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Unsaturated Long-Chain *N*-Acyl-vanillyl-amides (N-AVAMs): Vanilloid Receptor Ligands That Inhibit Anandamide-Facilitated Transport and Bind to CB1 Cannabinoid Receptors

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We investigated the effect of changing the length and degree of unsaturation of the fatty acyl chain of *N*-(3-methoxy-4-hydroxy)-benzyl-*cis*-9-octadecenoamide (olvanil), a ligand of vanilloid receptors, on its capability to: (i) inhibit anandamide-facilitated transport into cells and enzymatic hydrolysis, (ii) bind to CB1 and CB2 cannabinoid receptors, and (iii) activate the VR1 vanilloid receptor. Potent inhibition of [^{14}C]anandamide accumulation into cells was achieved with C20:4 n-6, C18:3 n-6 and n-3, and C18:2 n-6 *N*-acyl-vanillyl-amides (N-AVAMs). The saturated analogues and Δ^9-*trans*-olvanil were inactive. Activity in CB1 binding assays increased when increasing the number of *cis*-double bonds in a n-6 fatty acyl chain and, in saturated N-AVAMs, was not greatly sensitive to decreasing the chain length. The C20:4 n-6 analogue (arvanil) was a potent inhibitor of anandamide accumulation (IC_{50} = 3.6 μM) and was 4-fold more potent than anandamide on CB1 receptors (Ki = 0.25–0.52 μM), whereas the C18:3 n-3 N-AVAM was more selective than arvanil for the uptake (IC_{50} = 8.0 μM) vs CB1 receptors (Ki = 3.4 μM). None of the compounds efficiently inhibited [^{14}C]anandamide hydrolysis or bound to CB2 receptors. All N-AVAMs activated the cation currents coupled to VR1 receptors overexpressed in *Xenopus* oocytes. In a simple, intact cell model of both vanilloid- and anandamide-like activity, i.e., the inhibition of human breast cancer cell (HBCC) proliferation, arvanil was shown to behave as a "hybrid" activator of cannabinoid and vanilloid receptors.

Key Words: **cannabinoid; endocannabinoid; capsaicin; analgesics; breast cancer cells; carrier.**

Studies on the mechanism of action of a natural product widely used in pharmacological studies, the pungent 'hot' chili pepper ingredient, capsaicin [*N*-(3-methoxy-4-hydroxy)-benzyl-8-methyl-6-*trans*-nonenamide], underwent a major breakthrough with the cloning and structure determination of the rat capsaicin receptor, named vanilloid receptor 1 (VR1) (1). This protein is structurally related to members of the transient receptor potential (TRP) and TRP-like (TRPL) family of cation channels, is expressed uniquely in sensory ganglia, and, apart from capsaicin, is activated by noxious heat and protons and probably functions as a transducer for painful thermal stimuli (1, 2). Earlier studies had shown that capsaicin exhibits potent vasodilatory properties, probably mediated by the release of neurotransmitters such as substance P and the calcitonin gene related peptide, and exerts analgesic and anti-inflammatory actions through desensitization of sensory nociceptors (see [3] for a review). These receptor-mediated effects of capsaicin are

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Abbreviations used. N-AVAM, *N*-acyl-vanillyl-amide; VR1, vanilloid receptor type 1; RBL, rat basophilic laukemia; CB1, CB2, cannabinoid receptor type 1 and 2; TRP, transient receptor potential; TRPL, transient receptor potential-like; RTX, resiniferatoxin; FAAH, fatty acid amide hydroxylase; AM404, *N*-(4-hydroxyphenyl)-arachidonylamide; anandamide, *N*-arachidonoyl-ethanolamine; olvanil, *N*-(3-methoxy-4-hydroxy)-benzyl-*cis*-9-octadecenoamide; palvanil, *N*-(3-methoxy-4-hydroxy)-benzyl-hexadecanamide; arvanil, *N*-(3-methoxy-4-hydroxy)-benzyl-arachidonylamide; pseudocapsaicin, *N*-(3-methoxy-4-hydroxy)-benzyl-nonanamide; HBCC, human breast cancer cell.

