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# RAPID COMMUNICATION

## METHYL ARACHIDONYL FLUOROPHOSPHONATE: A POTENT IRREVERSIBLE INHIBITOR OF ANANDAMIDE AMIDASE

Dale G. Deutsch,[#†] Romelda Omeir,[†] Gladys Arreaza,[†] David Salehani,[†] Glenn D. Prestwich,[#] Zheng Huang* and Allyn Howlett**

[†]Department of Biochemistry and Cell Biology and Institute for Cell and Developmental Biology, State University of New York at Stony Brook, Stony Brook, NY 11794, U.S.A.; *Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada H9R 4P8; and ** Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, MO 63104, U.S.A.

**ABSTRACT.** Anandamide amidase (EC 3.5.1.4) is responsible for the hydrolysis of arachidonoyl ethanolamide (anandamide). Relatively selective and potent enzyme reversible inhibitors effective in the low micromolar range, such as arachidonyl trifluoromethyl ketone (Arach-$CF_3$), have been described (Koutek *et al.*, *J Biol Chem* **269**: 22937-22940, 1994). In the current study, methyl arachidonyl fluorophosphonate (MAFP), an arachidonyl binding site directed phosphonylation reagent, was tested as an inhibitor of anandamide amidase and as a ligand for the $CB_1$ cannabinoid receptor. MAFP was 800 times more potent than Arach-$CF_3$ and phenylmethylsulfonyl fluoride (PMSF) as an amidase inhibitor in rat brain homogenates. In intact neuroblastoma cells, MAFP was also approximately 1000-fold more potent than Arach-$CF_3$. MAFP demonstrated selectivity towards anandamide amidase for which it was approximately 3000 and 30,000-fold more potent than it was towards chymotrypsin and trypsin, respectively. MAFP displaced [$^3$H]CP-55940 binding to the $CB_1$ cannabinoid receptor with an $IC_{50}$ of 20 nM vs 40 nM for anandamide. It bound irreversibly and prevented subsequent binding of the cannabinoid radioligand [$^3$H]CP-55940 at that locus. These studies suggest that MAFP is a potent and specific inhibitor of anandamide amidase and, in addition, can interact with the cannabinoid receptors at the cannabinoid binding site. This is the first report of a potent and relatively selective irreversible inhibitor of arachidonoyl ethanolamide amidase.

***KEY WORDS***: anandamide; anandamide amidase; amidohydrolase; cannabinoid receptor; methyl arachidonyl fluorophosphonate; sleep-inducing factor

Anandamide amidase (EC 3.5.1.4; arachidonoyl ethanolamide amidohydrolase), an enzyme that is responsible for the degradation of anandamide, has been described [1-6]. A series of "transition-state" inhibitors were synthesized (trifluoromethyl ketone, α-keto ester, and α-keto amide derivatives) and tested *in vitro* and in intact cells as amidase inhibitors [7]. The trifluoromethyl ketones and, in particular, arachidonyl trifluoromethyl ketone was found to inhibit anandamide hydrolysis, in brain homogenates and in intact cells in culture. Inhibition was relatively potent, i.e. in the low micromolar range.

MAFP[††], a relatively stable arachidonyl binding site directed phosphonylation reagent, was initially designed and developed by Huang and coworkers [8, 9] as the active-site directed inactivator of the calcium-sensitive and arachidonyl selective $cPLA_2$. $cPLA_2$ is a phospholipid hydrolase using the hydroxyl of serine-228 residue as its catalytic nucleophile.

[#]Corresponding author: Dale Deutsch, Ph.D., Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215. Tel.(516)632-8595; FAX (516)632-8575; E-mail: DDEUTSCH@CCMAIL.SUNYSB.EDU

[††]Abbreviations: MAFP, methyl arachidonyl fluorophosphonate; $cPLA_2$, cytosolic phospholipase $A_2$; $iPLA_2$, calcium-independent arachidonic acid selective phospholipase $A_2$; Arach-$CF_3$, arachidonyl trifluoromethyl ketone; and PMSF, phenylmethylsulfonyl fluoride.

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In addition to the stoichiometric inhibition of $cPLA_2$, MAFP has been found to inhibit the catalytic activity of the calcium-independent, arachidonic acid selective phospholipase $A_2$ ($iPLA_2$) and cyclo-oxygenases (Huang *et al.*, unpublished results). MAFP is also a cell permeable phosphonylation reagent that potently blocks the calcium ionophore mediated arachidonic acid release in platelets, U-937 cells, and polymorphonuclear leucocytes [10].

