells to DAK results in concentration-related inhibition of AEA amidohydrolase activity in cellular membranes with an IC_{50} value of 0.3 μM. In summary, these studies demonstrate that DAK is an irreversible inhibitor of AEA amidohydrolase in its native membrane and provides a useful tool with which to study the role of AEA amidohydrolase in the termination of action of AEA.

N-Arachidonylethanolamine was isolated from porcine brain and identified as an endogenous agonist of the brain cannabinoid receptor (Devane *et al.*, 1992). AEA shares many of the physiological properties of the classical cannabinoids, including production of hypothermia and analgesia (Fride and Mechoulam, 1993) and inhibition of electrically induced contractions of the mouse vas deferens (Devane *et al.*, 1992). AEA binds and activates the brain cannabinoid receptor (CB1) resulting in inhibition of adenylyl cyclase activity (Vogel *et al.*, 1993; Felder *et al.*, 1993) and inhibition of the opening of voltage-operated calcium channels (Mackie *et al.*, 1993).

The incubation of AEA with brain membranes results in its rapid hydrolysis to arachidonic acid and ethanolamine (Deutsch and Chin, 1993). The hydrolysis is mediated by an enzyme, AEA amidohydrolase, that is present in microsomal membrane fractions of rat brain (Ueda *et al.*, 1995; Desarnaud *et al.*, 1995; Hillard *et al.*, 1995a) and is expressed by both neurons and glial cells (Beltramo *et al.*, 1997). AEA amidohydrolase can be solubilized with Triton X-100 and the detergent-solubilized protein retains enzymatic activity (Ueda *et al.*, 1995). AEA amidohydrolase also hydrolyzes other long-chain, unsaturated N-acylethanolamines (Schmid *et al.*, 1985; Ueda *et al.*, 1995) and the fatty acyl amide, oleoylamide (Maurelli *et al.*, 1995; Cravatt *et al.*, 1996). AEA amidohydrolase is inhibited by nonspecific serine esterase and protease inhibitors, including PMSF and DFP (Deutsch and Chin, 1993; Desarnaud *et al.*, 1995; Hillard *et al.*, 1995a), which suggests that the active site involves a serine residue. Sequence data demonstrate the presence of an amidase domain containing a GXSXG motif (Cravatt *et al.*, 1996) that is found in known serine proteases and amidases (Rawlings and Barrett, 1994). In addition to the hydrolysis of AEA, AEA amidohydrolase also catalyzes the synthesis of AEA *via* condensation of free arachidonic acid and ethanolamine (Ueda *et al.*, 1995; Arreaza *et al.*, 1997).

Received for publication July 15, 1997.
[1] Supported by USPHS grant DA09155. A preliminary report of these findings was presented at the 1997 meeting of the International Cannabinoid Research Society.

ABBREVIATIONS: AEA, N-arachidonylethanolamine; BSA, bovine serum albumin; BTNP, (E)-6-(bromoethylene) tetrahydro-3-(1-naphthalenyl)2H-pyran-2-one; CB1, neuronal cannabinoid receptor; DAK, diazomethyl arachidonyl ketone; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IC_{50}, concentration that produces 50% inhibition; NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonyl fluoride.

Several other irreversible inhibitors of AEA amidohydrolase have been described in addition to PMSF and DFP. A series of fatty acyl derivatives of PMSF, including palmitoyl sulfonyl fluoride (Lang *et al.*, 1996; Deutsch *et al.*, 1997a); lauryl- and myristoyl sulfonyl fluoride (Deutsch *et al.*, 1997a) inhibit AEA hydrolysis by brain microsomes with IC_{50} values between 5 and 50 nM. Although rigorous studies of the irreversibility of these agents have not been published, it is likely that they act *via* covalent modification of the active site serine (Deutsch *et al.*, 1997a). A second high-affinity inhibitor, MAFP, inhibits AEA hydrolysis with an IC_{50} value of 2.5 nM in brain homogenates (Deutsch *et al.*, 1997b) and an IC_{50} value of 1 to 3 nM in solubilized protein preparations (DePetrocellis *et al.*, 1997). The inhibition produced by MAFP is not reversed after ion exchange chromatography of the solubilized enzyme (DePetrocellis *et al.*, 1997). A third irreversible inhibitor also has been identified, BTNP (Beltramo *et al.*, 1997). BTNP has an IC_{50} value in brain membranes of 0.8 μM and in neurons of 100 nM, and inhibition is not reversed after dialysis.

In this study, we report the synthesis and characterization of another irreversible inhibitor of AEA amidohydrolase, DAK. DePetrocellis and co-workers (1997) have shown that DAK is an inhibitor of AEA amidohydrolase, and we have extended those studies here. The design of this inhibitor is based on the successful development of peptidyl diazomethanes as irreversible inhibitors of serine and cysteine proteases (Shaw, 1994). The rationale for this approach is that the addition of diazomethylketone to an arachidonate backbone should result in a selective, potent and irreversible inhibitor of the active site of AEA amidohydrolase. We find that DAK is an effective inhibitor of AEA amidohydrolase activity in membranes and cells and that the inhibition is irreversible when the enzyme is treated with DAK in its native membrane environment. In contrast, we find that DAK inhibition of detergent-solubilized AEA amidohydrolase is not irreversible, which suggests that the membrane is important for the tertiary structure of AEA amidohydrolase.

Materials and Methods

Materials. N-2-Hydroxyethyl (1′,2′-[^{14}C]) arachidonamide (A[^{14}C]EA; 120 mCi/mmol) was the generous gift of Dr. David Ahern (NEN Dupont, Boston, MA). [^{14}C(U)]AEA labeled in the arachidonyl portion of the molecule was synthesized as described previously (Hillard *et al.*, 1995a). [^{3}H]CP55940 (120 Ci/mmol) was purchased from NEN DuPont (Boston, MA) and [^{3}H]N-AEA (210 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL). Arachidonic acid was purchased from NuChek Prep (Elysian, MN) and AEA was purchased from Cayman Chemical Company (Ann Arbor, MI). All other drugs and chemicals were of the highest grade possible and were purchased from standard commercial sources.

