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The Pharmacological Activity of Anandamide, a Putative Endogenous Cannabinoid, in Mice

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ABSTRACT

The arachidonic acid derivative anandamide (arachidonylethanolamide) has been isolated from porcine brain and has been shown to bind competitively to the cannabinoid receptor. Although the pharmacological activity of this compound has not yet been fully determined, preliminary data suggest that it produces several effects similar to the cannabinoids. In the present experiments anandamide produced effects similar to those of Δ^9-tetrahydrocannabinol, including antinociception (as determined in a latency to tail-flick evaluation), hypothermia, hypomotility and catalepsy in mice after i.v., i.t. and i.p. administration. In general, the effects of anandamide occurred with a rapid onset, but with a rather short duration of action. Prominent antinociceptive effects (>80% maximal possible effect) were measured immediately after i.v. and i.t. administration. Anandamide produced significant decreases in rectal temperature (2–4°C) after either i.v. or i.t. injection. Maximal effects on motor activity (approximately 85% inhibition) were observed immediately after i.v. and i.p. administration and 10 min after i.t. administration. Maximum immobility observed after i.v. administration was over 80%, yet that produced after i.p. and i.t. administration was too small (≤20%) to be considered pharmacologically relevant. Anandamide was less potent (1.3 to 18 times) than Δ^9-tetrahydrocannibinol in all behavioral assays. Pretreatment with *nor*-binaltorphimine, a *kappa* opioid antagonist which blocks i.t. Δ^9-tetrahydrocannibinol-induced antinociception, failed to alter antinociception after i.t. anandamide administration. Binding studies demonstrated that anandamide displaces [^{3}H]CP-55,940 from rat whole brain P_2 membrane preparations with a K_D of 101 ± 15 nM. These findings demonstrate that anandamide produces effects in a tetrad of tests used to predict cannabimimetic activity and supports the contention of its role as an endogenous cannabinoid ligand. However, there appear to be distinct differences between anandamide and the cannabinoids with regard to their antinociceptive properties, and other properties vary as a function of route of administration.

Cannabinoids are a distinct class of psychoactive compounds which produce a wide array of effects in a large number of species. Until recently the mechanism by which the cannabinoids produce these effects was unknown. The discovery of the cannabinoid receptor was long hampered by lack of suitable radiolabeled ligand. However, with the advent of the extremely potent synthetic drug CP-55,940 a high affinity binding site has been characterized in rat cortical membranes which binds cannabinoids selectively (Devane *et al.*, 1988). Additional evidence has demonstrated that this CP-55,940 binding site is sensitive to guanine nucleotides, suggesting a G-protein-coupled receptor. This receptor has also been cloned, and its amino acid sequence has been found to be consistent with other members of the G-protein receptor super-family (Matsuda, 1992). Additionally, this receptor is found in brain and neural cell lines, and the binding of cannabinoids to this site inhibits adenylyl cyclase in an enantioselective, dose-dependent manner (Howlett *et al.*, 1986). At this time, the only confirmed cellular effect of cannabinoid binding to the cannabinoid receptor is the inhibition of adenylyl cyclase. However, preliminary data suggest that G-protein linked K^+ channel receptors may also be coupled to these receptors (Childers *et al.*, 1992).

The structure activity profile suggests that the site which binds CP-55,940 is identical to the receptor that mediates many of the pharmacological and behavioral effects of the cannabinoids (Compton *et al.*, 1993). All other psychoactive drugs, neurotransmitters, steroids and eicosanoids that have been tested have failed to bind to this receptor at physiologically relevant concentrations (Howlett *et al.*, 1992). Autoradiographic studies have demonstrated that there is a heterogeneous distribution of the cannabinoid receptor in brain (Herkenham *et al.*, 1991). This distribution pattern conforms to

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ABBREVIATIONS: THC, tetrahydrocannabinol; DMSO, dimethyl sulfoxide; i.t., intrathecal; i.v., intravenous; i.p., intraperitoneal; *nor*-BNI, *nor*-binaltorphimine; dH_2O, distilled water; ICI 174,864, N,N-diallyl-Try-Alb-Phe-Leu; %MPE, percent maximal possible effect; PMSF, phenylmethylsulfonylfluoride; %IMM, percent immobility; CB-R, cannabinoid receptor; Δ°C, change in rectal temperature in degrees Celsius.

cytoarchitectural and functional subdivisions in the brain and is unique to the cannabinoids. Cannabinoid receptor patterns are similar across several mammalian species, including human, suggesting phylogenetic stability and evolutionary conservation (Matsuda *et al.*, 1990; Gérard *et al.*, 1991). The location of cannabinoid receptors correlates well to pharmacological effects of the drug. High densities of receptors are in the hippocampus and cerebral neocortex, suggesting involvement in cognition and thought processing. High densities are present in GABAergic striatal neurons in the basal ganglia and in the glutaminergic granule cells of the cerebellum, implicating a modulatory role in movement. Finally, very sparse densities in the lower brainstem (in areas controlling cardiovascular and respiratory functions) would explain why high doses are not generally lethal (Herkenham *et al.*, 1991).

Arachidonylethanolamide, more commonly known as anandamide, is an ethanolamine derivative of arachidonic acid which was first isolated in porcine brain (Devane *et al.*, 1992). The structure of this compound (fig. 1) has been determined by both mass spectrometry and nuclear magnetic resonance spectroscopy. Several lines of evidence suggest that anandamide may function as an endogenous ligand for the cannabinoid receptor. Anandamide competitively inhibited the specific binding of [^{3}H]HU-243, a radiolabeled cannabinoid probe, to synaptosomal membranes and produced a dose-dependent inhibition of the electrically evoked twitch response in the mouse vas deferens (Devane *et al.*, 1992). In preliminary studies, it also produced effects in the ring immobility test, open field test, rectal temperature assay and hot-plate test after i.p. administration (Fride and Mechoulam, 1993).

At the present time, the pharmacological profile of anandamide is inadequate. The goals of this study were to characterize more completely the pharmacological effects of anandamide *in vivo* and *in vitro*, and compare those effects with that of Δ^9-THC, the prototypical cannabinoid. Specifically, the potency, onset and duration of action of anandamide were determined after different routes of administration. Additionally, binding characteristics were determined. Ultimately, this work permitted us to verify that there are distinct similarities and differences between anandamide and Δ^9-THC.

