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Neurobehavioral activity in mice of *N*-vanillyl-arachidonyl-amide

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Abstract

We studied the cannabimimetic properties of *N*-vanillyl-arachidonoyl-amide (arvanil), a potential agonist of cannabinoid CB_1 and capsaicin VR_1 receptors, and an inhibitor of the facilitated transport of the endocannabinoid anandamide. Arvanil and anandamide exhibited similar affinities for the cannabinoid CB_1 receptor, but arvanil was less efficacious in inducing cannabinoid CB_1 receptor-mediated GTPγS binding. The K_i of arvanil for the vanilloid VR_1 receptor was 0.28 μM. Administered i.v. to mice, arvanil was 100 times more potent than anandamide in producing hypothermia, analgesia, catalepsy and inhibiting spontaneous activity. These effects were not attenuated by the cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · HCl (SR141716A). Arvanil (i.t. administration) induced analgesia in the tail-flick test that was not blocked by either SR141716A or the vanilloid VR_1 antagonist capsazepine. Conversely, capsaicin was less potent as an analgesic (ED_{50} 180 ng/mouse, i.t.) and its effects attenuated by capsazepine. The analgesic effect of anandamide (i.t.) was also unaffected by SR141716A but was 750-fold less potent (ED_{50} 20.5 μg/mouse) than capsaicin. These data indicate that the neurobehavioral effects exerted by arvanil are not due to activation of cannabinoid CB_1 or vanilloid VR_1 receptors.

Keywords: Cannabinoid receptor; Vanilloid receptor; Anandamide; Capsaicin; Anti-nociception; Behavior; (Mouse)

1. Introduction

Arachidonoylethanolamide (anandamide) was isolated from porcine brain and shown to function as a ligand of cannabinoid receptors (Devane et al., 1992). Since the discovery of anandamide, other polyunsaturated fatty acid derivatives including two anandamide congeners (Hanus et al., 1993) and 2-arachidonoyl-glycerol (Mechoulam et al., 1995; Sugiura et al., 1995) were shown to bind to cannabinoid receptors and to elicit cannabimimetic responses in vivo and in vitro (for reviews see Di Marzo, 1998; Felder and Glass, 1998; Mechoulam et al., 1998; Martin et al., 1999). Although anandamide's capability to functionally activate cannabinoid CB_1 and, to a lesser extent, cannabinoid CB_2 subtypes of cannabinoid receptors is supported by several findings (for a recent review on cannabinoid receptor pharmacology, see Pertwee, 1999), numerous reports have suggested that this lipid behaves as a partial agonist at these receptors (Breivogel et al., 1998; Griffin et al., 1998; Sugiura et al., 1999). Furthermore, recent data (reviewed by Di Marzo et al., 1999) suggest that anandamide may have also other molecular targets different from the cannabinoid receptor subtypes reported so far. For example, anandamide was shown to interact with 5-hydroxy-tryptamine receptors (Fan, 1995; Kimura et al., 1998), *N*-methyl-D-aspartate receptors (Hampson et al., 1998), vanilloid receptors (Zygmunt et al., 1999) as well as with L-type Ca^{2+} channels (Johnson et al., 1993; Jarrahian and Hillard, 1997) and Shaker-related K^+ channels (Poling et al., 1996). Most of these effects are quite selective for anandamide over other fatty acid derivatives, although, in some cases, they are observed at concentrations higher than those required to activate cannabinoid CB_1 receptors.

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Many pharmacological actions of anandamide are attenuated by the cannabinoid CB_1 receptor antagonist (*N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · HCl (SR141716A). However, the selectivity of this compound, especially when used at concentrations higher than those required to bind to cannabinoid CB_1 receptors, has been recently questioned (White and Hiley, 1998; Zygmunt et al., 1999). Indeed, SR141716A was recently shown to counteract also those pharmacological actions of anandamide or non-psychotropic cannabinoids that are not mediated by cannabinoid CB_1 receptors (Chaytor et al., 1999; Járai et al., 1999; Wagner et al., 1999). On the other hand, in mice, anandamide typical tetrahydrocannabinol-like effects on spontaneous activity, body temperature and nociception are not affected by SR141716A (Adams et al., 1998). On the basis of our current knowledge, it is reasonable to assume that, although the rapid degradation of this compound both in vitro (Deutsch and Chin, 1993) and in vivo (Willoughby et al., 1997) may explain some of the controversy regarding its mechanism of action, the pharmacological actions of anandamide may be due also to the interaction with macromolecules other than the cannabinoid CB_1 and CB_2 receptors. Indeed, evidence for a non-CB_1, G-protein-coupled anandamide receptor in rat astrocytes (Sagan et al., 1999) as well as for a novel endothelial site of action for anandamide (Járai et al., 1999) was recently reported.

We have recently synthesized a series of unsaturated fatty acid derivatives of the hot chili pepper ingredient, capsaicin, and assessed their biochemical properties in intact cells and cell-free homogenates (Di Marzo et al., 1998; Melck et al., 1999). At low μM concentrations, these unsaturated *N*-acyl-vanillyl-amides, and in particular the arachidonic acid homologue, that we named arvanil (Fig. 1), bind to cannabinoid CB_1, but not CB_2, receptors, inhibit the uptake (and therefore the inactivation) of anandamide by intact cells, and activate the vanilloid VR_1 receptors for capsaicin. We proposed that arvanil could behave as a 'hybrid' ligand for cannabinoid CB_1 and vanilloid VR_1 receptors and gained in vitro evidence that this compound could be up to five-fold more potent than 'pure' agonists of either receptor type (Melck et al., 1999). Indeed, other recent observations showed that anandamide also activates vanilloid receptors resulting in an endothelium-independent vasodilator action in rat mesenteric arteries (Zygmunt et al., 1999). Given the known pharmacological properties in vivo of both anandamide (Di Marzo, 1998; Pertwee, 1999) and capsaicin (Szallasi and Blumberg, 1999), it is likely that arvanil may have a high potential for therapeutic use as an analgesic agent. Therefore, we undertook the present study with the aim of investigating for the first time the capasicin-like and cannabinoid pharmacological activity of arvanil in vivo. Furthermore, we carried out binding assays with membrane preparations from rat and mouse brain and cells transfected with vanilloid VR_1 receptors to re-assess the ability of arvanil to bind and/or activate cannabinoid CB_1 and vanilloid receptors. We report that arvanil exhibits a pharmacological profile that is more similar to that of anandamide than capsaicin. However, arvanil is much more potent in vivo than could be expected from its affinity and efficacy at cannabinoid CB_1 receptors, thus suggesting that this compound may act, inter alia, through novel sites of action for anandamide.