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usually antagonized by the ion channel blocker ruthenium red and by the competitive antagonist capsazepine (3). Another natural compound, the phorbol derivative resiniferatoxin (RTX), has a pharmacological profile similar to that of capsaicin (see [4] for review) and has been shown to activate both native vanilloid receptors and recombinant VR1 even more potently than capsaicin. Several studies, however, based on both qualitatively and quantitatively different responses of some tissue preparations to either capsaicin or RTX, have suggested that vanilloid receptors may be pharmacologically heterogeneous (as reviewed in [5]). Non pungent long fatty acyl chain analogues of capsaicin, such as olvanil [*N*-(3-methoxy-4-hydroxy)-benzyl-*cis*-9-octadecenoamide], were synthesized and also found to exert vasodilatory, analgesic and anti-inflammatory properties in vitro and, to a lesser extent, in vivo (6, 7, 8). Based on a series of pharmacological differences between olvanil [and other *N*-acyl-vanillyl-amides (N-AVAMs)] and capsaicin, it was proposed that this synthetic compound activates an additional vanilloid receptor subtype (6). However, since the cloning of VR1, no study has addressed yet the possibility that long chain N-AVAMs bind to this receptor.

Another natural fatty acid amide, anandamide (*N*-arachidonoyl-ethanolamine), was originally isolated from porcine brain and suggested to play a role as the endogenous ligand of cannabinoid receptors (9). Two such receptors have been identified so far in mammals, the CB1 receptor, present in both nervous and certain peripheral tissues, and the CB2 receptor, predominantly expressed in immune cells (see [10] for a recent review on the pharmacology of cannabinoid receptors). Anandamide was shown to bind to and activate, to a different extent, both receptors, and to exert pharmacological actions similar, although not identical, to those of marijuana's active principle (−)-Δ^9-tetrahydrocannabinol (for a recent review on anandamide pharmacology and metabolism see [11]). Apart from its pharmacological profile, a role as a cannabimimetic neuromodulator for anandamide was also suggested by the discovery of molecular mechanisms for its biosynthesis and inactivation in intact neurons (12). In particular, it was found that anandamide is enzymatically hydrolyzed to arachidonic acid and ethanolamine following its 'carrier'-mediated facilitated transport into neurons. More recent studies have succeded in characterizing the enzyme catalyzing anandamide hydrolysis, named 'fatty acid amide hydrolase' (FAAH) (13), and identifying—in rat basophilic leukaemia RBL-2H3 cells (14), cerebellar granule cells (15), cortical neurons and astrocytes (12, 16), and human lymphoma cells (17)—protein(s) responsible for anandamide facilitated diffusion through the cell membrane. Aromatic anandamide analogues, such as *N*-benzyl-arachidonyl-amide (15) and *N*-(4-hydroxyphenyl)-arachidonyl-amide (AM404) (16), as well as the anandamide congener oleoylethanolamide (15), were shown to inhibit anandamide re-uptake. This prompted a recent study on the possible interactions between olvanil and proteins of the 'endocannabinoid system', where we found that olvanil is a potent inhibitor of anandamide facilitated diffusion into RBL-2H3 cells, as well as a weak functional agonist of CB1, but not CB2 receptors (18). The former finding was later confirmed also in a human astrocytoma cell line expressing the anandamide 'carrier' (19). In the present study we have addressed the question of whether other N-AVAMs could be designed with higher inhibitory activity on the anandamide 'carrier' or with higher affinity for CB1 receptors, or both, while preserving their capability to activate vanilloid receptors. Answers to this question could provide supplementary information on the mechanisms underlying the molecular recognition of: (i) anandamide-like ligands by cannabinoid receptors and the anandamide transporter, and (ii) synthetic vanilloids by the VR1 receptor. Furthermore, given the well documented involvement of spinal/sensory cannabinoid receptors and endocannabinoids in the down-regulation of pain (20, 21), such N-AVAMs could be used as templates for the development of novel analgesic substances with multiple mechanisms of action, i.e. substances capable of: (i) desensitizing polymodal nociceptors, (ii) activating antinociceptive CB1 receptors, and (iii) potentiating the anti-hyperalgesic action of anandamide by inhibiting its inactivation. We have screened eleven N-AVAMs (Fig. 1), including capsaicin, and report that slightly different structural determinants of their fatty acyl chains are necessary to these compounds for an optimal interaction with either the anandamide membrane 'carrier' or the CB1 receptor, and that long chain N-AVAMs like olvanil do activate VR1 receptors. Moreover, we report that some of these substances, and in particular the C20:4 n-6 analogue, that we named arvanil, may behave as 'hybrid' CB1/VR1 functional agonists.

