BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 82–88 (1997)
ARTICLE NO. RC976000

Novel Inhibitors of Brain, Neuronal, and Basophilic Anandamide Amidohydrolase

L. De Petrocellis,* D. Melck,† N. Ueda,‡ S. Maurelli,† Y. Kurahashi,‡ S. Yamamoto,‡ G. Marino,§ and V. Di Marzo†,[1]

**Istituto di Cibernetica and †Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R.,[2] Via Toiano 6, 80072, Arco Felice (NA), Italy; ‡Department of Biochemistry, Tokushima University School of Medicine, Kuramoto-cho, Tokushima 770, Japan; and §Dipartimento di Chimica Organica e Biologica, Università di Napoli 'Federico II,' Via Mezzocannone 16, Naples, Italy*

Received December 10, 1996

Mammalian brain as well as mouse neuroblastoma ($N_{18}TG_2$) and rat basophilic leukaemia (RBL) cells were previously shown to contain 'anandamide amidohydrolase', a membrane-bound enzyme sensitive to serine and cysteine protease inhibitors and catalyzing the hydrolysis of the endogenous cannabimimetic metabolite, anandamide (arachidonoyl-ethanolamide). With the aim of developing novel inhibitors of this enzyme, we synthesized three arachidonic acid (AA) analogues, i.e. arachidonoyl-diazo-methyl-ketone (ADMK), arachidonoyl-chloro-methyl-ketone (ACMK) and *O*-acetyl-arachidonoyl-hydroxamate (AcAHA), by adding to the fatty acid moiety three functional groups previously used to synthesize irreversible inhibitors of serine and cysteine proteases. The three compounds were purified and characterized by proton nuclear magnetic resonance and electron impact mass spectrometry. Their effect was tested on anandamide amidohydrolase partially purified from $N_{18}TG_2$ and RBL-1 cells and porcine brain. Pre-treatment of the enzyme with each compound produced a significant inhibition, with ADMK being the most potent (IC_{50}= 3, 2 and 6 μM) and AcAHA the weakest (IC_{50}= 34, 15 and 25 μM) inhibitors. The inactivated enzyme regained its full activity when chromatographed by anion-exchange chromatography, suggesting that none of the compounds inhibited the amidohydrolase in a covalent manner. Accordingly, Lineweaver-Burk profiles showed competitive inhibition by each compound. Conversely, the irreversible inhibitor of cytosolic phospholipase A_2, methyl-arachidonoyl-fluoro-phosphonate (MAFP), covalently inhibited the amidohydrolase. MAFP was active at concentrations 10^3 times lower than those reported for phospholipase A_2 inhibition, and is the most potent anandamide amidohydrolase inhibitor so far described (IC_{50}= 1-3 nM). MAFP, ADMK and ACMK, probably by inhibiting anandamide degradation, produced an apparent increase of the *in vitro* formation of anandamide from its biosynthetic precursor *N*-arachidonoyl-phosphatidyl-ethanolamine.

Anandamide (arachidonoyl-ethanolamide) has been isolated from mammalian brain (1-4) and suggested to act as a physiological mediator at the central CB1 cannabinoid receptor (for reviews see[5-8]). In agreement with its putative role as a neuromodulator, a simple mechanism for the inactivation of anandamide, through the hydrolysis of its amide bond and subsequent formation of arachidonic acid (AA) and ethanolamine, has been described to occur in intact rat central neurons (9) and to be catalyzed by the enzyme 'anandamide amidohydrolase'. This enzyme has been characterized so far in rat (10, 11), porcine (12) and, more recently, mouse (13) brain microsomes. Apart from the breakdown of anandamide, the amidohydrolase from mouse neuroblastoma cells (14) was shown to catalyze also the hydrolysis of the novel sleep-inducing factor oleamide (15), whereas the enzyme from porcine brain microsomes was proposed to behave also as an 'anandamide synthase' by catalyzing, in the presence of high concentrations of ethanolamine, the condensation between the latter compound and AA to form anandamide (12). Finally, anandamide amidohydrolase-like enzymes have been recently found also in non-neuronal cells, i.e. in porcine ocular tissues (16), rat basophilic leukaemia (RBL) cells (17) and sea urchin ovaries (18). Therefore, among the several membrane proteins that are being currently described in the literature, anandamide amidohydrolase is receiving increasing attention by lipid biochemists, thus requiring the development of novel techniques for its purification and full characteriza-

[1] Author to whom all correspondence should be addressed. Fax: +39-81-8041770.

[2] Affiliated with the National Institute for the Chemistry of Biological Systems, C.N.R.

