

Cannabinoid Properties of Methylfluorophosphonate Analogs¹

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ABSTRACT

Methylarachidonylfluorophosphonate (MAFP) and related analogs have been shown to inhibit fatty acid amidohydrolase activity (FAAH), the enzyme responsible for hydrolysis of the endogenous cannabinoid ligand anandamide. To fully characterize this class of compounds, methylfluorophosphonate compounds with saturated alkyl chains ranging from C8 to C20 along with C20 unsaturated derivatives were synthesized and evaluated for their ability to interact with the CB1 receptor, inhibit FAAH, and produce in vivo pharmacological effects. These analogs demonstrated widely varying affinities for the CB1 receptor. Of the saturated compounds, C8:0 was incapable of displacing [³H]CP 55,940 binding, whereas C12:0 exhibited high affinity (2.5 nM). The C20:0 saturated analog had low affinity (900 nM), but the introduction of unsaturation into the C20 analogs restored receptor affinity. However, none of the

analogs were capable of fully displacing [³H]CP 55,940 binding. On the other hand, all compounds were able to completely inhibit FAAH enzyme activity, with the C20:0 analog being the least potent. The most potent FAAH inhibitor was the short-chained saturated C12:0, whereas the other analogs were 15- to 30-fold less potent. In vivo, the C8:0 and C12:0 analogs were highly potent and fully efficacious in producing tetrahydrocannabinol (THC)-like effects, whereas the other analogs were either inactive or acted as partial agonists. None was capable of attenuating the agonist effects of THC. Conversely, the C20:0 analog potentiated the effects of anandamide but not those of 2-arachidonoyl-glycerol and THC. The high in vivo potency of the novel short-chain saturated MAFP derivatives (C8:0 and C12:0) underscores the complexity of manipulating the endogenous cannabinoid system.

Discovery of the endogenous cannabinoid anandamide provided crucial evidence for the existence of a naturally occurring cannabinoid system (Devane et al., 1992). It is well known that anandamide produces most of the same pharmacological effects as Δ^9 -tetrahydrocannabinol (Δ^9 -THC), including sedation, hypothermia, analgesia, and catalepsy in mice (Fride and Mechoulam, 1993; Smith et al., 1994), drug discrimination in rats and monkeys (Wiley et al., 1995, 1997; Jarbe et al., 1998), and overt behavior in dogs (Lichtman et al., 1998). Some differences have also been reported between anandamide and Δ^9 -THC. Clearly, anandamide has a much shorter duration of action and is much less potent than Δ^9 -THC (Smith et al., 1994). These characteristics can be explained in part by the rapid metabolism of anandamide and its brief presence in brain after the i.v. administration in rodents (Willoughby et al., 1997).

As for other differences between anandamide and Δ^9 -THC, it was shown that in contrast to Δ^9 -THC, the antinociceptive effects of anandamide could not be altered by modulators of cAMP and by κ -opioid receptor agonists and antagonists (Welch et al., 1995). Studies from our laboratory have failed to demonstrate antagonism of the effects of anandamide in mice with the CB1-selective antagonist SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide], a compound that completely blocks the effects of Δ^9 -THC (Compton and Martin, 1997). Moreover, there have been two reports of low doses of anandamide attenuating the effects of Δ^9 -THC (Fride et al., 1995; Welch et al., 1995). Closer examination of the effects in the dog behavioral model revealed qualitative differences between anandamide and Δ^9 -THC (Lichtman et al., 1998). Although it is reasonable to speculate that the pharmacological effects produced by the exogenous administration of cannabinoids reflect the physiological role of endogenous cannabinoids, these pharmacological differences underscore the necessity of caution in making such an extrapolation. It may well be that the exogenous administration of cannabinoids

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ABBREVIATIONS: THC, tetrahydrocannabinol; MPE, maximum possible effect; MAFP, methylarachidonylfluorophosphonate; PMSF, phenylmethylsulfonyl fluoride; FAAH, fatty acid amidohydrolase activity; 2-Ara-Gl, 2-arachidonoyl-glycerol; CL, confidence limits; i.t., intrathecal; DMSO, dimethyl sulfoxide.

elicits an exaggerated response, such as the behavioral "high", which is atypical of the endogenous system.

Administration of the CB1 receptor antagonist SR 141716A provides another approach to manipulation of the endogenous system. It produces no behavioral effects in rodents at low to moderate doses (Rinaldi-Carmona et al., 1994; Compton et al., 1996), whereas higher doses produced motor stimulation that could be due to either a noncannabinoid effect of SR 141716A or blockade of endogenous anandamide (Compton et al., 1996). It also improved memory (Terranova et al., 1996) and attenuated pain threshold at an extremely low dose (Richardson et al., 1998), effects that could be due to manipulation of the endogenous anandamide or to inverse agonist effects.

Manipulation of the synthesis and metabolism of anandamide represents still yet another approach to exploring the endogenous cannabinoid system. Metabolic inhibitors of anandamide include phenylmethylsulfonyl fluoride (PMSF; Deutsch and Chin, 1993; Childers et al., 1994), methylarachidonylfluorophosphonate (MAFP; De Petrocellis et al., 1997; Deutsch et al., 1997b), arachidonyl serotonin (Bisogno et al., 1998), and fatty acid sulfonyl fluorides (Deutsch et al., 1997a). PMSF has been shown to potentiate the effects of anandamide as well as produce some anandamide-like effects at high doses (Compton and Martin, 1997). Unfortunately, PMSF is a nonselective enzyme inhibitor, and no efforts were made to measure endogenous anandamide levels. The pharmacological profile of the other metabolic inhibitors has not been evaluated thoroughly.

MAFP was initially designed and developed as the active-site directed inactivator of the calcium-sensitive and arachidonyl-selective cytosolic phospholipase A₂ (Huang et al., 1966; Street et al., 1993). It has been shown to be a highly potent and selective inhibitor of fatty acid amidohydrolase activity (FAAH), the major enzyme responsible for anandamide degradation (De Petrocellis et al., 1997; Deutsch et al.,

1997b). Based on studies with related reagents (disopropylfluorophosphonate and ethoxyoleoyl fluorophosphonate) and site-directed mutagenesis studies, the mechanism of inhibition was found to involve the stable phosphorylation of an active-site serine of FAAH (Hillard et al., 1995; Omeir et al., 1999; Patricelli et al., 1999).

MAFP bound irreversibly to the cannabinoid (CB1) receptor and prevented the subsequent binding of CP 55,940 (Deutsch et al., 1997b). In another study, it was found that MAFP acted as an irreversible cannabinoid receptor antagonist in the electrically evoked isometric contractions of the myenteric plexus-longitudinal muscle preparation of guinea pig small intestine (Fernando and Pertwee, 1997). Furthermore, in cardiac and invertebrate neural tissue, it enhanced anandamide-stimulated nitric oxide release (Bilfinger et al., 1998; Stefano et al., 1998). The goal of this study was to explore both in vivo and in vitro pharmacological actions of structural analogs of MAFP. Furthermore, efforts were made to determine whether the administration of MAFP analogs could elevate anandamide and 2-arachidonoyl-glycerol (2-Ara-Gl) levels in brain and spinal cord.

