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Biphasic Effects of Anandamide

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SULCOVA, E., R. MECHOULAM AND E. FRIDE. *Biphasic effects of anandamide*. PHARMACOL BIOCHEM BEHAV **59**(2) 347–352, 1998.—Effects of the endogenous cannabimimetic anandamide were assessed over a wide dose range in a series of physiological and behavioral assays. These included the tetrad of tests in mice commonly used to assess cannabinoid-induced effects (motor activity, ring catalepsy, hypothermia, and analgesia tests), as well as a model for agonistic behavior on dyadic interactions of singly housed males with nonaggressive group-housed partners. Anandamide-induced effects on leukocyte phagocytosis were measured in a chemiluminescence assay. Results indicated that the higher doses tested (10–100 mg/kg) produced the well-known inhibitory effects in all of the above parameters as well as inhibition of phagocytosis. The lowest dose of anandamide tested (0.01 mg/kg) stimulated behavioral activities in the open field, on the ring and aggressive behavior in timid singly housed mice. This dose of 0.01 mg/kg, also stimulated phagocytosis. We suggest several possible mechanisms to explain these findings such as a differential involvement of a Gs and a Gi protein activated at low and high doses, respectively, allosteric modulation of the cannabinoid, and activation of presynaptic cannabinoid receptors by low doses of anandamide. © 1998 Elsevier Science Inc.

Cannabinoids Anandamide Open field Catalepsy Agonistic behavior Phagocytosis Biphasic effects

AN endogenous ligand for the cannabinoid receptor was identified as the ethanolamide of arachidonic acid and named anandamide (9,27,28). It was found to display pharmacological effects similar to those of the cannabinoids, both in vivo (5,15, 40) and in vitro on receptor activation (13,48), as well as on immune functions (25,38). Anandamide was recently identified as the lipid constituent of the endothelial vasorelaxant, "endothelium-derived hyperpolarizing factor" (EDHF)(34). However, differences between anandamide and plant-derived or synthetic cannabinoids have been noticed in various experimental situations (12,15,25,40,48). Thus, the way in which this "anandamide-cannabinoid receptor system" functions in normal or pathologic conditions remains largely unknown. Indeed, there is evidence for additional mechanisms by which anandamide-induced effects may be explained, such as inhibition of gap junction permeability (46) and nicotinic α_7 receptor function (31).

A biphasic dose dependence of cannabinoid action was suggested more than 2 decades ago [(32), see also (10)]. Our previous studies with anandamide suggested to us that the effects of low doses of anandamide may in fact be discernible and different from those of high doses. Thus, we recently showed that very low doses of anandamides counteract or cause the opposite effects of higher doses of anandamide or cannabinoids in two separate studies: we observed that chronic in vivo administration of a very low dose of anandamide induces a tendency to sensitize the animal to an acutely administered high dose of anandamide, as opposed to the tolerance induced by chronic high doses of anandamide (14). Further, we have reported inhibition of activity of Δ^9-tetrahydrocannabinol (Δ^9-THC)-induced effects on behavior by 0.0001–0.1 mg/kg and adenylate cyclase activity by 0.1–1 nM of anandamide, whereas very low doses of Δ^9-THC did not produce these effects (16).

In the present study, effects of high and low doses of anandamide were compared in a series of tests commonly used to assess cannabinoid-induced activity: "open-field," "ring catalepsy," "hypothermia," and "analgesia" tests (26). Because relatively low doses of cannabinoid drugs have been shown to reduce attack behavior in a variety of animal species (29), we investigated effects of low doses of anandamide in a mouse model of agonistic behavior on dyadic social interaction. Furthermore because CB1 receptors had ben identified on leukocytes (2), we also measured phagocytic activity in a chemiluminescence assay in mouse leukocytes. We present evidence

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that low doses of anandamide have the opposite effect of high doses, in a wide range of experimental conditions.

METHOD

Animals

Female Sabra mice (6–7 weeks old, Harlan–Sprague–Dawley, Jerusalem) were used for the open-field, ring test, body temperature, and hot-plate tests. Males of albino outbred ICR mice bred at VELAZ, Prague (8 weeks old), were used for the model of agonistic behavior. Female mice of the inbred strain C57BL/10 (8 weeks old) were used for testing of leukocyte phagocytic activity. Animals received food and water ad lib and were maintained at constant temperature (20–22°C) on a 12 L:12 D cycle. Tests were performed during the light phase in the same room in which the mice were housed.

