0022-3565/97/2833-1138$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 283, No. 3

Printed in U.S.A.
JPET 283:1138–1143, 1997

The Effect of the Enzyme Inhibitor Phenylmethylsulfonyl Fluoride on the Pharmacological Effect of Anandamide in the Mouse Model of Cannabimimetic Activity[1]

DAVID R. COMPTON and BILLY R. MARTIN

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Accepted for publication August 22, 1997

ABSTRACT

Anandamide is an putative endogenous cannabinoid ligand that produces pharmacological effects similar to those of Δ^9-tetrahydrocannabinol, the principle psychoactive constituent in marijuana. There is considerable evidence that the enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF) is capable of altering the actions of anandamide *in vitro* by blocking its metabolism. Therefore, studies were conducted in mice to determine whether PMSF could produce cannabinoid effects by altering endogenous levels of anandamide as well as determining whether PMSF could potentiate the effects of exogenously administered anandamide. Mice receiving i.p. injections of PMSF exhibited cannabinoid effects that included antinociception, hypothermia and immobility with ED_{50} values of 86, 224 and 206 mg/kg, respectively. Spontaneous activity was reduced at doses greater than 100 mg/kg. However, none of these effects was blocked by the cannabinoid antagonist SR 141716A. On the other hand, pretreatment with an inactive dose of PMSF (30 mg/kg) potentiated the effects of anandamide on tail-flick response (antinociception), spontaneous activity and mobility by 5-, 10- and 8-fold, respectively. PMSF did not alter anandamide's hypothermic effects. Overall, these findings with PMSF underscore the importance of metabolism in the actions of anandamide. It still must be established whether metabolites of anandamide contribute to its pharmacological activity.

Arachidonylethanolamide, more commonly known as anandamide, is an ethanolamine derivative of arachidonic acid which was first isolated in porcine brain (Devane *et al.*, 1992). Several lines of evidence have suggested that anandamide may function as an endogenous ligand for the cannabinoid receptor. Anandamide competitively inhibited the specific binding of a radiolabeled cannabinoid probe to synaptosomal membranes, and it produced a dose-dependent inhibition of the electrically evoked twitch response in the mouse vas deferens (Devane *et al.*, 1992). In preliminary behavioral studies, anandamide produced moderate effects similar to that of Δ^9-THC (the prototypical psychoactive cannabinoid) after i.p. administration (Fride and Mechoulam, 1993). Subsequently, thorough dose-response analysis also indicated that anandamide produced effects similar to those of Δ^9-THC in a tetrad of tests used to predict cannabimimetic activity, including antinociception (as determined in a latency to tail-flick evaluation), hypothermia, hypomotility and catalepsy in mice after i.v., i.p. and intrathecal administration (Smith *et al.*, 1994). In general, the effects of anandamide occurred with a rapid onset and with a rather short duration of action. Also, it was 1.3 to 18 times less potent than Δ^9-THC in all behavioral assays.

Binding studies have demonstrated that anandamide interacts with the CB1 cannabinoid receptor with a K_D of approximately 100 nM (Childers *et al.*, 1994; Smith *et al.*, 1994). Because anandamide can be hydrolytically cleaved by a membrane-bound enzyme in brain tissue to arachidonic acid and ethanolamine (Deutsch and Chin, 1993), PMSF (50 μM) has typically been included in the incubation medium of *in vitro* binding assays to obtain true estimates of receptor affinity for anandamide (Childers *et al.*, 1994; Hillard *et al.*, 1995; Smith *et al.*, 1994) and related analogs (Adams *et al.*, 1995). PMSF is a nonspecific inhibitor of various proteases and other enzymes, including acetylcholinesterase, palmityl coenzyme A deacylase, arylsulfatase A, chymotrypsin and trypsin (James, 1978; Moss and Fahrney, 1978). Generally, PMSF acts by sulfonylating the hydroxyl groups of active site serine residues of enzymes, which causes an irreversible inhibition. Although not yet identified, the enzyme responsible for the metabolism of anandamide would be considered an

Received for publication May 19, 1997.
[1] This research was supported by NIDA grants DA 09789 and DA 08677.

