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Hypolocomotor effects in rats of capsaicin and two long chain capsaicin homologues

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Abstract

Capsaicin and its analogue *N*-arachidonoyl-vanillyl-amine (arvanil) are agonists of vanilloid VR_1 receptors, and suppress spontaneous activity in mice through an unknown mechanism. Here, we tested in rats the effect on motor behavior of: (1) capsaicin; (2) *N*-linoleoyl-vanillyl-amine (livanil) and *N*-α-linolenoyl-vanillyl-amine (linvanil), which, unlike arvanil, have very little affinity for cannabinoid CB_1 receptors; and (3) the endocannabinoid anandamide (*N*-arachidonoyl-ethanolamine), which is a full agonist at both cannabinoid CB_1 and vanilloid VR_1 receptors. All compounds, administered i.p., dose-dependently (0.1–10 mg/kg) inhibited ambulation and stereotypic behavior and increased inactivity in the open field test. The rank of potency observed in vivo (livanil > capsaicin > linvanil > anandamide) bore little resemblance with the relative potencies in a functional assay for rat vanilloid VR_1 receptors (livanil = linvanil > capsaicin > anandamide) and even less with the relative affinities in rat CB_1 receptor binding assays (anandamide > livanil > linvanil > capsaicin). The vanilloid VR_1 receptor antagonist capsazepine (10 mg/kg, i.p.) blocked the effect of capsaicin but not of livanil or anandamide, whereas the CB_1 receptor antagonist (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide.HCl (SR141716A, 3 mg/kg, i.p.) antagonized the actions of the CB_1 receptor agonist Δ^9-tetrahydrocannabinol, but not of livanil, anandamide or capsaicin. Anandamide occluded the effects of livanil on locomotion, possibly suggestive of a common mechanism of action for the two compounds. Finally, stimulation with capsaicin of cells expressing rat vanilloid VR_1 receptors led to anandamide formation. These data suggest that motor behavior can be suppressed by the activation of: (1) vanilloid receptors, possibly via the intermediacy of anandamide; or (2) capsazepine- and SR141716A-insensitive sites of action for anandamide, livanil and linvanil, possibly the same that were previously suggested to mediate arvanil hypokinetic effects in mice.

Keywords: Cannabinoid; Vanilloid; Locomotion; Endocannabinoid; Receptor; Basal ganglia

1. Introduction

Anandamide (*N*-arachidonoyl-ethanolamine) was the first endogenous ligand of cannabinoid receptors to be discovered (Devane et al., 1992). This compound binds with moderate affinity to cannabinoid CB_1 and CB_2 receptors (Pertwee, 1997), but is a preferential functional agonist only for the CB_1 subtype. Anandamide exhibits a pharmacological profile (see Mechoulam et al., 1998; Di Marzo, 1998) similar, but not identical, to that of the psychotropic component of *Cannabis sativa*, (−)-Δ^9-tetrahydro-cannabinol (Gaoni and Mechoulam, 1964). Differences in the pharmacology of anandamide and Δ^9-tetrahydrocannabinol may be due to the facile degradation of the former compound in vivo (Willoughby et al., 1997), or also to its interactions with targets other than cannabinoid CB_1 or CB_2 receptors. Indeed, a selective antagonist of CB_1 receptors, (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide.HCl (SR141716A, Rinaldi-Carmona et al., 1994), does not block the typical cannabimimetic neurobehavioral effects of anandamide in mice (Adams et al., 1998), which

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consist of suppression of spontaneous activity and induction of immobility, analgesia and hypothermia, and are known as the mouse "tetrad" of tests. Furthermore, some of these effects of anandamide are still observed in mutant mice where the CB_1 receptor gene has been disrupted ("CB_1 knockouts") (Di Marzo et al., 2000a). Recent experiments (Zygmunt et al., 1999; Smart et al., 2000) showed that anandamide is also a full agonist at the capsaicin receptor, a ligand and heat-activated non-selective cation channel named "vanilloid" VR_1 receptor (Caterina et al., 1997). By acting in part through vanilloid receptors, anandamide relaxes both the buffer-perfused small artery preparations (Zygmunt et al., 1999) and the electrically contracted mouse vas deferens (Ross et al., 2001), and induces apoptosis of cancer cells (Maccarrone et al., 2000). Although vanilloid VR_1 receptors have been identified in several areas of the rodent brain, e.g. the basal ganglia, hypothalamus and brainstem (Mezey et al., 2000), there is still no evidence for their participation either in central nervous system (CNS) functions other than body temperature control (Szallasi and Di Marzo, 2000). The involvement of vanilloid VR_1 receptors in the control of spontaneous activity, body temperature and (supra)spinal nociception is supported by the observation that capsaicin exhibits moderate to strong activity in the four tests of the mouse "tetrad", even though it was not possible to counteract these effects of capsaicin with the vanilloid VR_1 receptor antagonist, capsazepine (Di Marzo et al., 2000b). Apart from VR_1 receptors, anandamide interacts also with non-CB_1, non-CB_2 G-protein-coupled receptors in mouse brain (Di Marzo et al., 2000a) which are activated also by the cannabinoid receptor ligand *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN 55212-2) (Sagan et al., 1999; Breivogel et al., 2001). Therefore, it is possible that the effects of anandamide in the mouse 'tetrad' are partly mediated by VR_1 receptors or novel cannabinoid receptors, although these possibilities have not yet been fully investigated.