Materials and Methods

ICR male mice (Harlan Laboratories, Indianapolis, IN) weighing 24 to 26 g were used in all experiments. Mice were maintained on a 14:10-h light/dark cycle with free access to food and water. Δ^9 -THC and SR 141716A were obtained from the National Institute on Drug Abuse (Bethesda, MD). Anandamide and 2-Ara-Gl were synthesized in our laboratory, and MAFP was obtained from Cayman Chemicals (Ann Arbor, MI).

Synthesis. The various methylfluorophosphonate analogs (Fig. 1) were prepared using a three-step synthetic sequence. Dimethyl phosphite was alkylated by the appropriate iodo alkane derivative in the presence of sodium hydride/*N,N*-dimethylformamide/tetrahydrofuran to give the corresponding dimethyl alkyl phosphonate (84–94% yield). Treatment of the phosphonate with sodium iodide in acetone

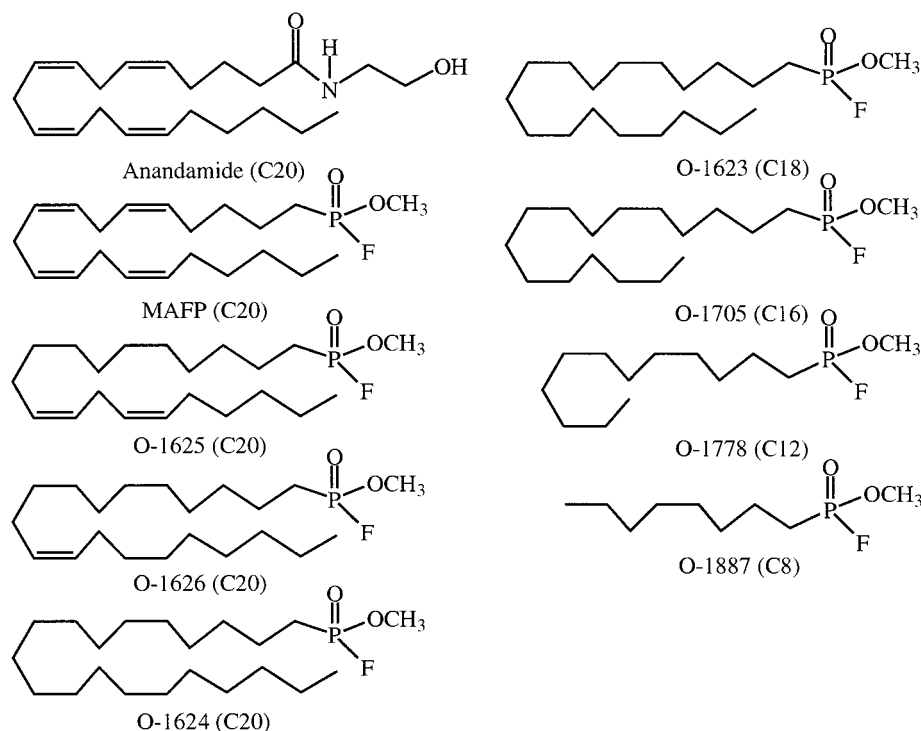


Fig. 1. Structures of methylfluorophosphonate analogs.

furnished the corresponding methyl alkyl sodium phosphonate (70–75% yield), which was then allowed to react with (diethylamino)sulfur trifluoride to give the final methylfluorophosphonate derivative (60–80% yield). The experimental procedure for the synthesis of methyl octadecyl fluorophosphonate (O-1623) is described below as a "general procedure" for all the analogs. The 1-iodo-(*Z,Z*)-11,14-eicosadiene and 1-iodo-(*Z*)-11-eicosene used in the preparation of O-1625 and O-1626 were synthesized from their respective alcohols (commercially available) using standard methodology by conversion to their mesylates (mesyl chloride/triethylamine/ CH_2Cl_2) followed by treatment with $\text{NaI}/\text{CH}_3\text{CN}/\text{reflux}$ (Ng et al., 1999). The iodo alkanes used in the synthesis of other analogs were all purchased from Aldrich Chemical Co. ^1H NMR spectra were recorded on a JEOL Eclipse 300 spectrophotometer using CDCl_3 as the solvent with tetramethylsilane as an internal standard. Flash chromatography was carried out on EM Science (Gibbstown, NJ) silica gel 60. Elemental analyses were performed by Atlantic Microlab, Inc. (Atlanta, GA).

Dimethyl Octadecyl Phosphonate. To a stirred suspension of sodium hydride (171 mg, 7.14 mmol) in DMF (9 ml) cooled to 0°C we added dropwise dimethyl phosphite (0.75 ml, 7.14 mmol). On addition, the mixture was warmed to room temperature and stirred for 1 h. A solution of 1-iodooctadecane (1.36 g, 3.59 mmol) in DMF/THF (1/1 8 ml) was then added dropwise, and the reaction mixture was stirred for 1 h. It was then quenched with water and extracted with ethyl acetate. The organic layer was dried over MgSO_4 and evaporated under vacuum to yield dimethyl octadecyl phosphonate as a white solid (1.1 g, 84%): ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.40 (m, 30 H), 1.51 to 1.79 (m, 4 H), 3.72 (d, 6 H, $J = 10.7$ Hz).

Methyl Octadecyl Sodium Phosphonate. A stirred solution of methyl octadecyl phosphonate (650 mg, 1.8 mmol) and sodium iodide (540 mg, 3.6 mmol) in acetone (7 ml) was refluxed for 17 h. It was cooled to room temperature, and the precipitated salt was filtered and washed with cold acetone. The salt was obtained as a white solid (486 mg, 73%) and used without further purification.

Methyl Octadecyl Fluorophosphonate (O-1623). To a stirred suspension of methyl octadecyl sodium phosphonate (200 mg, 0.54 mmol) in CH_2Cl_2 (9 ml) we added dropwise at room temperature (diethylamino)sulfur trifluoride (0.15 ml, 1.08 mmol). The reaction mixture was stirred for 1 h, quenched with water, and extracted with CH_2Cl_2 . The organic layer was dried over MgSO_4 , plugged through a pad of celite, and evaporated under reduced pressure. The residue was purified by flash chromatography (eluting with hexane/EtOAc 2:1) to give methyl octadecyl fluorophosphonate (141 mg, 75%) as a yellowish solid: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 30 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz). Analysis ($\text{C}_{19}\text{H}_{40}\text{O}_2\text{FP}$). Calculated: C 65.11, H 11.50. Found: C 65.07, H 11.54.

Methyl Octyl Fluorophosphonate (O-1887). Prepared in the same manner as methyl octadecyl fluorophosphonate in 84% yield: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 10 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz). Analysis ($\text{C}_9\text{H}_{20}\text{O}_2\text{FP}$). Calculated: C 51.42, H 9.59. Found: C 51.19, H 9.57.

Methyl Dodecyl Fluorophosphonate (O-1778). Prepared in the same manner as methyl octadecyl fluorophosphonate in 84% yield: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 18 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz). Analysis ($\text{C}_{13}\text{H}_{28}\text{O}_2\text{FP} \cdot 0.4 \text{ H}_2\text{O}$). Calculated: C 57.08, H 10.61. Found: C 57.21, H 10.34.

Methyl Hexadecyl Fluorophosphonate (O-1705). Prepared in the same manner as methyl octadecyl fluorophosphonate in 60% yield: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 26 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz). Analysis ($\text{C}_{17}\text{H}_{36}\text{O}_2\text{FP}$). Calculated: C 63.22, H 11.25. Found: C 63.42, H 11.18.