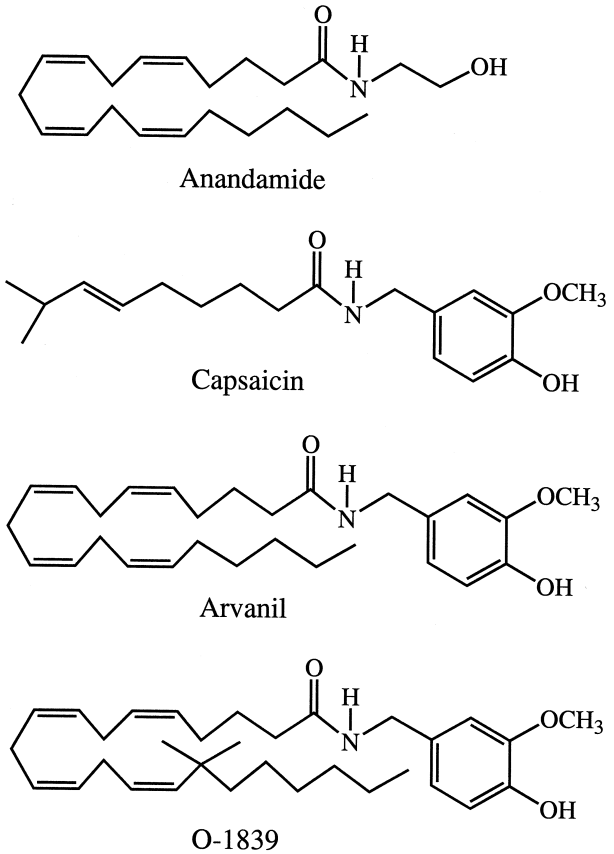

Fig. 1. Chemical structures of anandamide, capsaicin, arvanil and O-1839.

2. Materials and methods

2.1. Animals and reagents

ICR male mice (Harlan Laboratories, Indianapolis, IN) weighing 24 to 26 g were used in all in vivo experiments. Mice were maintained on a 14:10-h light/dark cycle with free access to food and water. Arvanil was synthesized as described previously (Melck et al., 1999). O-1839 and d_8-2-arachidonoyl-glycerol were synthesized in our laboratory (RKR) as will be described elsewhere. The chemical structure of O-1839 (Fig. 1) was confirmed by means of nuclear magnetic resonance. SR141716A was obtained from the National Institute of Drug Abuse (Bethesda, MD) or kindly donated by Sanofi Recherche, Montpellier, France, which also donated SR144528. d_8-anandamide

was purchased from Cayman Chemicals (Ann Arbor, MI). Other chemicals: phenylmethylsulfonylfluoride (Sigma, St. Louis, MO), adenosine deaminase (EC 3.5.4.4, Sigma), GDP and guanosine-5′-(3-thio)triphosphate (GTP-γ-S) (Roche Molecular Biochemicals, Chicago, IL), *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN 55212-2) (Research Biochemicals International, Natick, MA), and resiniferatoxin (Alexis, San Diego, CA).

2.2. Displacement and GTP-γ-S binding assays in brain membrane preparations

Displacement assays 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; De Petrocellis et al., 1998) on membrane preparations (0.4 mg/tube) from frozen male rat or mouse brains (Charles River, Italia). Membranes were incubated with increasing concentrations of arvanil, O-1839 and anandamide in the presence or absence of 100 μM phenylmethylsulfonylfluoride. K_i values were calculated from the IC_{50} values (obtained by GraphPad Software, San Diego, CA) for the displacement by the test compounds of the bound radioligand by using the Cheng–Prusoff equation. Specific binding was calculated with 1 μM SR141716A and was 93% and 84% for mouse and rat brain, respectively. O-1839 was also tested on the displacement of 1 nM [^{3}H]CP55,940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol] from rat brain P2 membranes as described previously (Compton et al., 1993).

For GTP-γ-S binding assays, whole brains of male ICR mice were removed and homogenized for 30 s in ice cold GTP-γ-S binding buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl, pH 7.4) using an Ultra-Turrax T25 (Janke and Kunkel) at 13,500 rpm. Homogenates were centrifuged at 40,000 × *g* for 10 min at 4°C, then the supernatants were discarded and the pellet resuspended using the Ultra-Turrax as before for 20 s. The centrifugation and resuspension were repeated twice more to wash the membrane pellet. Aliquots of the membranes were frozen at −80°C until use. On the day of each assay, aliquots were thawed, diluted in GTP-γ-S binding buffer and homogenized as above for 10 s. For phenylmethylsulfonylfluoride pretreatment, 50 μM phenylmethylsulfonylfluoride was added to membranes before centrifugation and resuspension as above. All membranes were preincubated with 0.004 units/ml adenosine deaminase for 10 min at 30°C before addition to the binding assay. Membranes (approximately 15 μg/tube) were incubated in GTP-γ-S binding buffer containing 0.1% (w/v) bovine serum albumin with 30 μM GDP, 0.1 nM [^{35}S] GTP-γ-S (1250 Ci/mmol, NEN, Boston, MA), and multiple concentrations of arvanil, anandamide or WIN 55212-2; 30 μM unlabelled GTP-γ-S was used to determine non-specific binding. For some assays, various concentrations of arvanil were assayed in the presence and absence of 2 nM SR141716A or 300 nM WIN 55212-2. Binding assays were incubated for 1 h at 30°C in a final volume of 1 ml, and were terminated by rapid filtration in a Brandel 96-well Cell Harvester (Gaithersburg, MD) onto Whatman GF/B glass fiber filters. Filters were transferred to scintillation vials and shaken for 1 h in Scintisafe Econo 1 scintillation fluid (Fisher Scientific, Pittsburgh, PA) before bound radioactivity was determined by liquid scintillation spectrophotometry at 92–95% efficiency for ^{35}S. Data were analyzed as net pmol of [^{35}S] GTP-γ-S binding stimulated by agonist per mg of membrane protein, by subtracting binding values obtained in the presence of agonist from basal binding values (those obtained in the absence of agonist). E_{max} and EC_{50} values were determined by non-linear regression analysis using Prism (GraphPad). The K_e value for SR141716A was determined by the equation:

$$K_e = \frac{[\text{ant}]}{\frac{EC_{50}+}{EC_{50}-} - 1}$$

where [ant] is the concentration of SR141716A, and EC_{50} + and EC_{50} − are the EC_{50} values obtained for anandamide in the presence and absence of SR141716A, respectively.

2.3. Assessing metabolic stability of arvanil and O-1839

Arvanil and O-1839 (200 μg/ml) were incubated with 1 ml of rat brain homogenates (2.5 mg total proteins) obtained by homogenizing a whole brain in 50 mM Tris–HCl, pH = 7.4. After a 30-min incubation at 37°C, the reaction was stopped by adding chloroform/methanol 2:1 by volume. Control incubations were carried out with homogenates boiled for 10 min. The organic extract was then analyzed for the presence of polyunsaturated fatty acids (e.g. arachidonic acid) by thin layer chromatography on silica gel plates (Merck) developed with chloroform/methanol/ammonia 85:15:1 by volume. Under these conditions polyunsaturated fatty acids exhibit an $R_f = 0.4$ different from arvanil and O-1839 (Rf = 0.8). Arvanil and O-1839 were also tested as inhibitors of FAAH in enzyme assays carried out at 37°C, pH 9.0 with membrane preparations from mouse neuroblastoma N18TG2 cells as previously described (Di Marzo et al., 1998).