ABBREVIATIONS: i.v., intravenous; i.p., intraperitoneal; THC, tetrahydrocannabinol; % MPE, percent maximal possible effect; ED_{50}, dose effectively producing 50% of maximal response; C.L., confidence limits; Δ°C, change in rectal temperature in degrees Celsius; PMSF, phenylmethylsulfonyl fluoride.

amidohydrolase. Metabolism of anandamide is greatest in the liver, also occurs to a significant degree in brain tissue, but only occurs to a much lesser extent in other tissues (Desarnaud *et al.*, 1995). This amidohydrolase can also be inhibited by *p*-bromophenacyl bromide (a histidine-alkylating agent), but not by other nonselective peptidase inhibitors such as ethylenediaminetetraacetic acid, bacitracin and *o*-phenanthroline (Desarnaud *et al.*, 1995). PMSF rapidly crosses the blood-brain barrier (Turini *et al.*, 1969), so *in vivo* administration would be expected to inhibit anandamide metabolism in brain tissue as well as in the periphery.

Very few data are available concerning the *in vivo* metabolism of anandamide. It is clear that it possesses a very short duration of action on various pharmacological measures (Smith *et al.*, 1994). This is assumed to be caused by rapid metabolic inactivation. If true, then it is also likely that the decreased *in vivo* potency of anandamide, relative to that of Δ^9-THC, is also caused by rapid metabolism. However, in such a scenario the maximum *in vivo* effect that could be produced by anandamide should not change, and the interaction of anandamide with the CB1 receptor (as evidenced by the shape of the dose-response curve) should not be altered by manipulations of metabolism. This research evaluated the *in vivo* pharmacological effects of anandamide in the mouse model of cannabinoid pharmacological activity after the *in vivo* administration of PMSF. This agent should attenuate the metabolism of anandamide thereby providing indirect evidence as to whether or not the weak potency of anandamide is caused by rapid metabolic inactivation.

Materials and Methods

Male ICR mice (Harlan Laboratories, Dublin, VA) weighing 18 to 25 g were used in all experiments. The mice were maintained on a 14:10 hr light/dark cycle with free access to food and water. Anandamide, 2-methylarachidonly-(2′-fluoroethyl)amide and SR141716A were obtained from Dr. Raj K. Razdan (Organix, Inc., Woburn, MA) and dissolved in 1:1:18 (emulphor/ethanol/saline) for *in vivo* administration. Emulphor (EL-620, a polyoxyethylated vegetable oil, GAF Corporation, Linden, NJ) is currently available as Alkmulphor. Anandamide and SR141716A injections were administered i.v. (tail vein) at a volume of 0.1 ml/10 g b.wt. PMSF was obtained from Sigma Chemical (St. Louis, MO), dissolved in sesame oil and administered i.p. at a volume of 0.1 ml/10 g b.wt. PMSF was always administered 10 min before i.v. anandamide or vehicle injections.

Mice were acclimated to the evaluation room overnight without interruption of food or water. After i.v. anandamide or vehicle administration each animal was evaluated as follows: tail-flick latency (antinociception) response at 5 min and spontaneous (locomotor) activity at 5 to 15 min; or core (rectal) temperature at 5 min and ring-immobility (catalepsy) at 5 to 10 min, as described elsewhere (Smith *et al.*, 1994). Where indicated, the cannabinoid antagonist SR141716A was administered (i.v.) 1 min before the i.p. administration of PMSF.

Spontaneous activity. Inhibition of locomotor activity was accomplished by placing mice in individual activity cages (6.5 × 11 inches) and recording interruptions of the photocell beams (16 beams per chamber) for a 10-min period with a Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH). Activity in the chamber was expressed as the total number of beam interruptions.

Tail-flick latency. Antinociception was assessed by the tail-flick procedure. The heat lamp of the tail-flick apparatus was maintained at an intensity sufficient to produce control latencies of 2 to 3 sec. Control values for each animal were determined before drug administration. Mice were then re-evaluated after drug administration and latency (sec) to tail-flick response was recorded. A 10-sec maximum was imposed to prevent tissue damage. The degree of antinociception was expressed as the % MPE which was calculated as:

$$\%\ \text{MPE} = \left[\frac{(\text{test latency} - \text{control latency})}{(10\ \text{sec} - \text{control latency})}\right] \times 100 \qquad (1)$$

Core temperature. Hypothermia was assessed by first measuring base-line core temperatures before drug treatment with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and a rectal thermistor probe inserted to a depth of 25 mm. Rectal temperatures were then measured after drug administration so that the temperature difference (°C) between values could be calculated for each animal.