MATERIALS AND METHODS

Materials, cell cultures and chemical synthesis of N-AVAMs. Mouse neuroblastoma N18TG2, rat basophilic RBL-2H3, and MCF-7, T-47D and EFM-19 cells were cultured as described previously (14, 18, 22, 23). [^{14}C]Anandamide (5 mCi/mmol) was synthesized as described previously (9) from [^{14}C]ethanolamine (50 mCi/mmol, Amersham) and arachidonoyl chloride (Sigma, UK). [^{3}H]SR141716A (55 Ci/mmol) and [^{3}H]WIN55,212-2 (43 Ci/mmol) were purchased from Amersham and NEN-Dupont, respectively. Capsaicin and its C9:0 analogue (pseudocapsaicin), forskolin and ruthenium red were purchased from Sigma, UK. Capsazepine was purchased from Biomol, and RTX from Alexis. SR141716A and SR144528 were kindly donated by Sanofi Recherche, Montpellier, France. N-AVAMs were synthesized by reacting 30 mg of 3-methoxy-4-hydroxy-benzylamine (obtained by dissolving the hydrochloride available from Sigma in Tris-HCl, pH 9.5, followed by repeated extractions with diethyl ether) with the corresponding fatty acyl

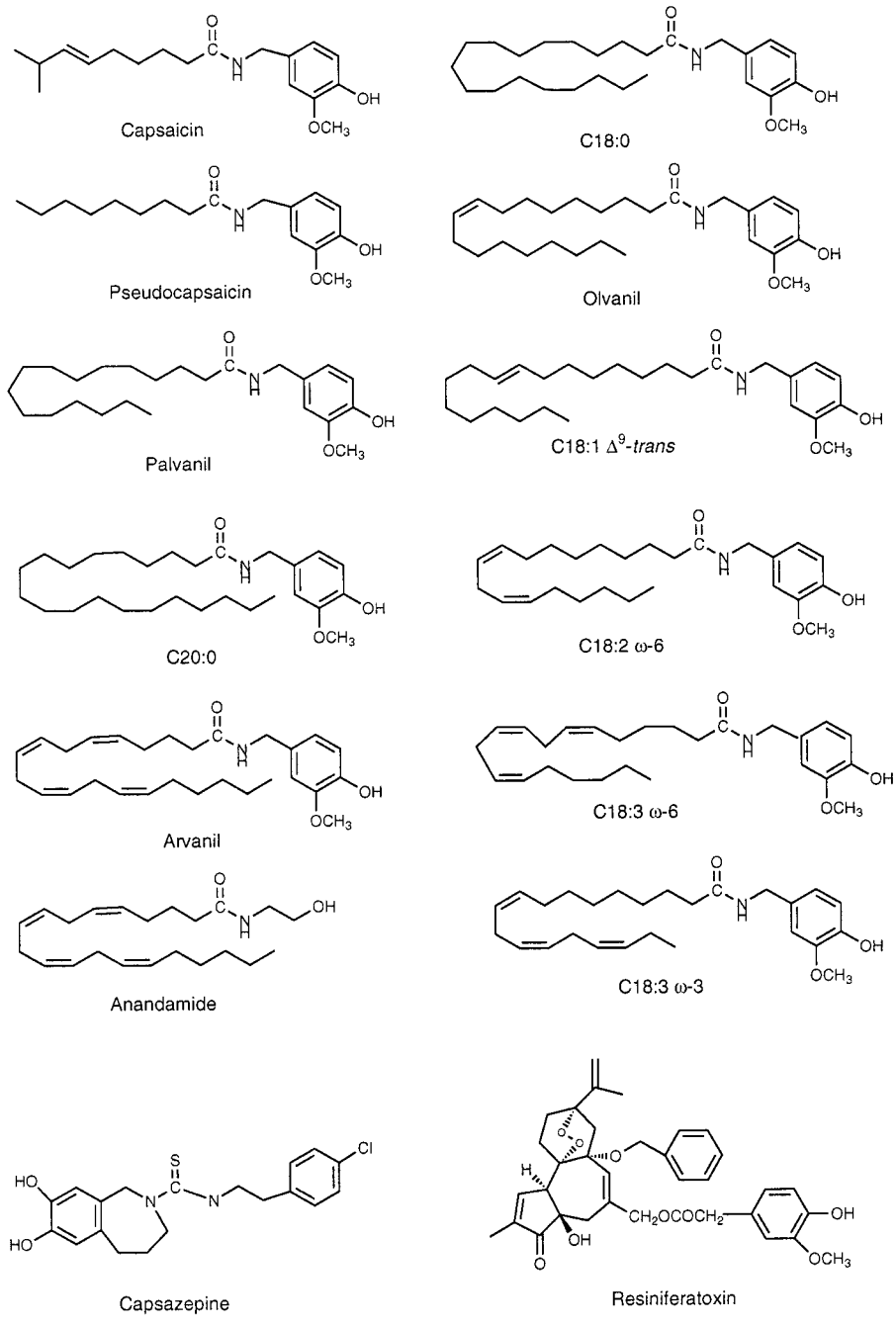

FIG. 1. Chemical structures of the N-AVAMs, vanilloids and anandamide utilized in this study.

chlorides, purchased from Sigma, at 4°C for 30 min. In some cases (i.e. for the C18:0, C20:0, Δ^9 *trans* C18:1 analogues) the chlorides were prepared by reacting the free fatty acids with oxalyl chloride as described in (9). The reaction