2.4. Vanilloid receptor VR_1 binding assays

Chinese Hamster Ovary (CHO)/rVR_1 cells were generated as described previously (Szallasi et al., 1999). A 2.7-kB cDNA with a verified sequence identical to rVR_1 (Caterina et al., 1997) was subcloned into pUHG102-3 (Clontech, Palo Alto, CA) for recombinant expression in CHO cells containing the pTet Off regulator plasmid

(Clontech). Ninety percent confluent cells were washed with phosphate-buffered saline, harvested in phosphate-buffered saline containing 5 mM EDTA, and pelleted by gentle centrifugation to be stored at −80°C until assayed. Binding studies with [^{3}H]resiniferatoxin (37 Ci/mmol; Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD) were carried out according to a published protocol (Szallasi et al., 1999). Binding assay mixtures were set up on ice and contained approximately 100,000 CHO/rVR$_1$ cells, 0.25 mg/ml bovine serum albumin (Cohn fraction V, Sigma), and [^{3}H]resiniferatoxin. The final volume was adjusted to 1000 μl with a buffer containing (in mM) KCl 5, NaCl 5.8, $CaCl_2$ 0.75, $MgCl_2$ 2, and HEPES 10; pH 7.4. Non-specific binding was determined in the presence of 1000 nM non-radioactive resiniferatoxin. The binding reaction was terminated after a 30-min incubation at 37°C by cooling the tubes on ice. Bovine α1-acid glycoprotein (100 μg/tube) was added to reduce non-specific binding (Szallasi et al., 1992). Membrane-bound resiniferatoxin was separated from the free as well as α1-acid glycoprotein-bound resiniferatoxin by rapid centrifugation at 4°C, and the radioactivity was determined by scintillation counting. Competition experiments were carried out with various concentrations of each analog in the presence of 100 pM [^{3}H]resiniferatoxin (the approximate K_d value). IC_{50} values were calculated by the curvilinear regression program LIGAND (Biosoft, Ferguson, MO). K_i values were obtained by the corresponding IC_{50} by using the Cheng–Prusoff equation and a K_d value of 100 pM.

2.5. Anandamide and 2-arachidonoyl-glycerol measurement in the mouse spinal cord by LC-MS

ICR mouse spinal cords were dissected and immediately frozen in liquid nitrogen. Lipid extraction and pre-purification was performed as described previously in the presence of 1 nmol d_8-anandamide and 2 nmol of d_8-2-arachidonoyl-glycerol (Bisogno et al., 1999 and papers cited therein). Pre-purified lipids were analyzed by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). The MS was equipped with a Z-Spray APCI source operating in the (+) APCI mode (source temp.: 120°C, probe temp: 110°C). N_2 was used as both drying and nebulizing gas (flow and probe position were adjusted daily for optimum sensitivity). The HPLC was equipped with a Supelco Supelcosil LC-18 column (15 cm × 4.6 mm, 5 μm particle size). The mobile phase was MeOH:H_2O:acetic acid 85:15:0.2 (by volume) at a flow rate of 1 ml/min. Both the column and the samples were maintained at 25°C. Retention of peaks of a selected m/z value was utilized to identify anandamide and 2-arachidonoyl-glycerol in their protonated (M + 1) form. Quantification of the two compounds was obtained by the isotope dilution method. Following each injection, a five-loop volume injection syringe purge was performed.

2.6. Measurement of spontaneous activity, antinociception and body temperature

Cannabinoids were dissolved in a 1:1:18 mixture of ethanol, emulphor and saline for i.v. administration. Mice received the analog by tail-vein injection and were evaluated for their ability to produce hypomotility, hypothermia, immobility and antinociception. These pharmacological measures were determined using a slight modification of our earlier approach (Compton et al., 1993). The primary difference is the shorter time to testing and only two measures were made in the same animal. In the first group of animals, antinociception and spontaneous activity was determined. Antinociception was determined using the tail-flick reaction time to a heat stimulus. Before vehicle or drug administration, the baseline latency period (2–3 s) was determined. Four minutes after the injection, tail-flick latency was assessed once more, and the differences in control and test latencies were calculated. A 10-s maximum latency was used to calculate the % maximal possible effect (MPE). These animals were then transferred immediately to individual photocell activity chambers (11 × 6.5 in.), and spontaneous activity was measured during the next 10-min period. The number of interruptions of 16 photocell beams per chamber was recorded, and the activity in the drug-treated groups was expressed as a percentage of the vehicle-treated animals. Some of these mice were also tested in the tail-flick assay 24 h later. Then a separate group of mice were used for measurement of drug-induced hypothermia and immobility. As for hypothermia, rectal temperature was determined prior to vehicle or drug administration with a telethermometer (Yellow Springs Instrument, Yellow Springs, OH) and a thermistor probe (model YSI 400, Markson) inserted at a depth of 2 cm. At 4 min after the injection, rectal temperature was measured again, and the difference between pre- and post-injection values was calculated. The animals were then placed on a metal ring (5.5 cm in diameter) that was attached to a stand at a height of 16 cm. The amount of time (s) that the mouse spent motionless during a 5-min test session was recorded. The criterion for immobility was the absence of all voluntary movements (excluding respiration, but including whisker movement). The immobility index was calculated as:

$$\%\,\text{immobility} = \left[\frac{\text{time immobile (s)}}{\text{length of session (s)}}\right]100$$

Mice that fell or actively jumped from the ring were allowed five such escapes. Following the fifth escape, the test for that animal was terminated and immobility was calculated as a percentage of time that it remained on the ring before being discontinued. Data from mice failing to remain on the ring at least 2.5 min were not included.

For intrathecal (i.t.) administration, the compounds were dissolved in 10% ethanol in dimethyl sulfoxide, and a 5 μl

solution was injected into the spinal column between L5 and L6 of unanesthesized mice with a 30-gauge, one-half inch needle (Hylden and Wilcox, 1980). The animals were tested for tail-flick response 5 min after the injection, or, in time-course experiments, at different times after the injection until 24 h later. ED_{50} and confidence limits were calculated by means of a modification of the methods of Tallarida and Murray (see Adams et al., 1998 and references reported therein).

Intracerbroventricular (i.c.v.) injections were performed in mice that were lightly anesthetized with ether. An incision was made in the scalp such that the bregma was exposed. Injections were performed using a 26-gauge needle with a sleeve of PE 20 tubing to control the depth of the injection. A 5-μl injection was made 2 mm rostral and 2 mm caudal to the bregma at a depth of 2 mm. The vehicle for these injections consisted of 10% ethanol in dimethyl sulfoxide. The animals were tested 10 min after the injection.

2.7. Statistics

Data were compared by analysis of variance followed by the Bonferroni/Dunn test, and the threshold for significance was $P \leq 0.05$.

