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Cannabinomimetic behavioral effects of and adenylate cyclase inhibition by two new endogenous anandamides

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Abstract

We have previously shown that the endogenous putative cannabinoid ligand arachidonylethanolamide (anandamide, 20:4, $n-6$) induces in vivo and in vitro effects typical of a cannabinoid agonist. We now report that two other endogenous anandamides, docosatetraenylethanolamide (anandamide, 22:4, $n-6$) and homo-γ-linolenylethanolamide (anandamide, 20:3, $n-6$), have similar activities. The new anandamides bind to SV40-transformed African green monkey kidney cells transfected with the rat brain cannabinoid receptor cDNA and display K_I values of 253.4 ± 41.1 and 244.8 ± 38.7, respectively. The value found for arachidonylethanolamide was 155.1 ± 13.8 nM. In addition, the new anandamides inhibit prostaglandin E_1-stimulated adenylate cyclase activity in Chinese hamster ovary-K_1 cells transfected with the cannabinoid receptor, as well as in $N_{18}TG_2$ mouse neuroblastoma cells that express the cannabinoid receptor naturally. The IC_{50} values for the inhibition of adenylate cyclase in transfected Chinese hamster ovary-K_1 cells were 116.8 ± 8.7 and 109.3 ± 8.6 nM for docosatetraenylethanolamide and homo-γ-linolenylethanolamide, respectively. These values were similar to that obtained with arachidonylethanolamide (100.5 ± 7.7 nM), but were significantly higher than the IC_{50} value observed with the plant cannabinoid Δ^9-tetrahydrocannabinol (9.2 ± 8.6 nM). The inhibitory effects of the anandamides on adenylate cyclase activity were blocked by pertussis toxin, indicating the involvement of pertussis toxin-sensitive GTP-binding protein(s). In a tetrad of behavioral assays for cannabinoid-like effects, the two new anandamides exerted similar behavioral effects to those observed with Δ^9-tetrahydrocannabinol and arachidonylethanolamide: inhibition of motor activity in an open field, hypothermia, catalepsy on a ring, and analgesia on a hot plate.

Keywords: Adenylate cyclase; Anandamide; Cannabinoid receptor; GTP-binding protein

1. Introduction

Recently, an endogenous ligand for the cannabinoid receptor was identified, isolated and characterized in our laboratory (Devane et al., 1992; Fride and Mechoulam, 1993; Vogel et al., 1993). This compound, arachidonylethanolamide (AEA), was designated anandamide (20:4, $n-6$). The pharmacological and behavioral activities of AEA have been studied in vitro and in vivo in various laboratories (for review see Mechoulam et al., 1994). AEA was shown to bind to the cannabinoid receptor and to inhibit adenylate cyclase activity in $N_{18}TG_2$ mouse neuroblastoma cells, which express the cannabinoid receptor naturally (Howlett et al., 1987, 1991), and in Chinese hamster ovary (CHO)-K_1 cells transfected with the cannabinoid receptor cDNA (Felder et al., 1993; Vogel et al., 1993). In addition, AEA was shown to inhibit the activity of the N-type Ca^{2+} channels in $N_{18}TG_2$ neuroblastoma cells (Mackie et al., 1993). In vivo, AEA elicited a characteristic tetrad of cannabinoid-induced pharmacological and behavioral effects, which include inhibition of motor activity in an open field, hypothermia, catalepsy on a ring, and analgesia on a hot plate (Crawley et al.,

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1993; Fride and Mechoulam, 1993). AEA has also been shown to activate the hypothalamo-pituitary-adrenal axis (Weidenfeld et al., 1994).

Recently, two additional members of the anandamide family (ethanolamides of unsaturated fatty acids) were identified and isolated from porcine brain. These new compounds are docosatetraenylethanolamide (DTEA, anandamide, 22:4, $n-6$) and homo-γ-linolenylethanolamide (HLEA, anandamide, 20:3, n – 6) (Hanus et al., 1993). The structure of the three anandamides is shown in Fig. 1. Pertwee et al. (1994) have recently shown that these compounds inhibit the twitch response of the mouse vas deferens. Here we report that the two newly isolated anandamides HLEA and DTEA exert in vitro and in vivo activities that are similar to those of AEA. These compounds bind to the cannabinoid receptor, inhibit prostaglandin E_1-stimulated adenylate cyclase activity, via a pertussis toxin-sensitive GTP-binding protein (G protein), and, in addition, elicit behavioral effects similar to those obtained with AEA and Δ^9-tetrahydrocannabinol.

