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Fatty Acid Sulfonyl Fluorides Inhibit Anandamide Metabolism and Bind to the Cannabinoid Receptor

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Arachidonoyl ethanolamide (anandamide) is an endogenous ligand for cannabinoid receptors (CB1, CB2) and a putative neurotransmitter. Phenylmethylsulfonyl fluoride (PMSF) is an inhibitor of the enzyme (an amidase) which hydrolyzes anandamide to arachidonic acid and ethanolamine. We report here that fatty acid sulfonyl fluorides are potent inhibitors of anandamide metabolism. In order to investigate the SAR of these anandamide amidase inhibitors we tested a series of fatty acid (C12 to C20) sulfonyl fluorides both as inhibitors of anandamide degradation and as ligands for the central cannabinoid receptor (CB1). AM374 (palmitylsulfonyl fluoride, C16) was approximately 20 times more potent than PMSF and 50 times more potent than arachidonyltrifluoromethyl ketone in preventing the hydrolysis of anandamide in brain homogenates. AM374 was over a thousand-fold more effective than PMSF in inhibiting the amidase in cultured cells. The C12 to C18 sulfonyl fluoride analogs were equipotent as inhibitors of the amidase and the reverse reaction (the synthase) with nanomolar IC_{50} values. These compounds generally showed decreasing affinity for the CB1 receptor as the chain length increased; thus, C12 sulfonylfluoride had an IC_{50} of 18 nM and C20 sulfonylfluoride had an IC_{50} of 78 μM. The C14, C16, and C18 sulfonyl fluorides showed high selectivity for the amidase over the CB1 receptor and thus are potentially useful selective anandamide amidase inhibitors.

Δ^9-Tetrahydrocannabinol (THC), the psychoactive marijuana plant-derived cannabinoid and numerous synthetic analogs bind to a specific brain receptor, named CB1 (1-4). Arachidonoyl ethanolamide (anandamide), homo-γ-linolenyl ethanolamide, and docosatetraenyl ethanolamide are naturally occurring brain constituents that bind to CB1 (5-7). Converging lines of evidence suggest that anandamide satisfies the essential criteria of a neurotransmitter or intracellular messenger for the central cannabinoid receptor (8-10).

Soon after the discovery of anandamide, an enzyme (E.C. 3.5.1.4, E.C. 3.5.1.60, called anandamide amidase, amidohydrolase, fatty acid amide hydrolase, N-arachidonoyl ethanolamine deacylase) responsible for its hydrolysis was described (11-13) and cloned (14). Anandamide is the preferred substrate for this enzyme although it reacts with a variety of other fatty acid ethanolamides (15,16) and fatty acid amides including oleamide, a putative sleep factor (17). A series of "transition-state" inhibitors (trifluoromethyl ketone, α-keto ester, and α-keto amide derivatives) were synthesized and tested *in vitro* as amidase inhibitors (18,19). The trifluoromethyl ketones (e.g., arachidonyltrifluoromethyl ketone) were found to inhibit anandamide hydrolysis at low micromolar concentrations. Phenylmethylsulfonyl fluoride (PMSF) a non-selective inhibitor of serine proteases was found to act as an irreversible inhibitor of this amidase and its inclusion in receptor binding assays, or in *in vitro* assays, increases the apparent potency of anandamide (11,15,16,20-27). However, the relatively low activity of the above inhibitors severely limits their effectiveness as biochemical and pharmacological tools or as therapeutic agents. In the present communication, a series of fatty acid sulfonyl fluorides were tested as inhibitors of anandamide metabolism and were found to be potent and specific.

MATERIALS AND METHODS

Synthesis of PMSF analogs. The fatty acid sulfonyl fluorides were synthesized from the respective alkyl bromides. Details of the syn-

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thesis will be described elsewhere (Lin and Makriyannis, unpublished data). Arachidonyl trifluoromethyl ketone was synthesized as described previously (18) or purchased from Biomol (Plymouth Meeting, PA).