3. Results

3.1. Arvanil as a ligand / agonist of cannabinoid CB_1 and vanilloid VR_1 receptors

The K_i values for the displacement of the high affinity cannabinoid CB_1 receptor ligand [^{3}H]SR141716A from rat and mouse brain membranes by anandamide, arvanil and the dimethylheptyl analogue of arvanil (O-1839, Fig. 1) are shown in Table 1. Arvanil's affinity was slightly less than that of anandamide, whereas O-1839's affinity was less than that of arvanil. If the hydrolase inhibitor phenylmethylsulfonylfluoride was omitted during the assay incubation, the ability of anandamide, but not arvanil or O-1839, to compete for binding was almost abolished. Furthermore, O-1839 failed to inhibit [^{14}C]anandamide hydrolysis by rat brain homogenates or mouse $N_{18}TG_2$ neuroblastoma cell membrane preparations even at a 100 μM concentration (89.3 ± 10.1% of control in $N_{18}TG_2$ cells, mean ± S.E.M., $n = 3$), and we found that less than 5% of either arvanil or O-1839 was hydrolyzed after a 30-min incubation with rat brain homogenates.

Table 1
Displacement (K_i, μM) by anandamide, arvanil and O-1839 of [^{3}H]SR141716A bound to membrane preparations from rodent brain

	Anandamide	Arvanil	O-1839
Mouse brain	0.9 ± 0.2	1.8 ± 0.3	7.3 ± 1.1
	(> 10)	(3.9 ± 0.4)	(9.2 ± 1.4)
Rat brain	1.1 ± 0.4	2.6 ± 0.6	5.9 ± 0.9
	(> 10)	(5.9 ± 1.2)	(8.5 ± 1.5)

Values are means ± S.E.M. of $n = 3$. B_{max} and K_d for [^{3}H]SR141716A calculated from Scatchard plots obtained with increasing concentrations of the radioligand were 2.1 ± 0.3 and 1.8 ± 0.2 pmol/mg protein, and 4.3 ± 0.6 and 1.4 ± 0.3 nM, for mouse and rat brain membranes, respectively (means ± S.E.M., $n = 3$). Competition experiments were carried out in the presence of 100 μM phenylmethylsulfonylfluoride, or in its absence (values in parentheses).

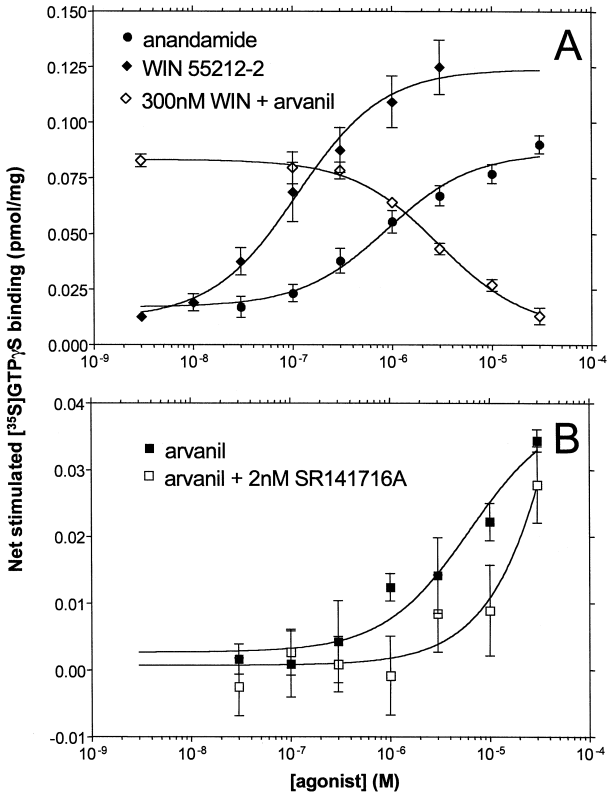

Fig. 2. Stimulation by various agonists of GTP-γ-S binding to mouse brain membranes. (A) Effects of different concentrations of WIN55212-2 (WIN) and anandamide, and of arvanil on the effect produced by 300 nM WIN55212-2. (B) Dose-dependent effect of arvanil in the presence or absence of 2 nM SR141716A (SR). Data are means ± S.E.M. of $n = 3$ independent experiments each carried out in triplicate. In (B) it was not possible to test a higher concentration of arvanil because of limited solubility at doses higher than 30 μM. The effect of SR141716A was significantly different from arvanil alone ($p < 0.05$ by analysis of variance) at 1 and 10 μM arvanil.

The cannabinoid ligand WIN 55212-2, anandamide and arvanil stimulated GTP-γ-S binding to mouse brain membranes in a dose-dependent manner (Fig. 2). The cannabinoid CB_1 receptor-selective antagonist SR141716A at 2 nM shifted the concentration–effect curve for arvanil to the right (Fig. 2B). WIN 55212-2 behaved as a full agonist (E_{max} 124 ± 10 fmol/mg, Fig. 2A). anandamide behaved as an intermediate partial agonist (E_{max} 83 ± 4 fmol/mg, 67% of WIN 55212-2, Fig. 2A), whereas arvanil behaved as a weak partial agonist (E_{max} 30 ± 8 fmol/mg, 24% of WIN 55212-2, Fig. 2B). In fact, arvanil counteracted dose-dependently ($IC_{50} = 3.0 \pm 0.7$ μM, $n = 3$, as as-

sessed by GraphPad data analysis, Fig. 2A) the stimulation of GTP-γ-S binding induced by 300 nM WIN 55212-2 (83 ± 3 fmol/mg in the absence of arvanil). The efficacies of anandamide and arvanil were not affected by pretreatment with phenylmethylsulfonylfluoride. However, the potency of anandamide, but not arvanil, decreased 4.4-fold in the absence of the inhibitor. EC_{50} values (μM) in the presence of phenylmethylsulfonylfluoride (means ± S.E.M., $n = 3$) were: arvanil, 1.22 ± 0.78; anandamide, 0.47 ± 0.16; WIN 55212-2, 0.12 ± 0.06.

We found that arvanil displaced the binding of [^{3}H]resiniferatoxin to membrane preparations from CHO cells transfected with vanilloid VR_1 receptor cDNA. The K_i value for this compound was 0.28 ± 0.11 μM (mean + S.D., $n = 3$).