Synthesis of DAK. Arachidonic acid was dissolved in dry methylene chloride under N_2. The solution was cooled to 0°C, and oxalyl chloride (2 M in methylene chloride, 5 equiv) was added slowly. The reaction mixture was warmed to 25°C and stirred for 1 hr. Solvent and excess reagent were removed with a stream of N_2, and the resulting oil was cooled to 0°C. Excess ethereal diazomethane was added and the reaction was stirred for 1 hr at 0°C. After the solvent was removed, diazomethylketone was purified by isocratic normal-phase, high-pressure liquid chromatography with use of a Nucleosil silica (Phenomenex, 5 μ, 250 × 10 mm) column with 0.5% isopropyl alcohol in hexane as the solvent. Flow rate was 4 ml/min and UV was monitored at 250 nm. DAK eluted at 15 min; the final yield was 80%.

We confirmed the structural identity of DAK by ^{1}H NMR: ($CDCl_3$, 300 MHz) d 5.37 (m, 8H), 5.24 (s, 1H), 2.82 (m, 6H), 2.33 (br, 2H), 2.07 (m, 4H), 1.71 (m, 2H), 1.30 (br, 6H), 0.89 (t, ^{3}H J = 6.6 Hz). The parameters obtained by ^{13}C NMR were: ($CDCl_3$, 75 MHz) d 194.9, 130.5, 129.0, 128.9, 128.7, 128.2, 128.1, 127.8, 127.5, 54.27, 40.27, 31.48, 29.28, 27.18, 26.53, 25.60, 25.91, 22.54, 14.05. Mass spectrometry (EI, 70 eV) 190,110, 97, 79, 67, 55, 41. Positive chemical ionization mass spectrometry of the synthesized DAK revealed the presence of the following major ions: 343 (M + 15), 329 (M + 1), 301, 191, 97. The NMR and mass spectrometry data demonstrate that the synthesized compound is DAK.

Rat brain membrane preparation and protein solubilization. The studies reported here were approved by the Medical College of Wisconsin Animal Care Committee and were carried out in accordance with the Declaration of Helsinki and following the NIH Guide for the Care and Use of Laboratory Animals. Before their experimental use, rats were maintained on a 12-hr light/dark schedule and had free access to food and water. Male, Sprague-Dawley rats (250–300 g) were used to prepare brain membranes for the assay of AEA amidohydrolase activity *in vitro*. Crude forebrain membranes were prepared by homogenization in TME buffer (50 mM Tris-HCl, 1.0 mM ethylenediaminetetraacetic acid and 3.0 mM $MgCl_2$, pH 7.4) followed by centrifugation at 11,300 × g for 20 min at 4°C. The pellet was resuspended in buffer and stored at −80°C until assay.

For those experiments with detergent-solubilized preparations of AEA amidohydrolase, partially purified membranes were prepared by homogenization of whole rat brain in 0.32 M sucrose containing 20 mM Tris-HCl (pH 7.5) and 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid. The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was recentrifuged at 22,000 × g for 30 min, and the resulting pellet was resuspended in TME buffer. Membrane proteins were extracted with Triton X-100 (1%) by the method of Ueda *et al.* (1995). After centrifugation at 105,000 × g for 1 hr, the solubilized proteins were stored at −80°C until use.

Protein concentrations were determined in each preparation by the dye binding method of Bradford (1976) with reagent and protein standard I obtained from BioRad Laboratories (Richmond, CA).

Ion exchange chromatography. Solubilized proteins were separated by ion exchange chromatography with a modification of the method of De Petrocellis *et al.* (1997). Solubilized enzyme prepared as described above was dialyzed overnight at 4°C against 20 mM citrate sodium phosphate buffer containing 0.05% Triton X-100 (Buffer A). A 2.0-ml aliquot of the dialyzed protein (containing approximately 20 mg of protein) was loaded onto an Econo Pac High Q cartridge (5 ml, BioRad) with a fast protein liquid chromatography system (Pharmacia). The chromatography was carried out at room temperature with a flow rate of 1.0 ml/min. The column was pre-equilibrated with Buffer A and, after loading the sample, was washed with 20 ml of Buffer A. Proteins were eluted with a 35-min linear gradient of NaCl (0–0.7 M) in Buffer A. Two milliliter fractions were collected, made 0.1% in BSA and assayed for AEA amidohydrolase activity by the method described below with [^{3}H]AEA as a substrate.

Cell culture. C6 rat glial tumor cells were obtained from American Type Tissue Culture (Rockville, MD) and were grown in Dulbecco's minimum essential medium containing 10% fetal bovine serum. For the amidohydrolase assay, the cells were washed in physiological salt solution and then were scraped into TME buffer. Membranes were isolated from lysates by centrifugation at 11,300 × g for 20 min. Cell viability was measured in treated cells using trypan blue exclusion. Trypan blue (0.25%) made in sterile phosphate-buffered saline was added to the media, and after 5 min, the number of blue cells was counted in three high-power fields.

Assay for AEA amidohydrolase activity. AEA amidohydrolase activity was measured by determining the conversion of AEA to arachidonic acid and ethanolamine. Three radiolabeled AEA sub-

strates were used in different experiments: AEA labeled with ^{14}C in the ethanolamine portion of the molecule (A[^{14}C]EA; Omeir *et al.*, 1995); AEA labeled with ^{14}C(U) in the arachidonate portion of the molecule ([^{14}C]AEA; Hillard *et al.*, 1995); and AEA labeled with ^{3}H in the arachidonate portion of the molecule. Regardless of the substrate, intact membranes or solubilized fractions were incubated in a final volume of 0.5 ml TME buffer containing 1.0 mg/ml of fatty acid-free BSA and 9 to 25 nCi radiolabeled AEA. Incubations were carried out at 37°C and were stopped with the addition of 2 ml of chloroform/methanol (1:2). After standing at room temperature for 30 min, 0.67 ml chloroform and 0.6 ml water were added. Aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min.