Immobility. Catalepsy was determined by a ring-immobility procedure. At 90 min after injection, mice were placed on a metal ring (5.5 cm in diameter) that was attached to a stand at a height of 16 cm. The amount of time (sec) that the mouse spent motionless during a 5-min test session was recorded. The criterion for immobility was the absence of all voluntary movements (excluding respiration, but including whisker movement). The immobility index was calculated as:

$$\%\ \text{Immobility} = \left[\frac{\text{time immobile (sec)}}{\text{length of session(sec)}}\right] \times 100$$

Mice that fell or actively jumped from the ring were allowed five such escapes. After the fifth escape, the test for that animal was terminated and immobility was calculated as a percentage of time that it remained on the ring before being discontinued. Data from mice failing to remain on the ring at least 2.5 min were not included.

Statistical analysis. Statistical analysis of all *in vivo* data was performed by ANOVA with Bonferroni/Dunn *post hoc* for comparison with vehicle with use of the StatView statistical package (Brainpower, Inc., Agoura Hills, CA). Differences were considered significant at the $P < .05$ level. ED_{50} values were determined by ALLFIT, a program for the simultaneous curve fitting of a family of sigmoidal curves.

Results

Agonist effects of PMSF. Although the primary objective was to determine the influence of PMSF pretreatment on anandamide's pharmacological potency, it was necessary to determine whether PMSF exhibited agonist effects. To mimic the treatment regimen involving the dual injections of PMSF and anandamide, evaluation of the effects of PMSF in the absence of anandamide was conducted by the dual-injection approach. All animals received an i.p. injection of PMSF followed by a 10-min period before the i.v. administration of vehicle. Thus, the pharmacological effects observed in these studies (figs. 1–3, table 1) correspond exactly to what would

TABLE 1

The effect of PMSF on locomotor activity in mice

PMSF was administered i.p., the mice were placed in the activity chambers 15 min later and interruptions of the photocells were recorded for the next 10 min.

PMSF	*n*	Photocell Interruptions	S.E.M.	Inhibition
mg/kg				%
0	6	1063	186	0
30	6	1096	142	0
60	6	1066	200	0
100	6	943	263	11
200	5	96*	19	91
300	6	277*	87	74

* Significantly different ($P < .05$) from control.

be the anticipated contribution of PMSF to the total response observed in the subsequent PMSF plus anandamide studies.

PMSF produced antinociception as indicated by the dose-responsive increase in % MPE in the tail-flick latency evaluation (fig. 1). Maximal antinociception was obtained with a dose of 300 mg/kg. Sigmoidal curve-fitting analysis of the % MPE log-dose response data ($n = 6$ per dose) indicated an ED_{50} value of 86 ± 10 mg/kg. These same animals were also evaluated (as described under "Materials and Methods") for alterations in spontaneous locomotor activity. Unlike that found for the antinociception measure, PMSF failed to produce a clear dose-responsive inhibition of locomotion. However, doses of 200 and 300 mg/kg produced a significant decrease in locomotor activity (table 1).

Rectal temperature was decreased after the administration of PMSF. Sigmoidal curve-fitting analysis of the temperature log-dose response data (fig. 2; $n = 4$–6 per dose) indicated an ED_{50} value of 224 ± 164 mg/kg. The large standard error for this value was primarily because of the limited number of doses (200 and 300 mg/kg) which produced a response greater than control. However, no statistically significant effect was observed at doses less than 200 mg/kg. No further attempts to characterize the dose-responsive relationship were attempted because the necessary doses would have been so large. Curve-fitting analysis suggested that the maximum effect on core temperature would be −4.0°C, whereas the basal response was −0.4 ± 0.3°C, which was nearly identical with experimentally obtained values.

Immobility was also a prominent effect produced by PMSF administration. Sigmoidal curve-fitting analysis of the percent immobility log-dose response data (fig. 3; $n = 6$ per dose) indicated an ED_{50} value of 206 ± 82 mg/kg. As with the production of hypothermia, no statistically significant effect was observed at doses less than 200 mg/kg, so these data were considered sufficient for meeting the purposes of this investigation, and no further attempts to characterize the dose-responsiveness were attempted. Curve-fitting analysis suggested that the maximum effect on ring-immobility would be 80%, whereas the basal response was 12 ± 5% immobility, which was nearly identical with experimentally obtained values.