Enzyme assay in vitro and in cell culture for inhibition of anandamide amidase. The *in vitro* assay of anandamide amidase was conducted using rat (Sprague Dawley) brain homogenate as recently described (28). Freshly dissected brain was immediately chilled on ice and homogenized in 5 volumes of ice cold 20 mM Tris, 1 mM EDTA, pH 7.6, with a polytron homogenizer. Aliquots of these brain homogenates were stored at −80°C. Incubations were performed in triplicate at 37°C in a water bath with shaking. Each incubation contained phosphate-saline (10 mM-160 mM), pH 7.6, 2.5 mg/ml defatted BSA (Calbiochem-Novabiochem Corp.), 120 μg rat brain homogenate protein, cold anandamide as indicated (Cayman Chemical Co.) containing 7.5×10^{-3} μCi of 120 mCi/mmol arachidonoyl ethanolamide [ethanolamine-1,2-^{14}C] (New England Nuclear, New Wilmington, Delaware) plus 3 μl of various concentrations of inhibitors dissolved in ethanol, in a final incubation volume of 200 μl. The blanks contained 1.5 mM phenylmethylsulfonyl fluoride (PMSF) freshly dissolved in ethanol, and the control tubes contained 3 μl ethanol without inhibitor. The reactions were terminated by addition of 2 volumes of chloroform:methanol (1:1). The radioactivity in the aqueous phase was determined by liquid scintillation counting.

Inhibition of anandamide amidase in cell culture was measured using intact neuroblastoma cells as described previously (18). Approximately 4×10^6 neuroblastoma cells (N18TG2) per 6cm dish were preincubated for 20 min in 1.5 ml media, consisting of Hams F12/DMEM (GIBCO, Grand, Island, NY) with penicillin (1000 U/ml), streptomycin (1mg/ml), gentamicin (1mg/ml), 10% bovine calf serum (HyClone, Logan, UT), plus 1 μl of ethanol containing various concentrations of the freshly prepared inhibitor solution. Control cells contained no inhibitor. Arachidonoyl [5, 6, 7, 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 one hour. At the end of the incubation, the cells were washed once with PBS and removed from the tissue culture plates after a brief incubation with 2 ml of a 0.05% trypsin in 0.53 mM EDTA solution at 37°C. The amidase catalyzed hydrolysis was terminated by the addition of two volumes of chloroform:methanol (1:1), and the aqueous samples dried under nitrogen gas after extraction from the organic phase. The extracts were redissolved in 50 μl of chloroform:methanol (1:1). Thin layer chromatography was performed on channeled silica gel coated plates, with a solvent system consisting of the organic layer of an ethyl acetate: hexane: acetic acid: water (100:50:20:100) mixture. 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.

Enzyme assay for inhibition of anandamide synthase. The reaction mixture for measurement of anandamide synthesis in the presence or absence of fatty acid sulfonyl fluoride inhibitors contained 200 μg/ml total protein from rat brain homogenate, 10 mM ethanolamine hydrochloride, 20 mM [^{14}C]-arachidonic acid (1 mCi/ml, 55 mCi/mmol), 20 mM Tris, pH 9.0, 1 mM EDTA in a total volume of 100 ml. The inhibitors were dissolved in ethanol which when added to the reaction mixture never exceeded 2% of the total volume. After incubation at 37°C for 60 minutes, the mixture was extracted with 250 ml of chloroform:methanol (2:1), mixed and centrifuged. Two hundred microliters of the organic phase were transferred to a clean tube and dried under nitrogen. The residue was redissolved in 20 ml of chloroform: methanol (2:1) and spotted on a TLC plate, along with standards. Anandamide quantification from the developed plate was performed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Receptor binding. Affinities of the amidase inhibitors for the cannabinoid receptor (CB1) were determined by a displacement assay using [^{3}H]CP-55,940 and rat forebrain membranes as previously described (5,18,24). Briefly, increasing concentrations of sulfonyl fluoride were incubated with 0.76nM [^{3}H]CP-55,940, in TME-0.1% BSA buffer (25mM Tris-HCl, 5mM $MgCl_2$, 1mM EDTA, pH 7.4, 0.1% essentially fatty acid free BSA) and 20 μg of rat forebrain microsomes, in a total volume of 200 μl. After 1 hour incubation at 30°C the protein bound [^{3}H]CP-55,940 was separated from the unbound by filtration and quantified by liquid scintillation counting. Non-specific binding was determined by 100nM cold CP-55,940 and was generally less than 15% of the total radioactivity bound. The concentration of sulfonyl fluoride required to displace 50% of specifically bound [^{3}H]CP-55,940 is reported as the IC_{50}. No attempt was made to determine whether the sulfonyl fluorides bound irreversibly to the receptor under the assay conditions.