In experiments in which the substrate was A[^{14}C]EA, the amount of ^{14}C in 1 ml each of the aqueous and organic phases was determined by liquid scintillation counting. For experiments in which the substrate was radiolabeled with either ^{3}H or ^{14}C in the arachidonate portion of the molecule, the radiolabeled species in the organic phase were separated by thin-layer chromatography as outlined previously (Hillard *et al.*, 1995). When the substrate was [^{14}C]AEA, the amounts of substrate and product were determined with an Ambis radioanalytic detector. When the substrate was [^{3}H]AEA, the thin-layer chromatogram was scraped, the silica was extracted and the extract was counted.

[^{3}H]CP55940 binding assay. [^{3}H]CP55940 binding to rat forebrain membranes was measured as reported previously (Hillard *et al.*, 1995a). Forebrain membranes were prepared as described above and were incubated in TME buffer containing 1 mg/ml fatty acid-free BSA with 0.7 to 0.9 nM [^{3}H]CP55940 for 60 min at room temperature. Nonspecific binding was defined with 10 μM Δ^9-THC. Bound and free [^{3}H]CP55940 were separated by filtration through Durapore 1.2-μm filters by a Multiscreen assay system (Millipore, Bedford, MA).

Data analysis. The parameters of K_m and V_{max} for AEA amidohydrolase hydrolysis of AEA were determined by measuring the initial rates of hydrolysis at five to six concentrations of AEA and fitting the data to the Michaelis-Menton kinetic equation by nonlinear regression (Prism, Graphpad Software). The IC_{50} values for DAK were determined from competition isotherms carried out at four to six concentrations between 10 nM and 10 μM by nonlinear curve fitting (Prism, Graphpad Software). In the [^{3}H]CP55940 binding experiments, K_i values were calculated from IC_{50} values with the formula of Cheng and Prusoff (1973).

Results

Pretreatment of rat brain membranes with DAK resulted in a concentration-related inhibition of AEA hydrolysis (fig. 1). The IC_{50} for DAK inhibition is 520 nM (95% confidence interval, 347–781 nM). We hypothesize that the diazomethyl moiety of DAK binds covalently the active site of AEA amidohydrolase. We have carried out a series of experiments to test the hypothesis that DAK-induced inhibition of AEA amidohydrolase activity is noncompetitive and irreversible.

The effects of DAK on the K_m and V_{max} of AEA amidohydrolase for [^{14}C]AEA substrate were determined. Rat brain membranes were preincubated with DAK or an equivalent amount of vehicle for 60 min before the addition of various concentrations of AEA and the determination of amidase activity (fig. 2). The K_m and V_{max} values for AEA hydrolysis were determined from the concentration-response isotherms and are reported in table 1. Pretreatment of the membranes with increasing concentrations of DAK resulted in both an increase in the K_m of AEA amidohydrolase for AEA and a decrease in the V_{max}. At a concentration of 1 μM, the kinetic parameters could not be determined because DAK inhibition

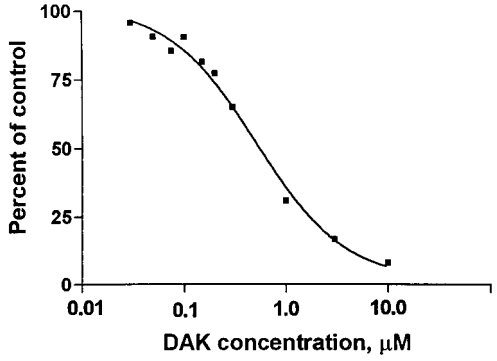

Fig. 1. DAK inhibits the hydrolysis of AEA in rat brain membranes. Forebrain membranes were prepared as outlined under "Materials and Methods" and were incubated for 60 min at 37°C with 16 nCi of A[^{14}C]EA labeled in the ethanolamine portion of the molecule. Values shown are the mean of three experiments. Untreated membranes hydrolyzed 85 ± 6% of the added A[^{14}C]EA during the incubation.

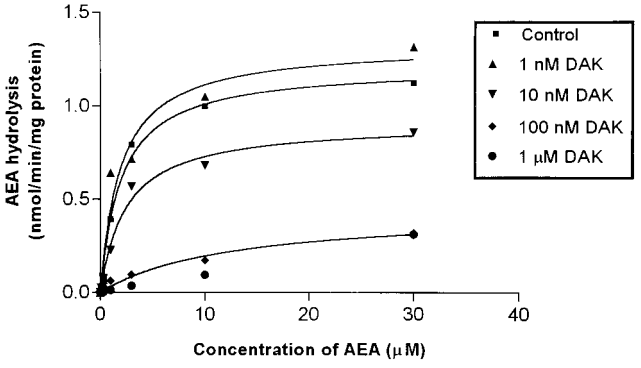

Fig. 2. DAK inhibition exhibits both competitive and noncompetitive kinetics. Forebrain membranes were preincubated with DAK at the concentrations indicated or DMSO ("control") for 60 min before the addition of AEA. AEA amidohydrolase activity was determined at eight concentrations of AEA between 0.01 nM and 30 μM; the concentration of [^{3}H]AEA was kept constant at 0.01 nM and the total concentration of AEA was adjusted with the addition of unlabeled AEA. Data shown are from a representative experiment that was repeated two times with equivalent results.