0006-291X/97 $25.00

tion as well as the synthesis of new inhibitors to be used in biochemical and pharmacological studies. Apart from non-specific esterase and protease inhibitors, such as phenyl-methyl-sulphonyl-fluoride (PMSF), *p*-hydroxy-mercuri-benzoate (*p*-HMB), *p*-bromo-phenacyl-bromide (*p*-BPB) (10-12, 14), only two types of reversible inhibitors have been so far described for anandamide amidohydrolase, i.e. the acyl-trifluoro-methyl-ketones and the acyl-α-keto-esters (19). These compounds were suggested to behave as transition-state inhibitors of the enzyme through the formation of tetrahedral intermediates similar to those utilized by serine and cysteine proteases (19). This suggestion, together with the inhibitory effect of PMSF, *p*-HMB and *p*-BPB, prompts the design of novel anandamide amidohydrolase inhibitors starting from our previous knowledge of serine and cysteine protease inhibitors. For example, the introduction of a sulphonyl-fluoride function in the place of the carboxylic group in palmitic acid has been recently shown to yield a potent anandamide amidohydrolase inhibitor (20). The aim of the present study was to explore the possibility that the addition to AA of chemical moieties known from previous studies to bind covalently to serine/cysteine protease active sites (21-23) may result in more potent, potentially selective and, possibly, covalent inhibitors of anandamide amidohydrolase. We report the synthesis and characterization of arachidonoyl-diazo-methyl-ketone (ADMK), arachidonoyl-chloro-methyl-ketone (ACMK) and *O*-acetyl-arachidonoyl-hydroxamate (AcAHA) (Fig. 1), and describe the inhibitory action, on anandamide amidohydrolase preparations from three different sources, of these compounds as well as of another AA derivative with potential anandamide amidohydrolase-alkylating activity, i.e. the irreversible inhibitor of cytosolic phospholipase A_2, methyl-arachidonoyl-fluoro-phosphonate (MAFP, Fig. 1, [24] and references cited therein).

MATERIALS AND METHODS

Materials. Mouse neuroblastoma $N_{18}TG_2$ and RBL-1 cells were purchased from DSM (Germany) and grown, respectively, in Dulbecco's modified Eagles Medium (DMEM, Sigma) and Minimum Eagle's Medium (MEM, Sigma) or in Dulbecco's Modified Eagle Medium (DMEM, Sigma), containing 10% fetal bovine serum (Sigma) plus penicillin:streptomycin (Sigma, 1%), at 37°C and 5% CO_2. Arachidonic acid (AA), arachidonoyl-chloride, Triton X-100, reduced Triton X-100 and PMSF were purchased from Sigma (U.K.). MAFP was purchased from Biomol (USA). Porcine brain was obtained at a local slaughterhouse (Tokushima, Japan). [^{14}C]-anandamide, labelled either on the AA (55 mCi/mmol) or on the ethanolamine moieties (5 mCi/mmol), were synthesized as described previously (25, 26), respectively from [^{14}C]-AA or [^{14}C]-ethanolamine purchased from Amersham International (Amersham, England), and diluted with unlabelled anandamide before enzymatic assay. Silica gel T.L.C. plates were purchased from Merck (Germany). [^{3}H]-*N*-arachidonoyl-phosphatidyl-ethanolamine (5 mCi/mmol) was synthesized from [^{3}H]-AA (230 Ci/mmol, NEN Dupont, MA, USA) and L-α-di-palmitoyl-phosphatidyl-ethanolamine (Sigma, UK) as described previously (9).

Synthesis, purification and chemical characterization of three novel anandamide amidohydrolase inhibitors. ADMK, ACMK and AcAHA were synthesized by a slight modification of the methodologies previously described for the synthesis of peptidyl diazomethyl-ketones, chloro-methyl-ketones and *O*-acetyl-hydroxamates (20-22). Briefly, ADMK was obtained by reacting 20 mg of arachidonoyl-chloride with an excess of ethereal diazomethane (2 ml) for 20 min at 4°C. The reaction mixture is then brought to dryness under a flow of N_2 and purified by T.L.C. on a semi-preparative silica gel-coated plate developed with petroleum ether/di-ethyl ether 85:15 (v/v). ADMK (Rf=0.5, 17 mg) is then extracted from silica with di-ethyl ether. ACMK was obtained by reacting 5 mg of ADMK with an anhydrous, saturated HCl methanolic solution (2 ml), at 4°C for 20 min. The reaction mixture is then brought to dryness under a flow of N_2 and purified by T.L.C. on an analytical silica gel-coated plate developed with petroleum ether/di-ethyl ether 85:15 (v/v). ACMK (Rf=0.6, 4 mg) is then extracted from silica with di-ethyl ether. Finally, AcAHA was obtained by reacting for 15 min at 4°C 10 mg of arachidonoyl-chloride with 400 μl of the surnatant of a saturated NH_2-OH solution. The latter was obtained by mixing 2 ml of a dry methanolic solution containing 118 mg of NH_2-OH · HCl with 2 ml of a dry methanolic solution containing 108 mg of CH_3ONa, and allowing NaCl to precipitate. The reaction mixture is then brought to dryness under a flow of N_2 and purified by T.L.C. on an analytical silica gel-coated plate developed with chloroform/methanol 95:5 (v/v). The purified arachidonoyl-hydroxamate (Rf=0.3, 8 mg) is then allowed to react overnight at room temperature with 50 μl of acetic anhydride in 200 μl of anhydrous pyridine. Finally, the *O*-acetyl-arachidonoyl-hydroxamate-containing solution is brought to dryness under a flow of N_2 and purified by T.L.C. on an analytical silica gel-coated plate developed with petroleum ether/di-ethyl ether 85:15 (v/v). AcAHA (Rf=0.35, 6.5 mg) is then extracted from silica with di-ethyl ether. [^{1}H]-Nuclear magnetic resonance (NMR) analysis of the compounds was carried out in $CDCl_3$ on a 300 MHz Bruker apparatus. Electron-impact mass spectrometric analysis of the compounds was carried out on a HP-MS 5989B quadrupole mass analyzer equipped with an electron impact source operating at 70 eV and 250 °C.

