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Pharmacological Activity of Fatty Acid Amides Is Regulated, but Not Mediated, by Fatty Acid Amide Hydrolase in Vivo

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ABSTRACT

Fatty acid amides (FAAs) represent a class of neuromodulatory lipids that includes the endocannabinoid anandamide and the sleep-inducing substance oleamide. Both anandamide and oleamide produce behavioral effects indicative of cannabinoid activity, but only anandamide binds the cannabinoid (CB1) receptor in vitro. Accordingly, oleamide has been proposed to induce its behavioral effects by serving as a competitive substrate for the brain enzyme fatty acid amide hydrolase (FAAH) and inhibiting the degradation of endogenous anandamide. To test the role that FAAH plays as a mediator of oleamide activity in vivo, we have compared the behavioral effects of this FAA in FAAH(+/+) and (−/−) mice. In both genotypes, oleamide produced hypomotility, hypothermia, and ptosis, all of which were enhanced in FAAH(−/−) mice, were unaffected by the CB1 antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-di-chlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) and occurred in CB1(−/−) mice. Additionally, oleamide displayed negligible binding to the CB1 receptor in brain extracts from either FAAH(+/+) or (−/−) mice. In contrast, anandamide exhibited a 15-fold increase in apparent affinity for the CB1 receptor in brains from FAAH(−/−) mice, consistent with its pronounced CB1-dependent behavioral effects in these animals. Contrary to both oleamide and anandamide, monoacylglycerol lipids exhibited equivalent hydrolytic stability and pharmacological activity in FAAH(+/+) and (−/−) mice. Collectively, these results indicate that FAAH is a key regulator, but not mediator of FAA activity in vivo. More generally, these findings suggest that FAAs represent a family of signaling lipids that, despite sharing similar chemical structures and a common pathway for catabolism, produce their behavioral effects through distinct receptor systems in vivo.

The nervous system is regulated by multiple classes of chemical transmitters, including amino acids, monoamines, and peptide hormones (Siegel et al., 1994). In recent years, several natural lipids have also emerged as candidate modulators of nervous system development and function. For example, *N*-arachidonoyl ethanolamine (anandamide) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) have been identified as endogenous ligands for the central cannabinoid (CB1) receptor, a G protein-coupled receptor that binds Δ^9-tetrahydrocannabinol, the active component of marijuana. Anandamide and 2-AG are prototype members of two classes of endogenous lipids, the fatty acid amides (FAAs) and monoacylglycerols (MAGs), respectively. In addition to anandamide, several natural FAAs have been found to produce neurobehavioral effects in rodents, including 9-(*Z*)-octadecenamide (oleamide), which promotes sleep (Cravatt et al., 1995; Basile et al., 1999); *N*-palmitoylethanolamine (PEA), which inhibits peripheral pain perception (Calignano et al., 1998; Jagger et al., 1998); and *N*-oleoylethanolamine (OEA), which suppresses food intake (Rodriguez de Fonseca et al., 2001). However, of these FAAs, only anandamide binds the CB1 receptor (Felder et al., 1993; Boring et al., 1996). Thus, the site(s) of action for non-CB1 binding, "orphan" FAAs remains unclear.

FAAs are rapidly hydrolyzed in vivo (Willoughby et al., 1997), hindering experimental efforts to determine their endogenous properties and sites of action. One candidate enzyme responsible for catabolizing FAAs in vivo is fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Giang and

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ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid; FAA, fatty acid amide; MAG, monoacylglycerol; PEA, *N*-palmitoylethanolamine; OEA, *N*-oleoylethanolamine; FAAH, fatty acid amide hydrolase; %MPE, percentage of maximum possible effect; PMSF, phenylmethylsulfonyl fluoride; 2-OG, 2-oleoylglycerol; GABA, γ-aminobutyric acid; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-di-chlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpho-linylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanonemesylate; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol.

Cravatt, 1997), a membrane-associated serine hydrolase enriched in brain and liver (Patricelli and Cravatt, 1999). FAAH hydrolyzes several bioactive FAAs in vitro, including both anandamide and oleamide (Cravatt et al., 1996), suggesting that this enzyme may serve as a general regulator of the FAA class of signaling lipids in vivo. Interestingly, FAAH also hydrolyzes MAGs in vitro (Goparaju et al., 1998), and thus, may also participate in the physiological inactivation of this class of lipids (Di Marzo et al., 1998).

The realization that FAAH hydrolyzes several natural FAAs and MAGs in vitro raises the possibility that these lipids may compete for access to FAAH's active site in vivo. If so, then some of the activities of orphan FAAs and MAGs may be the result of targeting FAAH, and thereby increasing the endogenous levels of endocannabinoids such as anandamide or 2-AG (Mechoulam et al., 1998). Indeed, such a model has been proposed to explain the pharmacological activity of oleamide (Mechoulam et al., 1997), which produces behavioral effects reminiscent of cannabinoids, but does not bind the CB1 receptor in vitro. In this model, oleamide would act as a competitive FAAH substrate in vivo, inhibiting the degradation of endogenous anandamide, which in turn would produce the observed cannabinoid behavioral effects through activation of the CB1 receptor.