TABLE 1
Effect of DAK on kinetics of AEA amidohydrolase with [^{3}H]AEA as a substrate

Treatment	Intact Membrane[a]		Detergent-Solubilized[b]	
	K_m[c]	V_{max}[c]	K_m[c]	V_{max}[c]
	μM		μM	
DMSO	2.0 ± 0.3	1.2 ± 0.1	7.5 ± 2.5	7.5 ± 0.8
1 nM DAK	1.9 ± 0.6	1.3 ± 0.1	4.5 ± 1.2	6.3 ± 0.6
10 nM DAK	2.5 ± 0.5	0.9 ± 0.1	6.0 ± 2.2	7.7 ± 1.0
100 nM DAK	12.2 ± 4.4	0.4 ± 0.1	36 ± 10	6.1 ± 0.5
1 μM DAK	n.d.	n.d.	318 ± 14[d]	7.0[d]

[a] Forebrain membranes were preincubated with DAK or DMSO for 60 min before the addition of AEA. AEA amidohydrolase activity was determined at eight concentrations of AEA between 0.01 nM and 30 μM; the concentration of [^{3}H]AEA was kept constant at 0.01 nM, and the total concentration of AEA was adjusted with the addition of unlabeled AEA.

[b] Forebrain membranes were solubilized with Triton X-100 and then incubated with DAK for 60 min before the addition of AEA. AEA amidohydrolase activity was determined at eight concentrations of AEA between 0.01 nM and 300 μM; the concentration of [^{3}H]AEA was kept constant at 0.01 nM and the total concentration of AEA was adjusted with the addition of unlabeled AEA.

[c] K_m and V_{max} were determined from saturation isotherms using nonlinear curve fitting. Units for V_{max} are nmol/min/mg protein. Parameters are shown ± S.E.M.

[d] V_{max} was fixed at 7.0 nmol/min/mg protein.

was completely noncompetitive in character. These results suggest that DAK has the characteristics of both a competitive and noncompetitive inhibitor of AEA amidohydrolase at low concentrations.

In a second experiment, forebrain membranes were preincubated with vehicle, 1 μM AEA or 1 μM DAK for 60 min at 37°C. After the incubation, membranes were either dialyzed overnight against 1000 volumes of TME buffer containing 1 mg/ml fatty acid-free BSA or were washed extensively. After dialysis or washing, the membranes were assayed for AEA amidohydrolase activity (table 2). After either dialysis or washing, membranes preincubated with vehicle or 1 μM AEA hydrolyzed [^{14}C]AEA, whereas membranes preincubated with 1 μM DAK exhibited very little hydrolysis activity.

In a third experiment, forebrain membranes were preincubated with vehicle, 1 μM AEA, 1 μM DAK or 100 μM PMSF for 60 min at 37°C. After incubation, the membranes were separated from the incubation buffer by centrifugation then solubilized with Triton X-100. The soluble fraction was dialyzed against 0.05% Triton X-100 in citrate buffer and then separated into fractions by anion exchange chromatography. The individual fractions were analyzed for AEA amidohydrolase activity. When the brain membranes had been pretreated with either DMSO or AEA before solubilization and dialysis, several of the protein fractions exhibited AEA amidohydrolase activity (fig. 3). However, preincubation of the membranes with either PMSF or DAK resulted in a complete loss of AEA amidohydrolase activity after chromatography of the detergent-solubilized preparation. Therefore, when the membranes are incubated with DAK before solubilization, DAK inhibition is not reversed by anion exchange chromatography of detergent-solubilized membrane proteins.

We have investigated the reversibility of DAK inhibition with a second incubation protocol. In this protocol, the membranes were solubilized with detergent before incubation with DAK or PMSF. DAK inhibits AEA hydrolase activity when incubated with detergent-solubilized preparations of forebrain membrane with an IC_{50} value of 0.4 μM. The kinetics of AEA hydrolysis in the presence of DAK suggest that DAK is a competitive inhibitor of the detergent-solubilized enzyme (table 1). In agreement with this kinetic profile, AEA amidohydrolase activity is restored to DAK-pretreated, detergent-solubilized protein after anion exchange chromatographic separation of the proteins (fig. 4). Therefore, when the detergent-solubilized protein is incubated with DAK, DAK inhibits, but the kinetic profile and reversibility after anion exchange chromatography indicate that DAK is a competitive inhibitor of the detergent-solubilized enzyme. These results agree with those of De Petrocellis and co-workers (1997) and suggest that the ability of DAK to covalently bind

TABLE 2
DAK inhibition of membrane-associated AEA amidohydrolase is not reversed by dialysis or extensive membrane washing

Treatment	Percent AEA Hydrolysis	
	Dialysis[a]	Extensive washing[b]
DMSO	23.6%	72%
1 μM AEA	20.6%	75%
1 μM DAK	2.8%	2.5%

[a] Forebrain membrane AEA amidohydrolase was prepared as described under "Materials and Methods" and was incubated with DMSO vehicle, DAK or AEA for 1 hr at 37°C in a total volume of 2 ml. After the incubation, each incubate in its entirety was placed into prewetted dialysis tubing (molecular weight cut-off, 12,000–14,000) and dialyzed at 4°C with stirring against TME buffer containing 0.1% BSA for 18 hr. AEA amidohydrolase activity was determined in 1.5 ml of the pretreated dialysate or washed membranes using [^{14}C]AEA as a substrate (final concentration, 11 μM). Data reported are the percent of the added [^{14}C]AEA hydrolyzed to [^{14}C]arachidonic acid during a 30-min incubation at 37°C.

[b] Rat brain membranes were prepared as outlined under "Materials and Methods" and were preincubated (final concentration 0.2 mg/ml) with either 1 μM DAK, 1 μM AEA or DMSO for 40 min at 37°C. The membranes were removed from the incubation mixture by centrifugation and were washed six additional times with buffer kept at 37°C. After the final wash, the membranes were incubated with 9 nCi of [^{14}C]AEA labeled in the ethanolamine portion of the molecule at 37°C for 5 min (final concentration of AEA, 0.2 nM).