Methyl Eicosanyl Fluorophosphonate (O-1624). Prepared in the same manner as dimethyl octadecyl phosphonate in 80% yield:

^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 34 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz). Analysis ($\text{C}_{21}\text{H}_{44}\text{O}_2\text{FP}$). Calculated: C 66.63, H 11.72. Found: C 66.73, H 11.83.

Methyl-(*Z,Z*)-11,14-eicosadienyl Fluorophosphonate (O-1625). Prepared in the same manner as methyl octadecyl fluorophosphonate in 60% yield: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.45 (m, 20 H), 1.58 to 1.72 (m, 2 H), 1.82 to 1.96 (m, 2 H), 2.04 (q, 4 H, $J = 6.6$ Hz), 2.76 (t, 2 H, $J = 5.8$ Hz), 3.85 (dd, 3 H, $J = 0.8, 11.3$ Hz), 5.28 to 5.43 (m, 4 H). Analysis ($\text{C}_{21}\text{H}_{40}\text{O}_2\text{FP} \cdot 0.5 \text{ H}_2\text{O}$). Calculated: C 65.76, H 10.77. Found: C 65.80, H 11.52.

Methyl-(*Z*)-11-eicosenyl Fluorophosphonate (O-1626). Prepared in the same manner as methyl octadecyl fluorophosphonate in 60%: ^1H NMR (CDCl_3) δ 0.87 (t, 3 H, $J = 6.6$ Hz), 1.20 to 1.40 (m, 26 H), 1.51 to 1.79 (m, 4 H), 2.00 (q, 4 H, $J = 6.6$ Hz), 3.85 (dd, 3 H, $J = 0.8, 12$ Hz), 5.34 (t, 2 H, $J = 5.9$ Hz). Analysis ($\text{C}_{21}\text{H}_{42}\text{O}_2\text{FP} \cdot 0.2 \text{ CHCl}_3$). Calculated: C 63.59, H 10.62. Found: C 63.96, H 10.71.

Pharmacological Assays. Cannabinoids were dissolved in a 1:1:18 mixture of ethanol, emulphor, and saline for i.v. administration. Mice received the analog by tail-vein injection and were evaluated for their ability to produce hypomotility, hypothermia, and antinociception. These pharmacological measures were determined in the same mouse as described earlier (Compton et al., 1993). To measure locomotor activity, mice were placed into individual photocell activity chambers (11×6.5 inches) 5 min after injection. Spontaneous activity was measured during the next 10-min period, and the number of interruptions of 16 photocell beams per chamber was recorded. Antinociception was determined using the tail-flick reaction time to a heat stimulus. Before vehicle or drug administration, the baseline latency period (2–3 s) was determined. At 15 min after the injection, tail-flick latency was reassessed, and the differences in control and test latencies were calculated. A 10-s maximum latency was used. Antinociception was expressed as percentage maximum possible effect (MPE) as described later. Regarding hypothermia, rectal temperature was determined before vehicle or drug administration with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and a thermistor probe (model YSI 400; Markson, Inc., Hillsboro, OR) inserted at a depth of 2 mm. At 20 min after the injection, rectal temperature was measured again, and the difference between preinjection and postinjection values was calculated. These pharmacological experiments were conducted between 8:00 AM and 11:00 PM.

For intrathecal (i.t.) administration, the methylfluorophosphonate derivatives were dissolved in 10% ethanol in dimethyl sulfoxide (DMSO). MAFP was prepared in DMSO alone. For i.t. administration, a 5- μl solution was injected into the spinal column between L5 and L6 of unanesthetized mice with a 30-gauge, 0.5-inch needle (Hylden and Wilcox, 1980). The animals were tested for tail-flick response 5 min after the injection.

The i.c.v. injections were performed in mice that were lightly anesthetized with ether. An incision was made in the scalp such that the bregma was exposed. Injections were performed using a 26-gauge needle with a sleeve of PE 20 tubing to control the depth of the injection. An injection (5 μl) was made 2 mm rostral and 2 mm caudal to the bregma at a depth of 2 mm. The vehicle for these injections consisted of 10% ethanol in DMSO. The animals were tested 10 min after the injection.

Receptor Binding. [^3H]CP-55,940 ($K_D = 690$ nM) binding to P_2 membranes was conducted as described elsewhere (Compton et al., 1993), except whole brain (rather than cortex only) was used. Displacement curves were generated by incubating drugs with 1 nM [^3H]CP-55,940. The assays were performed in triplicate, and the results represent the combined data from three individual experiments. The K_i values were determined from displacement data using EBDA (Equilibrium Binding Data Analysis; BIOSOFT, Milltown, NJ).

FAAH Activity. The in vitro assay for FAAH activity was conducted using rat brain homogenate essentially as described earlier

(Omeir et al., 1995). Frozen dissected brain (Pel-Freeze, Rogers, AR) was defrosted in 5 volumes of ice-cold Tris-EDTA, pH 7.6, and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). 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 $10\ \mu\text{l}$ of $50\ \text{mg/ml}$ defatted BSA in H_2O (Sigma Chemical Co., St. Louis, MO), $10\ \mu\text{l}$ of $20\ \text{mg/ml}$ rat brain homogenate protein, $30\ \mu\text{M}$ anandamide (Cayman Chemical Co., Ann Arbor, MI) plus $0.01\ \text{mCi}$ of $120\ \text{mCi/mmol}$ arachidonylethanolamide (ethanolamine-1,2- ^{14}C ; New England Nuclear, Boston, MA), and $2\ \mu\text{l}$ of various concentrations of inhibitors dissolved in ethyl alcohol, in a final $200\text{-}\mu\text{l}$ incubation volume of $0.1\ \text{M}$ Tris-HCl (pH 9.0). The control tubes contained $2\ \mu\text{l}$ ethyl alcohol without inhibitor, and the blanks contained everything except the rat brain. The reactions were terminated by the addition of 2 volumes of chloroform/methanol (1:1). The radioactivity in the aqueous phase was measured by liquid scintillation counting. In a separate set of experiments, FAAH activity was measured in ICR mouse spinal cord after i.t. treatment with O-1623 and O-1624.

Anandamide and 2-AG Measurement in Tissues by Liquid Chromatography-Mass Spectrometry. At 10 min after i.t. treatment with either O-1623 or O-1624 ($200\ \mu\text{g}/\text{mouse}$), ICR mouse spinal cords (one for each separate determination) were dissected and immediately frozen in liquid nitrogen, and so were the striata of ICR mice (two for each separate determination) after i.v. treatment with $30\ \text{mg/kg}$ O-1624. Lipid extraction and prepurification was performed as described previously in the presence of $1\ \text{nmol}$ of $^2\text{H}_8$ -anandamide and $2\ \text{nmol}$ of $^2\text{H}_8$ -2-AG (Bisogno et al., 1999). Purified lipids were analyzed by HPLC-chemical ionization (APCI)-mass spectrometry. The mass spectrometer was equipped with a Z-Spray APCI source operating in the (+) APCI mode (source temperature 120°C , probe temperature 110°C). N_2 was used as both drying and nebulizing gas (flow and probe position were adjusted daily for optimum sensitivity). The chromatograph was equipped with a Supelco Supelcosil LC-18 column ($15\ \text{cm} \times 4.6\ \text{mm}$, $5\ \mu\text{m}$ particle size). The mobile phase was $\text{MeOH}/\text{H}_2\text{O}/\text{acetic acid}$ (85:15:0.2 by volume) at a flow rate of $1\ \text{ml}/\text{min}$. Both the column and the samples were maintained at 25°C . Retention of peaks of a selected m/z value was used to identify anandamide and 2-AG in their protonated ($M + 1$) form. Quantification of the two compounds was obtained by the isotope dilution method. After each injection, a $5\ \text{loop}$ volume injection syringe purge was performed.