We recently reported the generation and initial characterization of mice with a targeted disruption of the FAAH gene [FAAH(−/−) mice] (Cravatt et al., 2001). Herein, we show that FAAH(−/−) mice exhibit augmented behavioral responses to oleamide, but not 2-AG, and these effects are independent of the CB1 receptor.

Materials and Methods

Subjects. The FAAH(+/+) and (−/−) mice used in this study were littermates from second- or third-generation intercrosses of 129SvJ-C57BL/6 FAAH(±) mice (Cravatt et al., 2001). The CB1(−/−) and (+/+) mice were littermate offspring from CB1(±) parents on a C57BL/6 background (Zimmer et al., 1999).

Drugs. SR141716A was provided by the National Institute on Drug Abuse (Bethesda, MD); WIN 55,212-2 and methanandamide were purchased from Tocris Cookson (Ballwin, MO). Fatty acid amides were chemically synthesized as described previously (Cravatt et al., 1996). Monoacylglycerols were synthesized as described previously (Han and Razdan, 1999). All drugs were administered i.p. in a mixture of 1:1:18 ethanol/Emulphor/saline (10 μl/g b.wt.).

Behavioral Studies. Locomotor activity was assessed by placing each mouse in a clear Plexiglas cage [18 × 10 × 8.5 inch (length × width × height)] that was marked in 7-cm square grids on the floor of the cage. The number of grids that was traversed by the hind paws was counted from 15 to 20 min postinjection, unless otherwise noted. Nociception was then assessed in the tail immersion assay, in which each mouse was hand-held with approximately 1 cm of the tip of the tail immersed into a water bath maintained at 56.0°C, and the latency for the animal to withdraw its tail was scored. The cutoff was 15 s, and the data are expressed as the percentage of maximum possible effect (%MPE), in which %MPE = 100 · (postinjection latency − preinjection latency)/(15 − preinjection latency). Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75 cm in diameter) that was elevated 4.5 cm above the surface. Mice that remained motionless with their paws on the bar for 10 s (with the exception of respiratory movements) were scored as cataleptic. Rectal temperature was determined by inserting a thermocouple probe 1.2 cm into the rectum, and temperature was obtained from a telethermometer. Eyelid ptosis was scored as follows: 0, eyes wide open; 1, one or both eyes partially closed; or 2, one or both eyes closed. Ptosis, catalepsy, and rectal temperature were assessed at 60 min postinjection unless otherwise stated. The data reported are from a combination of male and female mice (no significant sex differences were observed for either genotype).

FAAH Activity Assay. FAAH activity assays for FAAs and MAGs were measured by following the conversion of ^{14}C-labeled substrates to their respective fatty acids using a thin layer chromatography assay as described previously (Cravatt et al., 2001).

Radioligand Binding. The methods used for radioligand binding were similar to those described previously (Compton et al., 1993). Binding was initiated by the addition of 20 μg of membrane protein to siliconized tubes containing [^{3}H]CP 55,940 and a sufficient volume of buffer A (50 mM Tris-HCl, 1 mM EDTA, 3 mM $MgCl_2$, and 1 mg/ml fatty acid-free bovine serum albumin, pH 7.4) to bring the total volume to 0.5 ml. The competition experiments included anandamide (0.1–10,000 nM), anandamide (0.1–10,000 nM) with 50 μM PMSF to reduce metabolic degradation (Childers et al., 1994), methanandamide (1–10,000 nM), 2-AG (1,000–30,000 nM), and oleamide (1,000–10,000 nM). CP 55,940 (1 μM) was used to assess nonspecific binding. After incubation of the tubes (30°C for 60 min), the reaction was terminated by addition of 2 ml of ice-cold buffer B (50 mM Tris-HCl and 1 mg/ml bovine serum albumin, pH 7.4, followed by rapid filtration under vacuum through GF/C glass fiber [pretreated with polyethylenimine (0.1%) for at least 4 h; Whatman, Maidstone, UK]. The tubes were washed once with 2 ml of ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Bound radioactivity was determined by liquid scintillation counting after extraction in 5 ml of BudgetSolve scintillation fluid, having been shaken for 1 h.

Data Analysis. Analysis of variance was used to analyze locomotor activity, tail withdrawal, rectal temperature, and ptosis. Dunnett's test was used for post hoc analysis and the Bonferroni's t test was used for planned comparisons to assess genotype differences. Differences were considered significant at the $p < 0.05$ level.

In the binding studies, B_{max} and K_d values obtained from Scatchard analysis of saturation binding curves were analyzed by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ). Displacement IC_{50} values from competition experiments were determined originally by using Prism 2.0 software for the Macintosh (GraphPad Software, San Diego, CA) and then converted to K_i values using the method of Cheng and Prusoff (1973). Unpaired t tests were used to compare the K_i values between the genotypes ($p < 0.05$).