Arvanil (*N*-[3-methoxy-4-hydroxy-benzyl]-arachidonamide) is a recently developed anandamide/capsaicin structural "hybrid" (Melck et al., 1999) with an affinity for cannabinoid CB_1 receptors comparable to that of anandamide, and an activity at vanilloid VR_1 receptors that is stronger than that of capsaicin (De Petrocellis et al., 2000; Ross et al., 2001). Arvanil is also a potent inhibitor of anandamide-facilitated transport into cells (Melck et al., 1999). Its actions at these multiple sites, and its higher metabolic stability as compared to anandamide, may explain in part why arvanil is more potent than either anandamide or capsaicin in the mouse 'tetrad', even though its effects, like for anandamide, are insensitive to SR141716A (Di Marzo et al., 2000b). Although the time-course of arvanil actions in mice seem to differ from that observed with capsaicin, it is possible that this novel compound acts in the CNS via VR_1 receptors. Alternatively, since it induces potent analgesia in the tail-flick test in a manner insensitive to either SR141716A or capsazepine (Di Marzo et al., 2000b), arvanil may also activate non-CB_1, non-CB_2, and non-VR_1 receptors.

Here, we have addressed the question of whether vanilloid VR_1 receptors or non-CB_1, non-VR_1 sites of action are involved in the regulation of motor behavior in rats. We have studied the effect on locomotion of capsaicin, anandamide and of two arvanil analogues having weak activity, if any, at rat CB_1 receptors. We report that the activation of either VR_1 receptors or putative novel anandamide sites of action leads to suppression of motor behavior in rats, and suggest that vanilloid receptor stimulation may affect motor behavior also through the intermediacy of anandamide.

2. Materials and methods

2.1. Compounds

Capsaicin and capsazepine were purchased from Alexis Biochemicals or Sigma-Aldrich, and anandamide for in vivo studies from Tocris. Anandamide, *N*-linoleoyl-vanillyl-amine (livanil) and *N*-α-linolenoyl-vanillyl-amine (linvanil) (Fig. 1) were synthesized from the condensation of the corresponding fatty acids and amines as described previously (Melck et al., 1999). SR141716A was a kind gift from Sanofi Recherche (Montpellier, France). Δ^9-Tetrahydrocannabinol was kindly provided by the National Institute on Drug Abuse (Rockville, MD).

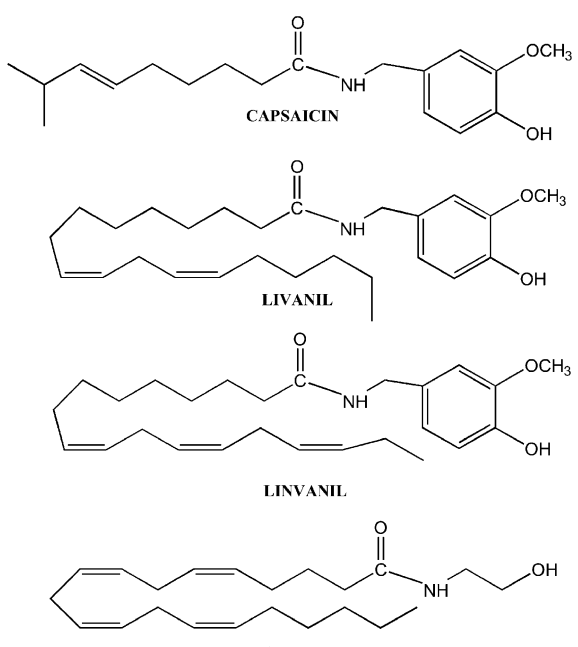

Fig. 1. Chemical structures of the compounds used in this study.

2.2. Cells and animals

Over-expression of rat VR_1 cDNA into human embryonic kidney (HEK) 293 cells was carried out as described previously (Smart et al., 2000). Transfected cells (HEK-rVR_1 cells) were grown as mono-layers in minimum essential medium supplemented with non-essential amino acids, 10% fetal calf serum and 0.2 mM glutamine, and maintained under 95%/5% O_2/CO_2 at 37°C. Male Wistar rats (> 10 weeks old; approx. 250 g) were used for pharmacological studies.