Data Analysis. For the production of hypomotility and hypothermia, the data were expressed as percentage of control activity and change in temperature ($^{\circ}\text{C}$), respectively. Antinociception was calculated as $\% \text{MPE} = [(\text{test latency} - \text{control latency}) / (\text{10 s} - \text{control latency})] \times 100$. At least six animals were treated with each dose so dose-response relationships could be determined for each analog. ED_{50} values were determined from least-squares unweighted linear regression analysis of the log dose-response plots. Maximal effects for all compounds combined on spontaneous activity, temperature, antinociception, and catalepsy were, respectively, 90% inhibition, -5°C , and 100% MPE. Thus, the ED_{50} values indicate response levels of 45% inhibition, -2.5°C , and 50% MPE. Statistical analysis was carried out using ANOVA and Bonferroni/Dunn post hoc analysis.

Results

Synthesis of Methylfluorophosphonate Analogs. Several analogs were prepared in which the alkyl moiety of the fluorophosphonate analog was varied as shown in Fig. 1. Initially, derivatives of MAFP were prepared by reducing the degree of unsaturation to either two double bonds to form methyl-(Z,Z)-11,14-eicosadienyl fluorophosphonate (O-1625), one double bond to form methyl-(Z)-11-eicosenyl fluorophosphonate (O-1626), or no unsaturation to form methyl eicosa-

nyl fluorophosphonate (O-1624). Then, three additional unsaturated analogs were prepared in which the alkyl group was reduced by 2, 4, 8, and 12 carbon atoms to form methyl octadecyl fluorophosphonate (O-1623), methyl hexadecyl fluorophosphonate (O-1704), methyl dodecyl fluorophosphonate (O-1778), and methyl octyl fluorophosphonate (O-1887), respectively.

Competition for [^3H]CP 55,940 Binding. The ability of these analogs to compete with [^3H]CP 55,940 binding is depicted in Fig. 2, top. MAFP was not analyzed because Deutsch et al. (1997b) previously reported that it displaced approximately 90% of [^3H]CP 55,940 binding with a IC_{50} value of $20\ \text{nM}$. All of the analogs, with the exception of O-1624, effectively bound to the receptor at relatively low concentrations. However, none of these analogs was able to completely displace [^3H]CP 55,940 binding, even at high concentrations. O-1625, the C20 analog with two double bonds, displaced approximately 70% of [^3H]CP 55940 binding at $10\ \text{nM}$ but was unable to displace a greater quantity at concentrations up to $300\ \text{nM}$ (Table 1). An estimated K_i value of $2.9 \pm 0.3\ \text{nM}$ could be obtained. The C20 analog with one double bond, O-1626, was somewhat less effective in competing for binding but presented a similar binding profile with 70% displacement at $1\ \mu\text{M}$. Again, analysis of the displacement curve yielded an estimated K_i value of $17 \pm 2.4\ \text{nM}$. Complete saturation of the C20 analog resulted in O-1624, a compound with dramatically reduced receptor affinity. The K_i value was determined to be $795 \pm 72\ \text{nM}$. Shortening the saturated chain resulted in a binding profile comparable to that of O-1625 and O-1626 (Table 1). Maximal displacement by O-1623 (C18) was less than 70%, and a K_i value was estimated to be $9.7 \pm 1.5\ \text{nM}$. O-1705 (C16) produced only 58% displacement at the highest concentration, and a K_i value could not even be estimated. The binding profile of the C12 saturated analog O-1778 was identical to that produced

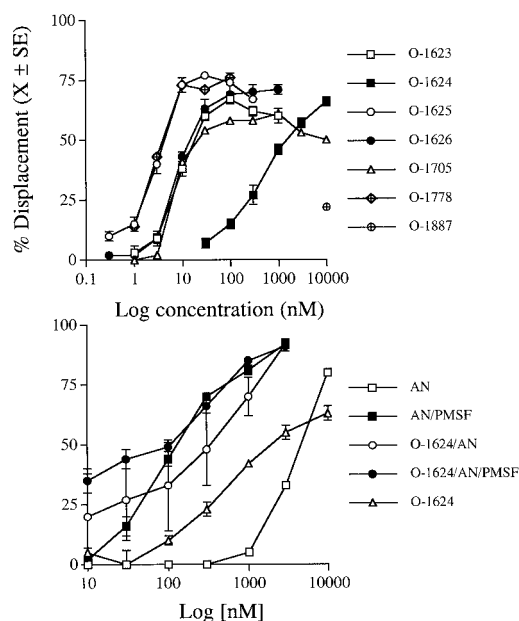


Fig. 2. Displacement of [^3H]CP 55940 binding to rat brain membranes. Top, competition of the methylfluorophosphonate analogs for [^3H]CP 55940 binding. Bottom, influence of O-1624 ($1\ \mu\text{M}$) and PMSF ($50\ \mu\text{M}$) on the ability of anandamide to displace [^3H]CP 55940 binding. The results in both experiments are presented as means $\pm$ S.E. of at least three separate displacements.

TABLE 1

Binding characteristics, FAAH inhibition, and pharmacological activity after i.v., i.t., and i.c.v. administration of the methylfluorophosphonate analogs

Analog	N ^a	KD ^b	FAAH ^c	S.A. ^d	R.T. ^d	% MPE ^d	% MPE ^e	% MPE ^f
O-1625	20:2 n-6	2.9 ± 0.3 (75)	55 ± 8	14 (5–36) (70)	13 (6–26) (–4.4)	N.D. ^g (1)	111 (28–445) (73)	190 (40–890) (67)
O-1626	20:1 n-6	17 ± 2.4 (70)	103 ± 7	N.D. ^g (38)	53 (38–75) (–2.5)	N.D. ^g (2)	68 (17–268) (72)	75 (28–204) (90)
O-1624	20:0	795 ± 72 (60)	137 ± 22	26 (11–64) (54)	N.D. ^g (–1.3)	N.D. ^g (7)	N.D. ^g (17)	N.D. ^g (15)
O-1623	18:0	9.7 ± 1.5 (58)	48 ± 13	N.D. ^g (48)	41 (13–120) (–3.0)	N.D. ^g (10)	N.D. ^g (56)	N.D. ^g (30)
O-1705	16:0	N.D. (58)	78 ± 12	18 (11–30) (60)	10 (8–12) (–6.2)	15 (9–25) (68)	N.D. ^g (45)	N.D. ^g (45)
O-1778	12:0	2.5 ± 0.3 (75)	3.0 ± 0.2	0.6 (0.2–1.5) (90)	3.2 (2.6–4.1) (–6.7)	2.2 (1.5–3.3) (100)	2.2 (1.5–3.3) (100)	20 (9–43) (84)
O-1887	8:0	>10,000	15 ± 3	0.6 (0.5–0.7) (90)	1.0 (0.8–1.2) (–3.8)	0.5 (0.5–0.6) (100)	7.7 (2.9–21) (91)	11 (4.8–27) (93)

^a Carbon length of the alkyl moiety: number of unsaturated bonds; n designates position of double bond from terminal carbon.