RESULTS

AM 374, the palmitylsulfonyl fluoride analog of PMSF, arachidonyl trifluoromethyl ketone and PMSF were tested as inhibitors of anandamide hydrolysis in rat brain homogenate (see structures in Figure 1A). The IC_{50} values for palmitylsulfonyl fluoride, phenylmethylsulfonyl fluoride, and arachidonyl trifluoromethyl ketone are 13, 290 and 900 nM, respectively (Figure 2). AM374, with an IC_{50} value of 13 nM, is a very potent inhibitor. It is approximately twenty times more potent than PMSF (IC_{50}, 290 nM) and fifty times more potent than ATFMK (IC_{50}, 900 nM) in preventing the hydrolysis of anandamide in brain homogenate.

The inhibition of anandamide breakdown in cell culture by AM374 was compared to PMSF (Figure 3). When anandamide is incubated with neuroblastoma (N18TG2) cells, it is taken up by the cells and hydrolyzed to arachidonate, which is then converted to other lipids containing arachidonate so that little free arachidonic acid is found in the cell (Figure 3). However, in the presence of 9 nM palmitylsulfonyl fluoride there is approximately a fifty-fold increase of anandamide levels from 1.3% in the control cells to 70% in the experimental. For comparison, the amount of anandamide in the experimental cells increases to approximately a twenty-fold maximum, relative to the control cells, at 12 μM PMSF. Accordingly, AM374 is over a thousand-fold more effective than PMSF when tested in cell culture.

A series of saturated fatty acid sulfonyl fluoride analogs (Figure. 1B) with chain lengths varying from C12 to C20 were tested as inhibitors of anandamide hydrolysis. The analogs from C12 to C18 were equipotent with IC_{50} values around 5 nM while the C20 analog was at least ten-fold less potent than the others (Fig 1). Similar results were obtained with the reverse reaction (synthase) where it was found that the C20 was the least potent inhibitor in the series (Figure 1B).

AM374 and the other sulfonyl fluorides were tested for their ability to displace [^{3}H]CP-55,940 binding specifically to the cannabinoid receptor (CB1) in rat forebrain synaptosomes. Assuming competitive displacement of [^{3}H]CP55,940 by these sulfonyl fluorides the IC_{50} for AM374 was 520 nM as shown in Figure 4.