Materials and Methods

Supplies. Male ICR mice (Dominion 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 was synthesized as described earlier (Devane *et al.*, 1992). Δ^9-THC was obtained from the National Institute on Drug Abuse. Both compounds were dissolved in 100% DMSO for i.t. administration and 1:1:18 (emulphor-ethanol-saline) for i.v. and i.p. admi[n]istration. Emulphor (EL-620, a polyoxyethylated vegetable oil, GA[F] Corporation, Linden, NJ) is currently available as Alkmulphor. *no*[r]BNI (Research Biochemicals Incorporated, Natick, MA) and naloxo[ne] hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved [in] dH_2O before injection. ICI 174,864 (Cambridge Research Biochemica[ls,] Cambridge, England) was prepared in saline. A standard procedure w[as] used for i.t. injection (Hylden and Wilcox, 1983). A 1/2 inch, 30 1/[2] gauge needle attached to a 25 μl Hamilton syringe was used to inject [5] μl of solution in the area of L_{5-6}. Both i.v. (tail vein) and i.p. injectio[ns] were administered at a volume of 0.1 ml/10 g b.wt. Mice were acclimat[ed] in the evaluation room overnight without interruption of food a[nd] water. After drug administration each animal was tested for effects [on] either tail-flick response, rectal temperature, catalepsy or motor acti[v]ity.

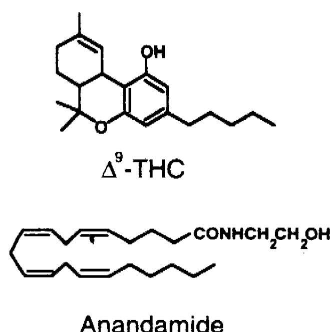

Fig. 1. The structures of Δ^9-THC and anandamide.

Evaluation for purity and authenticity. Samples containing [..] μg of anandamide in absolute ethanol were applied to silica gel th[in] layer chromatography plates and developed in solvent containing 5 [ml] of MeOH, 95 ml of $CHCl_3$ and 0.1 ml of acetic acid. Anandamide w[as] visualized with iodine vapor. Anandamide samples provided a sing[le] spot with an R_f value of 0.45. Arachidonic acid had an R_f value of 0.6[..] Samples of anandamide (2 μg) were analyzed by a Hewlett Packa[rd] Mass Spectrometer model 5988A using direct probe. The EI energy w[as] 70 cV and the source temperature was 200°C. The probe was heated [to] the source temperature for the first 5 min, after which the pro[be] temperature was increased at the rate of 5°C/min for the remainder [of] the analysis. A 40 to 400 a.m.u. scan was performed. The resulti[ng] mass spectrum was identical to that reported previously (Devane *et a[l.]*, 1992), except that the parent peak was observed under these condition[s.] Nevertheless, comparison of material in this original report to th[at] subsequently synthesized for these studies indicated identical spe[c]trums.

Antinociception. Antinociception was assessed using the tail-fli[ck] test (D'Amour and Smith, 1941; Dewey *et al.*, 1970). The heat lamp [of] the tail-flick apparatus was maintained at an intensity sufficient [to] produce control latencies of 2 to 4 sec. Control values for each anim[al] were determined before drug administration. Mice were then re-test[ed] after drug injection and latencies to tail-flick response were recorde[d.] A 10-sec maximum was imposed to prevent tissue damage. The degr[ee] of antinociception was expressed as the %MPE which was calculat[ed] as:

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

Hypothermia. Base-line rectal temperatures were determined b[e]fore drug or vehicle injection with a telethermometer (Yellow Sprin[gs] Instrument Co., Yellow Springs, OH) and a thermistor probe insert[ed] to 25 mm. Rectal temperatures were again measured after the injectio[n.] The difference between pre- and postinjection values was calculat[ed] for each animal.

Catalepsy. Catalepsy was determined by a modification of the ri[ng] immobility test (Pertwee, 1972). Mice were placed on a ring (5.5 cm [in] diameter) that was attached to a stand at a height of 10 cm. T[he] amount of time (sec) that the mouse spent motionless during a 5-m[in] test session was recorded. The criterion for immobility was the absen[ce] of all voluntary movements (excluding respiration, but including whi[s]ker movement). The immobility index was calculated as:

$$\%\ \mathrm{IMMOBILITY} = \left[\frac{\text{amount of time immobile}}{\text{length of test session}}\right] \times 100.$$

Mice that fell or actively jumped from the ring were allowed five suc[cessive] escapes. Following the fifth escape, the test for that animal was te[r]minated and immobility was calculated as a percentage of time that [it] remained on the ring before being discontinued. Data from mice faili[ng] to remain on the ring at least 2.5 min were not included.

Spontaneous activity. Mice were placed into individual activi[ty] cages (6.5 × 11 in) 5 min postinjection, and interruptions of t[he]

photocell beams (16 beams per chamber) were recorded for a 10-min period using a Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH). Activity in the chamber was expressed as the total number of beam interruptions.

Time course studies. Animals were injected either i.v., i.t. or i.p. with anandamide, Δ^9-THC or vehicle. At selected time points (1, 5, 10, 15, 20, 30, 40, 60, 90, 120 or 180 min) after injection, the animals were tested in 1 of the 4 assays including tail-flick, rectal temperature, ring immobility and spontaneous activity. Different mice were used for each test and time point.

Dose-response studies. Animals were injected either i.v., i.p. or i.t. with anandamide, Δ^9-THC or vehicle. Using the data from the time course studies, the mice were evaluated for each measure at a time in which maximal effects were demonstrated. After i.t. administration of anandamide, tail-flick latencies were measured at 1 min, rectal temperature at 5 min, spontaneous activity at 10 to 20 min and ring immobility at 10 to 15 min. After i.v. and i.p. administration of anandamide, tail-flick latencies were measured at 5 min, spontaneous activity at 5 to 15 min, ring immobility at 5 to 10 min and rectal temperature at 15 min.

Receptor binding. [^{3}H]CP-55,940 (K_D = 690 pM) 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 anandamide with 1 nM of [^{3}H]CP-55,940. The nonspecific enzyme inhibitor PMSF was added to the preparations in order to limit enzymatic destruction of anandamide as indicated elsewhere (Childers *et al.*, 1994). The assays were performed in triplicate, and the results represent the combined data from three individual experiments.

Statistical analysis. Statistical analysis of all *in vivo* data was performed using ANOVA with Dunnett's *t* test for comparison to vehicle or Scheffe's F test for comparisons among all groups. The ED_{50} values were calculated from dose-response curves analyzed using ALLFIT (De Lean *et al.*, 1987), a program for the simultaneous nonlinear fitting of a family of sigmoidal curves, and statistical comparisons between anandamide and THC curves (including parallelism) was evaluated within this application. The K_i value for anandamide was calculated from displacement data using EBDA (Equilibrium Binding Data Analysis; Biosoft, Milltown, NJ).