2. Materials and methods

2.1. Materials

[^{3}H-2]Adenine was purchased from Rotem Industries (Beer Sheva, Israel). The labeled cannabinoid [^{3}H]11-hydroxy-hexahydrocannabinol-3-dimethylheptyl (HU-243), (−)-Δ^9-tetrahydrocannabinol, AEA, DTEA and HLEA were synthesized in our laboratory as previously described (Devane et al., 1992; Hanus et al., 1993). For in vivo experiments, the cannabinoids were dissolved in ethanol and emulphor 620 (1:1) and mixed thoroughly with 9 volumes of sterile phosphate-buffered saline (PBS). Solutions were prepared on day 1 of the experiment and kept at 4°C up to 15 days after preparation without a decline in activity. Control animals received this formulation (vehicle) without the drug. Drugs were administered intraperitoneally in a volume of 0.1 ml/10 g mouse (Devane et al., 1992). For in vitro experiments, cannabinoid ligands were dissolved in absolute ethanol to a concentration of 10 mM. These solutions were kept at −70°C and diluted (just before the experiment) in medium containing fatty acid-free bovine serum albumin (Devane et al., 1992; Vogel et al., 1993). All dilutions of cannabinoid solutions were made in siliconized Eppendorf tubes (Sigma). Forskolin, 1-methyl-3-isobutylxanthine (IBMX), cyclic AMP (cAMP) and fatty acid-free bovine serum albumin were from Sigma.

2.2. Animals

Female C57/BL6 mice (Harlan-Sprague Dawley) received food and water ad libitum and were maintained at a constant temperature (20–22°C) on a 12-h light/dark cycle. Tests were performed during the light phase. Groups of 5–10 mice for each treatment were used throughout the study.

2.3. Cell cultures

The $N_{18}TG_2$ mouse neuroblastoma cell line and the CHO-K_1 cell line, transfected with the rat cannabinoid receptor plasmid SKR6, were kindly supplied by Drs. M.W. Nirenberg and T.I. Bonner (NIH, Bethesda, MD., USA), respectively. The control parental untransfected CHO-K_1 cell line and the SV40-transformed African green monkey kidney (COS-7) cells were from the American type culture collection (Rockville, MD., USA). COS-7 cells in 100-mm-diameter culture dishes were transfected according to the diethylamino-ethyl (DEAE)-dextran transfection procedure (Goeddel, 1990) with 10 μg/dish of the SKR6 cDNA cloned in the mammalian expression factor pCD (Matsuda et al., 1990). Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids and 8% fetal calf serum, in a humidified incubator containing 6% CO_2.

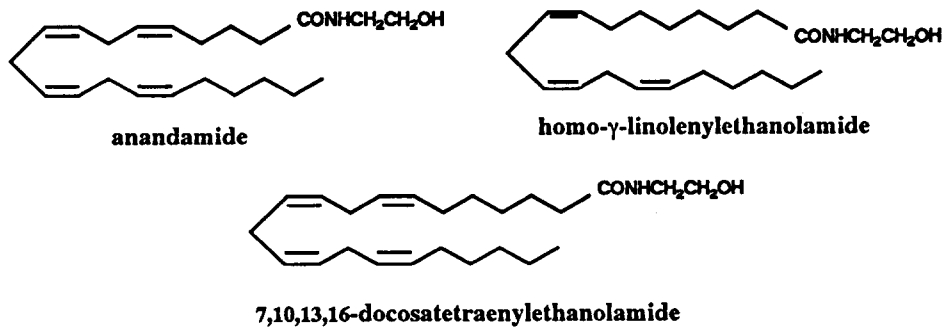

Fig. 1. Structures of the three anandamides arachidonylethanolamide (AEA), docosatetraenylethanolamide (DTEA) and homo-γ-linolenylethanolamide (HLEA).

2.4. Adenylate cyclase assay

The assay was performed essentially as described (Vogel et al., 1993). In brief, the assayed cells were cultured in 24-well plates (250 000 cells/well). Twenty-four hours later, the medium was removed and the cells were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml of [^{3}H]adenine. This medium was replaced with 0.4 ml/well of DMEM containing 20 mM Hepes (pH 7.4) and 0.1 mM IBMX, followed by the addition of 50 μl cannabinoid solution and 50 μl forskolin (1 μM, final) in the same medium. The cannabinoid solution also contained 20 mg/ml fatty acid-free bovine serum albumin and 1% ethanol. After 10 min at 37°C, the medium was removed and the reaction was terminated by the addition of 1 ml of 2.5% perchloric acid solution containing 0.1 mM of unlabeled cAMP. After 30 min at 4°C, volumes of 0.9 ml were removed into Eppendorf tubes and neutralized with 100 μl of a mixture of 3.8 M KOH and 0.16 M K_2CO_3. Aliquots of 0.9 ml of the supernatant, after pelleting the potassium perchlorate precipitate, were used in a two-step column separation procedure as described earlier (Salomon, 1991). The [^{3}H]cAMP was eluted directly into scintillation vials and counted.