Enzyme preparation. Anandamide amidohydrolase from porcine brain microsomes was solubilized, partially purified and assayed as described previously (12). For the preparation of the amidohydrolase from $N_{18}TG_2$ and RBL-1 cells, confluent cells were harvested and homogenized, and the enzyme solubilized, as described previously (14). The solubilized enzyme was diluted to 20 ml with 20 mM citrate-sodium phosphate buffer pH 6.0 containing 0.05% reduced Triton X-100 (buffer A) and loaded onto a Q anion exchange column (7 × 52 mm, Bio-Rad). The column, eluted by means of a Bio-Rad fast protein liquid chromatography system, was equilibrated with buffer A. After loading the samples, the column was first washed with 20 ml of buffer A and then eluted in 2.5 ml fractions first with a 35 ml linear gradient of NaCl (0-0.7 M) in buffer A and then with 10 ml of 1 M NaCl in buffer A. The chromatography was carried out at room temperature and at a flow rate of 1 ml/min. Fractions containing amidohydrolase activity, assayed as described previously (14, 17), were pooled and used for the experiments. Protein content was determined according to the method of Bradford. For inhibition studies carried out in the Tokushima laboratory, an approximately 40-fold purified porcine brain microsomal anandamide amidohydrolase (0.18 μg protein, 0.5 μmol min^{-1} mg protein $^{-1}$) was incubated for 20 min at 37°C with 100 μM [^{14}C]-anandamide (5,000 cpm, 10 nmol, labelled on the AA moiety) in 100 μl of 100 mM Tris-HCl, pH 9.0, after a 10 min pre-incubation with increasing concentrations of the inhibitors, and the formation of [^{14}C]-AA measured as described previously (12). For inhibition studies carried out in the Naples laboratory, an approximately 10-fold purified anandamide amidohydrolase from mouse neuroblastoma cells (27 μg protein, 0.94 nmol min^{-1} mg protein $^{-1}$) or RBL-1 cells (23 μg protein, 0.27 nmol min^{-1} mg protein $^{-1}$) was incubated for 30 min at 37°C with 100 μM [^{14}C]-anandamide (10,000 cpm, 50 nmol, labelled on the ethanolamine moiety) in 0.5 ml of 50 mM Tris-HCl, pH 7.4, after a 10 min pre-incubation with increasing concentrations of the inhibitors, and the formation of

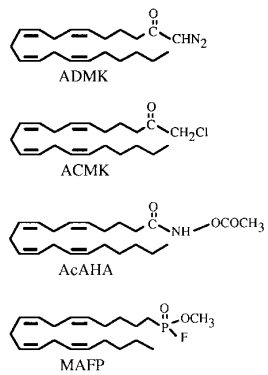

FIG. 1. Chemical structures of the three novel anandamide amidohydrolase inhibitors described in this study and of the previously described (27) irreversible inhibitor of cytosolic phospholipase A_2, MAFP.

[^{14}C]-ethanolamine measured as described previously (14, 17). Lineweaver-Burk profiles, in the absence or presence of the inhibitors, were calculated by measuring [^{14}C]-anandamide hydrolysis with increasing concentrations of the radiolabelled substrate. The irreversible or reversible modification of the amidohydrolase by the inhibitors was assessed by treating the enzyme either with a maximal concentration of inhibitors (100 μM for ADMK, ACMK, AcAHA and PMSF and 100 nM for MAFP) or with vehicle for 15 min at 37°C, and then by separating the enzyme from the inhibitors by means of anion exchange chromatography carried out as described above. The activities of the 2.5 ml fractions from each chromatographic analysis were then compared.

Effect of the inhibitors on phosphodiesterase-mediated biosynthesis of anandamide. The effect of a 10 min pre-incubation with ADMK, ACMK or MAFP, at concentrations corresponding to their IC_{50} for $N_{18}TG_2$ cell anandamide amidohydrolase inhibition, was tested on the enzymatic hydrolysis of [^{3}H]-*N*-arachidonoyl-phosphatidyl-ethanolamine to [^{3}H]-anandamide using whole homogenates from $N_{18}TG_2$ cells, prepared and incubated as described previously (27). The release of [^{3}H]-anandamide was measured by T.L.C. as described previously (26, 27).