In the current study, we describe the novel use of MAFP as an inhibitor of anandamide amidase. MAFP, a relatively stable arachidonyl binding site directed phosphonylation reagent, was found to be, by nearly three orders of magnitude, the most potent irreversible inhibitor of anandamide hydrolysis tested to date. In addition, MAFP appears to irreversibly bind to the $CB_1$ cannabinoid receptor, perhaps by occupying the space that the arachidonyl moiety of anandamide would occupy within the receptor binding cavity and possibly reacting with an amino acid moiety in the vicinity.

## MATERIALS AND METHODS

*Inhibitors.* MAFP (phosphonofluoridic acid, methyl-5,8,11,14-eicosatetraenyl ester) was obtained from the Merck Frosst Centre for Therapeutic Research or purchased from Cayman Chemical, Ann Arbor, MI. Arach-$CF_3$ was synthesized as described previously [7] or purchased from Biomol (Plymouth Meeting, PA).

*Effect of MAFP on Enzymes.* Anandamide amidase was measured in rat brain homogenates employing the substrate arachidonoyl ethanolamide [ethanolamine-1,2-$^{14}$C] (Dupont New England Nuclear, Boston, MA) as recently described [4]. The final reaction volume was 200 µL. Experimental tubes contained various concentrations of MAFP, blanks contained 1.5 mM PMSF, and the control tubes contained 3 µL of dimethyl sulfoxide, the vehicle used for MAFP. After 30 min, the reactions were terminated by the addition of 400 µL of chloroform:methanol (1:1), and the radioactivity in the aqueous phase was measured by liquid scintillation counting. Data were analyzed by linear regression (Sigma Plot, Jandel Scientific, San Rafael, CA). The $IC_{50}$ values were determined by inspection of the graph. The percent inhibition was calculated by [(rate of control - rate of experimental) x 100] / rate of control.

Inhibition of anandamide amidase in cell culture was measured using approximately 1 x $10^6$ N18TG2 intact neuroblastoma cells as described previously [2, 7]. Experimental cells were preincubated for 20 min in 1.5 mL medium, consisting of F12/DMEM (GIBCO, Grand Island, NY) with penicillin, streptomycin, gentamicin, 10% bovine calf serum (HyClone, Logan, UT), plus the inhibitor. Control cells contained no inhibitor. Arachidonoyl [5,6,8,9,11,12,14,15-$^3$H]ethanolamide (0.2 µCi of 221 Ci/mmol) from New England Nuclear was then added and the incubation continued for 1 hr. The amount of [$^3$H]anandamide in the cells was quantified by liquid scintillation counting of the silica scraped from the appropriate areas of the TLC plate identified by exposure to X-ray film. The control cells had an average of 194,240 ± 17,570 total dpm (N=4)

The effect of MAFP on the synthesis of anandamide from arachidonic acid plus ethanolamine was determined as follows. The reaction mixture containing 120 µg total protein from rat brain homogenate, 10 mM ethanolamine hydrochloride, 20 µM [$^{14}$C]arachidonic acid (1 µCi/mL, 55 mCi/mmol), 20 mM Tris-HCl, pH 9.0, in a total volume of 100 µL was incubated for 60 min at 37$^0$, extracted with 250 µL of chloroform:methanol (2:1), and centrifuged. Two hundred microliters of the organic phase was transferred to a clean tube and dried under nitrogen. The residue was redissolved in 20 µL of chloroform:methanol and was spotted on a TLC plate. Plates were analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA). To quantitate the amount of anandamide produced, the bands co-migrating with anandamide standards were quantitated against a standard curve of known amounts of [$^{14}$C]anandamide.

The effects of the amidase inhibitors, MAFP and PMSF, upon trypsin and chymotrypsin were measured using a resorufin-labeled casein assay according to the manufacturer (Boehringer Mannheim, Indianapolis, IN) with modifications as described below. This assay measures the release of resorufin-labeled peptides into the supernatant after proteolysis. A preincubation at 37° for 15 min was conducted with enzyme (0.05 mg/mL), various concentrations of inhibitor dissolved in DMSO (MAFP) or isopropanol (PMSF), and buffer (0.2 M Tris-HCl, 0.02 M $CaCl_2$, pH 7.8). The control (100% activity) contained vehicle in place of inhibitor (DMSO for MAFP, isopropanol for PMSF), while the blank tubes did not contain enzyme or inhibitor. Then the resorufin-labeled casein substrate (0.2%) was added, the assay was continued for 30 min at 37° with shaking, and the reaction was terminated by addition of 5% trichloroacetic acid, which precipitates undigested substrate. The tubes were centrifuged at 9630 *g* for 5 min, after incubation at 37° for 10 min. The supernatant (containing the resorufin-labeled peptides) was retrieved and mixed with buffer (0.5 M Tris-HCl, pH 8.8), and the resorufin-labeled peptides were measured spectrophotometrically at 574 nm (LKB Ultraspec II).