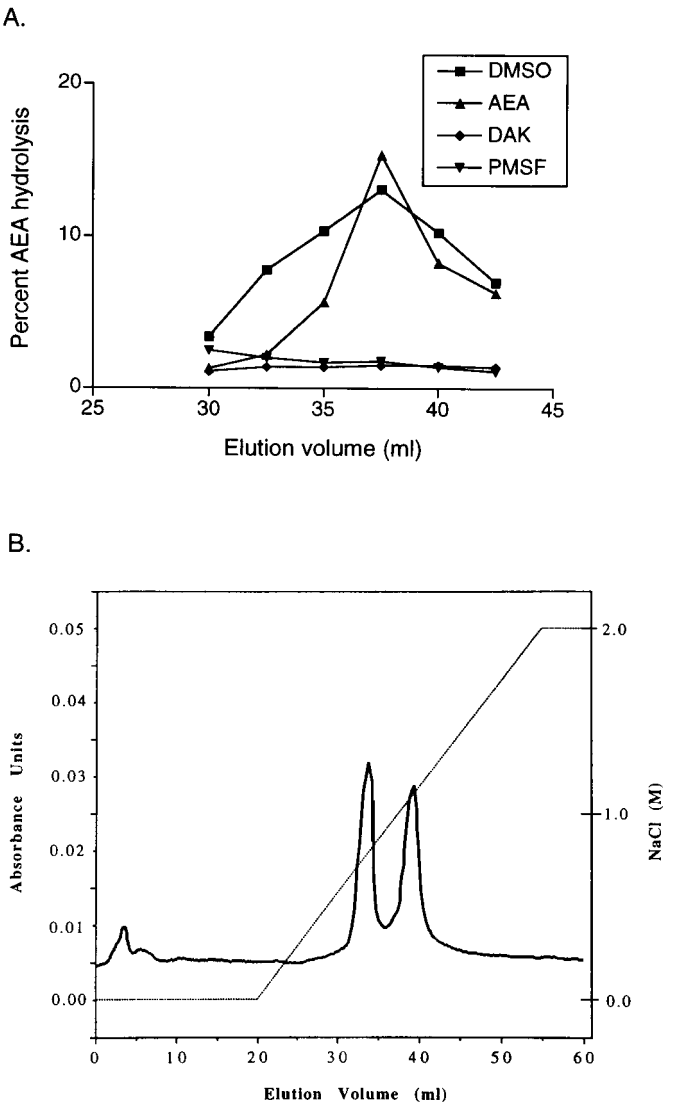

Fig. 3. (A) DAK inhibition of AEA amidohydrolase activity in brain membranes is not reversed after detergent solubilization and anion exchange chromatography. Forebrain membranes (0.05 mg/ml, final concentration) were prepared as described and were incubated with 1 μM AEA, 1 μ M DAK, DMSO or 100 μM PMSF for 1 hr at 37°C. Membranes were removed from incubation mixture by centrifugation, then were solubilized, dialyzed overnight and component proteins were separated by anion exchange chromatography. Each fraction (2.5 ml per fraction) was assayed for AEA amidohydrolase activity with [^{3}H]AEA as a substrate. The percent of the recovered ^{3}H that comigrated with [^{3}H]arachidonic acid was calculated for each fraction. (B) The UV absorbance for anion exchange effluent (solid line) and NaCl concentration (dashed line) for the protein preparation after preincubation with DMSO. Protein profiles after preincubation with DAK, AEA and PMSF were essentially identical.

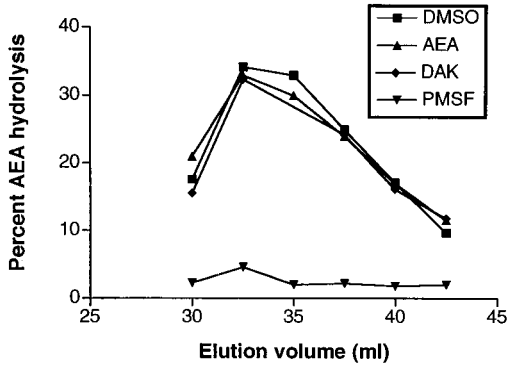

Fig. 4. DAK inhibition of AEA amidohydrolase activity in detergent-solubilized preparations is reversed after anion exchange chromatography. Forebrain membranes were prepared and incubated in Triton X-100 to solubilize membrane proteins. The solubilized preparation (12.35 mg/ml of protein in 2 ml) was incubated with 1 μM AEA, 1 μM DAK, DMSO or 100 μM PMSF for 1 hr at 37°C. After incubation, the entire incubate was placed onto the fast protein liquid chromatography column and AEA amidohydrolase activity was determined in each fraction with [^{3}H]AEA as a substrate. The percent of the recovered ^{3}H that comigrated with [^{3}H]arachidonic acid was calculated for each fraction.

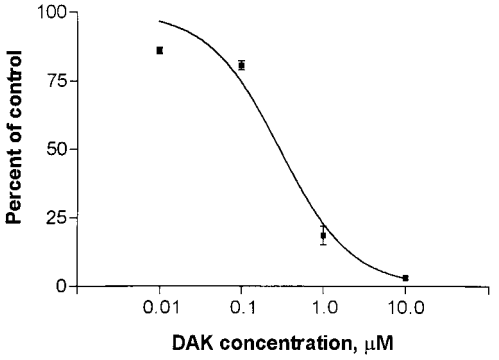

Fig. 5. AEA amidohydrolase activity of glioma cells *in vitro* is inhibited by DAK. C6 rat glioma cells were maintained in culture and, when confluent, were treated with either vehicle or DAK for 30 min. Cells were washed three times with warm buffer, and membranes were isolated and assayed for amidase activity. Membranes (0.05 mg/ml final concentration) were incubated with [^{3}H]AEA for 60 min at 37°C. Membranes from DMSO-treated cells hydrolyzed 82.2 ± 0.75% of the added AEA; data from DAK-treated cells as shown as a percent of this hydrolysis rate.

to AEA amidohydrolase is lost when the enzyme is removed from the biological membrane.