2.3. In vivo pharmacology

In a first set of experiments, we examined the animal response in the open-field test after the i.p. administration of a range of doses (0.1, 1.0 and 10 mg/kg weight) of anandamide, livanil, linvanil or capsaicin (the highest dose of capsaicin was toxic for animals and was not included in the study). In a second set of experiments, animals were pretreated with the vanilloid VR_1 receptor antagonist capsazepine (10 mg/kg weight, i.p.) or the cannabinoid CB_1 receptor antagonist SR141716 (3 mg/kg weight, i.p.) 30 min before the i.p. administration of capsaicin (1 mg/kg weight), anandamide (10 mg/kg weight), livanil (5 mg/kg weight) or Δ^9-tetrahydrocannabinol (5 mg/kg weight). In a third set of experiments, animals were subjected to i.p. administration of anandamide (1 mg/kg weight), livanil (1 mg/kg weight), or both. The same schedule was repeated for linvanil (1 mg/kg weight). All compounds were prepared in Tween 80-saline for administration and this was used for the corresponding vehicle-injected group in all experiments. In the three cases, animals were tested 10 min after the administration of the agonists in the open-field test. The open field consisted of a square (50×50 cm) with a surrounding wall (height: 40 cm). The square floor was divided into 25 small squares (10×10 cm) using transversal and longitudinal segments. Animals were placed in the middle of the structure and the spontaneous activity of each rat was recorded on a TV-video system for a period of 5 min. The apparatus was washed with a 5% acetic acid solution after each rat that had been tested. The following parameters were scored: (i) ambulation: number of sector crossings (a single line-crossing was defined as the rat placing the four paws into an adjacent quadrant); (ii) frequency of stereotypic behaviors (rearing, self-grooming and shaking); and (iii) time spent in inactivity. The scoring of the different behaviors was carried out by investigators who had no knowledge of the treatment of each rat.

2.4. Statistics

Means were compared by one-way (dose–response experiments) or two-way (antagonist or co-administration experiments) analysis of variance (ANOVA) followed by the Student–Newman–Keuls test.

2.5. Assay of vanilloid VR_1 receptor functional activity

The functional activation of rVR_1 by substances was studied by measuring their effect on cytosolic Ca^{2+} concentration by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. One day prior to the experiments, the cells were transferred into six-well dishes coated with Poly-L-lisine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment, the cells (50–60,000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes) in di-methylsulphoxide containing 0.04% Pluoronic. After the loading, cells were washed with Tyrode pH = 7.4, trypsinized, resuspended in Tyrode and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm) before and after the addition of the test compounds at various concentrations. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The size of the effect was measured as percent of the analogous effect observed with 4 μM ionomycin.

2.6. Cannabinoid CB_1 receptor binding assays

Displacement assays for cannabinoid CB_1 receptors were carried out by using [^{3}H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham) as the high affinity ligand, and the filtration technique previously described (Abood et al., 1997), on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Italia). Specific binding was calculated with 1 μM SR141716A (a gift from Sanofi Recherche, France) and was 84.0%. The K_i values for the various substances were calculated by applying the Cheng–Prusoff equation to the IC_{50} value (obtained by GraphPad) for the displacement of the bound [^{3}H]SR141716A by increasing the concentrations of the test compounds.

2.7. Study of the biosynthesis of anandamide in cultured cells

Eighty percent confluent HEK-rVR_1 cells, or non-transfected HEK cells, were incubated overnight with [^{14}C]ethanolamine (55 mCi/mmol, Amersham, 0.2 μCi/ml culturing medium), then washed with medium without serum and incubated with either 1 μM capsaicin or vehicle (0.2% methanol) in serum-free medium. After the incubation, cells plus medium were extracted with chloroform/methanol and the extracted lipids purified by open column silica chromatography as described previously (Bisogno et al., 1997). The pre-purified lipid fraction was fractionated by normal phase high-pressure liquid chromatography (NP-HPLC), carried out as described previously (Bisogno et al., 1997). Radioactivity in each NP-HPLC fraction was measured by liquid scintillation spec-

Table 1
Effect of capsaicin, livanil, linvanil and anandamide (AEA) on rat vanilloid VR_1 and cannabinoid CB_1 receptors
The potency (IC_{50}, nM) and efficacy (% of the maximal possible effect measured with 4 μM ionomycin) are shown for the effect of the four substances on VR_1-mediated increase in intracellular Ca^{2+} concentrations in HEK-rVR_1 cells. The K_i values (nM) for the displacement of [^{3}H]SR141716A from rat brain membranes by increasing concentrations of the four substances are also shown. Data are means ± S.D. of $n = 3$ independent determinations.