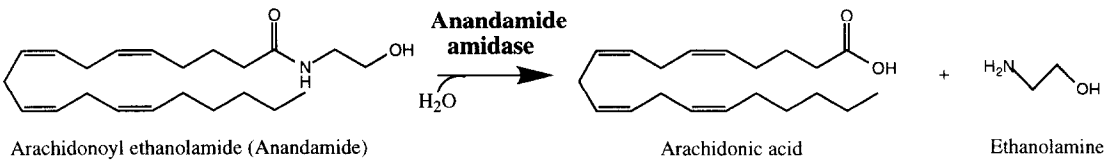
*Receptor Binding*. The affinity of MAFP for the $CB_1$ cannabinoid receptor in PMSF-pretreated rat brain membranes was determined by a radioligand displacement assay using [$^3$H]CP-55940 as described previously [7]. Modification of the ligand binding assay for the heterologous displacement of MAFP was that the reaction mixture consisted of 20 mM potassium diethylmalonate, pH 7.4, 3 mM $MgCl_2$, and 0.01% β-cyclodextrin. Bovine serum albumin was not present in the assay. Specific binding was defined as that which could be displaced by 100 nM desacetyllevonantradol.

For determination of irreversible actions of MAFP, membranes (1 mg protein/mL in potassium diethylmalonate, pH 7.4) were incubated at 30$^0$ for 30 min in the presence of the indicated concentrations of reagents. To terminate the pretreatment, 0.6 vol. of fatty acid-deficient bovine serum albumin (50 mg/mL) was added, and the reaction mixture was sedimented at 14,000 rpm for 20 min at 4$^0$. The pellet was resuspended in 20 mM Tris-HCl, 5 mM $MgCl_2$, and 2 mM EDTA, for a radioligand binding assay in that same incubation mixture.

## RESULTS AND DISCUSSION

MAFP was tested as an inhibitor of anandamide amidase activity in rat brain homogenates. MAFP, with an $IC_{50}$ value of 2.5 nM, is the most potent inhibitor of anandamide amidase reported to date (Fig. 1). It was approximately 800 times more potent than Arach-$CF_3$ ($IC_{50}$, 1.9 μM) in preventing the hydrolysis of anandamide in brain homogenate [7]. The potencies of MAFP and Arach-$CF_3$ are consistent with their proposed modes of action. That is, Arach-$CF_3$ functions as a reversible transition-state inhibitor and MAFP as an irreversible active-site inhibitor. That MAFP reacts irreversibly with anandamide amidase was demonstrated experimentally by the retention of 100% inhibition in homogenate treated with MAFP even after 20-fold dilution to a concentration below the $IC_{50}$ (data not shown).

A.




Arachidonoyl ethanolamide (Anandamide) Arachidonic acid Ethanolamine

B.