The inhibition of serine proteases by diazomethane peptide analogs is pH dependent in some cases (Kraut, 1977). AEA amidohydrolase activity is pH dependent; exhibiting one pH maximum at alkaline pH values (Hillard *et al.*, 1995; Desarnaud *et al.*, 1995) and another at pH 6.5 (Desarnaud *et al.*, 1995). Because of the pH dependence of substrate hydrolysis, we have investigated DAK inhibition and it's reversibility at incubation pH between pH 7.4 and 9.0 (table 3). These experiments were conducted under incubation conditions in which the hydrolysis of A[^{14}C]EA was essentially complete so that pH differences in substrate hydrolysis were not apparent. No differences in DAK inhibition were seen, and DAK inhibition was not reversible by membrane washing regardless of the pH of the incubation buffer.

To determine whether DAK is a useful inhibitor of AEA amidohydrolase in intact cells, C6 glioma were established in culture and incubated for 30 min with DAK or equivalent vehicle. The cells were washed extensively with Krebs-Ringer HEPES buffer containing 1% BSA followed by scraping in hypotonic buffer. Membranes were harvested and assayed for AEA amidohydrolase activity. DAK pretreatment inhibited the hydrolysis of [^{3}H]AEA by glioma cell membranes in a concentration-related manner (fig. 5). The IC_{50} for DAK in the cellular preparation was 298 nM (95% confidence interval, 121–736 nM). The treated cells exhibited greater than 90% viability with trypan blue exclusion as a measure.

TABLE 3
DAK inhibition of AEA amidohydrolase is not pH dependent

Incubation pH[b]	Percent AEA Hydrolysis[a]		
	DMSO	1 μM AEA	1 μM DAK
7.4	74.0	80.1	1.8
8.0	78.6	78.0	1.4
8.4	77.6	77.1	1.9
9.0	76.7	76.5	3.1

[a] Forebrain membranes were preincubated with DMSO, 1 μM AEA or 1 μM DAK for 60 min at 37°C. After the incubation, the membranes were removed from the buffer by centrifugation and were washed 6 times with warm buffer by incubation and centrifugation. AEA amidohydrolase activity was determined in the washed membranes with A[^{14}C]EA as a substrate and an incubation time of 60 min. Under these incubation conditions, the hydrolysis of added substrate by untreated membranes is essentially complete.

[b] Incubation buffer was Tris (50 mM) adjusted to the indicated pH with HCl.

A final goal of these studies was to determine whether DAK also binds to the CB1 receptor of rat brain membranes. The binding affinity of DAK for the brain cannabinoid receptor (CB1) was investigated with the selective CB1 receptor agonist [^{3}H]CP55940 as radioligand (fig. 6). DAK binds to the CB1 receptor with moderate affinity (K_i of 1.3 μM *vs.* 80 nM for AEA). DAK binds to the CB1 receptor in a noncompetitive manner; the B_{max} for [^{3}H]CP55940 is increased by 52% and the K_d is unchanged in the presence of 1 μM DAK.

Discussion

These results demonstrate that DAK is an inhibitor of AEA amidohydrolase in isolated rat brain membranes, the inhibition produced by DAK exhibits mixed kinetics (*i.e.,* both competitive and noncompetitive) and DAK-induced inhibition is not reversed on extensive washing of membranes, dialysis or anion exchange chromatography of detergent-solubilized membranes. Furthermore, treatment of cells with DAK results in complete loss of AEA amidohydrolase activity

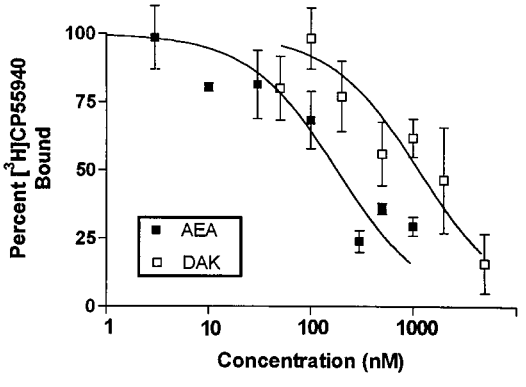

Fig. 6. DAK binds to the CB_1 receptor with moderate to low affinity. Rat forebrain membranes were prepared as outlined and were incubated (0.05 mg/ml final concentration) with DAK and 0.7 nM [^{3}H]CP55940 for 60 min at room temperature. Shown is the mean of three experiments; vertical lines represent S.E.M.

without producing cellular toxicity, which suggests that DAK is a useful tool for the study of the role of AEA amidohydrolase in the catabolism of AEA in cells.

The amidohydrolase that hydrolyzes AEA is the same enzyme that hydrolyzes other long-chain, unsaturated fatty acyl ethanolamides, such as oleoylethanolamide (Schmid *et al.*, 1985) and fatty acyl amides, including the putative sleep regulator, oleoylamide (Maurelli *et al.*, 1995; Cravatt *et al.*, 1996). AEA is a very good substrate for AEA amidohydrolase, with a K_m for unpurified, rat brain enzyme of 2 to 12 μM (Hillard *et al.*, 1995; Desarnaud *et al.*, 1995; results presented herein). AEA amidohydrolase has the characteristics of a protease; it results in the addition of water across an amide bond and is inhibited by several nonselective protease inhibitors, including PMSF (Deutsch and Chin, 1993; Hillard *et al.*, 1995; Desarnaud *et al.*, 1995). AEA amidohydrolase also is inhibited by the selective serine protease inhibitor DFP and not by the cysteine protease inhibitor, E64 (Hillard *et al.*, 1995), although another cysteine protease inhibitor, N-ethylmaleimide, produces partial inhibition (Schmid *et al.*, 1985; Desarnaud *et al.*, 1995). These data with inhibitors suggest that AEA amidohydrolase is a serine amidase with significant dependence on a free thiol. In addition, analysis of the amino acid sequence of AEA amidohydrolase has identified a region of the enzyme that is highly homologous to seven other amidases (Cravatt *et al.*, 1996). This region (called the amidase signature sequence) contains three conserved serine residues and a conserved asparagine (Cravatt *et al.*, 1996) in a motif that is very similar to serine proteases (Rawlings and Barrett, 1994).