2.5. Competition-binding assay with [^{3}H]HU-243

COS-7 cells were washed in PBS 2 days after transfection and crude membranes were prepared by homogenization in 50 mM Tris-HCl (pH 7.4), followed by a 1000 × g spin and recentrifugation of the supernatant for 20 min at 15 000 × g. The pellet was resuspended in the same buffer and aliquots of the suspension were used for binding of [^{3}H]HU-243 (specific activity, 48 Ci/mmol) as previously described (Vogel et al., 1993).

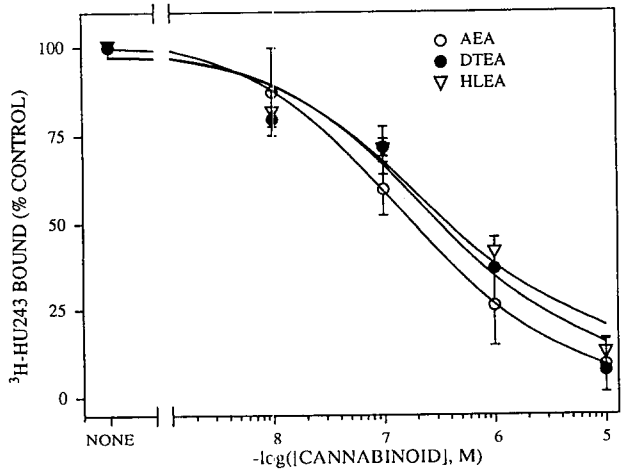

Fig. 2. AEA, DTEA and HLEA inhibit the binding of [^{3}H]HU-243 to membranes of COS-7 cells transfected with the cannabinoid receptor cDNA. Background binding, obtained in the presence of 1 μM of the cannabinoid HU-210, was subtracted. Data are the means ± S.E.M. of three experiments (performed in duplicate).

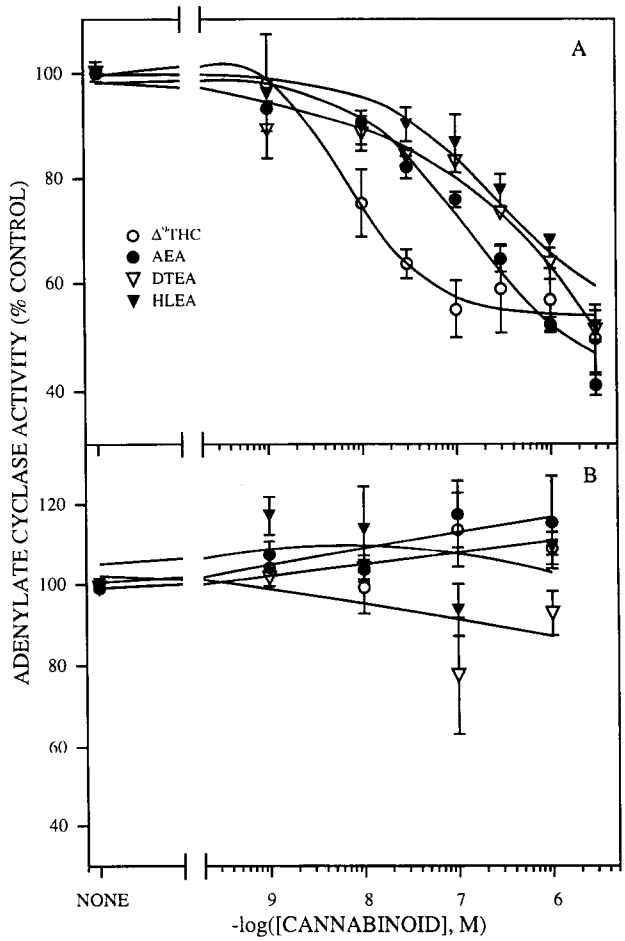

Fig. 3. Δ^9-Tetrahydrocannabinol, AEA, DTEA and HLEA inhibit prostaglandin E_1-stimulated adenylate cyclase activity in CHO-K_1 cells transfected with the cannabinoid receptor cDNA. The compounds (at the indicated concentrations) were added to transfected cells (A) or to control untransfected cells (B). Adenylate cyclase activity was determined as described in Materials and methods. Data are the means ± S.E.M. values from 3–4 experiments (performed in duplicate), expressed as the percentages of prostaglandin E_1-stimulated adenylate cyclase activity in the absence of the compounds. The IC_{50} values for AEA (100.5 ± 7.7 nM), DTEA (116.8 ± 8.1 nM) and HLEA (109.3 ± 8.6 nM) were significantly different from the IC_{50} value of Δ^9-tetrahydrocannabinol (9.2 ± 8.6 nM), $P < 0.05$.