Substance	ratCB_1 (K_i, nM)	ratVR_1 (EC_{50}, nM)	ratVR_1 (% ionomycin effect)
capsaicin	> 25,000	10.0 ± 1.6	67.0 ± 2.1
livanil	6200 ± 1500	3.8 ± 0.3	66.3 ± 1.9
linvanil	11,700 ± 2600	4.0 ± 0.3	65.2 ± 2.1
AEA	800 ± 200	350.0 ± 91.2	68.1 ± 1.9

trometry. HPLC fractions eluting at anandamide retention time (27 min), which contain all members of the *N*-acylethanolamine family of lipids, were analyzed by isotope-dilution gas chromatography-electron impact mass spectrometry (GC-EIMS) after appropriate derivatization (De Petrocellis et al., 1999) to confirm the presence of anandamide. Endogenous anandamide, palmitoylethanolamide and oleoylethanolamide produced from capsaicin-treated cells were quantitated by isotope dilution with 0.1 nmol each of d_8-anandamide, d_4-palmitoylethanolamide and d_4-oleoylethanolamide, all synthesized as described (De Petrocellis et al., 1999).

3. Results

3.1. Affinity of the compounds for cannabinoid CB_1 receptors

The two long chain, polyunsaturated capsaicin analogues synthesized here were chosen instead of arvanil because of their lower affinity for mouse cannabinoid CB_1 receptors and less potent inhibitory effect on the anandamide membrane transporter in rat cells (Melck et al., 1999). In a binding assay carried out with rat brain membranes, and, therefore, more relevant to the in vivo studies described here, we found that livanil and linvanil were very weak ligands of these receptors (Table 1). In the same binding assay, capsaicin was inactive up to 25 μM. The rank of potency in the binding assays was: anandamide > livanil > linvanil > capsaicin. Livanil was seven- and threefold, and linvanil 13- and sixfold, less potent than anandamide and arvanil ($K_i = 2$ μM; Di Marzo et al., 2000b), respectively.

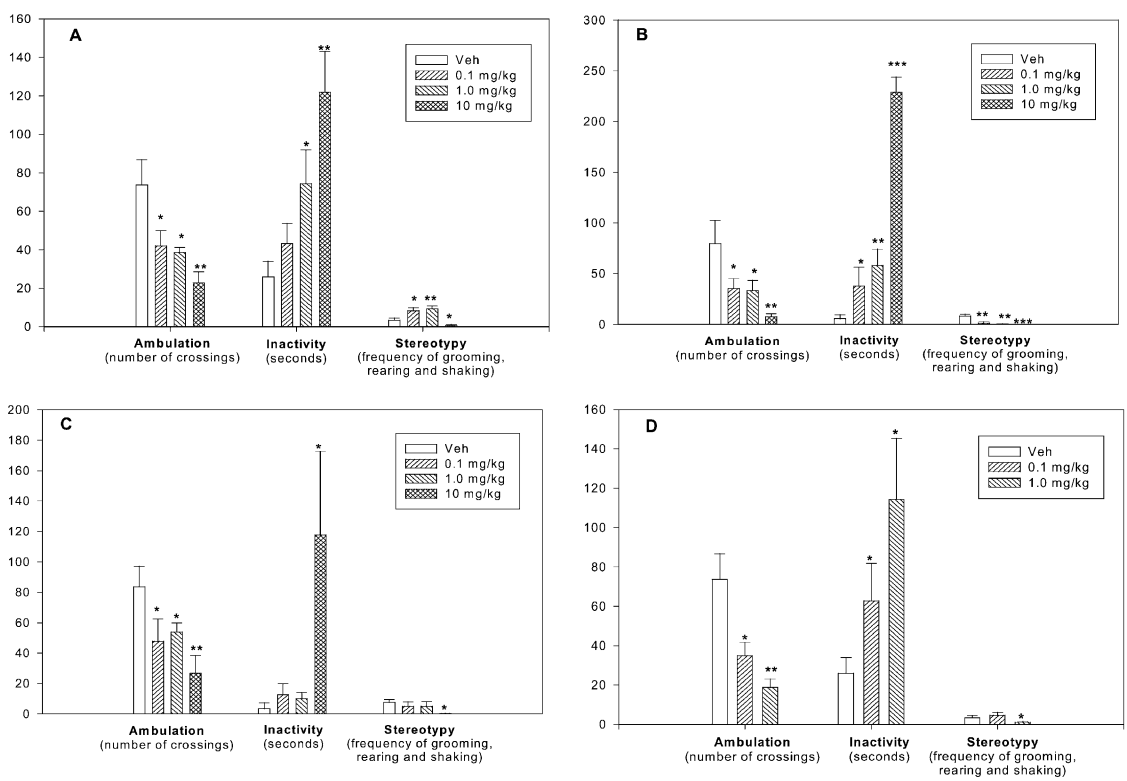

Fig. 2. Dose–response effect of (A) anandamide, (B) livanil, (C) linvanil and (D) capsaicin on rat motor behavior. Compounds were administered i.p. Results are means ± S.E.M. of $n = 4$. $^{*}P < 0.05$; $^{**}P < 0.0005$; $^{***}P < 0.00005$ vs. vehicle (ANOVA). The highest dose (10 mg/kg) of capsaicin was toxic to rats and could not be tested. Veh, vehicle.