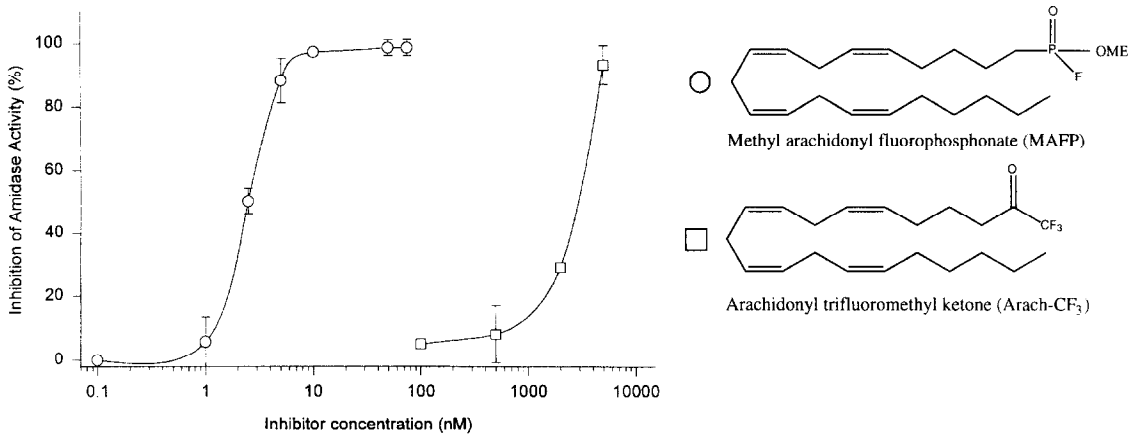



**Fig. 1.** Reaction catalyzed by anandamide amidase (A) and the inhibitory potencies of MAFP and Arach-$CF_3$ for anandamide breakdown (B). The $IC_{50}$ values for MAFP and Arach-$CF_3$ were determined as described in Materials and Methods. Values are means ± SD, N=3. One hundred percent anandamide amidase activity is 69.9 ± 9.5 nmol/hr/mg of protein.

The apparent $IC_{50}$ value for the inhibition of anandamide amidase by PMSF in brain homogenates was found to be 900 nM (Table 1). This is in agreement with previous studies showing that PMSF, at high concentrations, inhibits

**Table 1. Inhibitory potencies for anandamide amidase, anandamide synthase, chymotrypsin, and trypsin**

| Inhibitor | Apparent $IC_{50}$, nM ($r^2$) Amidase | Synthase | Chymotrypsin | Trypsin |
|---|---|---|---|---|
| MAFP | 2.5 (0.99) | 1.5 (0.90) | $7.6 \times 10^3$ (0.99) | $80 \times 10^3$ (0.99) |
| PMSF | $0.9 \times 10^3$ (0.99) | $17 \times 10^3$ (0.90) | $500 \times 10^3$ (0.94) | $>1 \times 10^6$ (0.96) |

The values of percent inhibition versus log of inhibitor concentration were plotted, and the value for 50% enzyme inhibition was determined. The correlation coefficient ($r^2$) for the linear regression analysis of the line was obtained using Sigma Plot (Jandel Scientific).

anandamide amidase nearly completely [2, 5, 11-15]. MAFP ($IC_{50}$, 2.5 nM) was therefore approximately 400 times more potent than PMSF as an inhibitor of anandamide amidase (Table 1). From the extrapolated $IC_{50}$ value of 30 μM for diisopropyl fluorophosphate to give 50% amidase inhibition [5], MAFP may be calculated to be approximately 12,000 times more effective than diisopropyl fluorophosphate.

The apparent $IC_{50}$ value for the inhibition of anandamide synthase by MAFP was 1.5 nM, which is nearly identical to that determined for the amidase (Table 1). One interpretation of this result is that the synthase is the reverse reaction of anandamide hydrolysis and that these two reactions are mediated by the same enzyme [5]. However, as shown in the present study (Table 1) and as recently reported [5], these two activities are not equally affected by all inhibitors. PMSF appears to be a somewhat better inhibitor of the amidase than of the synthase, suggesting that these may be two separate enzymes or that the rate of sulfonation of a single reversible enzyme varies with the different assay conditions (i.e. substrate concentrations) for the forward and reverse reactions.

The specificity of MAFP for other amide hydrolytic enzymes was tested using trypsin and chymotrypsin. MAFP was a poor inhibitor of trypsin and chymotrypsin, although better than PMSF (Table 1). These results are consistent with the expected increased specificity conferred upon MAFP by the arachidonyl moiety. Interestingly, MAFP is a better inhibitor of chymotrypsin than it is of trypsin (as is PMSF), and this is consistent with the substrate specificity of chymotrypsin for aromatic amino acids. Contrary to the chemically reactive non-selective phosphonylation reagents such as diisopropyl fluorophosphonate and the non-selective sulfonylation reagents such as PMSF, MAFP, designed by the tethering of the arachidonyl moiety to a reactive electrophilic phosphonylation center, is selective towards nucleophiles within the enzyme's arachidonyl binding site. This is the first report of a specific irreversible inhibitor for anandamide amidase. Another fatty acid amide, called oleamide (*cis*-9-octadecenamide), was recently discovered to be a sleep-inducing factor in rats [16,17]. It has been postulated that the enzyme (oleamide hydrolase), which hydrolyzes oleamide to oleic acid and ammonia, is identical to anandamide amidase [18]. MAFP may therefore serve as a general inhibitor for the breakdown of fatty acid amide signalling molecules. The data derived from experiments with MAFP further suggest that the anandamide amidase is likely to be a serine based amide hydrolase, as proposed previously from studies with PMSF [2, 11] and Arach-$CF_3$ [7], and as also reported for MAFP on $cPLA_2$ [9].