Drugs and Chemicals

Anandamide (ethanolamide of arachidonic acid) was synthesized as previously (9). The compound was dissolved in equal volumes of ethanol and emulphor 620 and mixed thoroughly with 9 volumes of sterile phosphate-buffered saline (final formulation 1:1:18) and administered in doses between 0.01 and 100 mg/kg in a volume of 0.1 ml/10 g mouse. Control animals received this vehicle without drug. In some experiments, solutions were prepared on day 1 of the experiment, kept at 4°C and used for up to 15 days after preparation without a noticeable reduction in effectiveness.

For the chemiluminescence assay, HSS (Hanks balanced salt solution) was used for the dilution of the blood samples. Luminol (Sigma Chemical Co., Germany) was solubilized in borate-buffer (pH 9.0) at the concentration of 1.7 mg/ml and stored at −20°C in the dark. Zymosan (Sigma-Aldrich s.r.o., Prague) was suspended in HSS at a concentration of 20 mg/ml and boiled for 20 min. The zymosan suspension was then centrifuged (10 min 2000 r.p.m.) and washed twice in HSS. The sediment was resuspended in mouse serum to the concentration of 20 mg/ml and incubated at 37°C for 45 min with moderate shaking. It was again centrifuged and washed twice in HSS. Finally, the opsonized zymosan was dissolved in HSS at a concentration of 20 mg/ml and stored at −20°C.

UNISOL I was obtained from Lachema a.s., and EKOGLOBIN from Hemax s.r.o. (Czech Republic).

Procedures

A series of four consecutive observations was performed on each mouse following a standard procedure employed to evaluate cannabinoid-induced effects in mice (26) with similar time intervals as described previously (15). Maximal pharmacological effects of anandamide are observed 10–15 min after IP injections (15). Hence, each mouse was observed starting 10 min after IP administration of anandamide. Horizontal (ambulation) and vertical (rearing) activity were measured for 8 min in an open-field (20 × 30 cm divided into 12 squares of equal size). Immediately after the open field test, catalepsy [immobility on a ring of 5.5-cm diameter, see (33)] was assessed for 4 min and expressed as % immobility. Body temperature (BT) was measured with a telethermometer (Yellow Springs Instruments Co.). Finally, analgesia on a hot plate (11) maintained at 55°C was measured as the latency (in seconds) until the first hindpaw lick or jump from the plate (the latter response was rarely observed) with a maximum of 45 s. It should be noted that no histopathological damage was observed when mice were kept for up to 60 s on a 59°C hot plate (1).

Agonistic behavior was assayed based on methods described by Krsiak (24). Thus, test animals were individually housed for 4 weeks in metal self-cleaning cages (8 × 16 × 13 cm). They were not handled except on the experimental days. This housing procedure is known to stimulate intensive agonistic behavior in male mice when tested on interactions with group-housed opponents of the same age and origin. The opponents were housed in groups of 10 in standard plastic cages (38 × 22 × 14 cm) with the floors covered with sawdust. Group housing nearly abolishes aggression of mice towards isolated males; hence, the competitive conditions during the encounter are unequal. The group-housed males behave as standard nonaggressive opponents. Agonistic behavior was tested in transparent observation boxes (20 × 30 × 20 cm) with clean sawdust provided before each encounter. After a 30-min adaptation period in the box, the opponent was introduced and interactions were videotaped for 4 min. The following behavioral categories of acts and postures, similar to those described previously (19), were recorded by computer using software for ethological observations: sociability—social sniffing, following the partner, climbing over the partner; timidity—defensive posture (upright), escape, alert posture; aggressivity—attack, aggressive unrest (threat), tail rattling; locomotion—walk, rear. The tested singly housed males show differential spontaneous behavior towards nonaggressive opponents. The tested singly housed mice were divided according to their behavior in control interactions into those that attack the opponents (aggressive mice), and those that show spontaneous defensive-escape behavior and no attacks (timid mice).

Chemiluminescence Assay of Leukocyte Phagocytosis

There is evidence that the chemical in vivo activation of the peripheral blood leukocytes leading to a burst of oxidative metabolism and, therefore, increased phagocytic capacity is more reliable after repeated doses of the drug under study (41,47). Thus, as in previous studies (22,23), seven daily injections of anandamide (0.01, 1.0, or 10.0 mg/kg) were used in the present experiments. Two hours after the last dose (given always between 0700–0800 h), 20 μl of blood was withdrawn from the retro-orbital plexus under ether anesthesia and placed in 500 μl of HSS. For leukocyte counts (Coulter Counter 2F, Coulter Electronics Ltd.) another 20 μl of blood were diluted in 10 ml UNISOL I including three drops of EKOGLOBIN.