^b Results (nM) presented as means ± S.E. with maximal displacement provided below in parentheses.

^c Expressed as IC₅₀ values in nanomolar.

^d Spontaneous activity (S.A.), rectal temperature (R.T.), and tail-flick response (% MPE) are expressed as ED₅₀ values (mg/kg) followed by confidence limits in parentheses after i.v. administration. Below the ED₅₀ values are the maximal effects produced.

^e Antinociceptive activity in the tail-flick procedure after i.t. administration. ED₅₀ (CL) expressed as micrograms per mouse with the maximal effect at doses up to 400 µg/mouse in parentheses.

^f Antinociceptive activity in the tail-flick procedure after i.c.v. administration. ED₅₀ (CL) expressed as micrograms per mouse.

^g N.D., could not be determined.

by O-1625 with regard to both maximal displacement (about 75%) and high receptor affinity ($K_i = 2.54 \pm 0.26$ nM). However, shortening the chain to only eight carbon atoms (O-1887) completely eliminated CB1 receptor affinity.

To compare the ability of PMSF and O-1624 to enhance anandamide binding to the cannabinoid receptor through FAAH inhibition, anandamide competition studies were conducted in the presence of O-1624, PMSF, or the combination of both compounds. The results in Fig. 2 (bottom) demonstrate that O-1624 is approximately as effective as PMSF in enhancing anandamide binding and that anandamide in the presence of the combination produced relatively the same amount of displacement.

FAAH Inhibition. All of the fluorophosphonates were highly potent inhibitors of FAAH in rat brain homogenates with IC₅₀ values in the nanomolar range (Table 1). Interestingly, the least potent, O-1624, could not yield greater than 70 to 80% inhibition even at 500 nM. The C12:0 compound, O-1778, was an extremely effective inhibitor. It yielded 50% inhibition of FAAH at 3 nM. Only two other compounds have been reported to match this ability to inhibit FAAH under comparable assay conditions: MAFP and laurylsulfonyl fluoride (De Petrocellis et al., 1997; Deutsch et al., 1997a,b; Di Marzo and Deutsch, 1998). Under the conditions of the assay with MAFP, it was estimated that approximately 10% of the cannabinoid receptors would be occupied at concentrations of MAFP that inhibit 90% of the enzyme. For laurylsulfonyl fluoride, the ratio of the IC₅₀ value for inhibition of FAAH versus competition for radioligand binding at the receptor was approximately 7-fold, whereas other sulfonyl fluoride analogs showed higher selectivity for FAAH over the CB1 receptor (Deutsch et al., 1997a).

Pharmacological Activity after i.v. Administration.

The analogs were administered at doses as high as 30 mg/kg i.v. to mice and assessed for reductions in spontaneous activity, antinociceptive activity, and hypothermia. The results in Fig. 3A and Table 1 show that the saturated C8:0 and C12:0 analogs O-1887 and O-1778 were highly potent and highly efficacious in depressing spontaneous activity. Its ED₅₀ value

[95% confidence limits (CL)] was 0.60 (0.5–0.7) and 0.60 (0.24–1.55) mg/kg, respectively. As for the other compounds, they depressed spontaneous activity at a dose of 30 mg/kg, but none produced maximal effects. Variable effects were obtained with lower doses. Although the maximal effects of O-1624 were only 54%, an ED₅₀ value (CL) of 26 (11–64) mg/kg could be calculated (Table 1). Likewise, comparable ED₅₀ value could be calculated for O-1625 and O-1705. All of the compounds, with the exception of O-1624, produced a dose-related reduction in rectal temperature (Fig. 3B). Considering the maximal 6°C drop produced by Δ^9 -THC, the only agonists that were as equally efficacious as Δ^9 -THC were O-1705 and O-1778 with ED₅₀ value of 10 and 3.2 mg/kg, respectively (Table 1). O-1625 exhibited similar potency, whereas O-1623 and O-1626 were considerably less potent and less efficacious. As for antinociception after i.v. administration, O-1887 and O-1778 were the only analogs that produced dose-related antinociception with maximal efficacy (Table 1). O-1705 was less potent but was capable of producing approximately 70% MPE at 30 mg/kg. None of the other analogs was active even at doses as high as 30 mg/kg (Fig. 3C).

Given the fact that several of the analogs were capable of competing for [³H]CP 55,940 binding at very low concentrations yet produced modest pharmacological effects, a series of studies were carried out to determine whether they would either enhance or block the effects of Δ^9 -THC. The results in Table 2 reveal that an i.v. dose of 10 mg/kg O-1623, O-1624, O-1625, and O-1626 produced some depression of motor activity, no antinociception, and very modest hypothermia. When these analogs were given before Δ^9 -THC, they failed to either potentiate or attenuate the actions of the latter.

The ability of O-1705 to produce hypoactivity, antinociception, and hypothermia suggested an interaction with the CB1 receptor despite its low receptor affinity. Therefore, SR 141716A was evaluated to determine whether it would antagonize O-1705. O-1705 (30 mg/kg) administered i.v. produced a 30% reduction in spontaneous activity, $69 \pm 16\%$ MPE, and a decrease of 4.7°C in body temperature. Pretreat-

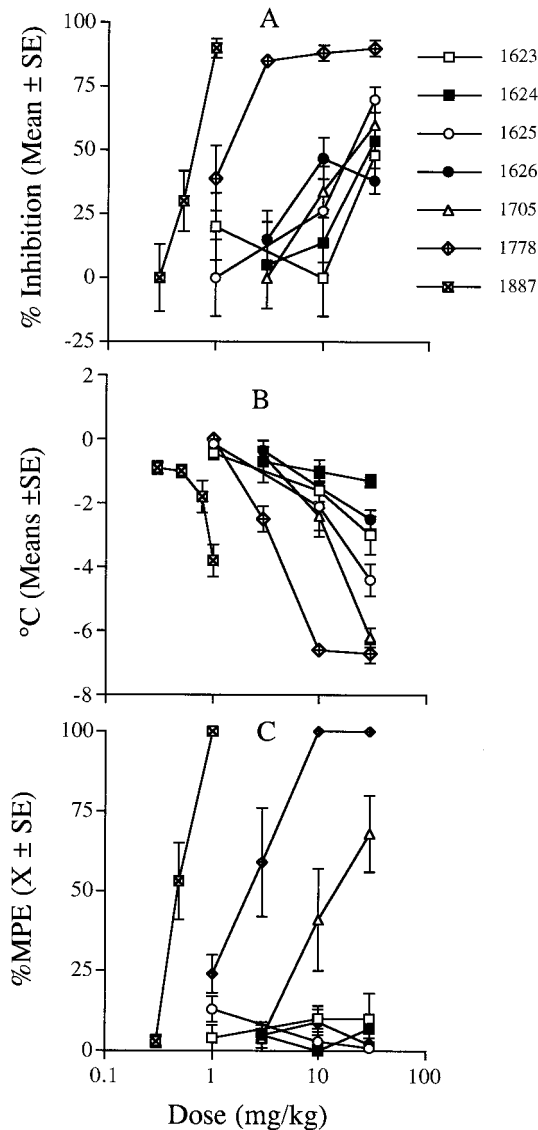


Fig. 3. The pharmacological effects of the methylfluorophosphonate analogs after i.v. administration in mice showing inhibition of spontaneous activity (A), depression of rectal temperature (B), and production of antinociception (C), respectively. The means ± S.E. represent at least six mice per group.

ment with SR 141716A (3 mg/kg i.v.) did not have a statistically significant influence on the effects of O-1705 on body temperature and spontaneous activity but did produce a statistically significant antagonism of its antinociceptive effect ($24 \pm 17\%$).