2.6. Behavioral procedures

A series of four observations was performed on each mouse following a standard procedure employed to evaluate cannabinoid activity in mice (Martin et al., 1991), using time intervals as previously described by Fride and Mechoulam (1993). Briefly, each mouse was observed, starting with an 8-min exposure to an open field (20 × 30 cm divided into 12 squares of equal size). Horizontal (ambulation) and vertical (rearing) activities were recorded every 2 min. Since no differential drug effects were noted between intervals of 2, 4, 6 and 8

min, activity is presented only for the full 8-min period. Immediately after the open field exposure, catalepsy (immobility on a ring of 5.5 cm diameter) was assessed for 4 min and expressed as percent immobility. Body temperature was measured with a telethermometer (Yellow Springs Instruments Co.) and subtracted from that measured immediately before the first injection (BT2 – BT1). Finally, hot plate analgesia (Eddy et al., 1953) was measured as the latency until the first hind paw licks or jumps from a surface maintained at 55°C (with a maximum of 45 s). The jump response was rarely observed. In this regard, Ankier (1974) did not observe any histopathological damage when mice were kept for up to 60 s on a 59°C hot plate. Hot plate latency was expressed as % maximum possible effect (MPE) = 100 × (test latency – baseline latency)/(45 – baseline latency).

2.7. Statistical analysis

Data were analyzed using Student's *t*-test. Adenylate cyclase activity (IC_{50} values) was determined by the Inplot 4 computer program (Graph Pad Software, San Diego, CA, USA). The competition-binding assays and adenylate cyclase inhibition curves were generated with the Sigma Plot 4.11 computer program (Jandel Scientific, Corta Madera, CA, USA), using an equation from the ALLFIT program (DeLean et al., 1988).

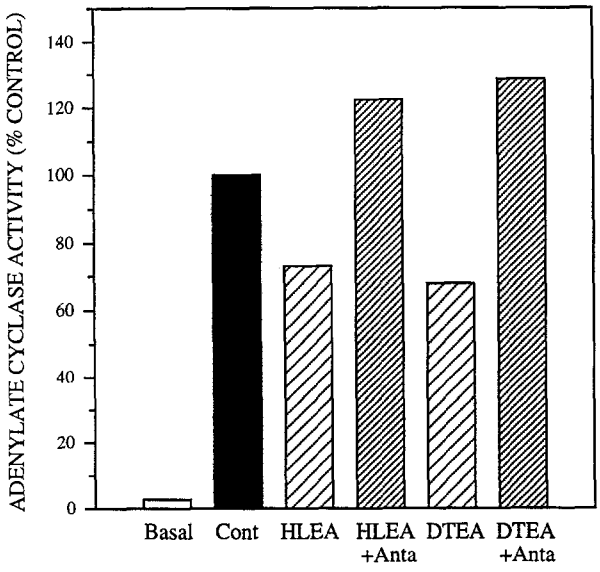

Fig. 4. DTEA and HLEA inhibit prostaglandin E_1-stimulated adenylate cyclase activity in $N_{18}TG_2$ mouse neuroblastoma cells. DTEA or HLEA (at 1 μM concentration) was added to $N_{18}TG_2$ cells in the presence or absence of 1 μM of the CB_1 antagonist SR141716A (added 10 min earlier). The data show a representative experiment replicated 3 times and are expressed as the percentages of prostaglandin E_1-stimulated adenylate cyclase activity in the absence of the compounds.