Chemiluminescence (CL) measurements were performed based on methods described previously (43,49). CL measurements were performed using Biolumat LB 9 500 C. Twenty microliters of blood were suspended in 500 μl of HSS and incubated at 37°C. Samples of 200 μl were mixed with 40 μl of luminol. Five minutes after the background chemiluminescence record, CL was initiated by adding 40 μl opsonized zymosan and CL was measured every 5th minute for 1 h.

Statistics

Data from the tetrad of assays (open-field, ring catalepsy, hypothermia, hot-plate tests) were analyzed by one-way analyses of variance (ANOVA). Post hoc comparisons were performed using Fisher`s protected least significant difference test ($p < 0.05$). Agonistic behavior was evaluated using two-tailed nonparametric Wilcoxon matched-pairs signed-ranks tests, separately for timid and aggressive mice (39). The kinetics of chemiluminescence was analyzed by multifactor analyses of variance—Tukey`s honest significant differences test ($p < 0.05$).

RESULTS

A dose of 0.01 mg/kg of anandamide significantly increased ambulation and rearing, as well as defecation in the open-field (Fig. 1). This dose also decreased the rate of immobility on the ring (Fig. 2A). A nonsignificant tendency to decrease analgesia (Fig. 2B) on a hot plate was also observed, but body temperature was not affected by this dose (data not shown). A dose of 0.1 mg/kg did not elicit significant changes. Ten and 100 mg/kg markedly decreased activity in the open field, increased immobility on the ring, and induced hypothermia and hypoalgesia (Figs. 1 and 2).

In the model of agonistic behavior, anandamide at doses of 0.01 or 0.1 mg/kg did not significantly affect agonistic behavior in aggressive mice (Fig. 3A). The highest dose tested (10 mg/kg) decreased occurrence of aggressive and locomotor activities and stimulated defense-escape behavior—timidity (Fig. 3A). In timid mice (Fig. 3B), the lowest dose of anandamide selectively stimulated aggressive behavior, while the highest dose (10 mg/kg) inhibited sociability and locomotion without significantly affecting either timid or aggressive behavior.

