Anandamide (N-arachidonoylthanolamine) and six fatty acid ethanolamides were synthesized and their pharmacological effects in mice were assessed using catalepsy, hypothermia and pentobarbital-induced sleep prolongation as indices. The effects of phenylmethylsulfonyl fluoride (PMSF) pretreatment on anandamide effects were also evaluated and discussed in relation to inhibition of anandamide amidohydrolase in mouse brain and liver. The cataleptogenic effect of anandamide (ED$_{50}$=6.0 mg/kg, i.v.) was 4 to 6 times more active than those of N-oleoyl- (ED$_{50}$=26.5 mg/kg, i.v.) and N-linoleoyl ethanolamine (ED$_{50}$=37.5 mg/kg, i.v.), although the peak time in the effect was observed within 1 min after i.v. administration. None of the saturated fatty acid ethanolamides (N-myristoyl-, N-palmitoyl-, N-stearoyl- and N-arachidoyl ethanolamine) showed a positive response in the cataleptogenic effect even at a dose up to 40 mg/kg i.v. Anandamide, N-linoleoyl-, N-oleoyl- and N-myristoyl ethanolamine (10 mg/kg, i.v.) produced a significant hypothermia (0.19 to 0.59 °C) at 5 to 15 min after administration. The duration of the effects of these ethanolamides was also relatively short. Anandamide, N-linoleoyl-, N-oleoyl- and N-palmitoyl ethanolamine (5 or 10 mg/kg, i.v.) significantly prolonged pentobarbital-induced sleeping time by 148—207% of control sleeping time. The cataleptogenic effect of anandamide was markedly potentiated by pretreatment of mice with PMSF (100 mg/kg, i.p.). The ED$_{50}$ (mg/kg, i.v.) of anandamide was 0.48 (0.24—0.96) in PMSF-pretreated mice. The pretreatment of mice with PMSF significantly decreased the metabolic clearance rate of anandamide in microsomal fractions of liver and brain. Thus, the $V_{max}$/K$_m$ values of brain and hepatic microsomes were 26 and 10%, respectively, as compared with those of control mice. The present study demonstrated that anandamide and N-acyl ethanolamines of unsaturated fatty acids exhibited cannabinoid-like effects in mice, and that anandamide amidohydrolase has an important role in the pharmacological effects of anandamide in vivo.

Key words anandamide; catalepsy; hypothermia; sleep prolongation; phenylmethylsulfonyl fluoride; anandamide amidohydrolase

Anandamide (N-arachidonoylthanolamine) was isolated from porcine brain and identified as an endogenous ligand for the cannabinoid receptor. The amide was also isolated from calf brain and identified as a regulator of L-type calcium channels. Frade and Mechoulam demonstrated that anandamide produced the same pharmacological effects as those of tetrahydrocannabinol (THC), an active constituent of marijuana. Several studies also demonstrated that anandamide parallels Δ$^2$-THC in competing with the binding of agonists to cannabinoid receptors and inhibits adenylate cyclase. Anandamide was also reported to be a potent inhibitor of gap junction conductance and a vasorelaxant in the endothelium suggesting that the amide has a biologically important role in the cardiovascular system.

Hanus et al. identified other fatty acid ethanolamides, homo-y-linoleoyl ethanolamine and docosatetraenoylethanolamine, in porcine brain that bind the cannabinoid receptor and inhibit the electrophoretically evoked twitch response of the mouse isolated vas deferens similarly to anandamide and cannabinoids. In vitro structure-activities studies, Felder et al. suggested that the end pentyl chain may play some role similar to the pentyl side chain of THC. Adams et al. demonstrated that the level of saturation of the arachidonic acid moiety of anandamide was critical to receptor affinity. Pinto et al. suggested that the bulk and length of the arachidonic acid moiety are important for affinity of the anandamide analogs to the central cannabinoid receptor (CB1). Wise et al. reported that a conjugated triene derivative of anandamide bound to the cannabinoid receptor. Palmityl ethanolamine has been reported to be an agonist for the peripheral cannabinoid receptor (CB2) on mast cells and may produce an antinflammatory effect. These data suggest that anandamide and other fatty acid ethanolamides may have some cannabimimetic effects, although more extensive pharmacological studies in vivo of fatty acid ethanolamides are required to give conclusive evaluation of the structural importance of anandamide.