RESULTS AND DISCUSSION

The successful addition of the diazo-methyl-, chloromethyl- and *O*-acyl-hydroxamate- moieties to the carboxy-group of AA (Fig. 1) was assessed by means of [^{1}H]-NMR (300 MHz, $CDCl_3$), which revealed the presence, respectively in ADMK, ACMK and AcAHA, of signals at δ=5.40 (1H, singlet), 4.07 (2H, singlet), and 2.31 (3H, singlet), corresponding to the -CH-N_2 proton, the two -CH_2-Cl protons and the three *O*-acetyl-hydroxamate methyl protons. The presence of these functional groups was confirmed by the chemical shift values of the two α-methylene protons of AA, which were shifted from δ=2.35 (in AA) to δ=2.10, 2.60 and 2.15 (2H, multiplets), respectively in ADMK, ACMK and AcAHA. Further evidence for the chemical structures of the three AA derivatives came from electron impact mass spectrometric analyses which, apart from the aliphatic fragmentation typical of anandamide (26), also showed signals at m/z= 328 and 300 for ADMK (molecular ion and fragment ion due to the loss of N_2), m/z= 336 and 286 for ACMK (molecular ion and fragment ion due to the formation of the chetene with loss of CH_3Cl), and m/z= 302 and 258 for AcAHA (fragment ions due to the loss of -O-CO-CH_3 and HCO-NH-O-CO-CH_3). The overall yields of the synthesis and purification of ADMK, ACMK, and AcAHA (which are stable for several months as dry compounds at −20°C and, respectively, for 1, 6 and 2 months in methanol solution at −80°C), relative to the starting reagent, arachidonoyl-chloride, were, respectively, 85%, 68% and 65%.

The effect on anandamide amidohydrolase activity of increasing concentrations of each of the newly synthesized inhibitors as well as of another (commercially available) AA derivative, the irreversible inhibitor of cytosolic phospholipase A_2, MAFP (24), was tested by using partially purified enzyme preparations from three different sources. Porcine brain microsomes and the 10,000 × *g* pellet fractions from mouse $N_{18}TG_2$ neuroblastoma and RBL-1 cells were shown previously to contain high levels of the enzyme (12, 14, 17, 19). The choice of these sources for the present study was prompted by the fact that, to date, they represent, respectively, the only nervous tissue from which the amidohydrolase has been partially purified (12), the only example of anandamide amidohydrolase-containing single neuronal cell type (14, 19), and the only example of anandamide amidohydrolase-containing non-neuronal cell type (17). We found that each of the three novel AA derivatives exhibited similar inhibition dose-dependency profiles (Fig. 2 A-C) independently from the enzyme source and type of protocols used for enzyme purification and assay. ADMK was always the most potent inhibitor, with IC_{50} values of 6, 3 and 2 μM respectively for porcine brain, $N_{18}TG_2$ cell and RBL-1 cell amidohydrolase. AcAHA was always the less active compound with IC_{50} values of 25, 34 and 15 μM, while the respective IC_{50} values for ACMK were 23, 10 and 3 μM. Although ADMK was as potent as the previously reported arachidonoyl-trifluoro-methyl-ketone (whose IC_{50} values are 1 μM, 3 μM and 2 μM respectively for porcine brain microsomes, $N_{18}TG_2$ and RBL cells [12, 14, 17]), its inhibitory effect was much smaller than that observed in this study with MAFP (24), which exhibited IC_{50} values of 3 and 1 nM, respectively with the $N_{18}TG_2$ and RBL-1 cell enzyme preparation (Fig. 2D). Interestingly, the inhibition of the enzyme by MAFP was observed at concentrations 500-1000 times lower than those previously reported to cause inhibition of cytosolic phospholipase A_2 ([24] and references cited therein), thus suggesting that this compound may selectively inhibit anandamide amidohydrolase when used at very low concentrations (see below).

Next, we investigated the nature of amidohydrolase inhibition by the three novel AA derivatives as well as by MAFP and PMSF. Enzyme preparations were