3.2. Effect of compounds on rat vanilloid VR_1 receptors in transfected cells

The four compounds examined in this study were also tested for their activity in a typical functional assay of vanilloid receptors in HEK cells over-expressing the rat vanilloid VR_1 receptor (Table 1). The rank of potency for the effect of the compounds on VR_1-mediated rise in cytosolic Ca^{2+} was as follows: livanil = linvanil > capsaicin > anandamide. Livanil and linvanil were 10-fold less potent than arvanil (EC_{50} = 0.4 nM; De Petrocellis et al., 2000) and 2.5- and 100-fold more potent than capsaicin and anandamide, respectively.

3.3. Effects of compounds on rat motor behavior

All four compounds tested dose-dependently affected the motor parameters examined in this study, by inhibiting ambulation and stereotypic behavior (grooming + rearing + shaking movements) and inducing inactivity (Fig. 2A–D). Capsaicin could be tested only up to a 1.0 mg/kg dose since it was toxic to rats at 10 mg/kg. However, no sign of toxicity was observed in rats treated with the 0.1 and 1.0 mg/kg i.p. doses and kept under observation for a week. The rank of potency observed in the ambulation test was capsaicin > livanil > linvanil = anandamide. For inactivity, the relative potency was similar (livanil > capsaicin > linvanil > anandamide), and so was in the stereotypy test. Therefore, the overall rank of potency for the three parameters was as follows: livanil > capsaicin > linvanil > anandamide.

The effect of the VR_1 receptor antagonist, capsazepine (10 mg/kg), and of the cannabinoid CB_1 receptor antagonist SR141716A (3 mg/kg) on the motor suppressant action of capsaicin (1 mg/kg), anandamide (10 mg/kg) and livanil (5 mg/kg) was also studied for the three parameters (Table 2). In the case of ambulation and inactivity, capsazepine significantly attenuated the effect of capsaicin ($P < 0.05$) but not of anandamide or livanil, whereas SR141716A significantly decreased the effect of Δ^9-tetrahydrocannabinol (5 mg/kg, $P < 0.05$), but not of anandamide, capsaicin or livanil. A more complex picture was observed for stereotypy, where SR141716A attenuated the effect of Δ^9-tetrahydrocannabinol ($P < 0.005$), anandamide ($P < 0.05$) and livanil ($P < 0.05$) but not capsaicin, whereas capsazepine was effective against both anandamide ($P < 0.05$) and capsaicin ($P < 0.05$), but not livanil. We also studied the effect of co-administration of livanil or linvanil with anandamide. At an effective, albeit sub-maximal, dose (1.0 mg/kg) of livanil and anandamide their effects appeared to be occlusive rather than additive when compared to the effects observed with either substance alone (Table 3). In fact, anandamide seemed to antagonize the effect of livanil. Similar results were obtained when co-administering linvanil and anandamide (data not shown).

Table 3
Effect on ambulation, inactivity and stereotypy of co-administration (i.p.) of livanil and anandamide (AEA) to rats
Data are means ± S.E.M. of $n = 4$ experiments.

Groups	Ambulation	Inactivity	Stereotypy
+ vehicle	134.5 ± 25.2	ND	11.3 ± 2.6
+ livanil (1 mg/kg)	12.3 ± 3.5[a]	75.7 ± 18.9[a]	0.3 ± 0.3[a]
+ AEA (1 mg/kg)	68.3 ± 16.2[b]	23.3 ± 10.3[b]	7.0 ± 3.1
+ both	37.3 ± 16.4[a]	49.3 ± 39.4	2.7 ± 1.4[b]

ND = not detectable.
[a] $P < 0.0005$ vs. vehicle (ANOVA), ($n = 4$).
[b] $P < 0.05$ vs. vehicle (ANOVA), ($n = 4$).

3.4. Effect of capsaicin on anandamide biosynthesis in cultured cells

When stimulated with capsaicin (1 μM) for 15 min at 37°C, intact HEK 293 cells over-expressing rat VR_1 receptors were found to contain a radioactive peak with the same retention time as synthetic anandamide and in amounts significantly higher than in cells stimulated with vehicle, or wild-type HEK 293 cells (Fig. 3). Analysis by GC-MS of the derivatized NP-HPLC fractions correspond-

Table 2
Effect of SR141716A (3 mg/kg, i.p.) and capsazepine (10 mg/kg, i.p.) on the actions of capsaicin (1 mg/kg, i.p.), livanil (5 mg/kg, i.p.), anandamide (AEA, 10 mg/kg, i.p.) and Δ^9-tetrahydrocannabinol (THC, 5 mg/kg, i.p.) on rat ambulation (number of sector crossings), time (s) spent in inactivity and stereotypical behavior (frequency of grooming + rearing + shaking movements)
The effect of each drug is expressed as % of vehicle (96.6 ± 24.4 crossings, 8.0 ± 7.2 s of inactivity, 8.7 ± 2.3 frequency of stereotypic behavior), or vehicle + SR141716A (47.5 ± 18.5, 34.6 ± 10.0, 7.3 ± 1.3, respectively) or vehicle + capsazepine (90.2 ± 9.3, 17.3 ± 5.4, 4.8 ± 2.0, respectively). Data are means ± S.E.M. of $n = 4$ experiments. All means were significantly different ($P < 0.05$, ANOVA) from the corresponding vehicle except for [a] $P > 0.05$; [b] $P < 0.005$ vs. vehicle + capsaicin. ND, not determined.