Results

FAA and MAG Hydrolysis Rates in Tissue Extracts from FAAH(+/+) and (−/−) Mice. To evaluate the relative contribution that FAAH makes to the catabolism of both FAAs and MAGs in vivo, the hydrolytic rates of these lipids were examined in brain and liver extracts from FAAH(+/+) and (−/−) mice. The hydrolytic rates for all FAAs examined, including the endocannabinoid anandamide and the orphan FAAs oleamide, OEA, and PEA were dramatically reduced in both brain and liver homogenates from FAAH(−/−) mice (Table 1). The values for FAA hydrolysis in FAAH(−/−) tissues ranged from 1% (brain anandamide hydrolysis) to 6% (brain PEA hydrolysis) of the respective rates of hydrolysis observed in wild-type tissues. In contrast, the hydrolysis rates for the MAG 2-oleoylglycerol (2-OG) were equivalent in FAAH(+/+) and (−/−) tissues. These results indicate that FAAH is the major enzyme responsible for FAA hydrolysis in vivo, but suggest that enzymes other than FAAH control the catabolism of MAGs.

TABLE 1
FAA and MAG hydrolytic activities in FAAH(+/+) and (−/−) mice (nanomoles of FAA/MAG per minute per milligram of protein)
Values represent means ± S.E. n = 6/group (see *Materials and Methods* for details).

	Brain		Liver	
	FAAH(+/+)	FAAH(−/−)	FAAH(+/+)	FAAH(−/−)
Anandamide[a]	0.33 ± 0.04	0.003 ± 0.002***	0.53 ± 0.06	0.01 ± 0.01***
Oleamide[a]	0.24 ± 0.03	0.004 ± 0.002***	0.54 ± 0.01	0.03 ± 0.01***
OEA	0.06 ± 0.01	0.001 ± 0.001***	0.11 ± 0.01	0.002 ± 0.001***
PEA	0.033 ± 0.002	0.002 ± 0.001***	0.098 ± 0.012	0.002 ± 0.001***
2-OG	19.7 ± 1.12	20.3 ± 2.0	33.8 ± 2.8	34.8 ± 2.9

[a] From Cravatt et al. (2001).
*** Significantly different from FAAH(+/+) mice (t test, $p < 0.001$).

Behavioral Effects of Orphan FAAs in FAAH(+/+) and (−/−) Mice. In our previous studies, we found that FAAH(−/−) mice display dramatically increased behavioral responses to anandamide (Cravatt et al., 2001), confirming the central role that FAAH plays in regulating the levels and activity of this FAA in vivo. If other FAAs are regulated by FAAH in vivo then one might expect that these lipids would also exhibit enhanced activity in FAAH(−/−) mice. On the other hand, if, as has been proposed previously for oleamide (Mechoulam et al., 1997), non-CB1 binding FAAs produce their behavioral effects by competitively inhibiting the FAAH-dependent degradation of endogenous anandamide, then these lipids should be inactive in FAAH(−/−) mice. To test the role that FAAH plays as a possible regulator and/or mediator of the behavioral effects of orphan FAAs, we examined the pharmacological properties of oleamide, PEA, and OEA in FAAH(+/+) and (−/−) mice using a tetrad test for cannabinoid behavior (Smith et al., 1994). In this test, measurements of spontaneous activity, thermal pain sensation, catalepsy, and rectal temperature are made, and compounds with cannabinoid activity should produce hypomotility, analgesia, catalepsy, and hypothermia. Post hoc analyses of these measures revealed that in both genotypes, oleamide (25 or 50 mg/kg i.p.) elicited significant hypomotility and hypothermia, OEA (50 mg/kg i.p.) produced only hypomotility, and PEA (50 mg/kg i.p.) did not differ from vehicle in any of these measures (Fig. 1, A and B). Oleamide and OEA were also found to produce ptosis in both genotypes (Fig. 1C). Although inspection of the data in Fig. 1, B and C, revealed that oleamide tended to elicit more hypothermia (p = 0.11, 50 mg/kg; p = 0.11, 25 mg/kg) and ptosis (p = 0.10, 50 mg/kg) in FAAH(−/−) mice than in FAAH(+/+) mice, there were no significant effects of genotype on any of the indices. Collectively, these data demonstrate that the behavioral effects of orphan FAAs are not dependent on FAAH, indicating that this enzyme does not serve as a major site of action for these lipids in vivo.