Effect of SR141716A on PMSF. To determine whether the pharmacological effects of PMSF could be prevented by the cannabinoid receptor antagonist SR141716A, mice were treated i.v. with this antagonist 10 min before the i.p. administration of PMSF (table 2). The dose of SR141716A chosen was known to be completely effective in antagonizing the effects of either WIN-55,212–2 or Δ^9-THC by this same protocol. The cannabinoid antagonist failed to reduce the pharmacological effect of 100 mg/kg PMSF.

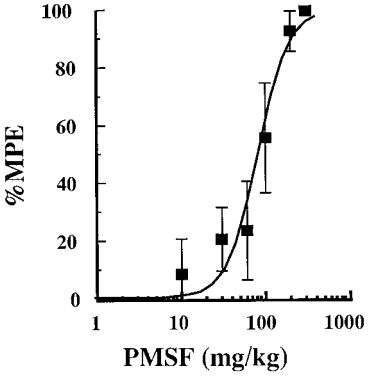

Fig. 1. The antinociceptive effects of PMSF administered i.p. to mice and tested 15 min later for tail-flick responsiveness. The results are presented as means ± S.E.M. for at least six mice per group.

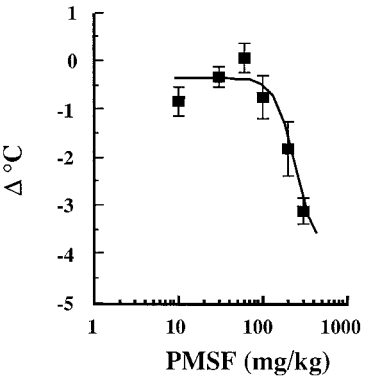

Fig. 2. The hypothermic effects of PMSF were determined 15 min after i.p. administration to mice. The results are presented as means ± S.E.M. for at least six mice per group.

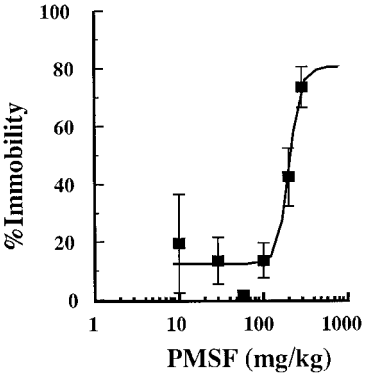

Fig. 3. Immobility produced by PMSF was determined for the 15- to 20-min period after i.p. administration to mice. The results are presented as means ± S.E.M. for at least six mice per group.

Effect of PMSF on anandamide. To evaluate the effect of PMSF on the pharmacological response to anandamide, the PMSF pretreatment dose chosen was 30 mg/kg. This dose of PMSF failed to produce a statistically significant effect on any measure, is much less than either the 100 or 200 mg/kg doses required to produce any pharmacological action and is sufficiently small that even in the tail-flick antinociception measure (see fig. 1) the response would be minimal (<20%). Thus, groups of mice were pretreated with either PMSF (30

TABLE 2

Effect of SR141716A on the pharmacological activity of PMSF

Mice were injected i.v. with SR 141716A 10 min before the i.p. administration of either PMSF or its vehicle (sesame oil) before testing as described under "Materials and Methods." Values (mean ± S.E.M., $n = 6$ per group) for tail-flick antinociception (% MPE), inhibition of locomotor activity (% inhibition), decrease in core temperature (Δ°C) and ring-immobility catalepsy (% immobility) after i.p. administration of PMSF. PMSF alone (in the absence of antagonist) was statistically different ($P < .05$) from both vehicle control (1:1:18 vehicle + sesame oil) (data not shown) and the drug control (SR141716A + sesame oil) but not from the SR 141716A/PMSF combination.