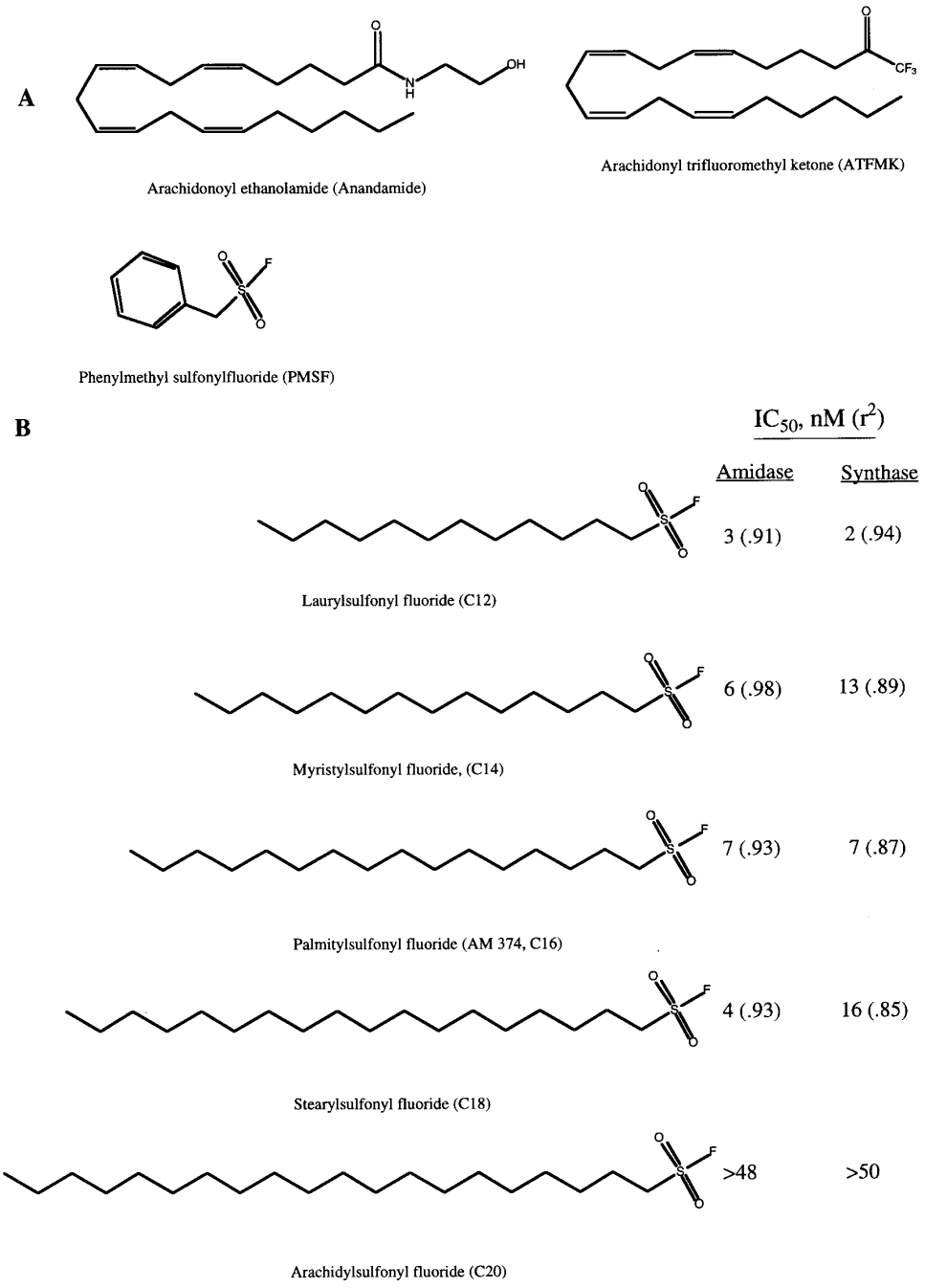

FIG. 1. Structure of anandamide, arachidonyltrifluoromethyl ketone, phenylmethylsulfonyl fluoride, and the fatty acid sulfonyl fluorides (C12, laurylsulfonyl fluoride; C14, myristyl sulfonyl fluoride; C16, palmitylsulfonyl fluoride; C18, stearylsulfonyl fluoride, and C20, arachidylsulfonyl fluoride). IC_{50} values for the series of saturated sulfonylfluorides (C12-C20) tested as inhibitors of anandamide amidase (30 μM anandamide substrate concentration) and anandamide synthase. The correlation coefficient (r^2) for the linear regression analysis was obtained using Sigma Plot (Jandel Scientific).