3.2. Effects of i.v. administration of arvanil to mice

When administered i.v., arvanil dose-dependently induced in mice typical tetrahydrocannabinol-like behavioral responses (Fig. 3A,B), e.g. inhibition of spontaneous activity in an open field, induction of immobility on a ring, analgesia as assessed in the tail-flick assay, and decrease of rectal temperature, with EC_{50} values between 0.07 and 0.1 mg/kg. Injections of vehicle did not cause any significant effect (less than 15% effect in the spontaneous activity, antinociception and immobility test, and less than −0.5°C in the rectal temperature test). None of these effects was blocked by SR141716A (Fig. 3C) at a dose (3 mg/kg) fully blocking equi-effective doses of tetrahydrocannabinol, WIN55,212-2 and CP55,490 (Adams et al., 1998). This dose is still considered to be sub-threshold for the recently reported non-CB_1 receptor-mediated effects of SR141716A (White and Hiley, 1998; Járai et al., 1999). However, in this study 3 mg/kg SR141716A, when administered alone, produced a seemingly agonistic effect in the tail-flick ($P > 0.05$) and spontaneous activity ($P < 0.05$) tests. This latter effect, for which we have no reasonable explanation, could not be reproduced in subsequent experiments and had never been reported before with SR141716A. Therefore, it is unlikely that this effect may have masked SR 141716A's antagonism of arvanil's neurobehavioural actions. In this same experiment, SR141716A produced no hypothermia and did not attenuate that produced by arvanil. Furthermore, SR141716A produced no agonist effects on pain perception following i.t. (see below, Fig. 5) or i.p. administration; yet, the antagonist still failed to block the arvanil's analgesic effects (SR141716A, 3 mg/kg, i.p., 7.0 ± 2.5% MPE; arvanil, 0.5 μg/mouse, i.t., 87 ± 10% MPE; arvanil 10 min after SR141716A, 86 ± 12.0% MPE, means ± S.E.M., $n = 6$, see also below).

In the neurobehavioral tests performed in this study, anandamide was at least 100 times less potent than arvanil (Fride and Mechoulam, 1993; Smith et al., 1994), and its effects were also not blocked by SR141716A (Adams et al., 1998). Capsaicin was very active in the tail flick and the spontaneous activity tests ($ED_{50} = 0.03$ and 0.3 mg/kg), but, as compared to arvanil, induced little hypothermia (not dose-related and maximal effect of −1.75°C at 1 mg/kg) or catalepsy (maximal effect 35% inhibition at 1 mg/kg). The analgesic effects of arvanil and O-1839 disappeared after 24 h, whereas nociception in mice treated with capsaicin was still observed 1 day after treatment (5.0 ± 5.6, 9.2 ± 5.5, and 40 ± 15 %MPE with 0.3 mg/kg of arvanil, O-1839 and capsaicin, respectively, means ±

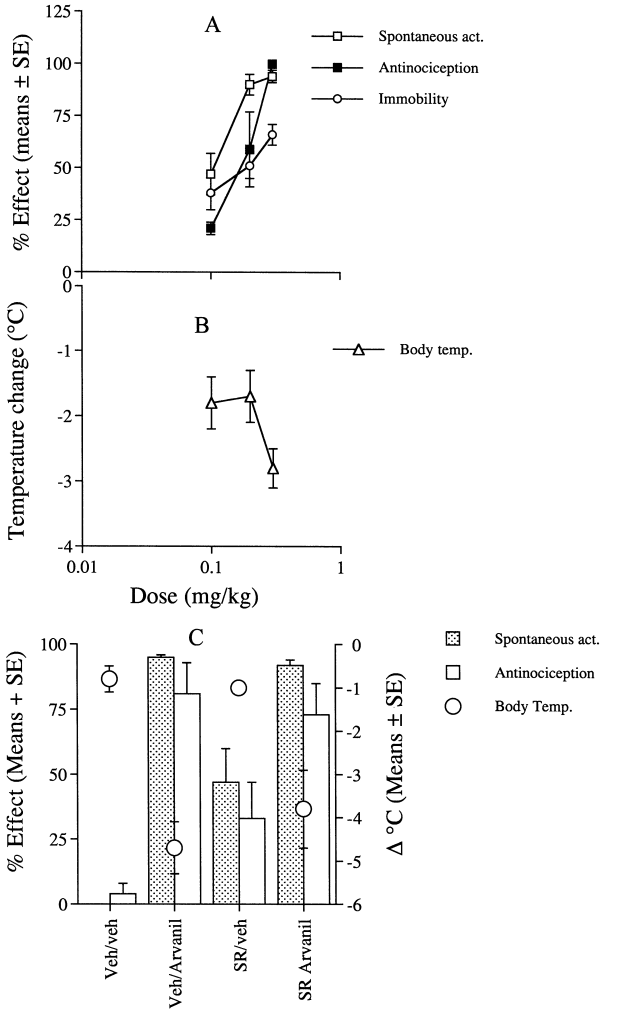

Fig. 3. Pharmacological effects of arvanil following i.v. administration. Arvanil was administered i.v. to mice and tested for spontaneous activity and antinociception in one group of mice and immobility (panel A) and rectal temperature (panel B) in a second group of mice. SR141716A (SR, 3 mg/kg, s.c.) was administered 10 min before arvanil (0.2 mg/kg) and tail-flick was measured at 4 min, rectal temperature immediately thereafter, and spontaneous activity from 5–15 min after injection (panel C). The results are presented as means ± S.E.M. for six mice per group. In (C) immobility was not tested, and data in the Veh/Arvanil group were significantly different from the Veh/veh group ($p < 0.01$), but not from the SR/Arvanil group. Data from the SR/Arvanil group were significantly different from the SR/veh group ($p < 0.05$), but not from the Veh/Arvanil group. Antinociception data from the SR/Veh group were not different from the Veh/veh group.

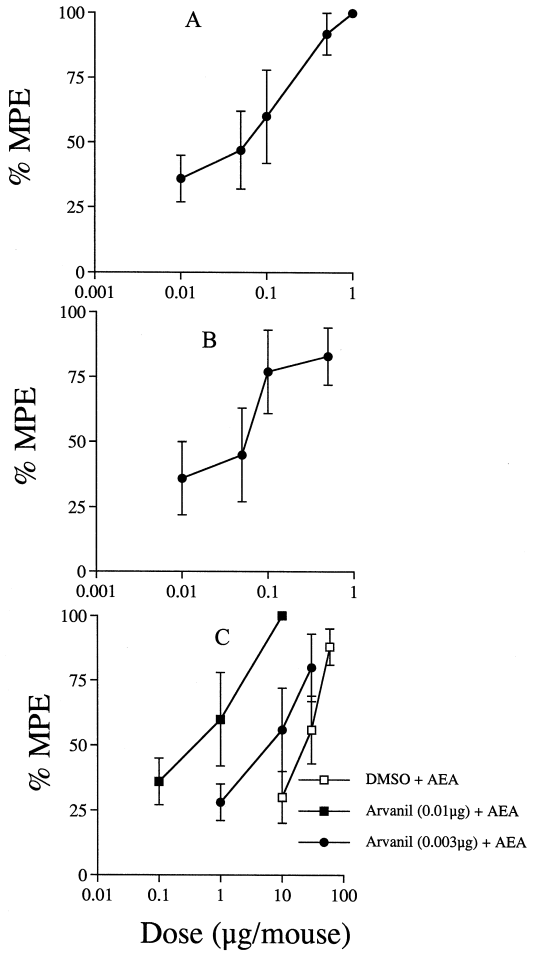

Fig. 4. Antinociceptive effects of arvanil following i.t. and i.c.v. administration. The mice were tested 5 and 10 min after i.t. (panel A) and i.c.v. (panel B) administration, respectively. For potentiation of anandamide, arvanil was administered i.t. 10 min prior to i.t. injection of anandamide (panel C). The results are presented as means ± S.E.M. for six mice per group.