Results

Time course and potency after i.v. administration. The time course of anandamide effects after i.v. administration is illustrated in figure 2. The results of the tail-flick assay (fig. 2A) show that anandamide produces a pronounced antinociceptive effect, and has an immediate onset when administered by this route. Significant antinociception continued until approximately 2 hr after the i.v. injection. Maximal hypoactivity (fig. 2B) was observed during the first testing interval as demonstrated by an 85% decrease in motor activity from that of vehicle-treated animals. Motor activity was not significantly different than vehicle at the 90 min time point. The hypothermic effect of anandamide (fig. 2C) had a somewhat shorter duration of action. A decrease of 2.2°C was measured immediately after injection and hypothermia lasted for approximately 30 min after injection. Effects in the ring immobility assay (fig. 2D) also were observed immediately after anandamide administration, but had essentially disappeared by 30 min. An immobility index of 68% was measured immediately after i.v. anandamide. In order to compare the anandamide time course to that of a prototypical cannabinoid, a dose of Δ^9-THC (3 mg/kg, which was approximately equieffective to that of 50 mg/kg of anandamide) was evaluated. As can be seen in figure 2, the time courses of Δ^9-THC and anandamide are similar for antinociception and hypothermia. Δ^9-THC has a somewhat longer duration of action for inhibition of spontaneous locomotor activity and immobility.

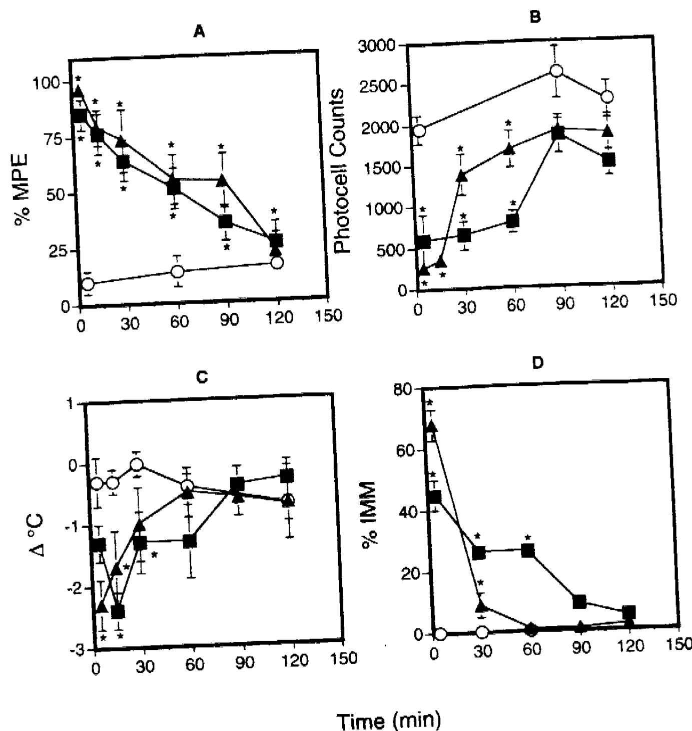

Fig. 2. The time course effects of either 50 mg/kg of anandamide (▲), 3 mg/kg of Δ^9-THC (■) or vehicle (O) after i.v. administration on tail-flick latency (A), motor activity (B), rectal temperature (C) and ring immobility (D). Animals received a single i.v. injection of either vehicle (1:1:18), Δ^9-THC or anandamide and were tested once at the indicated time. The means ± S.E. (N = 12) are presented. *Values found to be significantly different (P < .05) than groups receiving vehicle using Dunnett's *t* test.

Establishment of the time course for the anandamide effect allowed for potency determinations to be obtained during periods of maximal activity. The potency determination of anandamide after i.v. injection is presented in figure 3. Anandamide produced dose-related antinociceptive effects throughout the dose range tested. The ED_{50} of anandamide in the tail-flick assay was calculated to be 6.2 mg/kg, which was several-fold smaller than the ED_{50} values in the other three behaviors evaluated. In addition to antinociception, anandamide produced effects in the 3 other assays which were statistically significant at doses of 25 mg/kg and higher. Anandamide produced significant decreases in motor activity with an ED_{50} calculated to be 17.9 mg/kg. Profound hypoactivity (>85%) was observed at the highest dose tested (75 mg/kg). A maximal decrease in rectal temperature of 3.1°C was measured after 75 mg/kg of anandamide. The ED_{50} of anandamide in the rectal temperature assay was 26.5 mg/kg, which was greater than those in the other three measures. Prominent effects were observed in the ring immobility assay with a peak immobility index of 88% observed at the 75 mg/kg dose, and an ED_{50} of 19.1 mg/kg.

Time course and potency after i.p. administration. The time course of 25 mg/kg of anandamide given i.p. is shown in figure 4. Unlike the time course after i.v. administration, anandamide only produced a maximal %MPE of 43%, which occurred 5 min after i.p. injection. Tail-flick latencies only decreased slightly, remaining above 25% MPE, for up to 120 min after drug administration. In the spontaneous activity test,

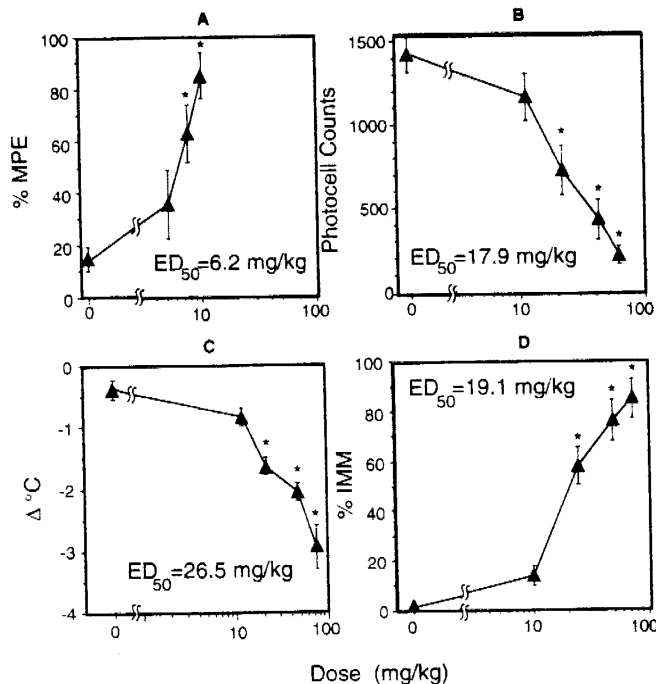

Fig. 3. The dose responsiveness of i.v. administered anandamide tested for tail-flick latency at 5 min (A), motor activity at 5 to 15 min (B), rectal temperature at 15 min (C) and immobility at 5 to 10 min (D). Animals received a single i.v. injection of either vehicle (1:1:18) or anandamide. The means ± S.E. (N = 12) are presented. *Values found to be significantly different (P < .05) than groups receiving vehicle as determined by Dunnett's t test.

profound hypoactivity (84% inhibition) was observed immediately after anandamide administration, but quickly subsided and was not significantly different than vehicle-treated animals at 30 min Maximal hypothermia occurred 15 min after anandamide administration with a decrease of approximately 2°C from base-line temperatures. Anandamide produced only weak effects in the ring immobility test (similar to that seen after i.t. administration). A maximal immobility index of 17% was measured immediately after injection. Ring immobility after i.p. anandamide was not significantly different than vehicle at 15 min.