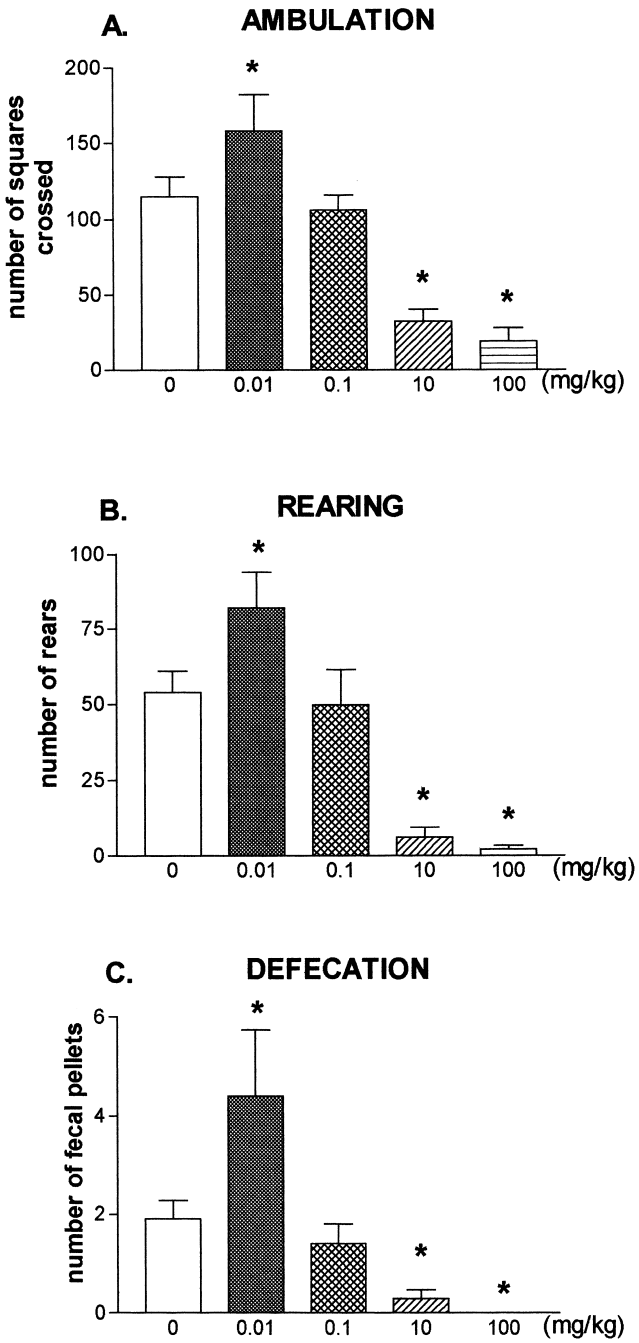


FIG. 1. Various doses of anandamide or vehicle, were injected (IP) into 6–7-week-old female Sabra mice. Ten minutes later they were exposed to an open-field (20 × 30 cm) and the number of squares crossed (A), the number of rearings (B) and the number of fecal pellets voided (C), were recorded for 8 min. *Significantly different from vehicle ($p < 0.05$, Fisher's protected least significant difference test.

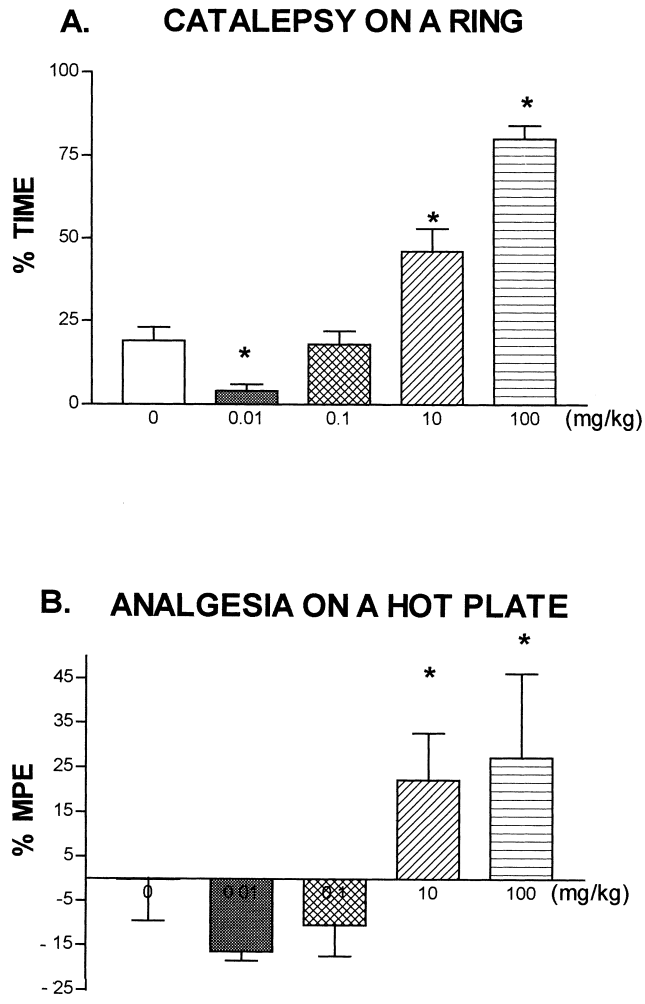
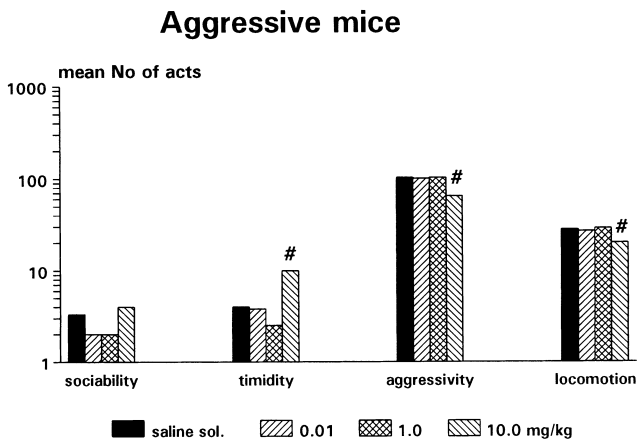


FIG. 2. Various doses of anandamide or vehicle, were injected (IP) into 6–7-week-old female Sabra mice. Immediately after open-field exposure, the time of immobility on a ring was recorded for 4 min (A). Following the ring test, analgesia on a hot plate was measured (B), where response latency was expressed as %MPE (% maximal possible response = 100 × ((test latency − baseline latency):(45 − baseline latency)). *Significantly different from vehicle ($p < 0.05$, Fisher's protected least significant difference test.