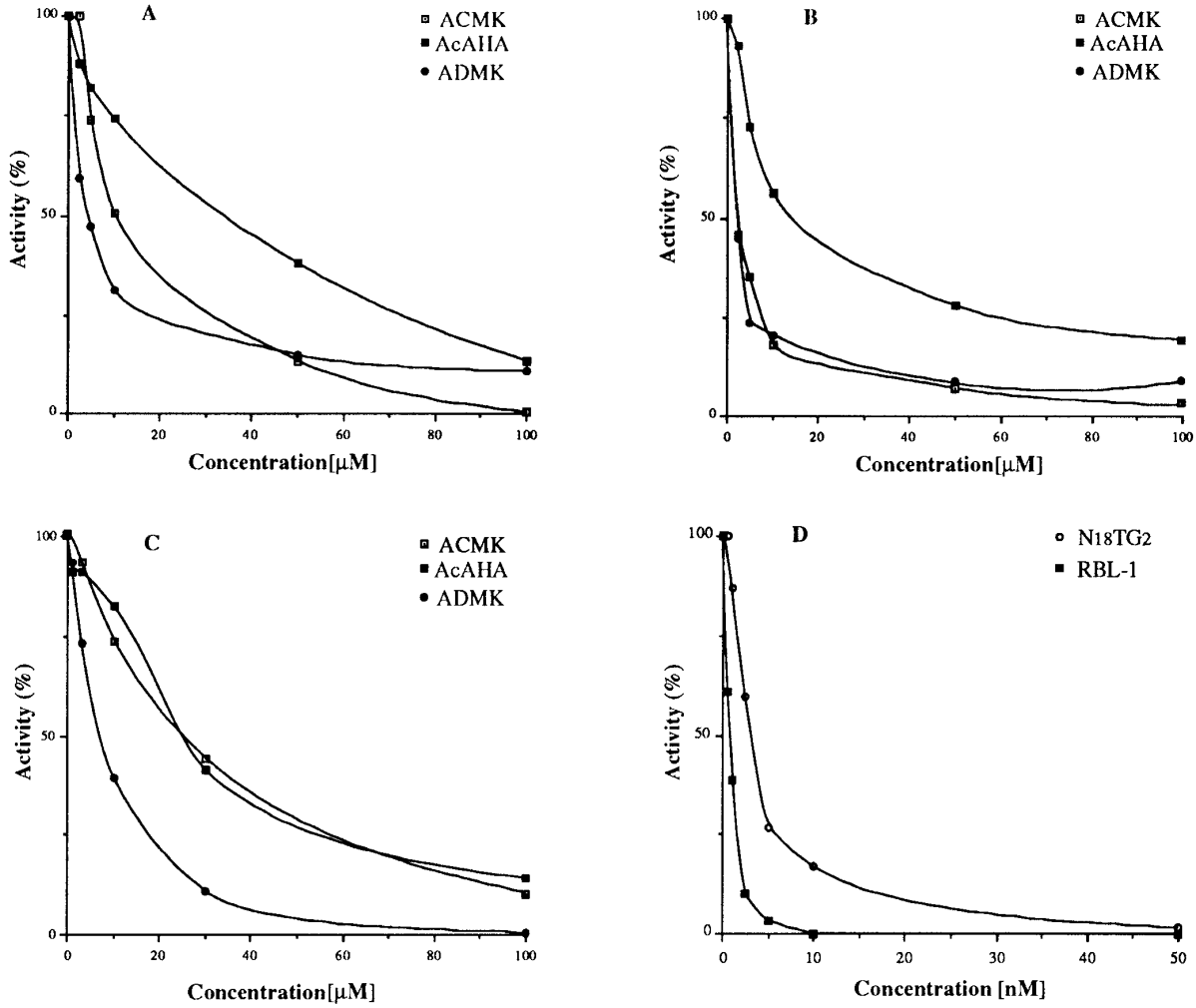

FIG. 2. Dose response curves for the inhibition by ADMK, ACMK and AcAHA of anandamide amidohydrolase from (A) mouse neuroblastoma $N_{18}TG_2$ cells, (B) rat basophilic leukaemia RBL-1 cells and (C) porcine brain, and for the inhibition by MAFP (D) of $N_{18}TG_2$ and RBL-1 cell anandamide amidohydrolase. The inhibitory effects are expressed as percent of enzyme activity (see Materials and Methods) in the absence of the inhibitors, and are means of three separate experiments.

divided into two identical aliquots and treated for 15 min either with vehicle or with maximal concentrations of each inhibitor. After the treatment, a small aliquot from each sample was assayed for amidohydrolase activity and the remainder analyzed by anion exchange chromatography, by assaying each fraction for amidohydrolase activity. As shown in Fig. 3 A-C, following the chromatographic step, enzyme preparations from each of the sources used, that had been inhibited by pre-treatment with the three novel inhibitors, completely recovered their initial amidohydrolase activity. Conversely, enzyme preparations that had been treated with either PMSF or MAFP did not recover their activity upon chromatographic separation of the enzyme from the inhibitors (Fig. 3 D, E and data not shown). This finding suggests that, unlike PMSF, none of the AA derivatives synthesized in this study works as a covalent anandamide amidohydrolase inhibitor, and that, conversely MAFP behaves as potent covalent inhibitor of the enzyme. Accordingly, while the inhibitory action of this latter compound was not counteracted by increasing concentrations of [^{14}C]-anandamide, Lineweaver-Burk profiles for [^{14}C]-anandamide hydrolysis by the enzyme preparations revealed a competitive inhibitory action by each of the three novel AA derivatives synthesized in this study (data not shown). When using $N_{18}TG_2$ cell anandamide amidohydrolase, ADMK (10 μM), ACMK (20 μM) and AcAHA (10 μM) caused a shift in the apparent Km value for anandamide from 15 μM to 28, 36 and 20 μM, respectively.