The lack of pronounced effects of anandamide after i.p. administration except for locomotor inhibition is in sharp contrast to the data obtained after 100 mg/kg of i.p. Δ^9-THC. In contrast to anandamide, the effects for Δ^9-THC are robust in all four measures. Additionally, Δ^9-THC clearly produces maximum effects for up to 60 min after administration, by which time there remains no anandamide effect on locomotion, and the remaining antinociception is minimal (<25% MPE). The Δ^9-THC effects return to control levels by 8 hr postadministration.

The dose-response of i.p. anandamide observed 5 min after administration is illustrated in figure 5. No ED_{50} values are calculated. The anandamide response in the tail-flick procedure never attained a 50% MPE level. The moderate degree of antinociception attained (approximately 40% MPE), in contrast to the nearly 100% obtained for Δ^9-THC (fig. 4), suggests that i.p. anandamide has limited pharmacological activity by this route of administration. This contention is supported by the fact that there is limited hypothermia and immobility (<2°C and <25%, respectively), plus the fact that the observed hypothermia is not dose-responsive. These data suggest that the i.p.

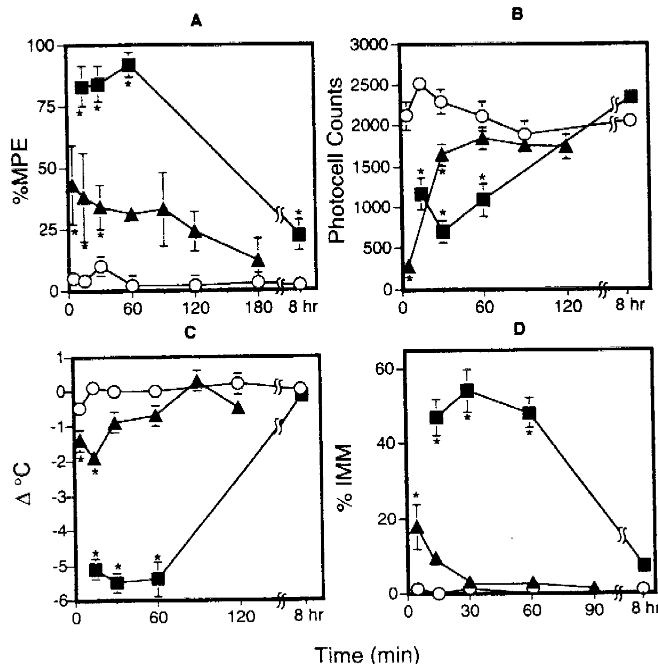

Fig. 4. The time course of effects of i.p. administered vehicle (○), 100 mg/kg of Δ^9-THC (■) and 25 mg/kg of anandamide (▲) on tail-flick latency (A), motor activity (B), rectal temperature (C) and ring immobility (D). The means ± S.E. (N = 12) are presented. *Values found to be significantly different (P < .05) than groups receiving vehicle using Scheffe F-test.

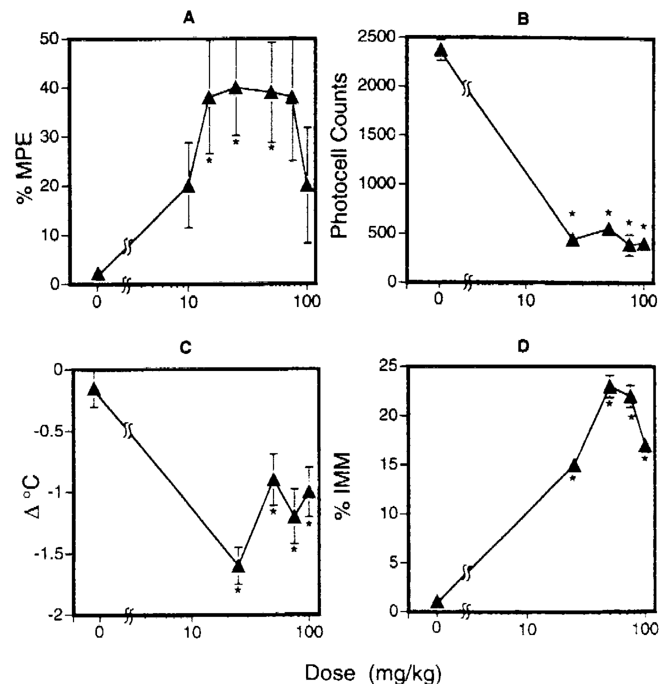

Fig. 5. The dose responsiveness of i.p. administered anandamide tested for tail-flick latency at 5 min (A), motor activity at 5 to 15 min (B), rectal temperature at 15 min (C) and immobility at 5 to 10 min (D). Animals received a single i.p. injection of either vehicle (1:1:18) or anandamide. The means ± S.E. (N = 12) are presented. *Values found to be significantly different (P < .05) than groups receiving vehicle as determined by Dunnett's t-test.

route is not one by which anandamide is likely to produce cannabimimetic responses. Inexplicably, i.p. anandamide produced profound inhibition of locomotor activity (approximately 85%) at all doses tested. Because anandamide effects after i.p. administration cannot be characterized as cannabimimetic, no attempts were made to characterize further the sedative effects of anandamide on locomotor activity.