When anandamide is added to neuroblastoma (N18TG2) cells in culture, without inhibitor, it is taken up and hydrolyzed to arachidonate, which is subsequently incorporated into other lipids [2, 6, 7]. Both MAFP and Arach-$CF_3$ were found to inhibit the breakdown of anandamide in intact cells in culture (Fig. 2). However MAFP was approximately 1000-fold more potent than Arach-$CF_3$ (50% inhibition at 20 nM MAFP vs approximately 40% at 12 μM Arach-$CF_3$).

MAFP is selective towards nucleophiles inside arachidonyl binding sites. For this reason, the ability of MAFP to interact with the cannabinoid receptor was tested. MAFP is able to bind to the $CB_1$ receptor in rat brain membrane preparations that had been pretreated with PMSF to covalently modify the active site of the amidase or other serine proteases. Under these conditions, the $IC_{50}$ values for anandamide and MAFP were 40 and 20 nM, respectively (Fig. 3). Thus, MAFP either exhibits a greater ability to bind to the cannabinoid receptor binding site, or else because of its ability to form a covalent bond, is binding irreversibly with an off-rate approaching zero.

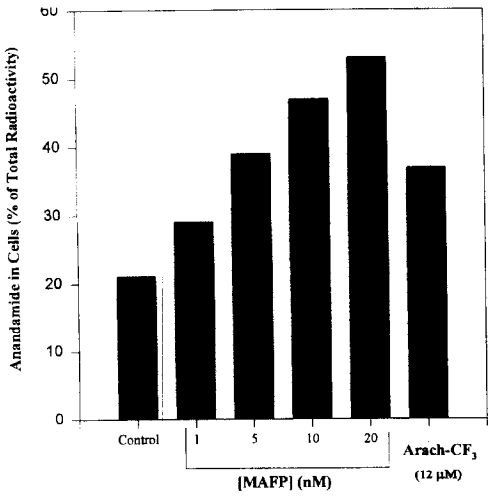



**Fig. 2.** Effects of MAFP and Arach-$CF_3$ on anandamide levels in neuroblastoma cells (N18TG2). The amount of [$^3$H]anandamide was determined in the control (no inhibitor) and experimental cultures containing 4 x $10^6$ cells, as described in Materials and Methods. $P<0.02$ was calculated (Spearman correlation, Instat, GraphPad Software, San Diego, CA) for the degree of linear correlation between MAFP concentration and the individual measurements of anandamide levels in the cells, shown in this experiment.

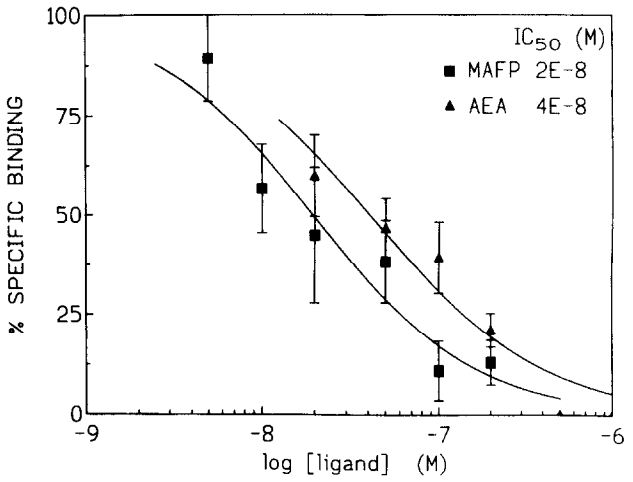



**Fig. 3.** Log concentration-response curve for MAFP and arachidonoyl ethanolamide (AEA) competition with [$^3$H]CP-55940 for binding to $CB_1$ receptors. The indicated concentrations of MAFP (■) and AEA (▲) were incubated with the radioligand in brain membranes that had been pretreated with PMSF, as indicated in the text. Each point on the curve represents the mean ± SEM of 3 experiments. $IC_{50}$ values (log base 10) were determined by nonlinear regression analysis using Graphpad Inplot.