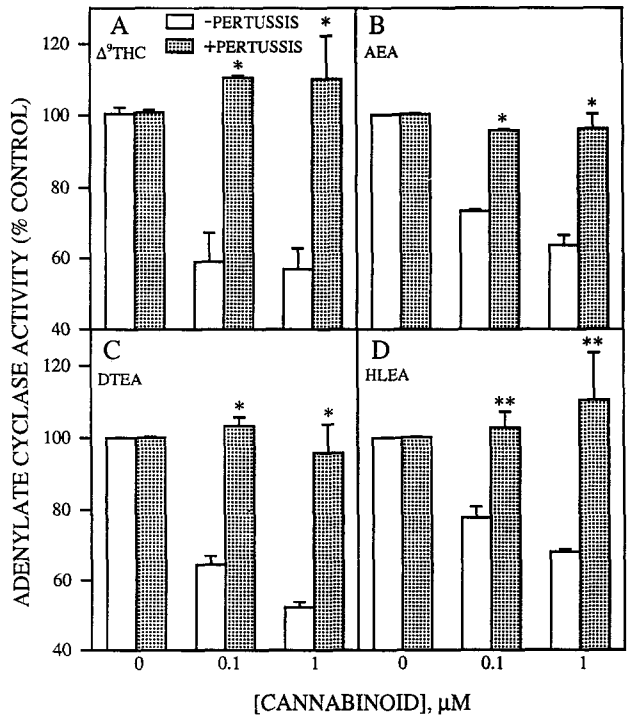

Fig. 5. Pretreatment with pertussis toxin blocks the inhibition by Δ^9-tetrahydrocannabinol, AEA, DTEA and HLEA of prostaglandin E_1-stimulated adenylate cyclase activity. Cannabinoid receptor-transfected CHO-K_1 cells were preincubated for 2 h in the presence or absence of 100 ng/ml of pertussis toxin (added together with the [^{3}H]adenine). Prostaglandin E_1-stimulated adenylate cyclase activity was determined in the presence of 0, 0.1 and 1 μM of the cannabinoid ligands. Data are the means ± S.E.M. values from 3–4 experiments (performed in duplicate), expressed as the percentages of prostaglandin E_1-stimulated adenylate cyclase activity in the absence of the cannabinoids. Significantly different from the corresponding pertussis toxin-treated cells: $^*P < 0.05$ and $^{**}P < 0.01$.

In vivo data were analyzed by one-way analysis-of-variance (ANOVA) or two-way ANOVA (time × anandamide). Post-hoc comparisons were performed using Fisher's protected least significant differences' test ($P < 0.05$).

3. Results

Crude membranes from COS-7 cells transiently transfected with the cannabinoid receptor cDNA were used to assess the potency of DTEA, HLEA and AEA to compete with binding of the labeled agonist cannabinoid [^{3}H]HU-243 to the cannabinoid receptor. As shown in Fig. 2, all three anandamides had similar competition curves. The K_i values obtained were 253.4 ± 41.1, 244.8 ± 38.7 and 155 ± 13.8 nM for DTEA, HLEA and AEA, respectively.

The question whether the newly discovered endogenous anandamides are functionally active as cannabinoid agonists was addressed by studying their ability to

inhibit adenylate cyclase activity. In CHO-K_1 cells transfected with rat cannabinoid receptor, DTEA and HLEA inhibited prostaglandin E_1-stimulated adenylate cyclase activity in a dose-dependent manner. As shown in Fig. 3A, the IC_{50} values of DTEA (116.8 ± 8.1 nM) and HLEA (109.3 ± 8.6 nM) were similar to the value obtained for AEA (100.5 ± 7.7 nM), but were significantly higher than the IC_{50} value obtained for Δ^9-tetrahydrocannabinol (9.2 ± 8.6 nM). No significant inhibition of prostaglandin E_1-stimulated adenylate cyclase activity was observed in the untransfected control CHO-K_1 cells (Fig. 3B). Inhibition of adenylate cyclase activity by DTEA and HLEA was also observed in