Studies from several laboratories have shown that anandamide is mainly degraded by hydrolysis with membrane-bound enzyme, anandamide amidohydrolase. Anandamide has been thought to be rapidly hydrolyzed by the in vivo system, since the pharmacological effects of anandamide are produced with rapid onset and a rather short duration of action. (R)-Methanandamide, a metabolically more stable derivative of anandamide, exhibited greater pharmacological effects such as hypothermia, hypokinesia, antinociception, ring immobility in mice than those produced by anandamide. Phenylmethylsulfonyl fluoride (PMSF) was found to be a potent inhibitor of the amidohydrolase. The addition of PMSF significantly decreased the K$_m$ value.

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for anandamide in competing \(^{[1]}\)HCP55940 binding to the cannabinoid receptor in rat brain P2 membrane preparations.\(^{23}\) These data suggest that PMSF may potentiate the biological effects of anandamide in vivo. Recently, Compton and Martin\(^{22}\) reported that PMSF potentiated antinociception and immobility produced by anandamide, although PMSF itself showed some pharmacological effects at rather high doses. However, the limited data available at present with respect to in vivo hydrolysis of anandamide and its pharmacological effects hinders the assessment of the importance of peripheral and central anandamide amidohydrolase in mediating the biological effects of anandamide.

The present paper describes the pharmacological effects of anandamide and six fatty acid ethanolamides, and the effects of pretreatment with PMSF on the cataleptogenic effect of anandamide in relation to inhibition of anandamide amidohydrolase in mouse brain and liver.

MATERIALS AND METHODS

**Pharmacological Experiments** Male ddY mice weighing 20 to 30 g were used for the experiments. The animals received food and water ad libitum. The pharmacological experiments were carried out at ambient temperature of 22 to 24 °C.

**Catalepsy** The cataleptogenic effect was assessed by the simple bar test as described previously\(^{23}\) after i.v. injection of anandamide and other six fatty acid ethanolamides at various time intervals. Mouse front paws were placed on a horizontal bar (0.5 cm in diameter), 5—6 cm height, and the mouse was forced to stand on its hind legs. When the mouse kept the unusual position for more than 30 s, the cataleptogenic effect was regarded to be positive. ED\(_{50}\) values of test compounds were evaluated by the method of Litchfield-Wilcoxon\(^{24}\) by using at least 3 different doses (5 to 40 mg/kg i.v.). Five groups consisting of 8 mice were used for the time course experiments at each time point (1, 5, 10, 30 and 60 min) for anandamide.

**Hypothermia** Rectal temperature of mice was measured with a thermometer (Natsume Seisakusho Ltd., Tokyo), recording for 30 min after i.v. injection of test compounds as described previously.\(^{23}\) The control mice received 1% Tween 80-saline only.

**Pentobarbital-Induced Sleep** Sodium pentobarbital (40 mg/kg, i.p.) was injected 1 min after i.v. injection of anandamide and other six fatty acid ethanolamides (5 or 10 mg/kg i.v.). The sleeping time was measured as loss of the righting reflex.\(^{23}\) Each group consisted of 8 to 25 mice.

**Cataleptogenic Effect in PMSF-Treated Mice** Mice were pretreated with PMSF at a dose of 100 mg/kg i.p. Mice pretreated with the dose of PMSF did not show any positive cataleptogenic effect, although Compton and Martin\(^{22}\) reported that PMSF reduced spontaneous activity of mice at doses greater than 100 mg/kg. Anandamide (10 mg/kg i.v.) was injected into 6 groups consisting of 8 mice each. In the time course experiments, the cataleptogenic effect was assessed at 1, 5, 10, 30, 60 and 90 min after the anandamide injection. ED\(_{50}\) values for anandamide in PMSF-treated mice was determined by the same procedure described as above, but the dose range was 1 to 10 mg/kg i.v.

**Drugs** Anandamide and its related fatty acid ethanolamides (N-myristoyl-, N-palmitoyl-, N-stearoyl-, N-oleyl-, N-linoleoyl-, N-arachidoyl-) were synthesized by the method described.\(^{26}\) Fatty acid chlorides were prepared by reacting free fatty acids with oxalyl chloride and then reacted with ethanolamine. The N-acylthanolamines synthesized were purified by column chromatography using a solvent system of methylene chloride/methanol (50:1). Sodium pentobarbital and PMSF were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and Sigma Chem. Co., respectively. Sodium pentobarbital (40 mg/kg) and PMSF (100 mg/kg) were dissolved in physiological saline and olive oil, respectively, and injected i.p.