Antinociceptive Activity after i.t. Administration. The activity of MAFP and the methylfluorophosphonate derivatives in the tail-flick procedure after i.t. administration is presented in Fig. 4. MAFP produced a dose-responsive antinociceptive effect with an ED_{50} value (CL) of 187 (156–224) $\mu\text{g}/\text{mouse}$. O-1625 and O-1626 also produced a dose-responsive tail-flick response with ED_{50} values of 111 (28–445) and 68 (17–268) $\mu\text{g}/\text{mouse}$. However, doses as high as 400 $\mu\text{g}/\text{mouse}$ failed to produce maximal antinociception. O-1623 and O-1705 were only partially active, and O-1624 was without effect. On the other hand, O-1778 and O-1887 were fully efficacious and highly potent with ED_{50} values (CL) of 2.2

TABLE 2

Failure of methylfluorophosphonate derivatives to antagonize the pharmacological effects of $\Delta^9\text{-THC}$

Mice received a 10 mg/kg i.v. injection of the methylfluorophosphonate analog 10 min before a second i.v. injection of $\Delta^9\text{-THC}$ (3 mg/kg), and the animals were evaluated for spontaneous activity (percentage of vehicle), tail-flick response (% MPE), and rectal temperature ($^{\circ}\text{C}$) at 5 to 15, 20, and 30 min after the last injection, respectively. The results are expressed as means ± S.E. for six mice per group.

Pretreatment	Treatment	Spontaneous Activity	Antinociception	Rectal Temperature
Vehicle	Vehicle		5 ± 2	-0.4 ± 0.1
Vehicle	$\Delta^9\text{-THC}$	83 ± 5	81 ± 12	-5.1 ± 0.4
O-1623	Vehicle	62 ± 11	19 ± 3	-2.4 ± 0.2
O-1623	$\Delta^9\text{-THC}$	77 ± 6	92 ± 8	-5.5 ± 0.3
O-1624	Vehicle	24 ± 16	5 ± 2	-1.4 ± 0.2
O-1624	$\Delta^9\text{-THC}$	84 ± 2	99 ± 1	-4.4 ± 0.4
O-1625	Vehicle	46 ± 7	14 ± 7	-1.7 ± 0.2
O-1625	$\Delta^9\text{-THC}$	90 ± 2	98 ± 2	-5.2 ± 0.3
O-1626	Vehicle	66 ± 6	5 ± 4	-1.7 ± 0.3
O-1626	$\Delta^9\text{-THC}$	90 ± 4	100	-4.8 ± 0.2

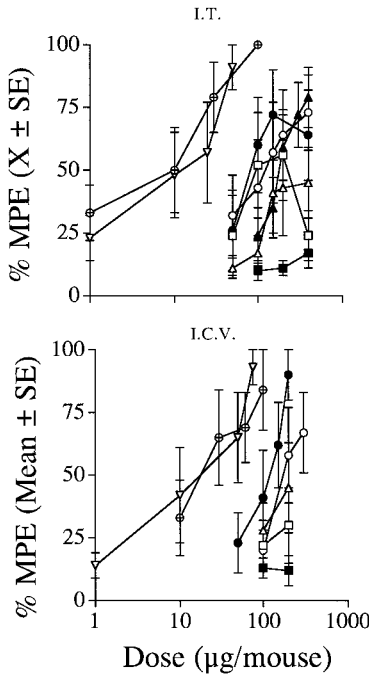


Fig. 4. The antinociceptive effects of the methylfluorophosphonate analogs after either i.t. or i.c.v. administration to mice. The means ± S.E. represent at least six mice per group. □, 1623; ■, 1624; ○, 1625; ●, 1626; △, 1705; ▲, MAFP; ⊕, 1778; ▽, 1887.

(1.5–3.3) and 7.7 (2.9–21) $\mu\text{g}/\text{mouse}$, respectively. We also determined that a 10-min pretreatment with SR 141716A (10 or 30 mg/kg i.p.) failed to block the antinociception produced by MAFP (300 $\mu\text{g}/\text{mouse}$). MAFP produced $92 \pm 8\%$ MPE in mice pretreated with vehicle and 79 ± 13 and $82 \pm 13\%$ MPE in mice receiving either 10 and 30 mg/kg SR 141716A (s.c.), respectively. An SR 141716A dose of 3 mg/kg completely blocks an ED_{84} dose of THC administered i.t.

To determine whether the inactive methylfluorophosphate analog O-1624 could potentiate the antinociceptive effects of anandamide, it was administered i.t. 10 min before an i.t. injection of anandamide. The results in Fig. 5, top, show that the ED_{50} value (CL) of anandamide [23 (15–36) $\mu\text{g}/\text{mouse}$] was decreased to 9.0 (4.2–20), 3.6 (1.8–7.1), and 2.6 (1.6–4.1) as the dose of O-1624 was increased to 50, 100, and 200 $\mu\text{g}/\text{mouse}$, respectively. The two higher doses of O-1624 produced statistically significant increases in that the CL values

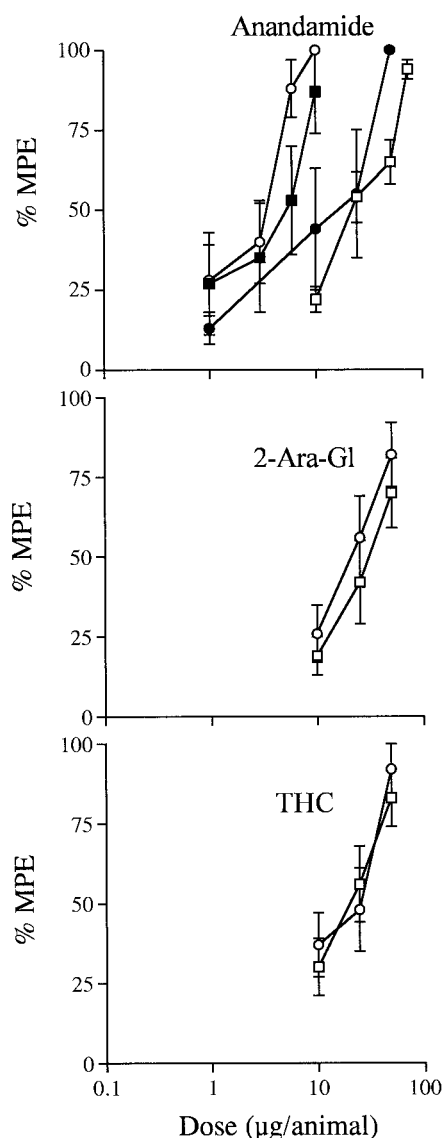


Fig. 5. The effect of O-1624 pretreatment on the antinociceptive effects of anandamide, 2-Ara-Gl, and Δ^9 -THC. Mice received a preinjection of either vehicle ($\square$) or O-1624 ($\bullet$, 50 μ g; $\blacksquare$, 100 μ g; $\circ$, 200 μ g) i.t. 10 min before a second i.t. injection of the cannabinoid and were tested 5 min after the last injection. The means $\pm$ S.E. represent at least six mice per group.