Arachidonyl trifluoromethyl ketone has an IC_{50} of 25 nM and anandamide (after the protein was pretreated with PMSF (24) yielded an IC_{50} value of 132.6 nM (Figure 4). The IC_{50} values for the series of PMSF fatty acid analogs tested for their ability to displace [^{3}H]CP-55,940 from CB1, are reported in Table 1. The affinity essentially decreased with increasing chain length, although the $C_{14}SO_2F$ analog displayed a slightly lower affinity than the $C_{16}SO_2F$. Thus $C_{12}SO_2F$ had the highest affinity with a IC_{50} of 18 nM. The IC_{50} values for the other analogs were $C_{14}SO_2F$ 1.39 μM, $C_{16}SO_2F$ 520 nM, $C_{18}SO_2F$ 18.5 μM, and $C_{20}SO_2F$ 78 μM.

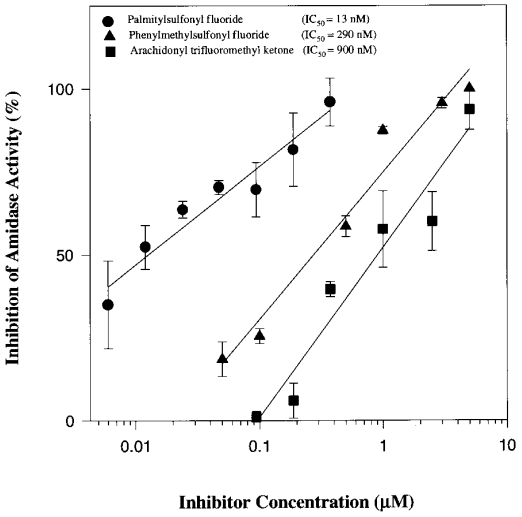

FIG. 2. Percentage inhibition of amidase activity by AM374 (palmitylsulfonyl fluoride), arachidonyltrifluoromethyl ketone, and phenylmethylsulfonyl fluoride as a function of inhibitor concentration as described in Experimental Methods. The anandamide substrate concentration was 100 μM. The data were analyzed by linear regression (Sigma Plot, Jandel Scientific). The IC_{50} values for the three inhibitors were determined by inspection of the graph. The percentage inhibition was calculated by [(rate of control − rate of experimental) × 100]/rate of control.

DISCUSSION

The rank order of these compounds as anandamide amidase inhibitors was found to be the fatty acid sulfonyl fluorides > PMSF > AFTMK. In cell culture,

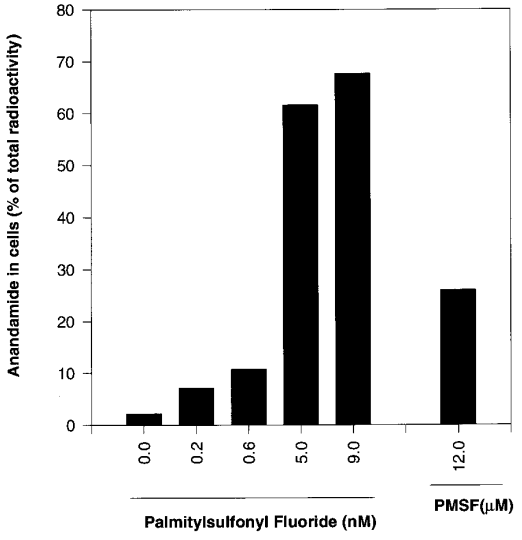

FIG. 3. The effect of AM374 (palmitylsulfonyl fluoride) and phenylmethylsulfonyl fluoride on anandamide levels in neuroblastoma cells (N18TG2). The amount of [^{3}H]-anandamide was determined in the controls (no AM374 or PMSF) and experimental cultures containing 4 × 10^6 cells as described in Experimental Methods.

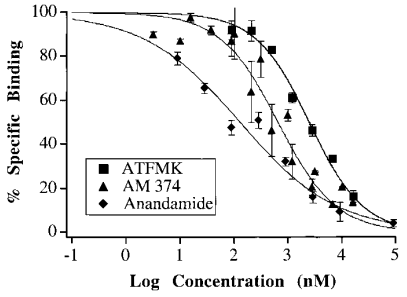

FIG. 4. Log dose-response curve for palmitylsulfonyl fluoride and arachidonyl trifluoromethyl ketone and arachidonoyl ethanolamide in competition with [^{3}H]CP-55,940 binding to CB1. The data shown are the IC_{50}s with the 95% confidence limits derived from three independent experiments conducted in duplicate. Assays were performed as described in Experimental Methods and the data were analyzed by non-linear least squares regression to a four parameter logistic equation.