**Assay of Anandamide Amidohydrolase Activity** Liver and brains of mice were removed at 60 min after i.p. injection of olive oil (control) or PMSF (100 mg/kg i.p.). Hepatic and brain microsomes were prepared from control and PMSF-pretreated ddY male mice. Anandamide amidohydrolase activity was assayed as described previously.\(^{27}\) A typical incubation mixture consisted of microsomes (0.02 g liver or 0.05 g brain equivalent) and anandamide (10—230 μM) in sodium potassium phosphate buffer (pH 7.4) to make a final volume of 1 ml. The incubations were carried out at 37°C for 20 min. The arachidonic acid formed was extracted with n-hexane (4 ml) after addition of 0.5 ml of 1 M KH\(_2\)PO\(_4\) and an internal standard (methyl linolate, 10 μg). The n-hexane extract was methylated with diazomethane in ethyl ether, and analyzed by GC.\(^{21,27}\)

**Analysis of Data** The data are expressed as means and limits of errors as standard errors for the hypothermic effect and pentobarbital-induced sleep prolongation. The ED\(_{50}\) values and their 95% confidence limits in the cataleptogenic effects of anandamide and fatty acid ethanolamides were calculated by the method of Litchfield-Wilcoxon.\(^{23}\) The effects of anandamide and other fatty acid ethanolamides on the body temperature of mice were evaluated by subtracting rectal temperature measured just before drug administration and thereafter for 30 min. The significance of difference between means was evaluated by ANOVA, and individual data were compared by F-test for the hypothermic effect and pentobarbital-induced sleep prolongation. The significance of differences in the time course experiment of cataleptogenic effect between control and PMSF-treated groups was calculated by \(\chi^2\)-test.

**RESULTS**

The time course in the cataleptogenic effect of anandamide is shown in Fig. 1. At the dose of 10 mg/kg i.v., anandamide exhibited a positive response in 75% of the mice at 1 min after the injection. The effect rapidly attenuated and none of the mice showed a positive response at 30 min after the injection of anandamide. Thus, the peak time in the cataleptogenic effect of anandamide was observed at 1 min after the injection. Therefore, the cataleptogenic effects of anandamide and other fatty acid ethanolamides were assessed at 1 min after their injections. The ED\(_{50}\) (with their 95% confidence limits) of anandamide, N-oleyl- and N-linoleylethanolamine were 6.0 (4.1—8.7), 37.5 (25.9—54.4) and 26.5 (20.7—33.9) mg/kg, respectively (Table 1). However, saturated fatty acid ethanolamides (N-myristoyl-, N-palmitoyl-, N-stearoyl- and N-arachidoylthanolamine) did not show any
positive cataleptic effect in mice even at a dose up to 40 mg/kg i.v.

The effects of anandamide and its related fatty acid ethanolamides (10 mg/kg, i.v.) on the rectal temperature of mice are illustrated in Fig. 2. Anandamide exhibited the highest potency in this parameter, while N-myristoyl-, N-oleoyl- and N-linoeloylethanolamine caused a significant hypothermic effect at 5 to 15 min after their injections. The maximal hypothermia produced were 0.59±0.08 (anandamide, 10 min), 0.39±0.07 (N-oleoylethanolamine, 5 min), 0.32±0.07 (N-linoeloylethanolamine, 5 min) and 0.19±0.07°C (N-myristoylethanolamine, 5 min). However, their hypothermic effects lasted only for 15 min and a significant hypothermia was not observed at 30 min after the administration. N-Palmitoyl- and N-arachidoylethanolamine did not show a significant hypothermic effect at 10 mg/kg, i.v.