do not overlap those of anandamide alone. The 200- μ g dose of O-1624 failed to alter the potency of either 2-ara-Gl or Δ^9 -THC as shown in the bottom two panels in Fig. 5. The ED_{50}

value (CL) values of 2-Ara-Gl after vehicle and O-1624 pretreatments were 29 (19–42) and 20 (14–29) μ g/mouse, respectively. The ED_{50} value (CL) values of THC after vehicle and O-1624 pretreatments were 19 (13–28) and 18 (11–26) μ g/mouse, respectively. Another compound that is capable of potentiating anandamide, PMSF, was not influenced by O-1624. An i.t. PMSF dose of 200 μ g/mouse produced $19 \pm 7\%$ and $26 \pm 8\%$ MPE in the absence and presence of O-1624 (200 μ g/mouse), respectively (data not shown). Two other analogs were also tested for their potential to augment the effects of anandamide. The ED_{50} value (CL) of anandamide alone was decreased to 3.6 (1.6–8) μ g/mouse when the mice were pretreated with a dose of 100 μ g/mouse of O-1705 and to 8.8 (5.5–13.9) μ g/mouse in mice pretreated with O-1623 (data not shown).

Antinociceptive Activity after i.c.v. Administration.

The results in Fig. 4, bottom, show that O-1887, O-1778, and O-1626 were full antinociceptive agonists with respective ED_{50} value (CL) values of 11 (5–27), 20 (9–43), and 75 (28–204) μ g/mouse (Table 1). O-1624 was without effect, and the other analogs produced less-than-maximal effects.

Influence of O-1623 and O-1624 on Endocannabinoid Levels and FAAH Activity. The i.t. administration of these two compounds dramatically reduced FAAH activity in the spinal cord with little activity detected with the more potent FAAH inhibitor O-1623. However, the anandamide levels were elevated only with the O-1624 analog, and neither compound altered 2-Ara-Gl levels. The i.v. administration of O-1624 produced an elevation in both anandamide and 2-Ara-Gl in the striatum, the only brain area studied (Table 3).

Time Course Studies. The duration of action of these analogs is of considerable interest because of their potential for binding irreversibly to either the receptor or to FAAH. The time course of O-1705, the relatively weak C16:0 analog, was evaluated after i.v. administration. The results in Fig. 6 demonstrate that this compound is still exerting sedative, antinociceptive, and hypothermic effects at 48 h after administration. The time course appears to consist of an early phase that partially recovers at 4 h, followed by a protracted second phase. Signs of toxicity were evident in the animals at both 24 and 48 h, which could be due either to direct effects of the drug or to indirect effects arising from prolonged hypothermia along with compromised food and water intake. These signs consisted primarily of loss of weight, lack of grooming, and lethargy.

TABLE 3

Effect of O-1623 and O-1624 on FAAH activity and endogenous levels of anandamide and 2-Ara-Gl in spinal cord and striatum

		Vehicle	O-1623	O-1624
10 min after i.t. injection of vehicle or a dose of 200 μ g				
FAAH ^a	Spinal cord	0.56 ± 0.08	$0.04 \pm 0.01^{***}$	$0.27 \pm 0.04^{***}$
Anandamide ^b	Spinal cord	21.1 ± 2.9	27.2 ± 1.5	$34.5 \pm 2.2^*$
2-Ara-Gl ^c	Spinal cord	3.5 ± 0.5	2.9 ± 0.4	3.5 ± 0.6
5 min after i.v. injection of vehicle or a dose of 30 mg/kg				
Anandamide ^b	Striatum	N.D. ^d		$30.4 \pm 11.7^{**}$
2-Ara-Gl ^c	Striatum	6.2 ± 1.0		$11.3 \pm 1.4^*$

^a Results are expressed in terms of nanomoles per minute per milligram of protein and presented as means $\pm$ S.E. for 8 to 10 animals per group.

^b The values are picomoles per gram of tissue and presented as means $\pm$ S.E. for at least three animals per group.

^c The results are expressed as nanomoles per gram of tissue and presented as means $\pm$ S.E. of at least three animals per group.

^d N.D., not detected.

Significantly different from the vehicle group at * $P < .05$, ** $P = .06$, and *** $P < .001$.

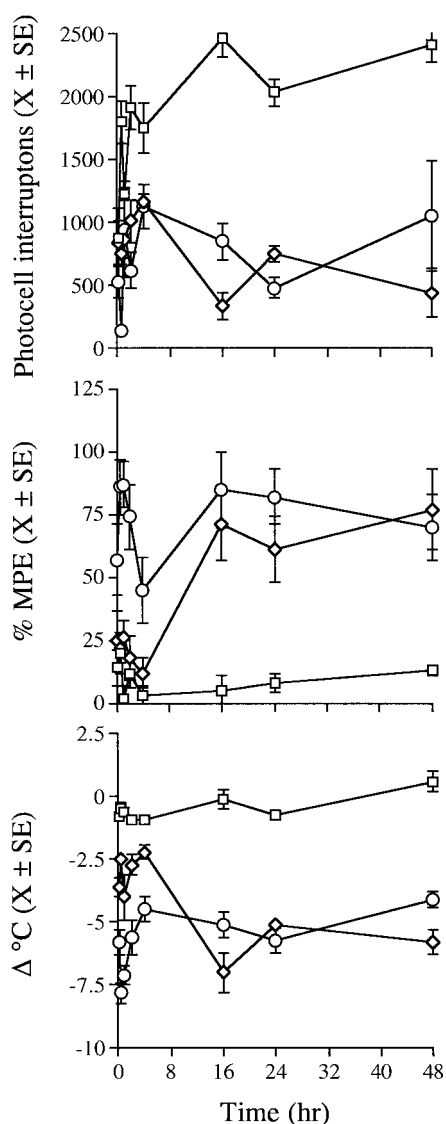


Fig. 6. The time course of O-1705 effects after i.v. administration. Separate groups of mice were treated with either vehicle (□) or 10 (◇) or 30 (○) mg/kg O-1705 and tested for spontaneous activity (top), tail-flick response (middle), and rectal temperature (bottom) at the indicated times. The means $\pm$ S.E. represent at least six mice per group.

Discussion

There are a large number of natural and synthetic inhibitors of endocannabinoid inactivation (see Di Marzo and Deutsch, 1998, for a recent review). Some of these agents interact with the CB1 receptor, thereby suggesting some similarities between the recognition sites on FAAH and the receptor. Previously, the structure-activity relationship of a series of saturated fatty acid sulfonyl fluorides was examined with regard to their ability to bind to the CB1 receptor and to inhibit FAAH (Deutsch et al., 1997a). The results obtained herein with the saturated methylfluorophosphonates resemble some of the properties of the corresponding sulfonyl fluorides, but striking differences exist. The C12:0 derivatives on both series had high affinity for the CB1 receptor, whereas there was a trend for decreasing receptor affinity as the length of the alkyl group increased. However, the important difference between the two series was that the methylfluoro-

phosphonates in the present study were not capable of complete displacement of [3 H]CP 55,940 binding, whereas the sulfonyl fluorides were. For example, the methylfluorophosphonate C16:0 at 10 μ M displaced approximately 50% of [3 H]CP 55,940 binding, whereas the corresponding sulfonyl fluoride displaced more than 90%. Presently, it is unclear how some of these methylfluorophosphonate analogs are competing for receptor binding at very low concentrations yet are incapable of maximal displacement. There are several possible explanations, the most intriguing of which would be CP 55,940 binding to both sensitive and nonsensitive methylfluorophosphonate sites. One cannot rule out as-yet-undefined factors that limit their accumulation at the binding site so that concentrations sufficient for complete displacement cannot be achieved.