Drug	Ambulation			Inactivity			Stereotypy		
	Vehicle	SR141617A	Capsazepine	Vehicle	SR141617A	Capsazepine	Vehicle	SR141617A	Capsazepine
Vehicle	100	100	100	100	100	100	100	100	100
Capsaicin	34.2 ± 7.6	42.7 ± 16.6	75.9 ± 26.1[a]	535 ± 139	377 ± 134	24.8 ± 16.1[b]	29.4 ± 15.3	58.3 ± 25.08	89.6 ± 37.5[a]
Livanil	35.2 ± 10.2	38.6 ± 5.9	14.2 ± 4.2	968 ± 177	568 ± 123	1002 ± 160	5.9 ± 5.9	75.0 ± 15.0[a]	10.4 ± 10.4
AEA	59.2 ± 16.1	41.2 ± 17.4	51.9 ± 12.1	517 ± 278	449 ± 152	288 ± 106	17.6 ± 10.6	70.0 ± 23.3[a]	110.4 ± 43.7[a]
THC	62.4 ± 4.5	101.0 ± 4.8[a]	ND	440 ± 73	141 ± 21[a]	ND	21.4 ± 5.5	103.5 ± 12.9[a]	ND

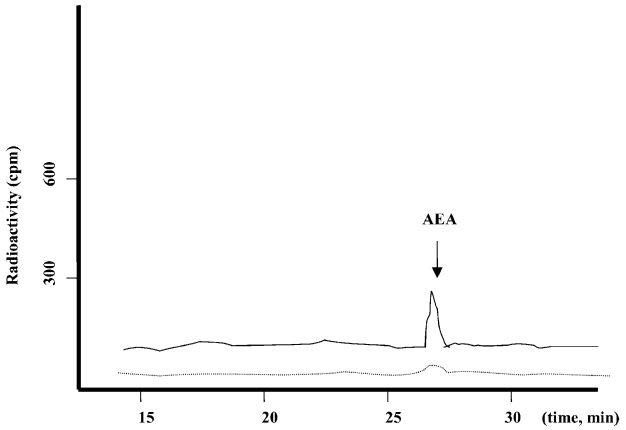

Fig. 3. Biosynthesis of anandamide in wild-type (dashed trace) or rat VR_1-over-expressing (upper trace) HEK cells stimulated with 1 μM capsaicin. Almost undetectable radioactivity was found at the retention time of anandamide (AEA, shown by an arrow) in either cell type when stimulated with vehicle. The radioactive peak was analyzed by GC-EIMS after opportune derivatization and confirmed the presence of anandamide as well as of two other *N*-acylethanolamines.

ing to anandamide, after appropriate derivatization, showed that anandamide, palmitoylethanolamide and oleoylethanolamide (13.0 ± 4, 145 ± 31, and 2.1 ± 0.5 pmol/10^7 cells) were produced from cells stimulated with capsaicin.