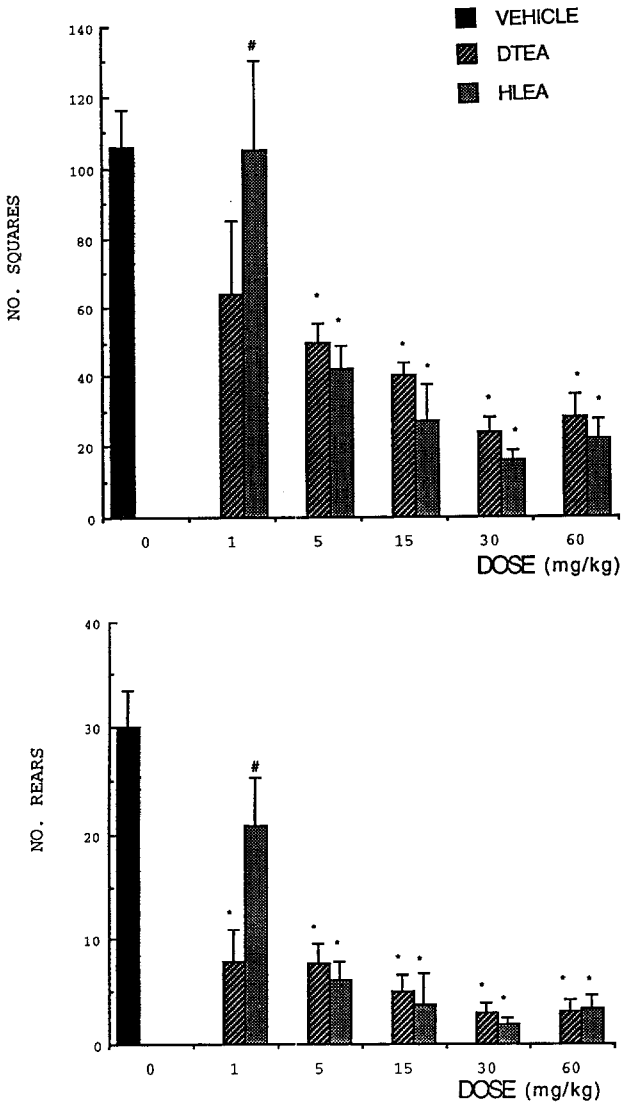

Fig. 6. Effect of various doses of DTEA and HLEA on (A) ambulation in an open field and (B) rearing in an open field by female C57/BL6 mice. Data are the means ± S.E.M., $n = 5–10$ in each group. * Significantly different from vehicle-injected mice. # Significantly different from mice injected with 1 mg/kg DTEA. Differences were tested by Fisher's protected least significant differences' test, $P < 0.05$.

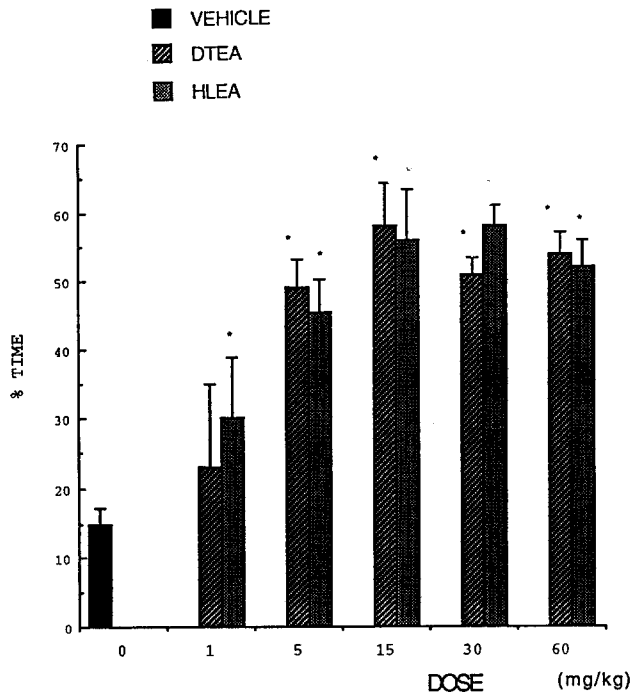

Fig. 7. Effect of various doses of DTEA and HLEA on the immobility in the ring test for catalepsy of female C57/BL6 mice. Data are the means ± S.E.M., $n = 5–10$ in each group. * Significantly different from mice injected with vehicle. Differences were tested by Fisher's protected least significant differences' test, $P < 0.05$.

prostaglandin E_1-stimulated $N_{18}TG_2$ neuroblastoma cells. For example, Fig. 4 shows that 1 μM of DTEA and HLEA inhibited adenylate cyclase activity by 32 and 37%, respectively. Moreover, using these cells and the CB_1 selective antagonist SR141716A, recently developed by Rinaldi-Carmona et al. (1994), we were able to show that the inhibition of prostaglandin E_1-stimulated adenylate cyclase by DTEA and HLEA was completely reversed by this antagonist. These findings strongly indicate that the new anandamides are functional agonists of the cannabinoid receptor. Pretreatment of the cells with pertussis toxin blocked the inhibitory effects of Δ^9-tetrahydrocannabinol, AEA, DTEA and HLEA on adenylate cyclase activity in CHO-K_1 cells (Fig. 5). These findings indicate that the new anandamides, like AEA and Δ^9-tetrahydrocannabinol, mediate their effects via pertussis toxin-sensitive G protein(s).