The finding of a reversible inhibition of anandamide amidohydrolase by ADMK, ACMK and AcAHA is consistent with the recent report (28) that diazo-methyl- and chloro-methyl-derivatives of oleic acid are non-covalent inhibitors of the amidohydrolase catalyzing the

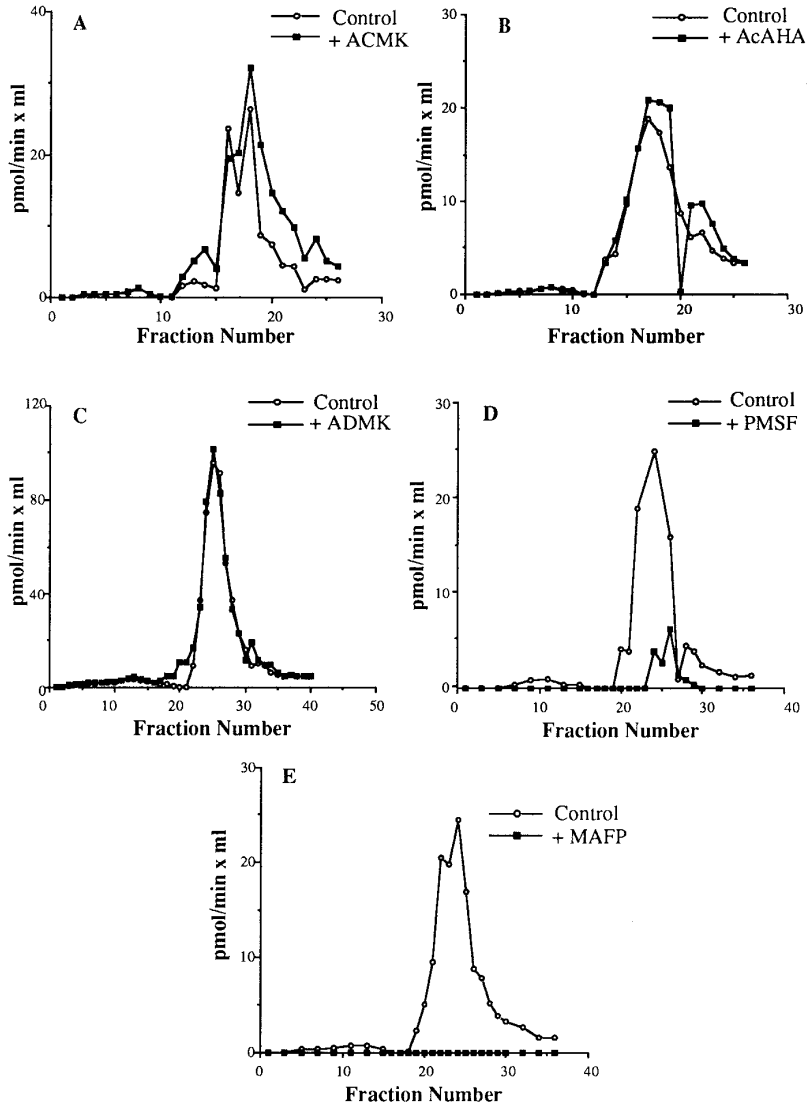

FIG. 3. Anandamide amidohydrolase activity profiles of anion exchange chromatograms of enzyme preparations from $N_{18}TG_2$ cells pre-treated for 15 min at 37 °C with 100 μM ACMK (A), 100 μM AcAHA (B), 100 μM ADMK (C), 100 μM PMSF (D) and 100 nM MAFP (E), in comparison with analogous profiles of enzyme preparations pre-treated with vehicle (methanol in A-D, and ethanol in E). Enzyme assay and chromatographic conditions are described in Materials and Methods. Data are representative of at least two separate experiments. Similar data were obtained using anandamide amidohydrolase preparations from RBL-1 cells.

hydrolysis to oleic acid of the sleep-inducing factor oleamide (15), an enzyme that we have previously suggested to be the same as anandamide amidohydrolase (14). The lack of covalent inhibition by these derivatives is somehow surprising considering that they bear functional groups previously reported to be extremely reactive towards serine and/or cysteine proteases (21-23), but may provide some interesting insights in the steric and functional requirements of the amidohydrolase catalytic site. According to the inhibitory mechanisms proposed previously for peptidyl-diazo-methyl-ketones, chloro-methyl-ketones and *O*-acyl-hydroxamates (21, 22), in order for these compounds to form a covalent adduct with the serine or cysteine protease active site, a rearrangement, following to the formation of the tetrahedral oxyanion intermediate, must occur. It is possible that the steric hindrance of anandamide amidohydrolase active site and/or the orientation of the aminoacid residues involved in the hydrolysis of the amide bond, do not allow this rearrangement to occur. This would explain why MAFP, which contains a strong electrophilic phosphonyl-group instead of the carbonylic group, and does not require such a rearrangement to form a relatively stable covalent bond with the active serine or cysteine residue, behaves as an irreversible inhibitor of the amidohydrolase. Conversely, ACMK, ADMK and, to a smaller extent, AcAHA are rather likely to function as reversible transition-state inhibi-

TABLE 1

Inhibitor	None	ADMK (3 μM)	ACMK (10 μM)	MAFP (3 nM)
Percent of anandamide release	100	136.4 ± 12.8	202.3 ± 3.9*	145.4 ± 5.4*