4. Discussion

Vanilloid VR_1 receptors have been identified in several nuclei of rat brain, including the substantia nigra compacta and the striatum, which are involved in the control of motor behavior (Mezey et al., 2000). Furthermore, it was also shown that i.v. administration of capsaicin to mice caused inhibition of spontaneous activity ($ED_{50} = 0.3$ mg/kg) and, to a lesser extent, immobility on a ring ($ED_{50} \sim 1$ mg/kg) (Di Marzo et al., 2000b). In mice, it was not possible to counteract capsaicin effects with capsazepine (i.v., 3–30 mg/kg). Here, we have shown that capsaicin, administered i.p to rats at slightly higher doses, induces immobility and inhibition of ambulation and of stereotypic motor behavior in a manner that could be reversed by capsazepine, thus providing unprecedented evidence for the involvement of vanilloid receptors in the control of motor behavior in rodents. To date, only sporadic studies carried out in the early 1980s had led to hypothesize that capsaicin could influence spontaneous activity in rats. In one of these studies, intra-nigral injection of capsaicin was shown to *enhance* motor activity (Dawbarn et al., 1981). Thus, it is possible that capsaicin causes opposite effects on motor behavior depending on the route of administration. Indeed, whilst activation of CB_1 receptors leads to inhibition of locomotion in rodents, for example by inhibiting the biosynthesis/release/action of dopamine in the basal ganglia (see Sanudo-Pena et al., 1999 for review and Giuffrida et al., 1999; Di Marzo et al., 2000c for recent data), acute treatment of rats with capsaicin increases 3,4-dihydroxyphenylacetic acid levels in the substantia nigra (Hajos et al., 1986). Also, if one looks at the intracellular signaling events coupled to VR_1 and CB_1 receptors, these are often in opposition with each other. Activation of vanilloid receptors is coupled to Ca^{2+} influx or increases of cAMP levels (Szallasi and Blumberg, 1999, for review), whereas stimulation of cannabinoid CB_1 receptors inhibits both voltage-operated Ca^{2+} channels and adenylyl cyclase (Howlett, 1995 for review). There are several possible explanations for the apparent paradox that stimulation of vanilloid VR_1 and cannabinoid CB_1 receptors leads to similar effects on locomotion. Firstly, these two receptors might exert opposing actions on neural circuits and/or neurotransmitter systems affecting in opposite ways the motor behavior, thus resulting in similar overall effects on locomotion. Indeed, cannabinoids themselves have opposite actions on motor behavior depending on the level of activity of the striatal and subthalamic inputs on the substantia nigra and globus pallidus (Sanudo-Pena et al., 1999). Secondly, since vanilloid receptors are easily desensitized by their agonists, the locomotor inhibitory effect of VR_1 receptor activation by capsaicin might be due to the rapid desensitization and subsequent inactivation of neurons positively controlling locomotion, or to the depletion of motor-inducing neurotransmitters (e.g. dopamine) synthesized from these neurons. A third possible explanation, which is supported by our present findings, is that the activation of vanilloid VR_1 receptors in the basal ganglia may lead to biosynthesis and release of anandamide in these brain nuclei. In support of this hypothesis, vanilloid receptor stimulation causes increases in cytosolic Ca^{2+} concentrations quantitatively similar to those observed with the calcium ionophore, ionomycin (De Petrocellis et al., 2000), which was previously shown to trigger the biosynthesis of anandamide and its congeners, the *N*-acylethanolamines (Di Marzo et al., 1994; Di Marzo et al., 1996; Bisogno et al., 1997). Accordingly, we have shown here that when cells expressing vanilloid VR_1 receptors are treated with capsaicin, the formation of *N*-acyl-ethanolamines, including anandamide, can be significantly stimulated. An anandamide tone in the striatum and globus pallidus of rats was recently correlated with inhibition of movement in normal (Giuffrida et al., 1999) and reserpine-treated (Di Marzo et al., 2000c) animals, respectively. It is, therefore, possible that stimulation of VR_1 receptors in the striatum leads to a local increase of anandamide levels with subsequent inhibition of spontaneous activity similar to that observed here following administration of exogenous anandamide. Accordingly, the cannabinoid CB_1 receptor antagonist SR141716A, at a dose fully effective against Δ^9-tetrahydrocannabinol, was ineffective with both capsaicin and anandamide-induced

hypokinesia, whereas capsazepine blocked the effect of capsaicin but not of anandamide. The possibility that capsaicin acts via anandamide is in agreement with the finding of increased anandamide blood levels and decreased ambulation following treatment of rats with the anandamide reuptake inhibitor, *N*-(4-hydroxy-phenyl)-arachidonamide (AM404; Giuffrida et al., 2000). The motor suppressing effect of this compound was attributed to its capability of increasing anandamide endogenous levels by inhibition of its reuptake, and subsequent degradation, by cells. However, it is also possible that AM404 enhances anandamide blood levels, at least in part, by stimulating vanilloid VR_1 receptors. In fact, several independent studies showed that AM404, which is chemically similar to arvanil, is a full agonist at rat and human VR_1, but not CB_1, receptors (Zygmunt et al., 2000; De Petrocellis et al., 2000; Ross et al., 2001). Furthermore, Giuffrida et al. found that administration of AM404 increased also the blood levels of the two other *N*-acylethanolamines, palmitoylethanolamide and oleoylethanolamide, which we have found here to be produced from HEK-rVR_1 cells stimulated by capsaicin. Unfortunately, the authors did not test the effect of capsazepine on AM404-induced inhibition of locomotion, although we have preliminary evidence that, under certain conditions, this VR_1 receptor antagonist does effectively counteract the hypokinetic effects of AM404 in rats (J.J. Fernandez-Ruiz and V. Di Marzo, unpublished observations). Whether AM404 increases anandamide levels by inhibiting anandamide inactivation or by stimulating its biosynthesis from VR_1-containing neurons, it remains to be explained why the inhibitory effect of AM404 on ambulation is reversed by SR141716A (Giuffrida et al., 2000), since this antagonist does not block the analogous effect of anandamide (this study), or it does so only when a very high dose of the endocannabinoid (20 mg/kg, i.p.) is used (Costa et al., 1999). Given the inhibition of capsaicin-, AM404- and anandamide-induced effects on VR_1 receptors observed with >1 μM concentrations of SR141716A (Zygmunt et al., 1999; De Petrocellis et al., 2001), the counteraction of AM404-induced hyperkinesia by SR141716A paradoxically supports the involvement of vanilloid receptors in this effect.