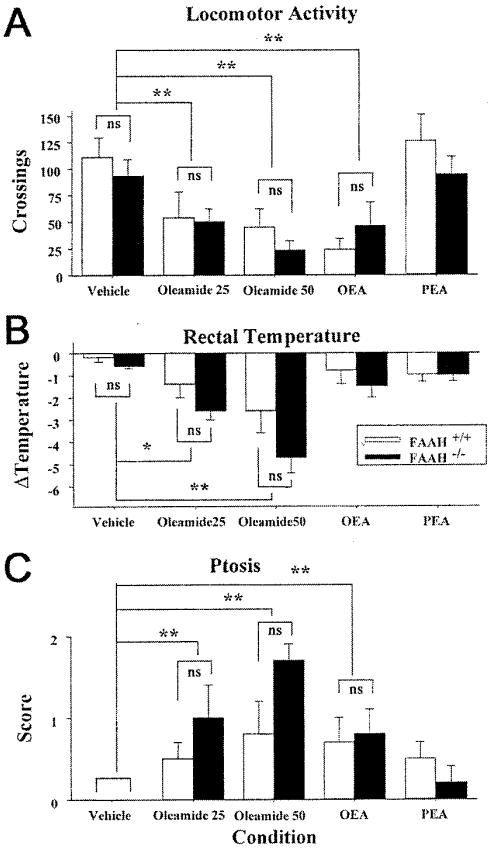

Fig. 1. Pharmacological activity of oleamide (25 and 50 mg/kg), OEA (50 mg/kg), and PEA (50 mg/kg) in FAAH(+/+) and (−/−) mice on locomotor activity from 15 to 20 min (A), hypothermia at 60 min (B), and eyelid ptosis at 60 min (C). Oleamide was active in all three measures, OEA elicited hypomotility and ptosis, and PEA failed to affect each of these measures. None of these FAAs induced catalepsy or antinociception (data not shown). **, $p < 0.01$ for drug-treated mice versus vehicle-treated mice, collapsed across genotype (Dunnett's test). There were no significant effects of genotype, as indicated by "ns". The results are presents as means ± S.E. (n = 6 to 8 mice/group).

Magnitude and Duration of Behavioral Effects of Oleamide in FAAH(+/+) and (−/−) Mice. After discovering that the behavioral effects of oleamide were not dependent on FAAH, we next considered whether this enzyme might regulate the pharmacological activity of oleamide in vivo. To examine this possibility in more detail, the magnitude and duration of the behavioral effects of oleamide were measured in FAAH(+/+) and (−/−) mice. The time course of the effects of oleamide (50 mg/kg i.p.) on locomotor activity, body temperature, and ptosis (Fig. 2, A–C, respectively) was determined in both genotypes. In contrast to the data represented in Fig. 1, in which oleamide only tended toward producing greater effects in FAAH(−/−) mice, increasing the sample size revealed significant genotype differences in the magnitude and duration of oleamide activity. For example, oleamide was found to produce hypomotility in both genotypes at 15 to 20 min, but by 120 min, only oleamide-treated FAAH(−/−) mice remained hypomotile (Fig. 2A). Similarly, oleamide caused greater hypothermia at 20, 60, and 120 min in FAAH(−/−) mice than in FAAH(+/+) mice (Fig. 2B), with

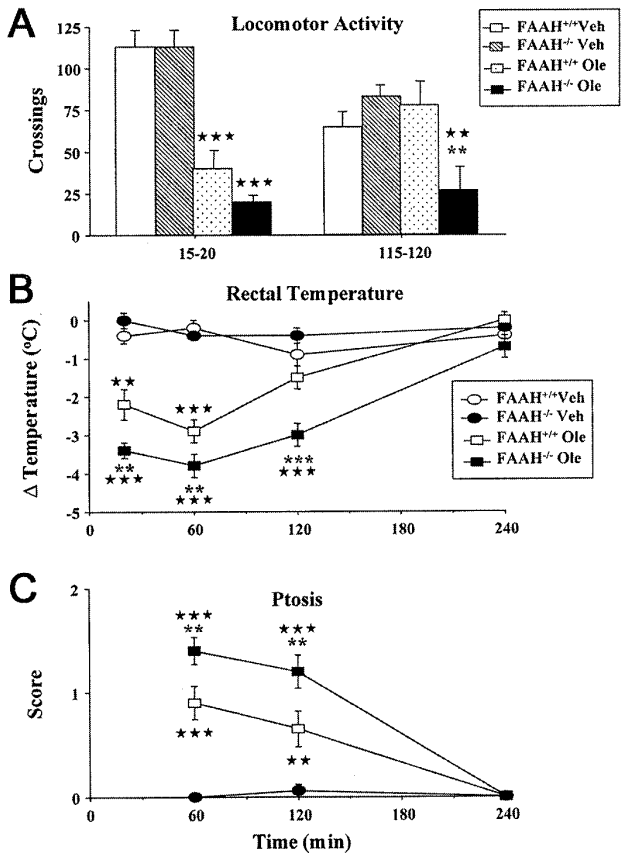

Fig. 2. Time course of hypomotility (A), hypothermia (B), and eyelid ptosis (C) in FAAH(+/+) and (−/−) mice treated with either vehicle or oleamide (50 mg/kg i.p.). **, $p < 0.01$ and ***, $p < 0.001$ for FAAH(+/+) versus (−/−) mice receiving oleamide (planned comparison). ★★, $p < 0.01$ and ★★★, $p < 0.001$ for oleamide-treated versus vehicle-treated in the same genotype (planned comparison). The results are presented as means ± S.E., $n = 8$ mice/group in the 120-min locomotor activity test and $n = 16$ to 20 mice/group in all other experiments.

the latter group of animals having returned to vehicle control levels by 120 min. Finally, oleamide induced ptosis in both genotypes at 60 and 120 min, and the magnitude of these effects was greater in FAAH(−/−) mice (Fig. 2C). By 240 min, oleamide-treated FAAH(+/+) and (−/−) mice did not differ from vehicle control mice in any of the tested behaviors. Collectively, these data indicate that FAAH regulates both the magnitude and duration of oleamide activity in vivo. More generally, the enhanced behavioral effects observed with both anandamide and oleamide in FAAH(−/−) mice support the notion that this enzyme is a primary catabolic regulator of the entire FAA family of neural signaling lipids.