S.E.M., $n = 6$). Pretreatment with capsazepine (3-30 mg/kg) either i.v. or subcutaneously did not block the effects of 0.3 mg/kg capsaicin (spontaneous activity, 56 ± 6.3 and 69 ± 8.3% inhibition, without and with 30 mg/kg capsazepine; antinociception, 98 ± 1.6 and 69 ± 20% MPE, without and with 30 mg/kg capsazepine; hypothermia, −0.87 ± 0.27 and −1.23 ± 0.13 Δ°C, without and with 30 mg/kg capsazepine, means ± S.E.M., $n = 6$). Finally, the other metabolically stable analogue O-1839 was very potent in reducing spontaneous activity (ED_{50} = 0.13 mg/kg, producing analgesia in the tail-flick assay (ED_{50} = 0.10 mg/kg), and reducing body temperature (ED_{50} = 0.18 mg/kg) following i.v. administration.

3.3. Analgesic effects of i.t. or i.c.v. administration of arvanil in mice

Arvanil also induced a potent analgesic response when administered either i.c.v. or i.t. [ED_{50} (C.L.) = 38 (20–70) or 30 (10–90) ng/mouse, respectively] in the tail-flick test (Fig. 4A,B), thus suggesting both spinal and supra-spinal mechanisms of action for this compound. Vehicle (dimethyl sulfoxide) always caused a non-significant response lower than 15%. In the same test, capsaicin was less potent [ED_{50} (C.L.) = 180 (90–350) ng/mouse, i.t.] while anandamide behaved as an analgesic only at 750-fold higher doses [(ED_{50} (C.L.) = 20.4 (14.0–29.8) μg/mouse, i.t.] (Fig. 4C). Interestingly, low doses of arvanil (i.t.)

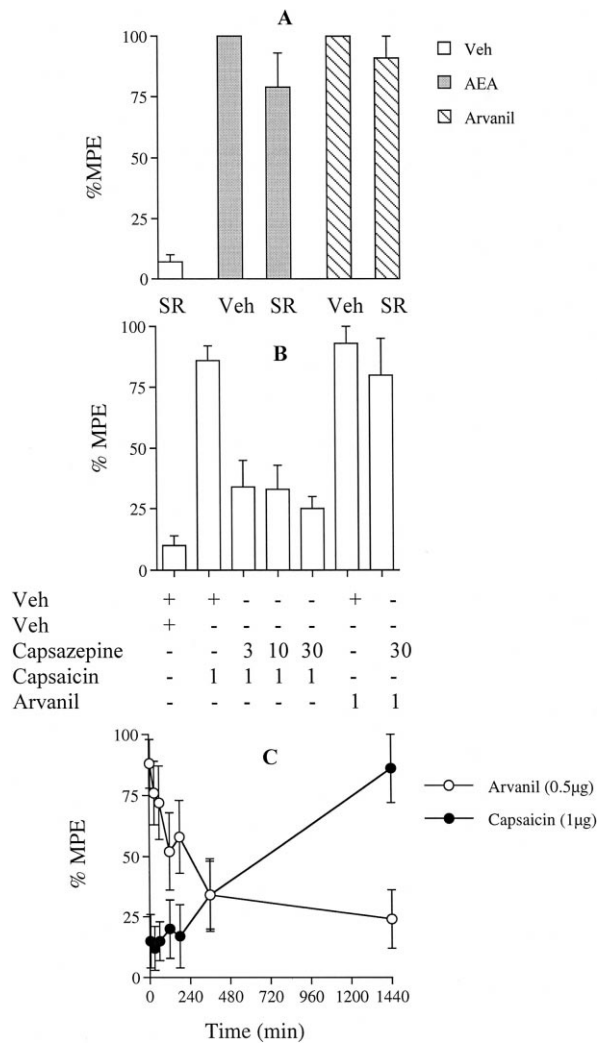

Fig. 5. Comparison of capsaicin and arvanil through antagonist studies and duration of action. Panel A: SR 141716A (50 μg/mouse) or its vehicle was administered i.t. 10 min prior to a second i.t. injection consisting of either arvanil (1.0 μg/mouse) or anandamide (60 μg/mouse). Panel B: Mice received an i.t. injection of either capsazepine (dose indicated in legend as μg/mouse) or its vehicle 10 min prior to a second i.t. injection of either vehicle, capsaicin or arvanil, the doses presented in μg/mouse. Latency was measured after 3 min for arvanil and after 24 h for capsaicin. Panel C: Comparison of the time course of arvanil (0.5 μg/mouse) and capsaicin (1 μg/mouse) following i.t. administration. A different group of mice were used for each time point. All results are presented as means ± S.E.M. for six mice per group. In (B) the effect of capsazepine was statistically significant against capsaicin ($P < 0.01$) at all doses.

administered 10 min before anandamide significantly enhanced the analgesic effects of the latter compound up to 60-fold. The ED_{50} for anandamide was decreased to 4.9 and 0.33 μg/mouse, respectively, with 3 and 10 ng/mouse of arvanil (Fig. 4C). The effects of arvanil (0.5 μg/mouse, i.t.) and anandamide (60 μg/mouse, i.t.) were not counteracted by pre-administration of an i.t. injection of a dose of 50 μg/mouse of SR141716A (Fig. 5A), which fully antagonizes a maximally effective i.t. dose of tetrahydrocannabinol (data not shown). Peripheral administration of this cannabinoid CB_1 receptor antagonist (3 mg/kg, i.p.) also did not attenuate anandamide administered i.t. (data not shown). Capsazepine (3 min pre-administration, 3–30 μg/mouse, i.t.) counteracted the analgesic effect of capsaicin (1 μg/mouse, i.t.) but not arvanil (1 μg/mouse, i.t.) (Fig. 5B). SR141716A (3 or 10 mg/kg, i.p.) did not influence the effect of 1 μg/mouse capsaicin (85.0 ± 15.0 and 76.0 ± 16.0, without or with 10 mg/kg SR141716A, means ± S.E.M., $n = 6$). We studied the time course for arvanil and capsaicin-induced analgesia in the tail-flick assay and found that while the effect of the former compound was maximal 3 min after i.t injection, the effect of capsaicin was maximal 24 h after treatment (Fig. 5C).

3.4. Effect of arvanil on anandamide and 2-arachidonoyl-glycerol levels in mouse spinal cord

We measured by LC-MS the levels of these compounds in ICR mouse spinal cord lipid extracts after i.t. injection of either vehicle or two different doses (10 and 50 ng/mouse) of arvanil (Table 2). We found that, after 3, 10 and 30 min, the levels of the two compounds were not significantly altered by arvanil (Table 2). We also measured anandamide levels in the spinal cord 5 min after i.t. injection of 30 μg/mouse of anandamide and found that only 1.54 ± 0.84 μg/mouse (or 4.45 ± 2.42 nmol/mouse, $n = 3$) of the compound could be recovered. These amounts were not increased if arvanil (10 ng/mouse) was administered i.t. 10 min prior to anandamide injection (1.59 ± 0.48 μg/mouse), i.e. under the same conditions used to observe enhancement of anandamide activity.