Note. Effect of ADMK, ACMK and MAFP on the levels of [^{3}H]-anandamide produced from the enzymatic hydrolysis of [^{3}H]-*N*-arachidonoyl-phosphatidyl-ethanolamine in $N_{18}TG_2$ cell whole homogenates (27). Incubations were carried out for 15 min at 37°C with 50,000 cpm (9.1 nmol) [^{3}H]-*N*-arachidonoyl-phosphatidyl-ethanolamine in 0.5 ml of homogenate (1.6 mg proteins) prepared as described previously (27) and pre-incubated for 10 min with the shown amounts of the inhibitors. Results are reported as percent of the rate of conversion in control incubations (5.4 ± 0.05 pmoles min^{-1} mg protein^{-1}) and are means ± S.D. of three separate experiments. Asterisks indicate statistically significant effects ($P < 0.05$, unpaired Student's T test).

tors of the enzyme, as previously suggested also for acyl-trifluoro-methyl-ketones and -α-keto-esters (19). It is worthwhile noting that, during the preparation of this manuscript, we became aware of two preliminary reports [29, 30] describing the inhibitory effect and the irreversible anandamide amidohydrolase inactivation respectively by MAFP and the PMSF-like fatty acid derivative, *cis*-13,14-[^{3}H,^{3}H]-octadecene-1-sulphonyl-fluoride, in agreement with the data presented here.

Finally, the possible use of ADMK, ACMK and MAFP in studies on anandamide biosynthesis was assessed by assaying the effect of these compounds on the *in vitro* conversion of *N*-arachidonoyl-phosphatidyl-ethanolamine into anandamide ([9] and, for review, [8]) in $N_{18}TG_2$ cell whole homogenates, where an enzymatic activity catalyzing this reaction has been described ([27] and references cited therein). We found that all the inhibitors tested caused an apparent potentiation of anandamide enzymatic formation (Table 1), very probably by inactivating anandamide amidohydrolase which was also present in the homogenates. This finding suggests that ADMK, ACMK and MAFP do not inactivate the phospholipase D-like enzyme previously suggested to catalyze the release of anandamide from its phospholipid precursor [8, 9, 27], and may be utilized to minimize anandamide degradation in biosynthetic studies. In such studies, bearing in mind that anandamide formation has been suggested to occur also through the reverse action of anandamide amidohydrolase (12, 16), these compounds may be also used to distinguish between the two biosynthetic routes proposed so far for anandamide biosynthesis (for a review [8]). Preliminary experiments carried out in our laboratories also showed that ADMK does not significantly inactivate sheep cycloxygenase-1, and inhibits porcine leukocyte-type 12-lipoxygenase and rabbit reticulocyte 15-lipoxygenase only at concentrations higher than those required for amidohydrolase inhibition. MAFP was already known not to affect significantly other enzymes of AA metabolism ([24] and references cited therein). However, further studies will be required in order to thoroughly assess the selectivity of the novel anandamide amidohydrolase inhibitors reported here.

In conclusion, the present study has led to the finding of two novel and potent competitive inhibitors of anandamide amidohydrolase, i.e. ADMK and ACMK, and has shown that MAFP, a previously reported cytosolic phospholipase A_2 inhibitor (24), is an irreversible as well as the most potent inhibitor so far described for the amidohydrolase. The possibility of using these three compounds for the purification and characterization of the enzyme, either through the preparation of affinity chromatography resins, or (in the case of MAFP) as selective affinity radio-labelling reagents, is under current investigation. Moreover, the present work opens the way to the use of these compounds both in studies on anandamide biosynthesis and pharmacological activity and as biochemical probes for the assessment of anandamide amidohydrolase role and distribution in mammalian tissues and cells.

ACKNOWLEDGMENTS

The authors thank Drs. T. Bisogno, A. Scaloni, N. Sepe, and F. Montanaro for their valuable assistance. This work was supported by grants from the Human Frontiers in Science Program (RG 26/95 to VDM and SY) and the C.N.R. (P.S. 'Tecnologie chimiche innovative', to VDM), and by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (to SY and NU). NMR spectra were obtained at the 'Servizio NMR dell'Area di Ricerca di Arco Felice del C.N.R.', whose staff is acknowledged.

Note added during revision. After submission of the present paper, Cravatt et al. (31) reported the purification, molecular characterization, cloning and expression of 'oleamide amidohydrolase', and showed that anandamide is a preferential substrate for this enzyme, thus providing conclusive evidence to our previous suggestion (14) that the same enzyme, i.e. a '(long chain) fatty acid amidohydrolase' (14, 31), catalyzes the degradation of neuroactive fatty acid amides. Following the important finding by Cravatt and co-workers, the inhibitors described herein may be now used also for pharmacological and biochemical studies on the 'sleep-inducing' factor oleamide (15).