The effects of anandamide and other fatty acid ethanolamides on pentobarbital-induced sleeping time are shown in Table 2. Five mg/kg i.v. of anandamide and N-oleoylethanolamine significantly prolonged pentobarbital-induced sleeping time, although other fatty acid ethanolamides examined in the present study did not prolong significantly the sleeping time at the same dose. At 10 mg/kg, i.v., anandamide, N-palmitoyl-, N-oleoyl- and N-linoeloylethanolamine significantly prolonged the sleeping time by 207, 145,
acid ethanolamides (N-myristoyl-, N-palmitoyl-, N-stearoyl- and N-arachidoyl-) produced little effects. Among fatty acid ethanolamides tested, anandamide showed the highest activity in all pharmacological indices. Frade and Mechoulam\(^3\) have reported the hypothermic effect of anandamide in mice. At a dose of 10 mg/kg i.p., maximal hypothermia produced by anandamide was reported to be 1.62 °C. However, the maximal hypothermia by anandamide was 0.59 °C in the present study. The discrepancy between the present result and the previous one may reflect some differences in the experimental conditions used. N-Oleoyl- and N-linoleoylthanolamine exhibited the pharmacological effects some extent, whereas ethanolamides of saturated fatty acids were far more less active. Felder et al.\(^7\) suggested the end pentyl chain of anandamide might play an important role in the receptor binding. Adams et al.\(^12\) also reported that the levels of saturation of anandamide in the fatty acid moiety were critical to receptor affinity and in vivo pharmacological effects. A loosed conformation of anandamide analogs has been suggested to be energetically favourable, and there is some structural similarity of anandamide to Δ^9-THC.\(^29\) These findings and the present results conclusively indicate that saturation of the fatty acid moiety is one of most important factors in exhibiting cannabimimetic effects for the anandamide analogs. The pharmacological experiments indicate that the effects of anandamide, N-oleoyl- and N-linoleoyl ethanolamine are qualitatively similar to those of THC, although the potency of these N-acylethanolamines is less than those of THCs reported previously from our laboratory under similar conditions.\(^25\)\(^28\) N-Palmitoylthanolamine exhibited a significant effect only on the prolongation of pentobarbital-induced sleep indicating that the ethanolamide may have some different effects from the other N-acylethanolamines examined in the present study.

The onset of catalepsy by anandamide was very rapid, and the ED_{50} value for anandamide was about half as active as those of Δ^9- and Δ^8-THC.\(^25\)\(^30\) Mechoulam et al.\(^31\) and Smith et al.\(^38\) reported that the pharmacological effects in mice of anandamide were less potent than those of Δ^9-THC. The rapid onset in the cataleptogenic effect indicates that anandamide rapidly crosses the blood-brain barrier. Thus, the peak time in the effect of anandamide was around 1 min after i.v. administration and faster than those of THC and its metabolites reported previously.\(^23\)\(^25\) Smith et al.\(^18\) reported that maximal antinociception was observed immediately after administration of anandamide in mice. The peak time (5 to 15 min) of anandamide in hypothermic effect is also rapid onset as compared with that of THC reported previously.\(^23\)\(^32\) The effect of anandamide rapidly disappeared after the injection, suggesting the rapid degradation or excretion of anandamide in mice in vivo. Crawley et al.\(^35\) reported that the sedative effect of anandamide was shorter in duration of action than that of THC in rats. Abadji et al.\(^28\) reported that (R)-methanandamide, possessing remarkable stability to hydrolysis, produced a relatively higher activity to inhibit electrically evoked contractions of the mouse vas deferens. These results suggest that anandamide is rapidly hydrolyzed in vivo to arachidonic acid and ethanolamine. Willoughby et al.\(^19\) reported that anandamide was quickly distributed, and bio-transformed to arachidonic acid and polar metabolites in brain of mice. They suggested that rapid metabolism of anan-
damide in brain may play a key role in the time course of its pharmacological effects. Anandamide amidohydrolase, which is the main enzyme to hydrolyze anandamide, is known to be present in liver and brain of mice.\(^2\) The presence of the enzyme in brain suggests that anandamide penetrating into brain from peripheral tissues by i.v. administration may also be rapidly hydrolyzed for inactivation.

It is well known that in vitro affinity of anandamide for the cannabinoid receptor is increased by PMSF, a potent inhibitor of anandamide amidohydrolase.\(^5\) At a concentration of 0.5 mm, PMSF decreased the \(K_d\) of anandamide for rat brain membrane from 1.3 \(\mu\)M to 143 \(\mu\)M. To examine the importance of anandamide amidohydrolase on the in vivo effects of anandamide, the catecholgenic effect of anandamide was assessed in mice pretreated with PMSF. The catecholgenic effect of anandamide was markedly enhanced by PMSF-pretreatment together with prolonging the duration of the effect. Recently, Compton and Martin\(^1\) reported that PMSF (30 mg/kg i.p.) highly potentiated the effects of anandamide on antinociception, spontaneous activity and mobility in mice. Kinetic parameters (\(V_{max}/K_m\)) for anandamide amidohydrolase in brain and liver of mice pretreated with PMSF were significantly decreased indicating a remarkable decrease in clearance of anandamide causing the high potentiation of the anandamide effect by PMSF-treatment.

The present study demonstrated that the short duration of action of anandamide is due to its rapid degradation by anandamide amidohydrolase, which has an important role for the regulation of the biological effect of anandamide in mice. Other possibilities that PMSF acts on other neuromodulatory molecules which may contribute to the enhancement of the anandamide effects by PMSF must also be evaluated.

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