Time course and potency after i.t. administration. The time courses of both 100 μg of anandamide and 50 μg of Δ^9-THC after i.t. administration are illustrated in figure 6. In the tail-flick assay, Δ^9-THC demonstrated very potent antinociceptive effects with a maximal %MPE of 92% at 10 min. Antinociceptive effects significantly greater than vehicle-treated animals continued from 1 to 90 min after i.t. injection. In contrast, a 100 μg dose of anandamide was less potent and had a faster onset. The peak %MPE of 63% was measured immediately after i.t. injection. The antinociceptive effect of anandamide then quickly decreased and was not significantly different than vehicle-treated animals 20 min after i.t. administration. In the spontaneous activity test, Δ^9-THC demonstrated a maximal drop in motor activity 20 min after injection, and this effect continued for approximately 90 min, at which time the effect was no longer significantly different than the vehicle group. Anandamide produced smaller decreases in motor activity which were statistically significant at the 5 and 10 min time points. Δ^9-THC produced a maximal decrease in rectal temperature (4.2°C) 20 min after i.t. injection. Anandamide produced a maximal drop (2.3°C) 5 min after i.t. administration and this effect was significantly different than vehicle at 1, 5 and 10 min after injection. Finally, Δ^9-THC produced very potent effects in the ring immobility test with a maximal immobility index (78%) 20 min after i.t. administration. These effects in ring immobility were statistically significant 90 min after injection. The immobility index attained after anandamide administration was 29%, measured 10 min after injection. However, this degree of immobility was not reproduced in subsequent dose-response studies (fig. 7), where the maximum effect obtained (at 10 min postinjection) was only 12% at the 200 μg dose, and was not statistically significant. Thus, immobility cannot be reliably produced by anandamide after i.t. administration.

The dose responsiveness of i.t. administered anandamide is shown in figure 7. Similar to results after i.v. administration, i.t. administration produced robust antinociception with a maximal MPE of 93% after a dose of 200 μg of anandamide. Statistically significant antinociceptive effects were observed at doses of 100 μg and higher. The ED_{50} of i.t. anandamide in the tail-flick assay was 51.8 μg (approximately 3 mg/kg). In the spontaneous activity test, significant decreases in motor activity were measured at doses of 50 μg and higher, and the ED_{50} value was 43.6 μg. Significant hypothermic effects were measured at doses of 50 μg and higher with an ED_{50} of 58.2 μg. However, unlike those results after i.v. administration, i.t. administered anandamide produced very little effect in the ring immobility assay. The highest dose of anandamide tested, 200 μg, did not produce a statistically significant increase in ring immobility.

Antagonism studies with *nor*-BNI. Recent experiments have demonstrated that an i.t. pretreatment with *nor*-BNI (potent *kappa* opioid antagonist) blocked cannabinoid-induced antinociception, whereas naloxone (nonselective antagonist) and ICI 174,864 (*delta* opioid antagonist) failed to block antinociception (Welch, 1993). Although *nor*-BNI is capable of blocking the antinociceptive effects of Δ^9-THC, it does so without altering other behavioral effects (Smith *et al.*, 1994). In preliminary experiments mice were pretreated i.t. with either dH_2O, naloxone (20 μg/mouse), *nor*-BNI (70 μg/mouse) or ICI 174,864 (20 μg/mouse) 10 min before an injection of anandamide (200 μg/mouse). The animals were tested for tail-flick response 3 min after anandamide administration. The %MPE

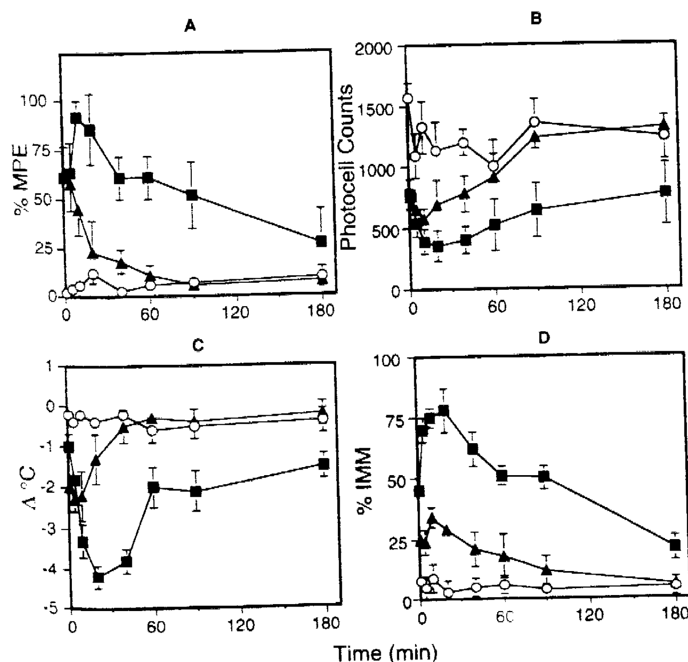

Fig. 6. The time course of effects of i.t. administered DMSO (○), 50 μg of Δ^9-THC (■) and 100 μg of anandamide (▲) on tail-flick latency (A), motor activity (B), rectal temperature (C) and ring immobility (D). The means ± S.E. (N = 12) are presented. Significant differences are described in the text in order to simply the figure.

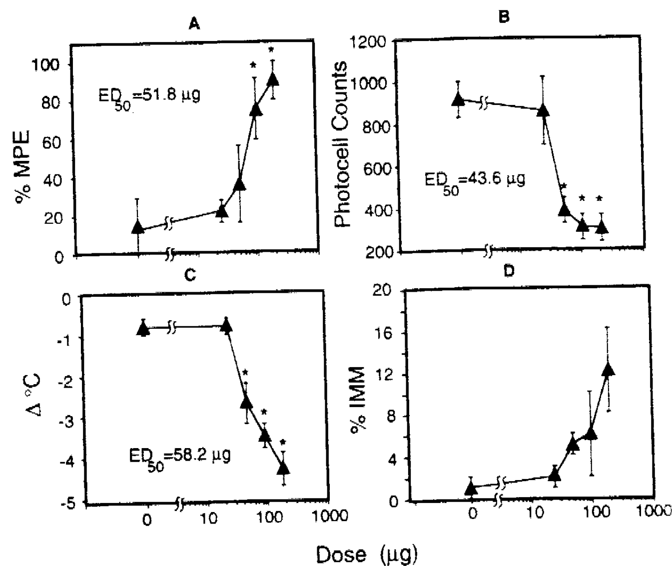

Fig. 7. The dose-responsive effect of i.t. administered anandamide was tested for tail-flick latency at 1 min (A), motor activity at 10 to 20 min (B), rectal temperature at 5 min (C) and immobility at 15 to 20 min (D). Animals received a single i.t. injection of either vehicle (DMSO) or anandamide. The means ± S.E. (N = 12) are presented. *Values found to be significantly different ($P < .05$) than groups receiving vehicle.

results (means ± S.E.M., $N = 8$) in animals pretreated with dH_2O (88 ± 8), naloxone (77 ± 16), *nor*-BNI (88 ± 8) or ICI 174,864 (80 ± 14) were not different. Efforts were undertaken to determine whether s.c. administration of a high dose (10 mg/kg) of naloxone would block the antinociception produced by anandamide (200 μg/mouse i.t.). Animals pretreated with dH_2O produced 100% MPE, whereas those pretreated with naloxone exhibited 84 ± 11 %MPE. Thus, extremely high doses of naloxone were not capable of antagonizing the effects of anandamide.