Binding of FAAs to CB1 Receptor in Brain Preparations from FAAH(+/+) and (−/−) Mice. Although the above-mentioned data discounted FAAH as a site of action for oleamide, they did not directly address the role that the endogenous cannabinoid system might play in mediating the behavioral effects of this FAA. To investigate further the potential relationship between oleamide and the endogenous cannabinoid system, the affinity of this FAA, anandamide, and related compounds for the CB1 receptor, reported as K_i values, was measured in whole brain homogenates from FAAH(+/+) and (−/−) mice by competition experiments with the radiolabeled CB1 ligand [^{3}H]CP 55,940. Both the binding affinity of [^{3}H]CP 55,940 to the CB1 receptor and CB1 receptor density were similar in FAAH(+/+) and (−/−) brains (Table 2), indicating that data from competition experiments could be directly compared between genotypes. At concentrations up to 20 μM, oleamide only marginally displaced [^{3}H]CP 55,940 from the CB1 receptor (less than 50%) for both genotypes (Table 3). In contrast, the K_i value of anandamide for the CB1 receptor in the FAAH(−/−) brains was 52 nM, a value over 15-fold lower than the K_i observed for this compound in FAAH(+/+) brains (Table 3). Interestingly, the K_i of anandamide for the CB1 receptor in FAAH(−/−) brains was found to be similar to 1) the K_i values for this FAA observed in FAAH(+/+) or (−/−) brains preincubated with the general serine hydrolase inhibitor PMSF; and 2) the K_i values of the catabolically more stable anandamide variant methanandamide (Abadji et al., 1994), in FAAH(+/+) and (−/−) brains (Table 3). Collectively, these data confirm that anandamide, but not oleamide binds to the CB1 receptor isolated from endogenous sources. Additionally, the dramatic increase in apparent affinity of anandamide for the CB1 receptor in brains from FAAH(−/−) mice further supports the primary role that this enzyme plays in regulating anandamide activity in the central nervous system.

Role of CB1 Receptor in Mediating Behavioral Effects of Oleamide. Although the above-mentioned data argue against oleamide acting as a direct CB1 ligand in vivo, the behavioral effects of this FAA could still result from the indirect activation of the CB1 receptor, if, for example, oleamide competed with the cellular uptake of anandamide. To test whether CB1 receptors mediate the behavioral effects of oleamide, the pharmacological activity of this FAA was examined in FAAH(+/+) and (−/−) mice pretreated with the CB1 antagonist SR141716A. In either group of animals, SR141716A (10 mg/kg i.p.) failed to antagonize any of the observed behavioral effects of oleamide, including hypomotility, hypothermia, and ptosis (Fig. 3, A–C, left). Additionally, oleamide was found to produce equivalent levels of hypomotility, hypothermia, and ptosis in CB1(+/+) and (−/−) mice (Fig. 3, A–C, right). These data indicate that oleamide produces behavioral effects in vivo through a mechanism(s) that is independent of the CB1 receptor.

Role of FAAH in Regulating 2-AG Activity in Vivo. The reduced hydrolysis and enhanced behavioral effects of both oleamide and anandamide in FAAH(−/−) mice indicate that this enzyme serves as a general catabolic regulator of the FAA class of neural signaling molecules. Considering that FAAH has also been found to hydrolyze several MAGs in vitro (Di Marzo et al., 1998; Goparaju et al., 1998), this enzyme may also control the activity of fatty acid ester-based endocannabinoids in vivo. To test this notion, we compared the CB1 binding affinity and behavioral effects of 2-AG, a putative endogenous CB1 ligand, in FAAH(+/+) and (−/−)

TABLE 2
CB1 receptor affinity of [^{3}H]CP55,940 and the concentration of CB1 receptors in brains of FAAH(+/+) and (−/−) mice
Values represent means ± S.E. $n = 3$/group (see *Materials and Methods* for details).

	FAAH(+/+)	FAAH(−/−)
K_d	0.49 ± 0.07 nM	0.66 ± 0.06 nM
B_{max}	5.4 ± 0.1 pmol/mg	5.9 ± 0.5 pmol/mg

TABLE 3

Binding profile of anandamide, methanandamide, 2-AG, and oleamide to CB1 receptors in brains of FAAH(+/+) and (−/−) mice

Inhibition constants (nanomolar) were derived from displacement studies of [^{3}H]CP55,940 at 0.5 nM (see *Materials and Methods* for details) (n = 3/group).