SR141716A	PMSF	% MPE	% inhibition	Δ°C	% immobility
mg/kg					
3	0	7 ± 5	4 ± 11	−1.6 ± 0.3	2 ± 1
0	100	49 ± 14	85 ± 6	−3.7 ± 0.4	22 ± 6
3	100	39 ± 15	74 ± 8	−4.1 ± 0.3	18 ± 5

mg/kg) or vehicle (1:1:18) before treatment with various doses of anandamide. The dose-response curves demonstrating the effect of PMSF on anandamide-mediated responses are demonstrated in figures 4 to 6 and summarized in table 3.

PMSF pretreatment produced a parallel shift to the left in the anandamide dose-response curve in the tail-flick antinociception measure (fig. 4). The ED_{50} value for anandamide was 17 ± 2 mg/kg. The potency of anandamide was somewhat less than that previously reported [6.2 mg/kg (Smith *et al.*, 1994)], although very similar to that observed for other measures. PMSF produced a statistically significant shift in that value to 3.3 ± .3 mg/kg. This 5-fold increase in the potency of anandamide was obtained in experiments in which the results for both vehicle and PMSF pretreatment at each dose of anandamide were generated within the same experiment.

PMSF pretreatment also produced a parallel shift to the left in the anandamide dose-response curve for attenuation of locomotor activity (fig. 5). The maximum effect of anandamide was 75% inhibition of activity, which was nearly identical with that obtained previously [85% inhibition (Smith *et al.*, 1994)], especially considering the error of 9% obtained here. The ED_{50} value for anandamide alone was found to be 10 ± 3 mg/kg. The potency of anandamide was somewhat greater than that reported previously [18 mg/kg (Smith *et al.*, 1994)]. PMSF produced a statistically significant shift in that value to 1.2 ± .6 mg/kg, corresponding to a 10-fold increase in potency. The dose-response curves were obtained from the same animals used to generate the tail-flick antinociception curves, which explains why the lower portion of the locomotor activity curves were not more complete.

PMSF pretreatment also produced a parallel shift to the left in the anandamide dose-response curve in the locomotor activity measure (fig. 6). The maximum effect was 77% immobility, which was nearly identical with that obtained previously [88% inhibition (Smith *et al.*, 1994)], especially considering the error of ±17% obtained here. The ED_{50} value for anandamide alone was found to be 35 ± 20 mg/kg. The large error may be attributed to the fact that only two doses produced response levels greater than control. However, given that statistical analysis indicated a maximum effect (for both curves) nearly identical with that already established in the literature, it was not deemed necessary to extend the anandamide dose-response curve to doses that would produce near-maximal responding. The potency of anandamide found here was somewhat less than that reported previously [19 mg/kg (Smith *et al.*, 1994)]. PMSF produced a statistically significant shift in that value to 4.2 ± 2.2 mg/kg, corresponding to a 8-fold increase in potency.

The only measure for which PMSF pretreatment did not produce a significant increase in the response was the anandamide-induced hypothermia (table 3). It is not yet clear whether increasing the dose of PMSF could alter the hypothermic response to anandamide. It is clear that there must be basic differences in the mechanism of action of anandamide-mediated hypothermia *versus* the other cannabimimetic measures. The temperature data obtained were from the same animals used to generate the ring-immobility curves (above), in which large increases in pharmacological response

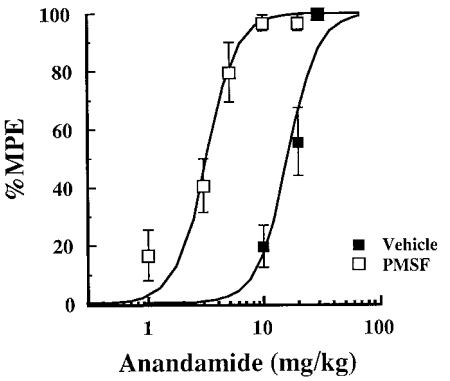

Fig. 4. Enhancement of anandamide-induced antinociception by a 10-min i.p. pretreatment with PMSF (30 mg/kg). The results are presented as means ± S.E.M. for at least six mice per group.

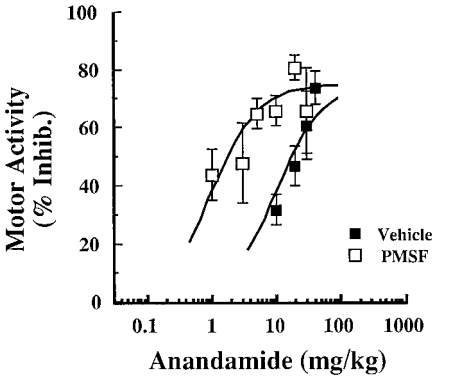

Fig. 5. Enhancement of anandamide-induced hypomotility by a 10-min i.p. pretreatment with PMSF (30 mg/kg). The results are presented as means ± S.E.M. for at least six mice per group.