Indeed, another observation of this study was that, like for the hypokinetic effects of anandamide in mice (Adams et al., 1998), the same effects of this endocannabinoid in rats are not influenced by pre-treatment of the animals with SR141716A, with the only exception of anandamide action on stereotypic movements, which, however, was inhibitory only at the highest anandamide dose used, and was also reversed by capsazepine. This lack of blockade with the CB_1 receptor antagonist is in agreement with the recent finding that anandamide is still active in inducing immobility and inhibiting spontaneous activity in CB_1 knockout mice (Di Marzo et al., 2000a). Therefore, it is possible that at least part of the motor suppressing effects of anandamide are due to the interaction with non-cannabinoid CB_1 receptors. These receptors are not likely to be CB_2 receptors, whose involvement in CNS functions has been ruled out by several observations (Pertwee, 1997). Furthermore, although anandamide is an agonist at VR_1 receptors (Zygmunt et al., 1999; Smart et al., 2000), whose activation we have suggested here leads to inhibition of locomotion, these proteins are also not likely to be involved in anandamide effects on ambulation and immobility since these effects were not reversed by a dose of capsazepine sufficient to block the actions of capsaicin. Also arvanil, the anandamide/capsaicin "hybrid", was previously suggested to induce hypolocomotor effects in mice independently from both cannabinoid CB_1 and vanilloid VR_1 receptors (Di Marzo et al., 2000b). Therefore, we have tested here two synthetic analogues of this compound, livanil and linvanil, which, unlike arvanil, have almost no activity at CB_1 receptors. The two compounds were equipotent as rat VR_1 receptor agonists and yet exhibited very different potencies as inhibitors of ambulation and inducers of inactivity, thus arguing against the involvement of vanilloid VR_1 receptors in their hypokinetic action. Accordingly, the effect of livanil on ambulation and time spent in inactivity was not significantly affected by a dose of capsazepine sufficient to block capsaicin hypokinetic actions, or by a dose of SR141716A sufficient to totally reverse Δ^9-tetrahydrocannabinol actions on locomotion. This observation suggests that this compound (and possibly its analogues) does not affect motor behavior via CB_1 or VR_1 receptors. Furthermore, when anandamide and livanil were co-administered to rats at effective doses, they occluded each other's effects on ambulation, stereotypy and inactivity. This latter finding suggests that anandamide and livanil (or linvanil) might control motor behavior in rats through the same mechanism(s). It is possible that anandamide and long chain capsaicin analogues, although capable to bind and activate to a varying extent both cannabinoid CB_1 and vanilloid VR_1 receptors (Melck et al., 1999; De Petrocellis et al., 2000), influence spontaneous activity in rodents by being recognized *preferentially* by specific non-CB_1, non-VR_1 sites of actions. Another possibility would be that livanil and linvanil, previously shown to inhibit anandamide reuptake by cells (Melck et al., 1999), act by enhancing endogenous anandamide, as suggested for AM404 (Giuffrida et al., 2000). However, this hypothesis is not supported by the observation that (1) livanil was more potent than anandamide in all the motor behaviors examined here, and (2) livanil and linvanil are equipotent on anandamide uptake but exhibited in this study significantly different potencies on rat motor behavior. At any rate, our data should not be seen as evidence against anandamide being a physiological agonist of CB_1, or even VR_1, receptors since in rodents several other pharmacological actions of this compound are mediated by either of (or both) these two receptors (e.g. stimulation of food-intake, inhibition of learning or hypotension for CB_1 receptors [Williams and

Kirkham, 1999; Brodkin and Moersch, 1997; Járai et al.,1999], or small artery vasodilation, cancer cell apoptosis, and *vas deferens* relaxation for VR_1 receptors [Zygmunt et al., 1999; Maccarrone et al., 2000; Ross et al., 2001]).

In conclusion, we have presented here pharmacological evidence for the involvement of vanilloid receptors in the control of motor behavior in rats. We have suggested that activation of these receptors may trigger the biosynthesis of anandamide, which, in turn, may exert a hypokinetic action by activating non CB_1–non-VR_1 sites of actions or, in some cases, cannabinoid CB_1 receptors. A molecular approach is now required to demonstrate the existence of novel receptors for anandamide and long chain capsaicin analogues, whereas further studies, possibly through the use of yet to be developed anandamide biosynthesis inhibitors, should address the possibility that vanilloid VR_1 receptor agonists like capsaicin act in vivo through the VR_1-mediated release of anandamide.

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