In order to more thoroughly determine whether *nor*-BNI might possibly antagonize anandamide effects other than the tail-flick assay, a protocol very similar to that used for the previous Δ^9-THC studies (Smith *et al.*, 1994) was used. *nor*-BNI (10 μg) or dH_2O vehicle was administered by i.t. injection to each animal. Ten minutes later, a second i.t. injection of either DMSO, 50 μg of Δ^9-THC or 100 μg of anandamide was administered. Tail-flick latencies were measured at 1 min, rectal temperature at 5 min, spontaneous activity at 5 to 15 min and ring immobility at 15 to 20 min after the second injection. The results of these experiments are illustrated in figure 8. After vehicle pretreatment, both Δ^9-THC and anandamide produced potent antinociceptive effects. Animals pretreated with *nor*-BNI followed by Δ^9-THC demonstrated no antinociceptive effects and were not significantly different than vehicle-vehicle-treated animals. However, *nor*-BNI pretreatment followed by anandamide still produced potent antinociceptive effects (approximately 60% MPE). The responses in these animals were not significantly different than those receiving vehicle pretreatment followed by anandamide. In the spontaneous activity test, both Δ^9-THC and anandamide produce significant decreases in motor activity after vehicle pretreatment. *nor*-BNI pretreatment had no significant effect on the hypoactivity produced by either compound. Both Δ^9-THC and anandamide produced decreases in rectal temperature of approximately 3°C from base line. Pretreatment with *nor*-BNI had no significant effect on the hypothermia produced by either Δ^9-THC or anandamide. Finally, ring immobility after both anandamide and Δ^9-THC in *nor*-BNI-pretreated animals was not significantly different than when administered after vehicle pretreatment.

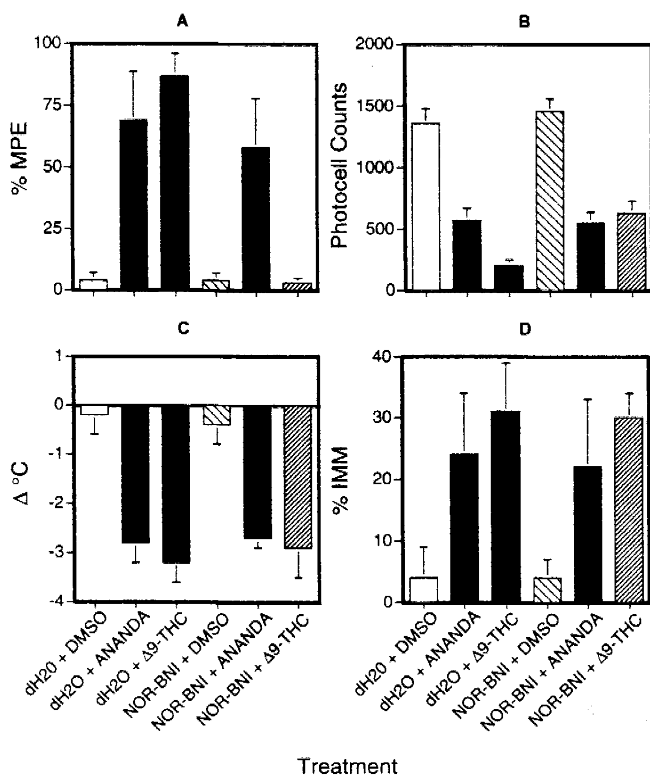

Fig. 8. The effects of 10 μg of i.t. *nor*-BNI pretreatment followed by 100 μg of i.t. anandamide or 50 μg of Δ^9-THC on tail-flick latency (A), motor activity (B), rectal temperature (C) and ring immobility (D). Animals received an i.t. injection of either dH_2O or *nor*-BNI followed 10 min later by a second i.t. injection of either DMSO, Δ^9-THC or anandamide. The means ($N = 6$) ± S.E. are presented.

Competition for [^{3}H]CP-55,940 receptor binding. Anandamide was evaluated for its ability to compete for [^{3}H]CP-55,940 binding to whole brain P_2 membranes. Unfortunately, anandamide exhibited very low affinity for binding. Other investigators obtained similar results when anandamide was evaluated for competition with [^{3}H]WIN 55,212-2 at 25°C for 90 min, which are normal incubation conditions (Childers *et al.*, 1994). However, these investigators discovered that anandamide effectively competed for receptor binding when 50 μM PMSF, an enzyme inhibitor, was added to the incubation medium. The displacement curve depicted in figure 9 was generated by incubating various concentrations of anandamide with 1 nM of [^{3}H]CP-55,940 when PMSF was added to the incubation medium. The average K_i (nM; mean ± S.E.) for anandamide with PMSF was 101 ± 15, whereas that without PMSF was 5400 ± 1.55. The assays were performed in triplicate and the results represent the combined data of three experiments.

Discussion

The isolation and identification of the putative endogenous cannabinoid ligand anandamide from porcine brain provides a new avenue for exploration of the cannabinoid system in the central nervous system. Although anandamide has been shown to compete for binding to the cannabinoid receptor (Devane *et al.*, 1992; Vogel *et al.*, 1993), to inhibit the electrically stimulated twitch of the mouse vas deferens and to produce cannabinoid effects in several behaviors in mice (Fride and Mechoulam, 1993), several questions remain unanswered. Obviously, the chemical structure of anandamide differs dramatically from that of Δ^9-THC, raising the question as to whether these two

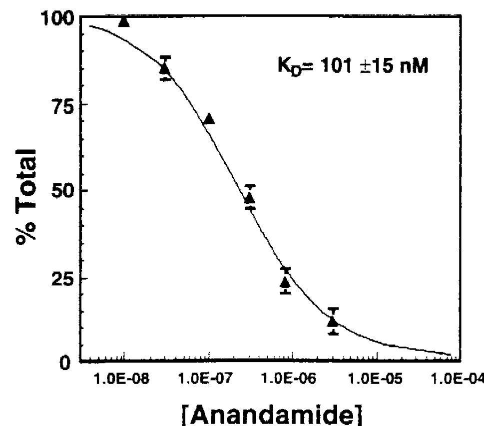

Fig. 9. Anandamide displacement of [^{3}H]CP-55,940 binding in the presence of 50 μM PMSF. The data are presented as percent displacement of total binding. K_D = mean ± S.E. of three experiments.

compounds are capable of producing identical pharmacological profiles. Even if both agents interact with the same receptor, it would not be unreasonable to expect each to exert some unique properties. Additionally, it would be expected that their pharmacokinetics may differ considerably, and therefore they may have very different time courses of action. It would be reasonable to expect anandamide to be hydrolyzed to arachidonic acid, whereas it is well known that the primary metabolism of Δ^9-THC occurs *via* the P450 system (Agurell *et al.*, 1986).