	FAAH(+/+)	FAAH(−/−)
	Apparent K_i (95% C.I.)	
Anandamide	794 (300–2104)	52 (22–127)**
Anandamide and PMSF	61 (17–221)	48 (15–157)
Methanandamide	75 (9–616)	92 (22–384)
2-AG	1,890 (980–3,650)	2,100 (1,230–3,560)
Oleamide (10,000 nM)	46 ± 23% displacement	38 ± 15% displacement

** Significantly different from FAAH(+/+) mice (t test, $p < 0.01$).

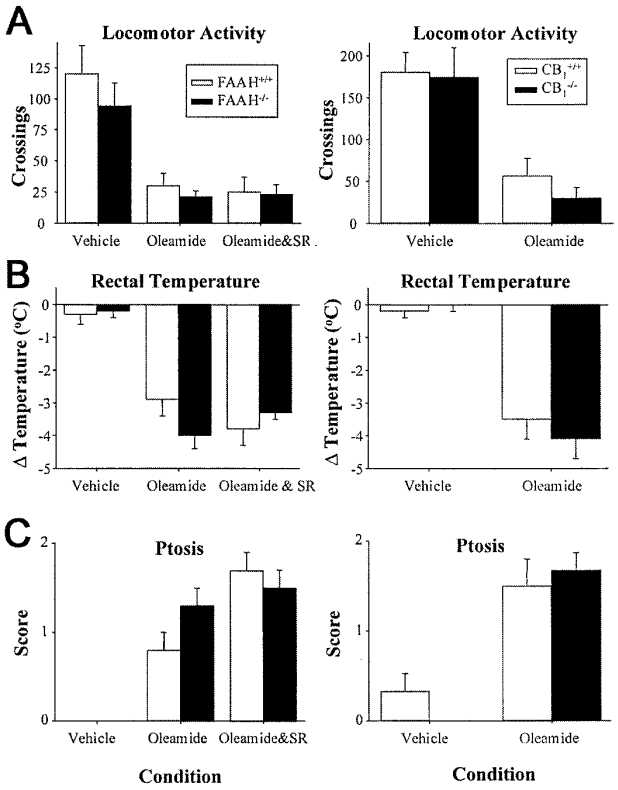

Fig. 3. Behavioral effects of oleamide are independent of the CB1 receptor. Left, CB1 receptor antagonist SR141716A (10 mg/kg) administered 10 min before oleamide (50 mg/kg) failed to block hypomotility from 15 to 20 min (A), hypothermia at 60 min (B), and eyelid ptosis at 60 min (C) in either FAAH(+/+) (□) or FAAH(−/−) (■) mice. The oleamide and oleamide + SR141716A (oleamide & SR) groups significantly differed from vehicle for each measure (Dunnett's test, $p < 0.01$). Right, CB1(+/+) mice (□) and CB1(−/−) mice (■) exhibited similar degrees of hypomotility from 15 to 20 min (A), hypothermia at 60 min (B), and eyelid ptosis at 60 min (C) after treatment with oleamide (50 mg/kg i.p.). Oleamide treatment elicited significant differences from the vehicle treatment for each measure (analysis of variance, $p < 0.0001$). The results are presented as means ± S.E. (n = 6 to 12 mice/group).

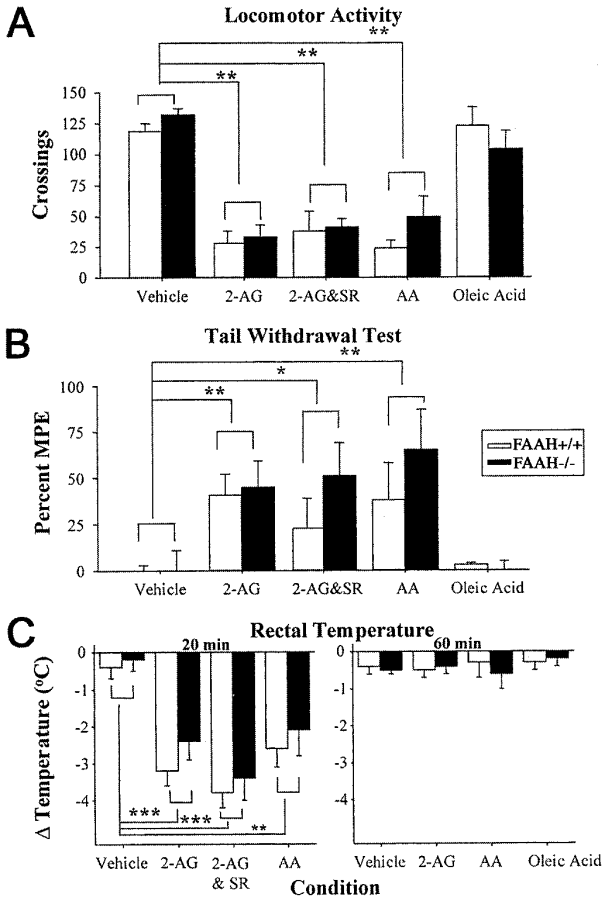

Fig. 4. Pharmacological effects of 2-AG (50 mg/kg) on hypomotility from 15 to 20 min (A), antinociception at 20 min (B), and hypothermia at 20 min (C) are mediated through a FAAH-independent and CB1 receptor-independent mechanism of action. FAAH(+/+) (□) and FAAH(−/−) (■) mice exhibited similar pharmacological responses to 2-AG and arachidonic acid (AA). SR141716A (10 mg/kg) failed to attenuate the pharmacological effects of 2-AG. 2-AG failed to induce catalepsy or ptosis in either genotype (data not shown). Oleic acid failed to elicit any significant effects. *, $p < 0.05$ and **, $p < 0.01$ for drug-treated mice versus vehicle-treated mice, collapsed across genotype (Dunnett's test). The results are presents as means ± S.E. (n = 6 to 15 mice/group).