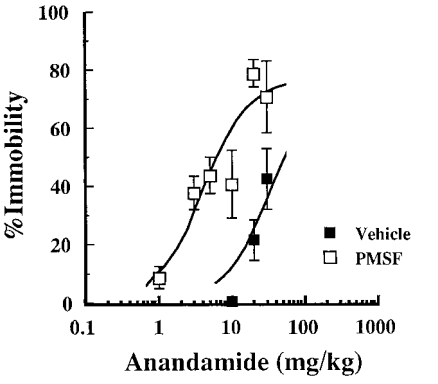

Fig. 6. Enhancement of anandamide-induced immobility by a 10-min i.p. pretreatment with PMSF (30 mg/kg). The results are presented as means ± S.E.M. for at least six mice per group.

TABLE 3

Failure of PMSF to modulate anandamide-mediated hypothermia

Mice were injected i.p. with PMSF 10 min before an i.v. injection of anandamide, and the animals were tested as described under "Materials and Methods." The differences between preinjection rectal temperatures and those after anandamide administration are presented as means and S.E.M.

PMSF	Anandamide	*n*	Δ°C	S.E.M.
mg/kg				
0	10	6	−1.2	0.8
30	10	6	−0.5	0.1
0	20	6	−0.5	0.3
30	20	6	−1.0	0.2
0	30	6	−2.4	0.7
30	30	6	−2.7	0.4

was observed after PMSF pretreatment. Thus, the lack of effect of PMSF on anandamide-mediated hypothermia can not be attributed to errors in the administration of either PMSF or anandamide. However, the unusually small drop in temperature observed even at 30 mg/kg of anandamide might be a factor.

Effect of PMSF on 2-methylarachidonly-(2′-fluoroethyl)amide. To determine whether PMSF would alter the potency of a metabolically stable analog of anandamide, 2-methylarachidonly-(2′-fluoroethyl)amide was evaluated in the presence and absence of PMSF using the protocol described for anandamide-induced hypoactivity and antinociception. As seen in figure 7, PMSF pretreatment resulted in only a slight enhancement of 2-methylarachidonly-(2′-fluoroethyl)amide potency. The ED_{50} values (C.L.) for 2-methylarachidonly-(2′-fluoroethyl)amide in vehicle and PMSF-pretreated mice were 3.0 (2.2–4.0) and 1.5 (0.8–2.7) mg/kg, respectively. The modest difference in potency is caused by a slight elevation in potency of the 1 mg/kg dose of 2-methylarachidonly-(2′-fluoroethyl)amide potency. Similar results were obtained in the tail-flick test with ED_{50} values (C.L.) in vehicle- and PMSF-pretreated mice of 2.6 (1.9–3.5) and 1.6 (1.0–2.6) mg/kg. The only difference in the two treated groups was an enhancement of the 3 mg/kg dose of 2-methylarachidonly-(2′-fluoroethyl)amide by PMSF.

Figure 7

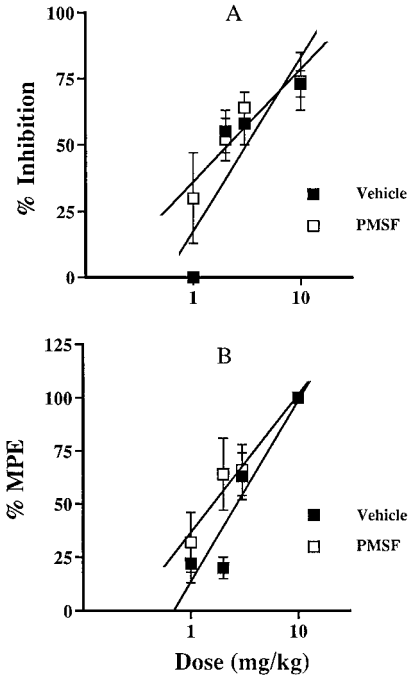

Fig. 7. Failure of PMSF to enhance the pharmacological activity of the metabolically stable 2-methylarachidonly-(2′-fluoroethyl)amide for inhibition of spontaneous activity (A) and antinocicepton (B). Mice were pretreated i.p. with either vehicle or PMSF (30 mg/kg) 10 min before the administration of the analog. The animals were tested for tail-flick response 5 min after the injection of the analog and spontaneous activity for 5 to 15 min. The results are presented as means ± S.E.M for at least six mice per group.