Table 2
Effect of arvanil on endogenous levels of anandamide and 2-arachidonoyl-glycerol levels in ICR mouse spinal cord

	Anandamide	2-Arachidonoyl-glycerol
Vehicle (i.t.)	After 3′ 17.7 ± 5.2[a]	After 3′ 3.5 ± 0.6[b]
	After 10′ 9.0 ± 4.0[a]	After 10′ 3.4 ± 0.1[b]
	After 30′ 11.7 ± 4.4[a]	After 30′ 3.9 ± 0.6[b]
Arvanil (i.t., 50 ng/mouse)	After 3′ 16.1 ± 1.0[a]	After 3′ 2.6 ± 0.6[b]
Arvanil (i.t., 10 ng/mouse)	After 10′ 5.5 ± 3.2[a]	After 10′ 4.0 ± 0.4[b]
	After 30′ 6.9 ± 3.0[a]	After 30′ 3.5 ± 0.6[b]

Anandamide and 2-arachidonoyl-glycerol were measured by isotope-dilution LC-APCI-MS in lipid extracts prepared from the spinal cord of each mouse treated as indicated, dissected after 3, 10 or 30 min after treatment. Data are means ± S.E.M. of $n = 3$ separate experiments. i.t., intrathecal, ND, not determined.

[a] Data are expressed as pmol/g tissue (wet weight).

[b] Data are expressed as nmol/g tissue.

4. Discussion

4.1. Arvanil is a partial agonist at stimulating GTP-γ-S binding to rat brain membranes

Previously, it was shown that μM concentrations of olvanil, a potent ligand of vanilloid receptors (Szallasi and Blumberg, 1999), could inhibit anandamide facilitated transport into cells and activate cannabinoid CB_1, but not CB_2, receptors as a partial agonist (Di Marzo et al., 1998). More recently, in a study carried out by using isolated cells in culture, the $C_{20:4}$ n-6 analogue of olvanil, which we named arvanil, was also shown to inhibit the anandamide transporter and to exhibit an affinity for cannabinoid CB_1 (but not CB_2) receptors at least comparable to that of anandamide (Melck et al., 1999). Since arvanil was three- to fivefold more potent than anandamide and about two-fold more potent than capsaicin as an inhibitor of human breast cancer cell proliferation, we proposed that this compound could act as a 'hybrid' agonist at cannabinoid CB_1 and vanilloid VR_1 capsaicin receptors. These findings provided the rationale for testing arvanil in models of cannabinoid and vanilloid activity. In this study, we confirmed that arvanil and anandamide have similar affinities for cannabinoid CB_1 receptors. The K_i values described herein are one order of magnitude higher than those described previously when using [^{3}H]CP55,490 or [^{3}H]SR141716A (Adams et al., 1998; Melck et al., 1999), but it should be noted that often great differences are observed when using different binding assay protocols and/or radiolabelled ligands and/or rat or mouse strains (see Pertwee, 1999, for review). For example, the K_i values for the dimethylheptyl analogue of arvanil, O-1839, determined by using [^{3}H]CP55,490 in rat brain P2 membranes (262 ± 90 and 201 ± 4 nM, means ± S.E.M., $n = 3$, without and with phenylmethylsulfonylfluoride, respectively), were considerably lower than those obtained herein with [^{3}H]SR141716A.

Arvanil also stimulated GTP-γ-S binding and was considerably less efficacious than either WIN 55,212-2 or anandamide. Although numerous receptors are coupled to G-proteins, the fact that arvanil binds weakly to the cannabinoid CB_1 receptor and is capable of blocking WIN 55212-2-stimulated GTP-γ-S binding suggests that arvanil interacts with low efficacy at the cannabinoid CB_1 receptor. This low efficacy may not necessarily prevent arvanil from acting as a cannabimimetic agonist in vivo. For example, tetrahydrocannabinol is a full agonist in vivo but behaves as a partial agonist in the GTP-γ-S binding assay

(Breivogel et al., 1998). Further evidence that arvanil stimulation of GTP-γ-S binding was at least in part mediated by cannabinoid CB_1 receptors rests with SR141716A. It counteracted arvanil's effect with an estimated K_e value of 0.82 nM similar to that reported for cannabinoid CB_1 receptor blockade by this compound. We did not test a higher concentration of the antagonist as arvanil efficacy in this assay was so low that, in the presence of a higher concentration of SR141716A, we would not have been able to approach a maximally effective concentration of agonist. Furthermore, arvanil was poorly soluble in water, which prevented the use of higher concentrations.

Finally, we found previously that arvanil activates vanilloid VR_1-mediated cation currents receptor with an efficacy at least comparable to that of capsaicin in a heterologous system expressing vanilloid VR_1 (Melck et al., 1999). Here we have established that this effect is due to the interaction with vanilloid VR_1 binding sites by using a displacement assay carried out with the potent ligand of these sites, [^{3}H]resiniferatoxin (Szallasi and Blumberg, 1999). We also found that arvanil and O-1839 are more stable than anandamide with respect to enzymatic hydrolysis. These data, taken together, suggest that arvanil's increased resistance to enzymatic hydrolysis and its capability to activate vanilloid VR_1 receptors, rather than its affinity for cannabinoid CB_1 receptors, may be responsible for its higher pharmacological activity than anandamide, particularly as an inhibitor of human breast cancer cell proliferation, where pharmacological evidence for the presence of vanilloid receptors was reported (Melck et al., 1999). In order to substantiate this concept, we investigated the cannabimimetic and capsaicin-like effects of arvanil in vivo.