mice. 2-AG displayed a weak affinity for the CB1 receptor that did not differ between genotypes (apparent K_i values of approximately 2 μM; Table 2), and produced equivalent behavioral effects in FAAH(+/+) and (−/−) mice (50 mg/kg i.p.), causing hypomotility (Fig. 4A), partial antinociception (Fig. 4B), and a brief decrease in rectal temperature (Fig. 4C). However, none of these behavioral effects were reduced by pretreatment with SR141716A (Fig. 4, A–C), suggesting that they might be mediated by the major 2-AG/anandamide metabolite arachidonic acid. Consistent with this notion, arachidonic acid produced nearly identical behavioral effects to those elicited by 2-AG in FAAH(+/+) and (−/−) mice (Fig. 4). In contrast, oleic acid was completely inactive in the tetrad test in either FAAH genotype (Fig. 4). Collectively, these data, in conjunction with the equivalent rates of 2-OG hydrolysis in tissue homogenates of FAAH(+/+) and (−/−) mice (Table 1), indicate that enzymes other than FAAH are primarily responsible for the catabolic regulation of the MAG family of signaling lipids in vivo.

Discussion

Several FAAs have been identified as endogenous constituents of nervous system tissues and fluids (Devane et al., 1992; Cravatt et al., 1995; Cadas et al., 1997). Most of these lipids produce neurobehavioral effects in rodents (Cravatt et

al., 1995; Calignano et al., 1998; Rodriguez de Fonseca et al., 2001), activate neuronal receptors and/or channels in vitro (Devane et al., 1992; Thomas et al., 1997; Smart et al., 2000), and are hydrolyzed by the brain enzyme FAAH in vitro (Cravatt et al., 1996). Although these findings suggest that FAAs may represent an important class of neural signaling molecules, major questions remain regarding their molecular and cellular site(s) of action in vivo. With the exception of anandamide, which has been shown to bind and activate CB1 receptors in vitro (Felder et al., 1993) and in vivo (Calignano et al., 1998; Hillard et al., 1999; Cravatt et al., 2001), most FAAs produce their behavioral effects through unknown mechanisms, and thus currently represent "orphan ligands" (endogenous bioactive compounds lacking cognate receptors).

Some orphan FAAs, such as oleamide, produce behavioral effects that are indicative of cannabinoid activity, despite failing to bind the CB1 receptor in vitro. These observations have led to the hypothesis that oleamide may produce its behavioral effects indirectly by competitively inhibiting the FAAH-mediated degradation of anandamide in vivo (Mechoulam et al., 1997). This "competitive substrate" model, also referred to as "the entourage effect" (Mechoulam et al., 1998; Lambert and Di Marzo, 1999), predicts that both FAAH and the CB1 receptor should be required for oleamide activity in vivo. Previous efforts to evaluate the roles played by these proteins in mediating the behavioral effects of oleamide have produced mixed results. For example, the CB1 antagonist SR141716A has been shown to block the hypnotic effects of oleamide (Mendelson and Basile, 1999), but fails to antagonize oleamide-induced hypothermia or hypomotility (Federova et al., 2001). Similarly, oleamide analogs that bind FAAH with high affinity, but are not substrates for the enzyme, fail to exhibit enhanced activity in animals (Federova et al., 2001). Collectively, these pharmacological studies have left the roles of FAAH and CB1 receptor as direct and/or indirect sites of action for oleamide open to debate.

To directly test the hypothesis that oleamide and related orphan FAAs produce behavioral effects through FAAH, we measured the pharmacological activity of a panel of these lipids in FAAH(+/+) and (−/−) mice. Each FAA was found to produce a unique spectrum of behavioral effects in the tetrad test for cannabinoid activity (Smith et al., 1994), with oleamide inducing hypomotility, hypothermia, and ptosis, OEA causing hypomotility and ptosis, and PEA being inactive. Importantly, all of these behavioral effects were observed in both FAAH(+/+) and (−/−) mice, demonstrating that this enzyme is not required for the pharmacological activity of orphan FAAs in vivo. Moreover, both the duration and magnitude of the behavioral effects of oleamide were significantly enhanced in FAAH(−/−) mice. These results, coupled with our finding that all of the FAAs tested exhibited dramatically reduced rates of hydrolysis in FAAH(−/−) tissues, indicate that FAAH is a primary catabolic regulator, but not mediator of FAA activity in vivo.