In order to determine if DTEA and HLEA also exert cannabinoid-like effects in vivo, we investigated the effects of these compounds on a tetrad of behavioral tests (ambulation, rearing in open field, immobility on the ring, and analgesia on hot plate) known to reflect cannabinoid-induced effects (Compton et al., 1993; Martin et al., 1991). In preliminary experiments we found that, similar to AEA (Fride and Mechoulam, 1993); DTEA and HLEA exerted their maximal in vivo

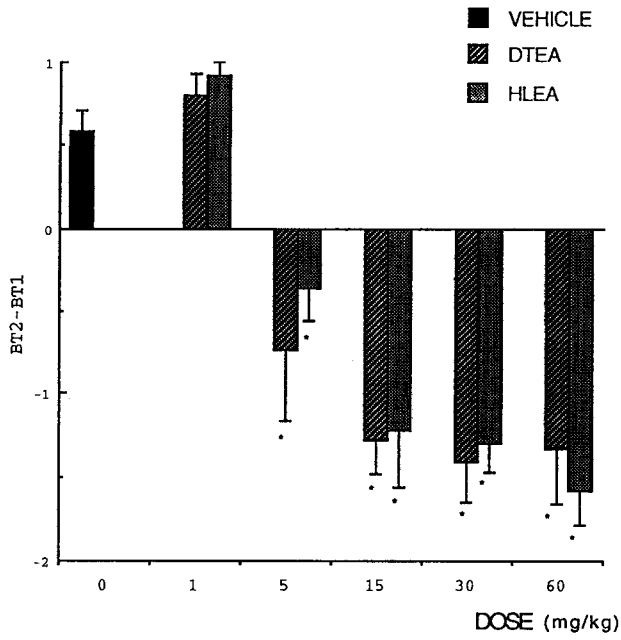

Fig. 8. Effect of various doses of DTEA and HLEA on the difference (°C) between the body temperature measured immediately before and 20 min after injection. Data are the means ± S.E.M., $n = 5$–10 in each group. * Significantly different from mice injected with vehicle alone. Differences were tested by Fisher's protected least significant differences' test, $P < 0.05$.

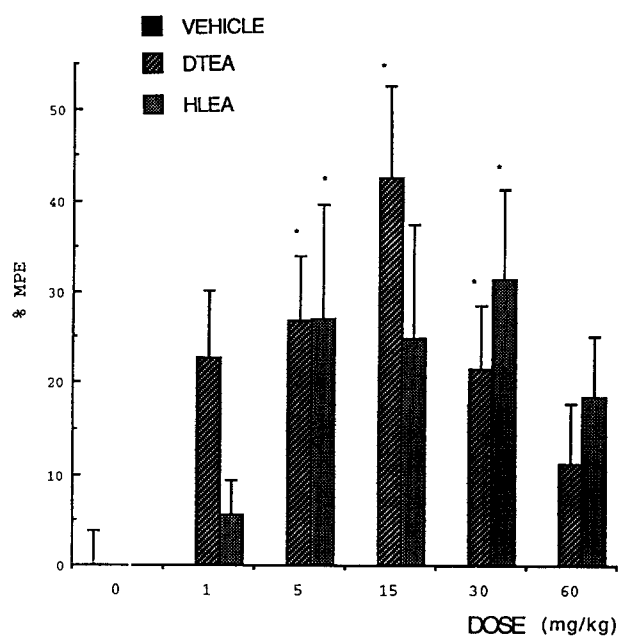

Fig. 9. Effects of various concentrations of DTEA and HLEA on analgesia on a hot plate, expressed as percent maximum possible effect (%MPE), using the latency to lick a hind paw or to jump from the hot plate as an analgesic criterion. Data are the means ± S.E.M., $n = 5$–10 in each group. * Significantly different from mice injected with vehicle alone. Differences were tested by Fisher's protected least significant differences' test, $P < 0.05$.

activity within 20 min after administration. Observations were therefore made 20 min after the injection of DTEA and HLEA at doses between 1 and 60 mg/kg. In all behavioral tests employed, both DTEA and HLEA showed a clear dose-response dependency. Thus, between 1 and 30 mg/kg, ambulation ($F(10,90) = 15.0$, $P < 0.0001$) and rearing ($F(10,90) = 19.7$, $P < 0.0001$) were increasingly suppressed compared with the responses of vehicle-injected controls (Fig. 6). A maximal effect was reached with a dose of 30 mg/kg. DTEA and HLEA also showed a dose-response relationship, with maximal effects being reached at doses between 15 and 30 mg/kg for immobility in the ring test ($F(10,90) = 14.2$, $P < 0.0001$) (Fig. 7), for change in body temperature ($F(10,90) = 12.7$, $P < 0.0001$) (Fig. 8) and for analgesia in the hot plate test ($F(10,87) = 2.0$, $P < 0.05$) (Fig. 9).

The in vivo effects of HLEA and DTEA were compared to those of AEA and Δ^9-tetrahydrocannabinol. We found that all compounds induced cannabinoid-like effects, but the maximal effects obtained by HLEA and DTEA were lower in their magnitude than those induced by Δ^9-tetrahydrocannabinol (Table 1). AEA induced intermediate responses.