4.2. Neurobehavioral effects of arvanil

In the mouse 'tetrad' of tests, which is highly indicative of cannabimimetic activity (Martin et al., 1991), arvanil was at least 100-fold more potent than anandamide, and as potent as (or more potent than) some of the most potent synthetic cannabinoid receptor ligands such as CP55,940 (Compton et al., 1992b) and WIN55,212-2 (Compton et al., 1992a). However, the effects of arvanil in these neurobehavioral tests were not affected by SR141716A. Given the lack of sensitivity also of anandamide, but not tetrahydrocannabinol or other cannabinoid CB_1 receptor agonists, to SR141716A in these tests (Adams et al., 1998), it is possible that arvanil, a substance whose chemical structure is similar to that of anandamide (Fig. 1), acts with high efficacy via non-CB_1 anandamide receptors in mouse central nervous system. Both anandamide and arvanil, like capsaicin, activate vanilloid VR_1 receptors (Zygmunt et al., 1999; Melck et al., 1999). Unfortunately, we could not find a way of blocking the neurobehavioral effects of i.v. capsaicin with the only vanilloid receptor antagonist known to date, i.e. capsazepine, and therefore we could not test the effect of this antagonist on the analogous responses induced by arvanil. Thus, we cannot rule out that a part of the neurobehavioral effects of the latter compound is due to activation of vanilloid VR_1 receptors. Indeed, discrepant results have been reported on the presence of these receptors in rodent central nervous system. Caterina et al. (1997), by using Northern blotting, did not find mRNA for this protein in any of the rat brain regions analyzed. On the other hand, Sasamura et al. (1998) found vanilloid VR_1 immunoreactivity in the hypothalamus and cerebellum, and Mezey et al. (2000) have recently reported the presence of this protein and the encoding mRNA in several other rat brain regions, including the hippocampus and the substantia nigra. However, capsaicin activates vanilloid VR_1 with a similar efficacy as arvanil (Melck et al., 1999), and yet exhibited here a different pharmacological and pharmacokinetic profile; its analgesic actions, for example, were still observable after several hours of treatment, whereas its cataleptic activity in the immobility test was hardly detectable. Conversely, one would have expected from the two compounds a qualitatively similar activity if their neurobehavioral effects were mediated by the same receptor. Furthermore, capsaicin activation of vanilloid receptors is coupled to glutamate release from central neurons (Sasamura et al., 1998), an effect that is not consistent with some of the 'inhibitory' neurobehavioral actions described here for arvanil. Capsaicin is also not likely to activate cannabinoid CB_1 receptors since it does not bind to these receptors (Di Marzo et al., 1998), and it induces little or no hypothermia and catalepsy in mice (present study). In fact, it should be noted that only a positive response in all four tests of the mouse 'tetrad' is highly suggestive of cannabimimetic activity (Martin et al., 1991). Also, the analgesic effect of capsaicin (i.t.) was not mediated by cannabinoid CB_1 receptors inasmuch as it was not blocked by SR141716A. The finding of little hypothermic effects by capsaicin was surprising, since this compound was reported to lower body temperature when administered subcutaneously to rats, although at doses somewhat higher than the maximal dose used here (Miller et al., 1982; Hayes et al., 1984). The different animal species, route of administration, doses and experimental protocol used in the present study probably account for this discrepancy. Doses of capsaicin higher than 1 mg/kg (i.v.) were toxic to mice and could not be tested here.

In order to further investigate the role for either cannabinoid CB_1 or vanilloid VR_1 receptors in the neurobehavioral effects of arvanil, the analgesic effects of this compound were examined in the tail-flick test after i.t. and i.c.v. administration. We found again a very potent analgesic response with either route of administration. These effects resembled the analogous action exerted by 750-fold higher doses of anandamide inasmuch as they were (i) maximal immediately after injection, (ii) unaffected by pre-treatment with a dose of SR141716A sufficient to block the effect of tetrahydrocannabinol, and (iii) unaf-

fected by a dose of capsazepine sufficient to attenuate the effect of capsaicin. In fact, capsaicin was also quite active (although at fivefold lower concentrations), but its analgesic effect was maximal after 24 h from treatment. Thus, in this as in the case of the effects observed after i.v. administration, it is possible that arvanil acts at novel sites of action for anandamide but with a much higher potency than the endogenous cannabinoid. Undoubtedly, arvanil's higher potency is due at least in part to its greater resistance to metabolic inactivation. However, blockade of in vivo metabolism of anandamide only potentiates its effects 5–10-fold (Compton and Martin, 1997), which is far less than the 750-fold potency difference between anandamide and arvanil.

4.3. The effects of arvanil in vivo are not due to its enzymatic hydrolysis

Anandamide is almost immediately hydrolyzed to arachidonic acid, particular after i.v. administration (Willoughby et al., 1997), which raises the possibility that arachidonic acid and subsequent oxidation of this fatty acid to eicosanoids contributes to anandamide's effects. However, there are several lines of evidence that argue against metabolites contributing to arvanil's effects. First, arvanil is more stable than anandamide toward enzymatic hydrolysis in vitro. Additionally, O-1839 is metabolically stable (Table 1) yet is as potent as arvanil at inhibiting spontaneous activity, and at inducing analgesia and hypothermia when administered i.v. Furthermore, enzymatic hydrolysis of O-1839 would not yield arachidonic acid. Based on these observations, it seems highly unlikely that hydrolysis of arvanil to arachidonic acid contributes to the neurobehavioral actions of this compound.

4.4. Arvanil does not act in vivo by enhancing endogenous anandamide levels

Although almost inactive as an inhibitor of anandamide hydrolysis, arvanil is a rather potent inhibitor of anandamide facilitated transport into cells at low μM concentrations ($IC_{50} = 3.6$ μM; Melck et al., 1999). Based also on the observation that neither anandamide nor arvanil neurobehavioral actions on mice are blocked by SR141716A, we wanted to assess whether the effects that we observed with this compound in vivo were due to enhancement of endogenous anandamide levels. We chose the tail-flick test and the i.t. treatment of mice with arvanil to address this problem. We found that the analgesic action of anandamide (i.t.) was significantly enhanced by arvanil (i.t.) even with a very low dose (3 ng/mouse, corresponding to 6.8 pmol) of the latter compound that alone produces negligible analgesic effects in the same test. This dose is not likely to yield the concentration expected to be necessary to block anandamide uptake by neurons. When we injected 30 μg/mouse of anandamide i.t., a dose close to that necessary for a half-maximal effect in the tail-flick test, we found that 5 min after treatment, when the analgesic effect of anandamide is maximal, 5% of the amount injected (i.e. 1.54 μg/mouse, corresponding to 4.45 nmol/mouse) was present in the spinal cord. We found that pre-treatment of mice with up to 10 ng (23 pmol) of arvanil did not increase these amounts of anandamide. We also wanted to determine the effect of i.t. arvanil on *endogenous* anandamide levels, at concentrations similar to those necessary to observe an analgesic effect in the tail-flick test. Again, we found that anandamide spinal cord levels measured after the injection of arvanil were not different from those measured after vehicle. The levels of the other proposed endocannabinoid, 2-arachidonoylglycerol, were also unaffected. These data strongly suggest that the analgesic effects of low doses of arvanil, as well as their facilitatory effect on anandamide analgesic response, are not due to inhibition of anandamide degradation.

4.5. Conclusions

We have reported here that arvanil is a very potent and efficacious tetrahydrocannabinol-like agent in vivo despite its low efficacy at cannabinoid CB_1 (and CB_2) receptors. The effects of this compound are not blocked by antagonists of cannabinoid CB_1 or (in the case of i.t. administration of arvanil) vanilloid receptors, nor are they due to its metabolism or to enhancement of endogenous anandamide levels. Molecular approaches are now necessary in order to assess whether arvanil effects are due to novel sites of action, which may be activated also by anandamide. In any event, whatever its mechanism of action, arvanil may serve as a template for the development of novel ultra-potent analgesic drugs.

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