Although the pronounced behavioral effects of oleamide in FAAH(−/−) mice excluded FAAH as a direct site of action for this FAA in vivo, these results did not address the role that the CB1 receptor might play in mediating the pharmacological activity of oleamide. Receptor binding studies failed to detect a direct interaction between oleamide and the CB1 receptor in brain homogenates of either FAAH(+/+) or (−/−) mice. In contrast, anandamide was found to display a high binding affinity for the CB1 receptor selectively in FAAH(−/−) brain preparations (K_i = 52 nM). Consistent with previous studies (Childers et al., 1994), this anandamide-CB1 interaction was only observed in wild-type samples after treatment with the general serine hydrolase inhibitor PMSF. These data showcase both the potency with which anandamide binds the CB1 receptor and the primary role that FAAH plays in regulating this interaction. In contrast, the failure of oleamide to bind the CB1 receptor in either FAAH(+/+) or (−/−) brain preparations indicates that this FAA does not act as a direct CB1 ligand in vivo.

We next considered the possibility that oleamide might act indirectly through the CB1 receptor, which could occur if, as has been suggested (Federova et al., 2001), this FAA competed with the cellular uptake of anandamide in vivo. However, we found that the behavioral effects of oleamide were not affected by the CB1 antagonist SR141716A and were of equivalent magnitude in CB1(+/+) and (−/−) mice. These results indicate that the observed pharmacological activity of oleamide was not mediated by CB1 receptors. Although the precise site(s) of action for the behavioral effects of oleamide remains unclear, this FAA has been shown to activate both serotonin (Huidobro-Toro and Harris, 1996; Thomas et al., 1997) and GABA receptors (Yost et al., 1998) in vitro. Consistent with the participation of GABA receptors in transmitting some of the behavioral effects of oleamide, the $GABA_A$ antagonist bicuculline has been shown to partially block the hypothermic, but not hypomotility effects of oleamide (Federova et al., 2001). Additionally, the sleep-inducing effects of oleamide are attenuated in mice lacking the β-subunit of the $GABA_A$ receptor (Laposky et al., 2001). Interestingly, we found that the structurally similar FAA OEA produced a subset of the behavioral effects observed with oleamide, causing hypomotility, but not hypothermia. Collectively, these data suggest that oleamide produces behavioral effects through multiple receptor systems, some of which respond selectively to this FAA, and others of which may be targeted by additional endogenous FAAs (e.g., OEA). Importantly, these results also reveal that oleamide and anandamide, despite both producing robust behavioral effects that are regulated in duration and magnitude by FAAH, possess nearly orthogonal sites of action in vivo. Indeed, our previous studies demonstrated that all of the observed behavioral effects of anandamide were blocked in FAAH(−/−) mice by pretreatment with SR141716A (Cravatt et al., 2001), a finding that now stands in striking contrast to the failure of SR141716A to reduce any of the pharmacological activities of oleamide in these animals.

FAAH has not only been proposed to participate in the physiological inactivation of FAAs (Patricelli and Cravatt, 2001) but also a second family of endogenous bioactive lipids, the MAGs (Di Marzo et al., 1998). However, tissue homogenates from FAAH(+/+) and (−/−) mice were found to hydrolyze MAGs at equivalent rates. Additionally, the endocannabinoid MAG 2-AG produced similar behavioral effects in FAAH(+/+) and (−/−) mice. Considering that none of these behavioral effects were blocked by the CB1 antagonist SR141716A, we tested whether they might be produced by the major 2-AG metabolite arachidonic acid. Arachidonic acid was found to elicit nearly identical behavioral effects to those caused by 2-AG in FAAH(+/+) and (−/−) mice. Collectively, these data demonstrate that enzymes other than FAAH are primarily responsible for catabolizing MAGs in vivo.

In this study, several bioactive lipids, including oleamide,

OEA, and arachidonic acid, produced CB1-independent behavioral effects in the tetrad test for cannabinoid activity. Although these data might suggest that this collection of assays is not highly predictive of compounds that act through the CB1 receptor, it is important to recognize that, of all the compounds analyzed, only the endogenous CB1 ligand anandamide was fully active in the tetrad test. The key behavioral assay that differentiated anandamide from other FAAs proved to be the catalepsy bar test (Sanberg et al., 1988), in which anandamide alone was active. These results suggest that compounds exhibiting partial activity in the tetrad test, but failing to produce catalepsy may not act through the CB1 receptor in vivo. In future studies, drawing such a distinction may assist in differentiating the pharmacological activities of endocannabinoids from those associated with their metabolites (e.g., arachidonic acid) and/or other bioactive FAAs (e.g., oleamide).

In summary, an analysis of the biochemical properties and behavioral effects of members of the FAA family in FAAH(+/+) and (−/−) mice has revealed that these lipids, despite possessing similar chemical structures, exhibit remarkably diverse pharmacological activities in vivo. Indeed, our results indicate that the two most bioactive FAAs tested, anandamide and oleamide, possess distinct and nonoverlapping endogenous sites of action, with the former FAA behaving as a pure CB1 agonist and the latter producing entirely CB1-independent behavioral effects. Nonetheless, anandamide and oleamide were both found to exhibit significantly enhanced pharmacological activity in FAAH(−/−) mice, supporting a role for this enzyme as a general catabolic regulator of FAA signaling in vivo. In this regard, FAAs may represent a family of neural signaling molecules reminiscent of the monoamine class of neurotransmitters, with members that act at distinct receptors, but share a common pathway for catabolism in vivo (Shih et al., 1999).

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