Although many pharmacological effects of cannabinoids have been described in several animal species, these behaviors are also shared by many other classes of compounds. One of the most reliable procedures for verifying cannabinoid properties in unknown compounds has been drug discrimination (Balster and Prescott, 1992). On the other hand, extensive use of a combination of unconditioned behaviors in mice, which includes spontaneous activity, body temperature, antinociception and catalepsy, has proven to be highly predictive of cannabinoid activity (Martin *et al.*, 1991; Compton *et al.*, 1992). The present results demonstrated that anandamide mimicked Δ^9-THC in most of these tests when administered by a variety of routes in mice. Pronounced hypoactivity occurred after all three routes of administration. Antinociception was also observed after all three routes of administration; however, only minimal levels were obtained by the i.p. route. Similarly, a robust hypothermic response was observed after i.v. and i.t. administration, but not after i.p. administration. In contrast, pharmacologically relevant immobility was only observed after i.v. administration, with levels after i.t. and i.p. administration often being similar to vehicle control. Additionally, ratings of approximately 20% IMM are obtained with sedatives, such as barbiturates, thus compounds are not typically considered cannabimimetic unless the results are both dose-responsive and the maximum effect attained at least a 30% IMM. For example, the observed effects in the time course studies with Δ^9-THC indicate immobility ratings of 55 to 75% are possible.

Early *in vivo* investigations also suggested similarity between anandamide and the cannabinoids. Anandamide produced hypoactivity, hypothermia and antinociception similar to that observed with Δ^8-THC after i.p. administration in the mouse (Fride and Mechoulam, 1993). Comparison to data in figure 5 generally indicates good agreement between these two studies. Fride and Mechoulam reported maximum pharmacological effects at 20 mg/kg with a loss of activity at a dose of 100 mg/kg (except for ring-immobility). Data in figure 5 similarly indicate a loss of activity at 100 mg/kg. Temperature decreases in figure 5 are not as great as those previously described, but are within 1°C. Inhibition of locomotor activity was previously reported to be 71% inhibition, which is very similar to that observed here. It is difficult to compare the results between hot-plate and tail-flick antinociceptive evaluations, as the latter involves a more intense stimulus and a 10-sec cut-off *vs.* the 45-sec cut-off time of the hot-plate test. However, the hot-plate data suggest the maximum effect of anandamide is a latency of only 11 sec, whereas that observed for 20 mg/kg of Δ^8-THC was 28.2 sec. This would suggest, as indicated in figure 5, that i.p. anandamide is not capable of producing robust antinociception. The most striking difference between these two reports is in the immobility data. A 54% rating was reported previously, yet levels not even as high as 25% were found here. This difference would initially appear to be great, especially given that 20 mg/kg of Δ^8-THC produced 70% IMM, which is at least comparable to the data reported for i.p. Δ^9-THC in figure 4. However, there are other considerations. The control immobility rating for mice in the previous study was 21%, which is much higher than that observed in these studies, and alone could explain most of the apparent difference between the 54% rating previously reported and the 25% rating observed here. In fact, it may be that the differences between these studies involve strain-related effects, because animals used previously (Fride and Mechoulam, 1993) were females of the Sabra strain. Though similar results were reportedly found in male Sabra and C57/Bl male and female mice, these data were not shown. Still, the possibility of strain differences appears very likely given the differences in control immobility scores.

One of the primary differences between Δ^9-THC and anandamide is their potency. As summarized in table 1, anandamide is considerably less potent than Δ^9-THC in all of the pharmacological tests after i.v. administration. Anandamide is also less potent than Δ^9-THC after i.t. administration, although the potency differences are not as great as those after i.v. On the other hand, either i.v. or i.t. anandamide appears to be a full agonist in all of the tests, producing maximal effects comparable to those reported for Δ^9-THC (Compton *et al.*, 1992; Smith *et al.*, 1994).

With regard to onset and duration of action, maximal antinociceptive effects were measured immediately after administration by all three routes. This suggests that anandamide gains access to the neuroanatomical sites responsible for pain modulation very quickly. Previous studies have indicated that cannabinoids may block pain transmission at the level of the spinal cord (Lichtman and Martin, 1991; Smith and Martin, 1992), although antinociceptive effects may also be mediated by actions in the brain. At this point it is not known whether anandamide is acting at the same sites as cannabinoids to produce antinociception. This question is also confounded by the fact that the specific neurochemical mechanism of cannabinoid-induced antinociception is also unclear at this point. Other cannabinoid effects, if observed, were also produced rapidly by the administration of anandamide by the i.v., i.t. and i.p. routes.

When equieffective doses of anandamide and Δ^9-THC were administered i.v., both compounds produced similar time courses for antinociceptive and hypothermic effects. Yet, the hypothermic effect was minimal at 30-min postinjection, whereas the antinociceptive properties of both drugs were still present beyond 90 min. In contrast, the time courses for an-

TABLE 1

Comparison of the potency of anandamide and Δ^9-THC when administered by different routes of administration

Route	Cannabinoid	ED_{50}			
		Hypoactivity	Hypothermia	Antinociception	Immobility
i.v.	Δ^9-THC[a]	1.0	1.4	1.4	1.5
i.v.	anandamide	17.9	26.5	6.2	19.1
	potency ratio[b]	18	19	4.4	13
i.t.	Δ^9-THC[c]	6.0	12	41	12
i.t.	anandamide	43.6	58.2	51.8	>200
	potency ratio	7.3	4.9	1.3	>17

[a] Previously reported by Compton *et al.* (1992) in terms of milligrams per kilogram.

[b] Potency ratio determined as the ED_{50} of anandamide divided by that of Δ^9-THC.

[c] Data are presented as micrograms per mouse. The dose-response curves generated herein are consistent with those previously reported by Smith *et al.* (1994).

andamide-induced hypoactivity and immobility were considerably shorter than those for Δ^9-THC, and had practically disappeared by 30-min postinjection, whereas Δ^9-THC effects were quite prominent at 60 min. One possibility for this might be due to differences in metabolism, because anandamide (an amide derivative) would be expected to be metabolized relatively quickly. However, if metabolic destruction of anandamide were responsible for the less profound effects and relatively shorter duration of action in the hypoactivity and immobility measures, then the relatively long duration of antinociception must be explained by other mechanisms. A higher rate of enzymatic degradation could limit the availability of anandamide to binding sites and prevent prolonged activation of these receptors. The [^{3}H] CP-55,940 displacement studies performed in the presence of PMSF, an enzyme inhibitor, supports the notion that metabolism may play an important role in the pharmacological actions of anandamide.