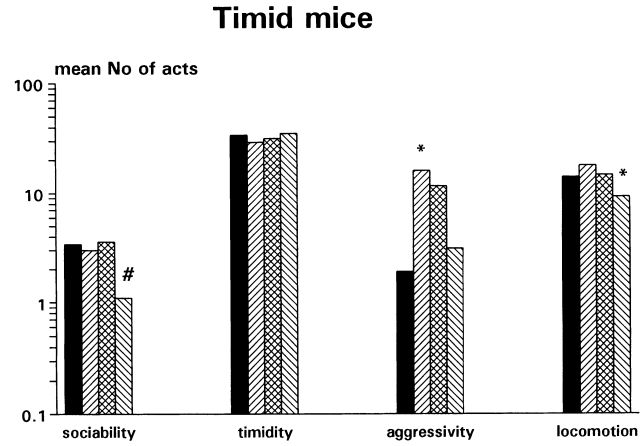


FIG. 3. Agonistic behavior was measured in 28 aggressive and 21 timid male ICR mice that had been treated with various doses of anandamide for 7 days. Two hours after the last injection, mice were observed for 4 min after a 30-min adaptation period to the test cage. *Significantly different from vehicle ($p < 0.05$, Wilcoxon matched-pairs signed-ranks test; #Significantly different from vehicle ($p < 0.01$, Wilcoxon matched-pairs signed-ranks test.

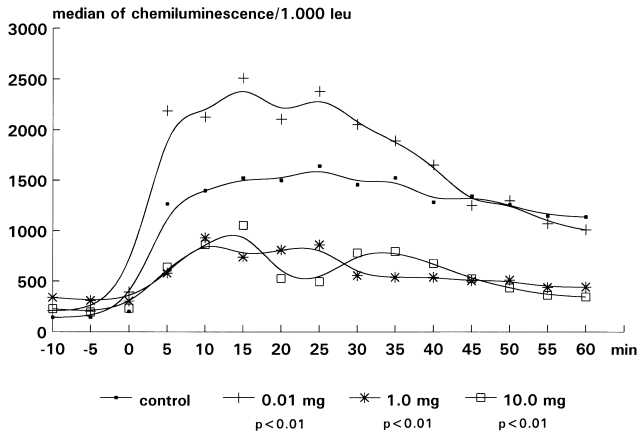


FIG. 4. Female C57BL/10 mice that had been treated with various doses of anandamide for 7 days, were sacrificed 2 h after the last injection. Chemiluminescence in blood leukocytes was recorded every 5 min for 1 h. Eleven to 12 mice were used for each dose. All three experimental curves were significantly different from the control curve ($p < 0.01$).

In the chemiluminescence assay for phagocytosis (Fig. 4), mouse leukocytes displayed stimulation after the lowest dose of anandamide (0.01 mg/kg) and inhibition after the two high doses (1 and 10 mg/kg).

DISCUSSION

The inhibitory effects of high doses of anandamide in the various tests are in agreement with previous data (15,40). In contrast, in most assays performed, a low dose (0.01 mg/kg) of anandamide had effects in the opposite direction to those found after high doses. Thus, we observed stimulated motor activity and rate of defecation in the open field, increased motility on the ring, and aggressive behavior in "timid" mice. Body temperature and analgesia were not significantly affected by low doses. However, a trend toward hyperalgesia was observed. The lack of significance in this test may be explained in terms of a ceiling effect: to detect hyperalgesia, the experimental animals must show a faster response time on the hot plate than controls. This may be impossible motorically, at least when the temperature of the plate was set at 55°C. Therefore, a lower temperature setting may enable expression of a significant hyperalgesic effect by a low dose of anandamide.

We have also found opposite effects of high and low doses of anandamide in an ex vivo study where a comparison of the zymosan-induced luminol-aided chemiluminescence curves showed that a dose of 0.01 mg/kg caused a marked stimulation of phagocytic activity in mouse leukocytes while higher doses (1.0 and 10.0 mg/kg) produced the opposite effects, namely inhibition of phagocytic activity. Thus, a dose of 0.01mg/kg of anandamide was required to display a stimulatory effect in all experimental conditions used in the present studies.