It is known that most cysteine and some serine proteases are inhibited irreversibly by peptidyl diazomethanes having the general structure R–C(=O)–CHN_2 (Shaw, 1994). The mechanism by which this class of inhibitors inactivates serine proteases was elucidated by Ermer and co-workers (1990) and is shown in figure 7. On binding of the carbonyl carbon to the active site serine, the diazomethyl C1 atom is protonated by the imidazolium residue of histidine. The next step is either simultaneous nucleophilic attack of the C1 atom of the diazonium ion by the imidazole nitrogen and release of nitrogen or a two-step process in which the carbonium ion is formed first by liberation of nitrogen followed by alkylation of the imidazole nitrogen by the carbonium ion carbon atom (Shaw, 1994). Some evidence supports the existence of an intermediary carbonium ion in the reaction of diazomethyl ketones with chymotrypsin (Watanabe *et al.*, 1979).

This reaction scheme has several implications. First, the initial binding of diazomethyl ketone to the enzyme is reversible. Second, covalent binding of diazomethyl ketone depends on steric factors, in particular the positions of the histidine and serine residues of the active site. This dependence was demonstrated by Stone and co-workers (1992), who found that irreversible incorporation of peptidyl diazomethanes into prolyl endopeptidase was slowly reversible when the enzyme was in its native state but irreversible after denaturation.

The results of our experiments support this model for the interaction of DAK with AEA amidohydrolase. In particular, DAK produces both an increase in the K_m of the enzyme for AEA and a substantial decrease in the V_{max} of the enzyme. These results suggest that DAK is capable of binding reversibly to the substrate binding site but that some of the interactions result in the formation of a very stable complex that does not reverse even after detergent solubilization, dialysis and ion exchange chromatography. We hypothesize that the

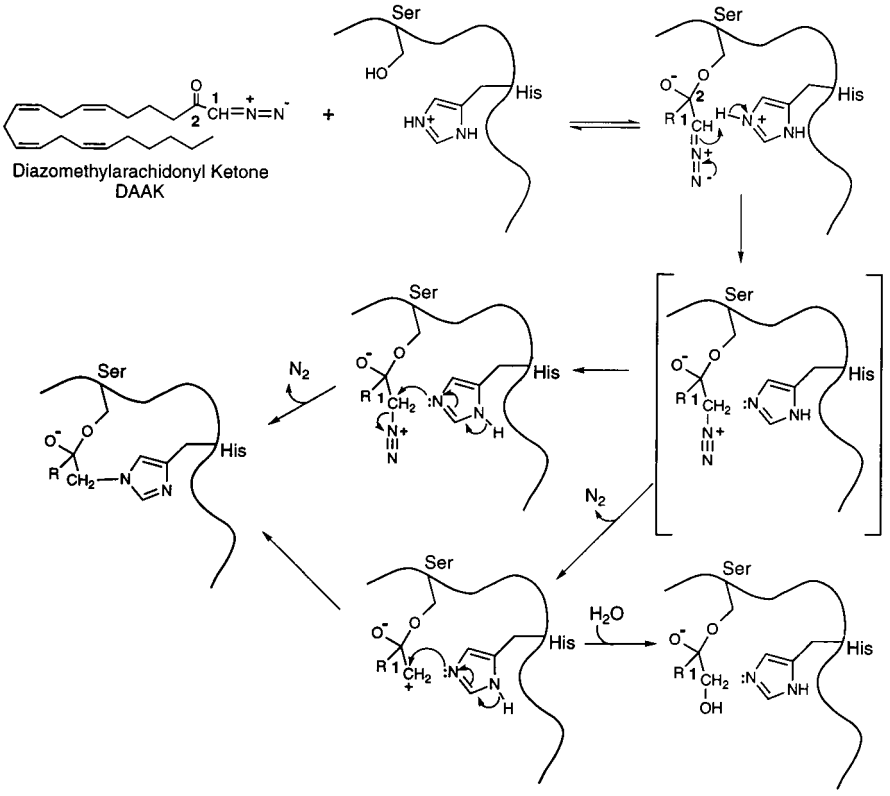

Fig. 7. Proposed mechanism of enzyme inactivation by DAK. This scheme is based on the mechanisms by which diazomethyl ketones inhibit serine peptidases as outlined by Ermer *et al.* (1990).

arachidonate portion of DAK is covalently associated with both the histidine and serine in the active site, which inhibits their participation in AEA hydrolysis.

Two other groups have synthesized and characterized diazomethane-based inhibitors of AEA amidohydrolase; one group studied DAK (De Petrocellis *et al.*, 1997) and one group studied the diazomethyl ketone of oleic acid (Patterson *et al.*, 1996). Both of these groups concluded that the diazomethane inhibitors were competitive and reversible. The discordance between these results and ours is likely caused by differences in methodology. In particular, the De Petrocellis group studied the effects of DAK with detergent-solubilized preparations of the enzyme. Like these investigators, we find that AEA amidohydrolase that has been detergent solubilized is inhibited by DAK but that the inhibition is reversed after anion exchange chromatography of the proteins. Solubilization and ion exchange chromatography themselves do not reverse DAK inhibition when membrane-associated enzyme is incubated with DAK. Therefore, the most likely explanation of these results is that the conformation of AEA amidohydrolase is sufficiently different in detergent micelles to prevent covalent binding of DAK. In this regard, however, it is puzzling that DAK inhibition of detergent-solubilized enzyme is not reversible by dialysis.