One interesting feature of the anandamide time courses after i.v. administration is that they differ depending upon the pharmacological measure. The greatest contrast can be found between that for antinociception and immobility. Although differences between the time courses for Δ^9-THC and anandamide can be argued on metabolic terms, such cannot be the case when multiple effects of the same compound are being compared. These differences suggest that different mechanisms are involved in these actions of anandamide, a notion which has not been easy to demonstrate for Δ^9-THC. The experiments with the *kappa* opioid antagonist, *nor*-BNI, suggest that anandamide may not be eliciting its effects *via* the same mechanisms as Δ^9-THC. The antinociceptive effects of Δ^9-THC are clearly blocked after pretreatment with 10 μg of *nor*-BNI. Anandamide-induced antinociception is not affected by *nor*-BNI pretreatment. In addition, the studies using higher doses of *nor*-BNI were also unable to block antinociception produced by i.t. anandamide. This distinction represents the first critical difference between anandamide and Δ^9-THC. They could be acting on different pain pathways or at different sites within the central nervous system. For example, anandamide could be migrating to supraspinal structures, whereas it is known that Δ^9-THC alters pain transmission by action at spinal sites after i.t. administration. The administration of *nor*-BNI directly at the level of the spinal cord may block these sites and inhibit the antinociceptive effects. If anandamide is producing antinociception at sites in the brain and in the spinal cord, the administration of an antagonist at the level of the spinal cord would be insufficient to block antinociception. Recent studies demonstrate that the antinociceptive effects of the cannabinoids are mediated through mechanisms distinct from those responsible for other behavioral effects (Smith *et al.*, 1993). The behavioral effects of both Δ^9-THC and anandamide after i.t. administration suggest that they act, at least in part, in the brain and/or spinal cord. However, the use of *nor*-BNI to distinguish between these two drugs is purely empirical because it currently is unclear whether this is truly an opiate (*kappa* receptor) effect indicating that Δ^9-THC induces release of endogenous opiate peptides, or by an unknown receptor mechanism. Regardless, it is clear that this antagonism by *nor*-BNI of Δ^9-THC, but not anandamide, is a unique pharmacological distinction between these two cannabimimetics.

In vitro studies with anandamide have confirmed several similarities to previous cannabinoid compounds. Anandamide has been demonstrated to bind to the cannabinoid receptor on cell membranes (Devane *et al.*, 1992; Vogel *et al.*, 1993). It also inhibited forskolin-stimulated adenylyl cyclase in both transfected cells and cells naturally expressing the cannabinoid receptor and this inhibition was blocked by pertussis toxin (Vogel *et al.*, 1993). Thus the putative endogenous cannabinoid anandamide resembles both Δ^9-THC and the highly potent synthetic compound HU-210 in binding to the cannabinoid receptor as well as inhibiting adenylyl cyclase. Considering the differences in chemical structure, it may be difficult to perceive how anandamide might interact with the same receptor as Δ^9-THC (see fig. 1). Previous investigators have demonstrated that several structural characteristics are associated with the pharmacological activity of the cannabinoids (Martin, 1985; Razdan, 1986; Makriyannis and Rapaka, 1990). Presently, it is not apparent how these characteristics apply to anandamide. Anandamide interaction with the cannabinoid receptor will require a close re-examination of the cannabinoid pharmacophore.

The existence of multiple CB-Rs must also be considered. Besides the widely recognized CB-R described in brain tissue (referred to as CB-1), another receptor (CB-2) has been described (Munro *et al.*, 1993) which was observed in spleen tissue. However, CB-2 is not found in brain tissue, or even in other peripheral tissues such as liver, nasal epithelium, thymus, lung or kidney. Anandamide also binds to CB-2, although possibly with a lower affinity than CB-1 (Munro *et al.*, 1993). Thus, it is possible that some of the differences between the pharmacology of Δ^9-THC and anandamide are due to actions upon different receptors, as well as for possibly different actions (*e.g.*, partial agonist activity) upon the same neuronal receptor. Potential novel, as yet undescribed, receptors could exist in peripheral tissue only, or co-exist within neuronal tissue with CB-1. Although CB-2 is not found in the brain, it is unknown whether it (or another receptor) exists in spinal cord. Because both CB-1 and CB-2 bind [^{3}H]CP-55,940 with similar affinities, then it is also possible that novel receptors cannot be distinguished from these two recognized receptors based solely upon ligand binding. Currently described binding sites both within and outside of brain or spinal tissue may actually represent both CB-1 and other molecular species of CB-R. If this were the case, then there would appear to be great potential to exploit the pharmacology of anandamide for the production o[illegible] therapeutically useful drugs. For example, if we assume tha[illegible] the limited effects observed after i.p. anandamide are due t[illegible] activation of a novel peripheral receptor, and not CB-1 becaus[illegible] of the rapid metabolism of anandamide, then this would sugges[illegible] it is possible to develop cannabinoid sedatives devoid of othe[illegible] side effects, because only inhibition of locomotion is observe[illegible] in mice after i.p. anandamide. However, at this time there ar[illegible] no other data supporting such contentions.

In conclusion, this study provides convincing evidence th[illegible] the arachidonic acid derivative, anandamide, produces effect[illegible] in mice which are consistent with that of the cannabinoid[illegible] This is demonstrated by the ability of anandamide to produ[illegible] antinociception, hypothermia, decreases in motor activity an[illegible] catalepsy in mice. Differences between Δ^9-THC and anandan[illegible] ide include a lower potency and shorter duration of action f[illegible] anandamide in some, but not all, measures. The specific *kap*[illegible] opioid antagonist, *nor*-BNI, which blocks Δ^9-THC-induced a[illegible] tinociception, failed to alter antinociception after anandami[illegible] administration. This antagonism provides support for a distin[illegible] mechanism of action of anandamide-induced antinocicepti[illegible]

from that of Δ^9-THC. Similarly, anandamide is unique in that it is capable of producing all cannabinoid effects after i.t. administration except immobility, and that after i.p. administration the only effect anandamide produces to a significant degree is inhibition of locomotor activity. Given these differences, further exploration of anandamide and recently discovered related ethanolamides which bind to the cannabinoid receptor (Hanus *et al.*, 1993) may reveal an entire class of endogenous compounds which possess unique properties that could be exploited as research tools or therapeutic agents.

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