Discussion

The data presented in this manuscript are consistent with the hypothesis that the *in vivo* administration of amidohydrolase inhibitors like PMSF can prevent the rapid *in vivo* metabolism of anandamide. The result is a parallel shift to the left in the dose-response curves for anandamide on pharmacological measures such as antinociception, the inhibition of locomotor activity and the production of catalepsy. PMSF administration had no effect on the hypothermic effects of anandamide, however, which perhaps suggests that hypothermia, unlike the other measures of this tetrad of pharmacological measures, is not produced by anandamide directly, or perhaps that anandamide is still rapidly metabolized within the brain region that provides the neural substrates for this response. This could be caused by either incomplete inhibition of the unidentified amidohydrolase or by the existence of an isozyme or novel enzyme that can also metabolize anandamide, but there are no data to support this contention. The shift in potency of anandamide by PMSF pretreatment varied slightly, but was 5 to 10 times that of anandamide alone. The result was that the ED_{50} values obtained for anandamide fell into a 1 to 4 mg/kg range, almost exactly that observed for Δ^9-THC. The notion that PMSF enhances the potency of anandamide by diminishing its metabolism was supported by the findings that PMSF had little effect on the potency of 2-methylarachidonly-(2′-fluoroethyl)amide. Although PMSF has a dramatic effect on the binding affinity of anandamide in receptor binding assays, it has little influence on that of 2-methylarachidonly-(2′-fluoroethyl)amide (Adams *et al.*, 1995).

The dose of PMSF used in these studies to manipulate the pharmacological effect of anandamide was 30 mg/kg. Although PMSF produced effects on all four pharmacological tests, it did so only at doses of 100 mg/kg or greater. The only measure at which PMSF produced dose-responsive effects below 100 mg/kg was in antinociception, which necessitated the choice of the lower dose of 30 mg/kg for interaction studies with anandamide. These results are similar to the general depression, impairment of righting reflexes and unresponsiveness to noxious stimuli described by others (Turini *et al.*, 1969).

Additionally, the agonist activity of PMSF is not likely caused by actions at the cannabinoid receptor, because the antagonist SR141716A did not alter the PMSF responses. Thus, it seems unlikely that PMSF administration is either increasing levels of endogenous anandamide or another as-yet-unidentified endogenous cannabinoid ligand, or acting directly on the receptors. On the other hand, recent studies in our laboratory have failed to demonstrate SR 141716A-blockade of some of anandamide's pharmacological effects in mice (Adams, I. B., Compton, D. R. and Martin, B. R., unpublished observations). Therefore, it will be important to actually measure endogenous levels of anandamide in blood and brain after PMSF administration before a definitive conclusion can be reached. Such an endeavor is beyond the scope of the present investigation.

A possibility that cannot be ruled out is that PMSF enhancement of anandamide is caused by the inhibition of other enzymes. Inhibition of esterases, thus increasing levels of endogenous acetylcholine, could be responsible for some the effects observed after the administration of PMSF alone.

However, a PMSF dose of 85 mg/kg produces only about 30% inhibition of brain acetylcholinesterase activity, and this effect occurs 18 hours after drug administration (Moss *et al.*, 1985). Thus, it seems unlikely the interaction of 30 mg/kg of PMSF with anandamide is compromised by changes in esterase activity, but it does seem plausible that the effects observed at 100 to 300 mg/kg of PMSF could be caused by esterase inhibition.

Although the primary emphasis of the present investigation was directed toward inactivation of anandamide *via* hydrolysis, both inactivation and activation *via* other metabolic pathways is a possibility. Relatively little is known regarding the metabolic profile of anandamide. One report revealed that anandamide can serve as a substrate for brain lipoxygenase with the resultant product being 12-hydroxyanandamide (Hampson *et al.*, 1995). Studies in our own laboratories have demonstrated that there is a discordance between the time courses of brain levels of exogenously administered anandamide and pharmacological effects (Willoughby *et al.*, 1997). Essentially, brain levels of anandamide fell despite the persistence of pharmacological effects implying the formation of active metabolites. One cannot rule out the possibility that PMSF-blockade of anandamide results in increased formation of active metabolites, the actions of which may or may not be antagonized by SR 141716A.