One drawback to the use of DAK as an irreversible inhibitor of AEA amidohydrolase is that it binds to the neuronal CB1 receptor in the same concentration range that inhibits AEA amidohydrolase. An ideal AEA amidohydrolase inhibitor would be devoid of effects on other proteins involved in actions of AEA. In this regard, other AEA derivatives that inhibit AEA amidohydrolase also bind to the CB1 receptor, including arachidonyl trifluoromethyl ketone (Koutek *et al.*, 1994), the sulfonylfluoride of palmitic acid (Deutsch *et al.*, 1997) and methyl arachidonyl fluorophosphate (Deutsch *et al.*, 1997a) whereas both methyl arachidonyl fluorophosphate (De Petrocellis *et al.*, 1997) and BTNP (Beltramo *et al.*, 1997) also inhibit phospholipase A_2.

In this study, we have demonstrated that DAK is a moderately potent, irreversible inhibitor of AEA amidohydrolase. DAK adds to the available inhibitors of AEA amidohydrolase. Because it is irreversible, it may be used for unique studies of the turnover of AEA amidohydrolase as well as identification of the amino acid residues of the catalytic site. In addition, these studies are the first demonstration of the usefulness of diazomethyl ketones for the inactivation of a serine amidase.

Acknowledgments

The authors are grateful to Bruce Peltier for technical assistance.

References

Arreaza G, Devane WA, Omeir RL, Sajnani G, Kunz J, Cravatt BF and Deutsch DG (1997) The cloned rat hydrolytic enzyme responsible for the breakdown of anandamide also catalyzes its formation via the condensation of arachidonic acid and ethanolamine. *Neurosci Lett* **234:**59–62.

Beltramo M, di Tomaso E and Piomelli D (1997) Inhibition of anandamide hydrolysis in rat brain tissue by (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one. *FEBS Lett* **403:**263–267.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–252.

Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3110.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA and Guila NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384:**84–87.

DePetrocellis L, Melck D, Ueda N, Maurelli S, Kurahashi Y, Yamamoto S, Marino G and Di Marzo V (1997) Novel inhibitors of brain, neuronal, and basophilic anandamide amidohydrolase. *Biochem Biophys Res Commun* **231:**82–88.

Desarnaud F, Cadas H and Piomelli D (1995) Anandamide amidohydrolase activity in rat brain microsomes. *J Biol Chem* **270:**6030–6035.

Deutsch DG and Chin SA (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **47:**711–715.

Deutsch DG, Lin S, Hill WAG, Morse KL, Salehani D, Arreaza G, Omeir RL and Makriyannis A (1997) Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem Biophys Res Commun* **231:**217–221.

Deutsch DG, Omeir R, Arreaza G, Salehani D, Prestwich GD, Huang Z and Howlett A (1997a) Methyl arachidonyl fluorophosphate: A potent irreversible inhibitor of anandamide amidase. *Biochem Pharmacol* **53:**255–260.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258:**1946–1949.

Ermer A, Baumann H, Stuede G, Peters K, Fittkau S, Dolaschka P and Genov NC (1990) Peptide diazomethyl ketones are inhibitors of subtilisin-type serine proteases. *J Enzym Inhib* **4:**35–42.

Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K and Devane WA (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.

Fride E and Mechoulam R (1993) Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. *Eur J Pharmacol* **231:**313–314.

Hillard CJ, Wilkison DM, Edgemond WS and Campbell WB (1995) Characterization of the kinetics and distribution of *N*-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim Biophys Acta* **1257:**249–256.

Hillard CJ, Edgemond WS and Campbell WB (1995a) Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: application to anandamide. *J Neurochem* **64:**677–683.

Koutek B, Prestwich GD, Howlett AC, Chin SA, Salehan D, Akhavan N and Deutsch DG (1994) Inhibitors of arachidonyl ethanolamide hydrolysis. *J Biol Chem* **269:**22937–22940.

Kraut J (1977) Serine proteases: structure and mechanism of catalysis. *Annu Rev Biochem* **46:**331–358.

Lang W, Qin C, Hill WAG, Lin S, Khanolkar AD and Makriyannis A (1996) High performance liquid chromatographic determination of anandamide amidase activity in rat brain microsomes. *Anal Biochem* **238:**40–45.

Mackie K, Devane WA and Hille B (1993) Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol Pharmacol* **44:**498–503.

Maurelli S, Bisogno T, De Petrocellis L, Di Luccia A, Marino G and Di Marzo V (1995) Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase.' *FEBS Lett* **377:**82–86.

Omeir RL, Chin S, Hong Y, Ahern DG and Deutsch DG (1995) Arachidonyl ethanolamide-[1,2-^{14}C] as a substrate for anandamide amidase. *Life Sci* **56:**1999–2006.

Patterson JE, Ollmann IR, Cravatt BF, Boger DL, Wong C-H and Lerner RA (1996) Inhibition of oleamide hydrolase catalyzed hydrolysis of the endogenous sleep-inducing lipid *cis*-9-octadecenamide. *J Am Chem Soc* **118:**5938–5945.

Rawlings ND and Barrett AJ (1994) Families of serine peptidases. *Methods Enzymol* **244:**19–61.

Schmid PC, Zuzarte-Augustin ML and Schmid (1985) Properties of rat liver *N*-acylethanolamine amidohydrolase. *J Biol Chem* **260:**14145–14149.

Shaw E (1994) Peptidyl diazomethanes as inhibitors of cysteine and serine proteinases. *Methods Enzymol* **244:**649–656.

Stone SR, Rennex D, Wikstrom P, Shaw E and Hofsteenge J (1992) Peptidyldiazomethanes. A novel mechanism of interaction with prolyl endopeptidase. *Biochem J* **283:**871–876.

Ueda N, Kurahashi Y, Yamamoto S and Tokunaga T (1995) Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem* **270:**23823–23827.

Vogel Z, Barg J, Levy R, Saya D, Heldman E and Mechoulam R (1993) Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J Neurochem* **61:**352–355.

Watanabe H, Green GD and Shaw E (1979) A comparison of the behavior of chymotrypsin and cathepsin B towards peptidyl diazomethyl ketones. *Biochem Biophys Res Commun* **89:**1354–1360.

Send reprint requests to: Cecilia J. Hillard, Ph.D., Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.