4. Discussion

The first endogenous ligand of the cannabinoid receptor was isolated from porcine brain and identified

Table 1
Comparison of maximal effects between Δ^9-tetrahydrocannabinol and anandamides

	Δ^9-THC [a]	Anandamides		
		AEA [a]	DTEA [b]	HLEA [b]
Ambulation in open field (no. of squares)	0 ± 0	5 ± 3.5	24 ± 4	16 ± 2.8
Immobility on the ring (% of total time)	82 ± 5.4	77 ± 3.8	58 ± 6.2 [c,d]	58 ± 3 [c,d]
Change in body temperature (°C)	−4.7 ± 0.28	−2.4 ± 0.3 [c]	−1.4 ± 0.24 [c,d]	−1.6 ± 0.21 [c]
Analgesia on the hot plate (% MPE)	100 ± 0	66 ± 21	43 ± 9.9 [c]	31 ± 10 [c,d]

Previous data from our laboratory had indicated that the maximally effective dose of Δ^9-tetrahydrocannabinol is 50 mg/kg, of AEA 100 mg/kg (Mechoulam and Fride, 1995), and of DTEA and HLEA between 15 and 60 mg/kg (see Figs. 6–9). Hence the maximal effects reflect data for these doses. [a] $n = 5$. [b] $n = 10$. [c] Significantly different from Δ^9-tetrahydrocannabinol (Δ^9-THC) in the same row. [d] Significantly different from AEA in the same row.

as AEA (anandamide, 20:4, $n-6$) (Devane et al., 1992). Within a short period, two analogs of anandamide DTEA (anandamide, 22:4, $n-6$) and HLEA (anandamide, 20:3, $n-6$) were identified and isolated from porcine brain (Hanus et al., 1993). These two analogs were evaluated in this study for their functional activity on the cannabinoid receptor both in vivo and in vitro. The binding experiment and the pattern of inhibition of adenylate cyclase activity by the cannabinoids indicate that both DTEA and HLEA exhibit similar effects to those of AEA. The effects of DTEA and HLEA on inhibition of adenylate cyclase activity were shown with $N_{18}TG_2$ mouse neuroblastoma cells, as well as with CHO-K_1 cells transfected with rat brain cannabinoid receptor cDNA. No inhibition of adenylate cyclase was evident in untransfected control CHO-K_1 cells, suggesting that both compounds act via the cannabinoid receptors. Moreover, the inhibition of adenylate cyclase activity by DTEA and HLEA could be blocked by the CB_1 antagonist SR141716A. The effect of DTEA and HLEA on adenylate cyclase activity was pertussis toxin-sensitive, indicating that this functional response is mediated through the G_i/G_o type of G proteins.

The recent characterization of the peripheral type of cannabinoid receptor (CB_2) (Munro et al., 1993), the isolation of DTEA and HLEA, and the isolation of an endogenous ligand which interacts with both the central and peripheral receptors (Mechoulam et al., 1995), indicate the multiplicity of receptors and ligands of the cannabinoidergic system.

A strong correlation was observed between the binding properties of various cannabinoids and their pharmacological effects in vivo (Compton et al., 1993). Indeed, DTEA and HLEA displayed dose-response relationships for all the behavioral tests applied. The ED_{50} values of these compounds were comparable to those of 5 mg/kg AEA or 15 mg/kg Δ^9-tetrahydrocannabinol (data not shown). However, the maximal effects obtained with these compounds were smaller than the maximal effects obtained with a high dose of Δ^9-tetrahydrocannabinol (50 mg/kg). These results are in line with the observation that, under certain conditions, anandamides function as partial agonists when compared with Δ^9-tetrahydrocannabinol. We and others have previously shown that AEA may serve as a partial agonist in assays for analgesia and hypothermia in experimental animals, as well as for the inhibition of N-type Ca^{2+} channels in neuroblastoma cells (Fride et al., 1995; Mackie et al., 1993; Mechoulam and Fride, 1995). In the present study, the maximal effect of a high dose of AEA (100 mg/kg) was intermediate between that of HLEA or DTEA and that observed with Δ^9-tetrahydrocannabinol (see Table 1). Thus, at least in vivo, the partial agonist properties of anandamides are even more evident for DTEA and HLEA than for AEA. Differential pharmacokinetics or susceptibility to degradation may explain the partial agonist effect of DTEA and HLEA in vivo.

In conclusion, we have shown that the newly discovered anandamides DTEA and HLEA mimic the effects of Δ^9-tetrahydrocannabinol and AEA both in vivo and in vitro. These anandamides may function as endogenous agonists at the neuronal cannabinoid receptor.

Acknowledgements

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