To determine the effectiveness of covalent modification of the receptor, the brain membrane preparation was incubated with increasing concentrations of MAFP and then washed to remove MAFP that was not covalently bound to the proteins. Under these conditions, MAFP at both 1 and 10 μM attenuated specific binding of [$^3$H]CP-55940 with no effect on the nonspecific binding component (Fig. 4A). The effectiveness of the washing procedure was demonstrated by the use of anandamide as a control for these studies. Figure 4B demonstrates that only the specific component of the [$^3$H]CP-55940 binding was eliminated by MAFP pretreatment. None of the radioligand associated with the MAFP-pretreated membranes was dissociable by the cannabinoid agonist desacetyllevonantradol.

These data suggest that the MAFP interacts irreversibly with the $CB_1$ cannabinoid receptor, and that this interaction precludes the subsequent binding of cannabinoid agonists. One explanation is that the arachidonyl moiety is positioned such that the site within the receptor that would be occupied by the cannabinoid ligand is occluded. An alternative explanation is that the presence of the arachidonyl moiety induces a conformational change in the protein such that the ability of the receptor to interact with the cannabinoid agonist is no longer possible.

The ratio of the $IC_{50}$ values for inhibition of anandamide amidase versus competition for radioligand binding was approximately 1 order of magnitude. Thus, under the assay conditions described here, approximately 10% of the cannabinoid receptors would be occupied at a concentration of MAFP expected to inhibit 90% of the enzyme activity.

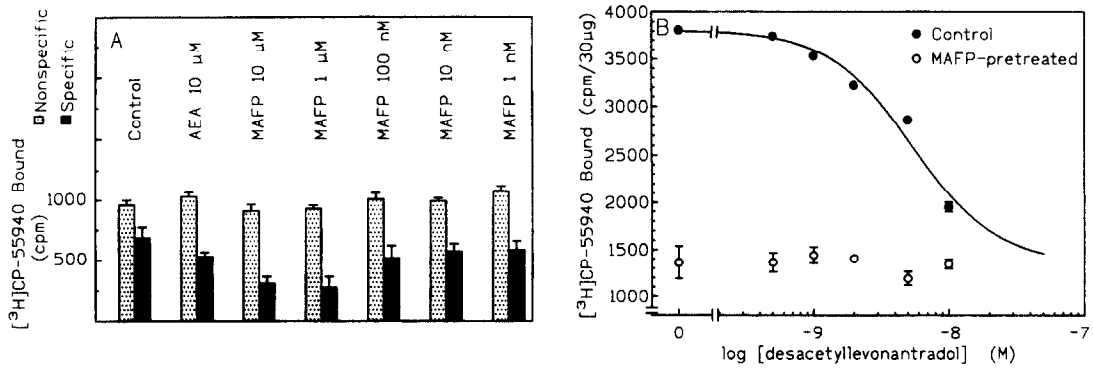



**Fig. 4.** Covalent modification of the $CB_1$ cannabinoid receptor by MAFP. (A) Concentration-dependent attenuation by MAFP of [$^3$H]CP-55940 binding to the cannabinoid receptor. PMSF-pretreated rat brain membranes were incubated with vehicle (0.05% β-cyclodextrin), anandamide (AEA), or MAFP at concentrations ranging from 1 nM to 10 μM. After sequestering the lipid reagents with fatty acid-deficient bovine serum albumin and sedimentation, the membranes were resuspended, and the ability of [$^3$H]CP-55940 to bind to cannabinoid receptors (30 μg membrane protein) was determined. Data are the averages ± range of two independent experiments. (B) Specificity of the MAFP modification for the cannabinoid receptor. After pretreatment with vehicle (●) or 1 μM MAFP (O) as described in panel A, a heterologous displacement radioligand binding assay was performed using the cannabinoid agonist desacetyllevonantradol. Data are the means ± SEM of triplicate determinations from a single representative experiment.

Clearly, greater concentrations of MAFP could potentially interact at both sites. The functional significance of the covalent occupancy of the cannabinoid receptors by the arachidonyl moiety is unclear at the present time.

In conclusion, MAFP, an arachidonyl binding site directed phosphonylation reagent, affects activities of $cPLA_2$, $iPLA_2$, and cyclo-oxygenase. Herein, we demonstrated that it binds irreversibly and with greater affinity to arachidonoyl ethanolamide amidase than it does to other amide hydrolytic enzymes or to the cannabinoid receptor $CB_1$. This is the first report of a specific irreversible inhibitor that, when used in a radiolabeled form, will be a useful tool for anandamide amidase purification and cloning.

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