Biphasic effects of cannabinoids have been suggested previously (7,10,32). More recent reports include excitatory and depressant effects of Δ^9-THC on cortical evoked responses (over an average dose range of about 0.5–3.5 mg/kg (44)) and on muscimol-induced circling behavior (after intracerebral injections of Δ^9-THC (1–10 μg) (50)), and a biphasic anxiolytic/anxiogenic effect induced by 4 or 100 mg/kg, respectively, of the synthetic cannabinoid HU-210 (36). In these experiments however, the dose range required to induce excitatory effects was close to that used to obtain depression (3–25-fold lower). In our studies on anandamide we found that the stimulatory dose was 100–1000-fold lower that the "inhibitory" dose. Hence, a different mechanism may account for the stimulation-depression dichotomy for anandamide in our experiments, compared to the experiments on cannabinoids quoted above. Indeed, we did not find evidence for an effect of very low doses of Δ^9-THC [(16), and data not shown].

Very low doses of anandamide have also been shown to counteract effects of high doses of cannabinoids. Thus, 0.0001–0.1 mg/kg or 0.1–1.0 nM of anandamide inhibited Δ^9-THC–induced effects on behavior and cannabinoid receptor activation, respectively (16). These data were supported by a report indicating that low doses of anandamide (0.01–0.56 mg/kg) inhibit Δ^9-THC–induced amnesia (3).

It is possible that a similar mechanism underlies both the antagonism to Δ^9-THC–induced effects and the stimulatory effects of very low doses of anandamide as described here.

Two types of cannabinoids receptors have been identified: CB1, which is found in the brain, but also peripherally, such as on leukocytes (2,17), and CB2, found primarily in immune tissues (17,30).

Several mechanisms may be suggested to explain our data: for example, allosteric modulation of the CB1 receptor by low doses of anandamide or the involvement of a Gs protein as opposed to a Gi protein, which is activated by higher doses of cannabinoids or anandamide (13,18,20). A similar mechanism has been proposed to explain the dual effects of opioids. Thus, Crain and co-workers (4,6) have proposed that opioid receptors are directly coupled to both Gs and Gi proteins to stimulate or inhibit opioid-stimulated adenylate cyclase activity, respectively. Indeed, they reported that cholera toxin (CTX) attenuated, while pertussis toxin (PTX) enhanced activation of opioid stimulated adenylate cyclase activity in F-11 neuroblastoma-sensory neuron hybrid cells (6). Hence, effects of CTX and PTX on low and high dose-induced effects of anandamide will be studied in future experiments. A previous study (8) showed that low (nanomolar) concentrations of cannabinoids such as Δ^9-THC and CP55,940, stimulated B cell growth in vitro. In that study however, anandamide was not active. Moreover, the effect was blocked by PTX but not by SR 171416A, a specific CB1 receptor antagonist (35), thus suggesting a CB2 receptor-mediation of the growth enhancing effect on B cells (8). Thus, these observations seem to differ from those reported here.

Additional explanations may also be offered. Thus theoretical models have been presented (37,42), describing the action of an agonist on two different, functionally opposing receptors to induce stimulatory and inhibitory effects, depending on the dose used. It has also been suggested (21) that opposite activities of the same ligand may be the result of its ability to activate two separate sites on the same receptor molecule, acting in opposite directions. These models are consistent with recent findings on anandamide-induced cardiovascular function in rats, which include a brief pressor effect by anandamide, not affected by a selective CB1 receptor antagonist and a more prolonged depressor effect, which could be inhibited by the antagonist (45).

Presynaptic responsiveness to low doses of anandamide, analogous to that found in the dopamine system, should also be considered. Thus stimulation of such a putative presynaptic cannabinoid receptor by low doses of anandamide could act to inhibit anandamide release from the terminals and, hence, inhibit intrinsic anandamide activation of the postsynaptic CB1 receptor. Recent evidence has suggested that central effects of anandamide, in addition to its activation of the CB1 receptor, may also be explained by inhibitory gap junction permeability between neurons and astrocytes (46) or by inhibiting nicotine α_7 receptor activation (31).

In conclusion, we have demonstrated in three separate exprimental paradigms that low doses of the endogenous cannabinoid ligand anandamide may induce opposite effects from high doses of anandamide. We did not observe this phenomenon with the plant-derived Δ^9-THC.

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