In conclusion, the pharmacological effects of the enzyme inhibitor PMSF generally occur in the range of 100 mg/kg of drug or higher, with the exception of the antinociceptive properties which occur at lower doses. These effects cannot be attributed to direct actions at the cannabinoid receptor. An inactive dose of PMSF significantly increased the pharmacological activity of anandamide and produced parallel shifts to the left in the dose-response curves for all anandamide-mediated measures evaluated, except for hypothermia. The use of PMSF or other specific amidohydrolase inhibitors should be used to evaluate the *in vivo* potencies of anandamide analogs, just as for *in vitro* studies, to establish an appropriate correlation between *in vivo* potency and *in vitro* affinity to the cannabinoid receptor. It seems likely that the correlation between *in vivo* potency and receptor affinity for a variety of anandamide analogs could be enhanced with the inclusion of PMSF (Adams *et al.*, 1995). These studies underscore the importance of metabolism in the expression of pharmacological actions of anandamide.

Acknowledgments

The authors wish to thank Renee G. Jefferson and Ramona Winckler for their technical expertise.

References

Adams, I. B., Ryan, W., Singer, M., Thomas, B. F., Compton, D. R., Razdan, R. K. and Martin, B. R.: Evaluation of cannabinoid receptor binding and *in vivo* activities for anandamide analogs. J. Pharmacol. Exp. Ther. **273:** 1172–1181, 1995.

Childers, S. R., Sexton, T. and Roy, M. B.: Effects of anandamide on cannabinoid receptors in rat brain membranes. Biochem. Pharmacol. **47:** 711–715, 1994.

Desarnaud, F., Cadas, H. and Piomelli, D.: Anandamide amidohydrolase activity in rat brain microsomes. J. Biol. Chem. **270:** 6030–6035, 1995.

Deutsch, D. G. and Chin, S. A.: Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem. Pharmacol. **46:** 791–796, 1993.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R.: Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science **258:** 1946–1949, 1992.

Fride, E. and Mechoulam, R.: Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. Eur. J. Pharmacol. **231:** 313–314, 1993.

Hampson, A., Hill, W. A., Zan-Phillips, M., Makriyannis, A., Leung, E., Eglen, R. and Bornheim, L.: Anandamide hydroxylation by brain lipoxygenase: Metabolite structures and potencies at the cannabinoid receptor. Biochim. Biophys. Acta **1259:** 173–179, 1995.

Hillard, C. J., Edgemond, W. and Campbell, W.: Characterization ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: Application to anandamide. J. Neurochem. **64:** 677–683, 1995.

James, G. T.: Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Anal. Biochem. **86:** 574–579, 1978.

Moss, D. E. and Fahrney, D.: Kinetic analysis of differences in brain acetylcholinesterase from fish or mammalian sources. Biochem. Pharmacol. **27:** 2693–2698, 1978.

Moss, D. E., Rodriguez, L. A. and McMaster, S. B.: Comparative behavioral effects of CNS cholinesterase inhibitors. Pharmacol. Biochem. Behav. **22:** 479–482, 1985.

Smith, P. B., Compton, D. R., Welch, S. P., Razdan, R. K., Mechoulam, R. and Martin, B. R.: The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. J. Pharmacol. Exp. Ther. **270:** 219–227, 1994.

Turini, P., Kurooka, S., Steer, M., Corbascio, A. N. and Singer, T. P.: The action of phenylmethylsulfonyl fluoride on human acetylcholinesterase, chymotrypsin and trypsin. J. Pharmacol. Exp. Ther. **167:** 98–104, 1969.

Willoughby, K. A., Moore, S. F., Martin, B. R. and Ellis, E. F.: The biodisposition and metabolism of anandamide in mice. J. Pharmacol. Exp. Ther. **282:** 243–247, 1997.

Send reprint requests to: Billy R. Martin, Ph.D., P.O. Box 980613, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298-0613.