Regarding FAAH inhibition, the saturated sulfonyl fluoride C12:0 to C18:0 derivatives were all highly effective and of comparable potency in blocking FAAH (Deutsch et al., 1997a). The C20:0 derivative appeared to be least effective in blocking FAAH. Also, the C20:0 methylfluorophosphonate was the least active FAAH inhibitor in the present study. In contrast, the C12:0 methylfluorophosphonate was considerably more potent than all of the other analogs, which were of comparable potency regardless of alkyl length and degree of saturation. On the other hand, the introduction of either one or two unsaturated bonds to form either O-1626 or O-1625, respectively, enhanced receptor affinity with relatively little influence on FAAH inhibitory activity. It is therefore evident that the receptor prefers methylfluorophosphonates with short alkyl chains or long alkyl chains that are constrained, whereas the enzyme is considerably less restrictive. Unfortunately, the influence of unsaturation of the alkyl group was not examined in the sulfonyl fluoride series (Deutsch et al., 1997a).

On i.v. administration, some of the methylfluorophosphonate analogs displayed potency and efficacy consistent with this binding profile. The finding that selected compounds were able to interact with the CB1 receptor and act as either partial agonists or agonists extends the cannabinoid structure-activity relationship. Most fatty acid derivatives that have been evaluated for cannabinoid effects have been arachidonyl analogs. In this study, the C20 analog with unsaturation at C11 and C14 (O-1625) exhibited the highest receptor affinity and produced some effects on spontaneous activity and hypothermia after i.v. administration. Saturating the C14 position (O-1626) slightly reduced affinity and pharmacological potency. Obviously, the degree of unsaturation influences receptor affinity for C20 analogs. The remaining compounds were saturated with varying chain length. Earlier, we had found that a fully saturated anandamide derivative (C20:0) exhibited pharmacological potency comparable to that of anandamide itself, but shorter chain derivatives were not explored (Adams et al., 1995). In contrast, the C20:0 saturated methylfluorophosphonate analog O-1624 exhibited very low affinity for the receptor and produced very little pharmacological activity regardless of the route of administration. Shortening the chain by two carbons (O-1623) seemed to increase its ability to bind to the receptor, but this analog was still quite weak pharmacologically. Reducing the chain length by two additional carbons led to O-1705, a compound that exhibited little affinity for the receptor; yet, it was capable of producing dose-related effects in all three

assays after i.v. administration with potency comparable to that of anandamide. It appears that this derivative is not acting in a fashion identical to that of anandamide, because it was fully efficacious in reducing rectal temperature, whereas anandamide derivatives act as partial agonists in producing hypothermia. The high receptor affinity, in vivo potency, and efficacy resulting from further reduction of the alkyl chain to merely C12:0 (O-1778) were unexpected, particularly in light of our recent observations that extending and branching the alkyl chain in anandamide, to mimic a dimethylheptyl analog, enhanced CB1 receptor affinity and pharmacological potency (Ryan et al., 1997). This C12:0 derivative provides the clearest distinction between the structural requirements of methylfluorophosphonates and anandamide-like derivatives for interaction with the CB1 receptor. On the other hand, the finding that the C8:0 analog failed to bind to the CB1 receptor, yet retained high potency, demonstrates that this cannabinoid pharmacological profile is not confined to this single receptor subtype. Furthermore, these observations raise the possibility that the other methylfluorophosphate analogs are producing their effects through a combination of CB1 and non-CB1 sites or solely non-CB1 sites.

If the unsaturated analogs are indeed binding to the cannabinoid receptor to act as partial agonists, then they could act as antagonists of Δ^9 -THC. None of the analogs were able to attenuate the effects of Δ^9 -THC. However, it is important to point out that we have not been able to reliably antagonize the in vivo effects of Δ^9 -THC unless we use antagonists with high receptor affinity.

As mentioned earlier, the pharmacological effects of these compounds could be due to either CB1 receptor activation, FAAH inhibition, or to yet another mechanism. O-1623 and O-1624 produced only minimal pharmacological effects after the i.v. administration, which is not surprising given their relatively weak receptor affinity, as discussed earlier. In particular, O-1624 elevated anandamide in both the spinal cord and the striatum (where 2-AG levels were also elevated) under the same conditions necessary for this compound to produce a small inhibitory effect on spontaneous activity but not on the nociceptive response in the tail-flick test. These findings suggest that the treatment of mice with this compound is sufficient to raise endogenous anandamide levels in some tissues but not always to an extent necessary to exert a cannabimimetic action. However, the observation that O-1623 was more potent than O-1624 in inhibiting FAAH in the spinal cord after i.t. administration but paradoxically less potent in raising anandamide levels in this tissue may suggest that the effects of these compounds on endogenous cannabinoid levels are not explained solely by their inhibition of FAAH. On the other hand, O-1705 exerts activity at the receptor as well as FAAH inhibition similar to that of O-1624 and O-1624, yet it is capable of producing pharmacological activity after i.v. injection. This comparison suggests that O-1705 is producing its effects through a non-CB1 receptor mechanism, as suggested earlier. This notion is further supported by the potent C8:0 analog, which lacks affinity for CB1. The differences between i.t. and i.v. administration of these analogs are also quite striking. The opposite pharmacological effects occurred with O-1705, which was inactive when administered i.t., whereas O-1625 and O-1626 were active.

There is no question that O-1624 greatly potentiated the pharmacological effects of exogenously administered anandamide as well as increased the ability of the latter to compete for CB1 receptor binding, much in the same manner we demonstrated for PMSF (Compton and Martin, 1997). However, it appears that this action may have more relevance to metabolism of exogenous anandamide than to endogenous cannabinoids. Although all of these MAFP analogs inhibit FAAH in brain homogenates, the degree to which they inhibit the enzyme in vivo remains to be fully established. Their efficacy depends on their ability to be taken up into cells containing FAAH as well as their own metabolic stability. Furthermore, exogenous anandamide may be hydrolyzed by different FAAH-containing cells than the endogenous substance.

In conclusion, both saturated and unsaturated methylfluorophosphonate analogs inhibit FAAH. A short saturated alkyl chain (C12:0) derivative resulted in an highly potent agonist and a unique receptor probe. Failure of these analogs to completely displace [3 H]CP 55,940 binding raises the possibility of subpopulations of sites labeled by this ligand. The finding that the C8:0 analog was highly potent in vivo yet failed to interact with the CB1 receptor supports this notion. The inhibitory activity of these analogs at FAAH effectively retards the metabolism of exogenously administered anandamide, but this action does not appear to contribute to their own pharmacological effects. It appears that these analogs are capable of producing at least some of their effects through CB1 as well as non-CB1 receptors.

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