REFERENCES

1. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949.
2. Schmid, P. C., Krebsbach, R. J., Perry, S. R., Dettmer, T. M., Maasson, J. L., and Schmid, H. H. O. (1995) *FEBS Lett.* **375,** 117–120 plus (1996), **385,** 125–126 (correction).
3. Sugiura, T., Kondo, S., Sukagawa, A., Tonegawa, T., Nakane,

S., Yamashita, A., Ishima, Y., and Waku, K. (1996) *Eur. J. Biochem.* **240,** 53–62.

4. Felder, C. C., Nielsen, A., Briley, E. M., Palkovits, M., Priller, J., Axelrod, J., Nguyen, D. N., Richardson, J. M., Riggin, R. M., Koppel, G. A., Paul, S. M., and Becker, G. W. (1996) *FEBS Letts.* **393,** 231–235.
5. Devane, W. A. (1994) *Trends Pharmacol. Sci.* 40–41.
6. Iversen, L. L. (1994) *Nature* **372,** 619.
7. Mechoulam, R., Hanus, L., and Martin, B. R. (1994) *Biochem. Pharmacol.* **48,** 1537–1544.
8. Di Marzo, V., De Petrocellis, L., Bisogno, T., and Maurelli, S. (1995) *J. Drug Devl. Clin. Pract.* **7,** 199–219.
9. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.-C., and Piomelli, D. (1994) *Nature* **372,** 686–691.
10. Desarnaud, F., Cadas, H., and Piomelli, D. (1995) *J. Biol. Chem.* **270,** 6030–6035.
11. Hillard, C. J., Wilkison, D. M., Edgemond, W. S., and Campbell, W. B. (1995) *Biochim. Biophys. Acta* **1257,** 249–256.
12. Ueda, N., Kurahashi, Y., Yamamoto, S., and Tokunaga, T. (1995) *J. Biol. Chem.* **270,** 23823–23827.
13. Watanabe, K., Kayano, Y., Matsunaga, T., Yamamoto, I., and Yoshimura, H. (1996) *Biol. Pharm. Bull.* **19,** 1109–1111.
14. Maurelli, S., Bisogno, T., De Petrocellis, L., Di Luccia, A., Marino, G., and Di Marzo, V. (1995). *FEBS Letts.* **377,** 82–86.
15. Cravatt, B. F., Prospero-Garcia, O., Siuzdak, G., Gilula, N. B., Henriksen, S. J., Boger, D. L., and Lerner, R. A. (1995) *Science* **268,** 1506–1509.
16. Matsuda, S., Kanemitsu, N., Nakamura, A., Mimura, Y., Ueda, N., Kurahashi, Y., and Yamamoto, S. (1997) *Exp. Eye Res.*, in press.
17. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., and Di Marzo, V. (1997) *J. Biol. Chem.* in press.
18. Bisogno, T., Ventriglia, M., Mosca, M., Milone, A., Cimino, G., and Di Marzo, V., submitted.
19. Koutek, B., Prestwick, G. D., Howlett, A. C., Chin, S., Salehani, D., Akhavan, N., and Deutsch, D. G. (1994) *J. Biol. Chem.* **269,** 22937–22940.
20. Lang, W. S., Qin, C., Hill, W. A. G., Lin, S. Y., Khanolkar, A. D., and Makriyannis, A. (1996). *Anal. Biochem.* **238,** 40–45.
21. Shaw, E. (1994) *Methods Enzymol.* **244,** 649–656.
22. Bromme, D., Demuth, H.-U. (1994) *Methods Enzymol.* **244,** 671–685.
23. Kettner, C., and Shaw, E. (1981) *Methods Enzymol.* **80,** 826–841.
24. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765.
25. Ueda, N., Yamamoto, K., Yamamoto, S., Tokunaga, T., Shirakawa, E., Shinkai, H., Ogawa, M., Sato, T., Kudo, I., Inoue, K., Takizawa, H., Nagano, T., Hirobe, M., Matsuki, N., and Saito, H. (1995) *Biochim. Biophys. Acta.* **1254,** 127–134.
26. Fontana, A., Di Marzo, V., Cadas, H., and Piomelli, D. (1995) *Prostaglandins Leukot. Essent. Fatty Acids* **53,** 301–308.
27. Di Marzo, V., De Petrocellis, L., Sugiura, T., and Waku, K. (1986) *Biochem. Biophys. Res. Commun.* **227,** 281–288.
28. Patterson, J. E., Ollmann, I. R., Cravatt, B. F., Boger, D. L., Wong, C.-H., and Lerner, R. A. (1996) *J. Am. Chem. Soc.* **118,** 5938–5945.
29. Deutsch, D. G., Arreaza, G., Omeir, R., Salehani, D., Prestwich, G. D., and Howlett, A., Proceedings of the 1996 International Cannabinoid Research Society Meeting, West Dennis, Massachusetts, USA, p. 55.
30. Fang, J. C.-W., Prestwich, G. D., and Deutsch, D. G., Proceedings of the 1996 International Cannabinoid Research Society Meeting, West Dennis, Massachusetts, USA, p. 29.
31. Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) *Nature* **384,** 83–87.