AM374, in the nanomolar range, inhibited the amidase approximately one thousand-fold more effectively than PMSF and AFTMK (18,29). The potency of AM374 (22) and ATFMK are consistent with the proposed mechanism of inhibition as discussed below. ATFMK was designed as a transition-state inhibitor, capable of forming a long-lived tetrahedral intermediate on the path to the acyl enzyme intermediate characteristic of all ester and amide hydrolases. This tight-binding inhibitor is nonetheless a reversible inhibitor and is therefore released or hydrolyzed *in vitro*. In contrast, the alkylsulfonyl fluorides are active site directed electrophilic reagents and function by covalent modification of an active site serine to give an alkyl sulfonate ester. This modification is essentially irreversible; that is, hydrolysis to generate the active site serine hydroxyl is extremely slow relative to the time scale of this experiment. The fatty acid sulfonyl fluorides from C12 to C18 appear to be equipotent in preventing breakdown or synthesis of anandamide while the saturated C20 exhibited decreased activity for both the amidase and

TABLE 1

IC_{50} Values with the 95% Confidence Limits for the C12 to C20 Alkylsulfonyl Fluorides, Anandamide Phenylmethylsulfonyl Fluoride, and Arachidonyltrifluoromethyl Ketone Binding to a Rat Forebrain Synaptosomal Preparation (CB1) Obtained as Described in Experimental Methods

Compound	CB1 affinity (IC_{50})	95% confidence limits
$C_{12}SO_2F$	18.4 nM	(16–21.2 nM)
$C_{14}SO_2F$	1.39 μM	(1.15–1.19 μM)
$C_{16}SO_2F$	520 nM	(462–586 nM)
$C_{18}SO_2F$	18.5 μM	(14.8–22.0 μM)
$C_{20}SO_2F$	78 μM	(60.5–101.7 μM)
Anandamide	132.6 nM	(115.6–152.2 nM)
PMSF	>10 μM	
ATFMK	2.509 μM	(2.327–2.705 μM)

synthase. These findings strengthen the postulate that the amidase and the so-called synthase are the same enzyme (16). Interestingly, the C12 to C18 analogs were equipotent under our experimental conditions, an observation that may be attributed to the fact that the reaction is essentially irreversible and differences in their reaction rates are not detected over the relatively long incubation period. Irreversibility was experimentally demonstrated by the inability of high concentrations of anandamide substrate to prevent the inhibition of the amidase by palmitylsulfonyl fluoride (data not shown). The C20 analog appears to be less effective at inactivating the enzyme and CB1 presumably because of steric constraints. Interestingly, the enzyme appears less selective than the receptor in interacting with the C12 - C18 series of sulfonyl fluorides. Ideally, a selective amidase inhibitor would antagonize the enzyme at concentrations less than those needed to interact at the cannabinoid receptors. The C14-, C16- and C18-sulfonyl fluorides fulfill this criterion since they exhibit relatively low affinities for CB1 (IC_{50} = 520 nM to 18.5 μM) corresponding to low receptor occupancy at concentrations (approximately 10 nM) that inhibit amidase activity in cell free preparations and in intact cells. Although these inhibitors may interact with other enzymes or receptors, the only other known target for palmitylsulfonyl fluoride is the Escherichia *coli* outer-membrane phospholipase A (30,31). Further modifications on this basic structural motif may enhance the separation between amidase and CB1 binding and selectivity for the anandamide amidase (32). These compounds may be useful to probe the molecular properties of anandamide amidase and to study the physiological roles of anandamide. Furthermore, they may serve as prototypes for the development of novel analgesic agents.

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