mixture was then brought to dryness and purified by normal phase high pressure liquid chromatography as described in (18). The chemical structure of the purified compounds was checked by gas chromatography-electron impact mass spectrometry as previously described (18).

Anandamide uptake and FAAH assays. The effect of N-AVAMs on the accumulation of anandamide in intact RBL-2H3 cells was studied according the procedures described in (14, 18). Briefly, confluent cells in six-well dishes (1×10^6 cells/well) were incubated for 30 min at 37°C with 0.5 ml of serum-free media containing 20,000 cpm [^{14}C]anandamide (4 μM) plus vehicle or one of the N-AVAMs shown in Fig. 1, at various (0, 1, 5, 10, 50 μM) concentrations. After the incubation, the medium was removed, the cells washed three times with 3 ml of medium containing 0.2% bovine serum albumin, and extracted with serum-free medium/chloroform/methanol 1:2:1 (by vol.). [^{14}C]Anandamide accumulated in cells was determined as described (14). FAAH assays were carried out using a procedure similar to that described in previous papers (14, 18). Briefly, 10,000 $\times$ *g* pellets (0.05–0.1 mg proteins) from N18TG2 cell homog-

TABLE 1

Effect of N-AVAMs on [^{14}C]Anandamide Accumulation into RBL-2H3 Cells and on [^{14}C]Anandamide Hydrolysis by N18TG2 Cell Membranes

	[^{14}C]Anandamide accumulation into RBL-2H3 cells	[^{14}C]Anandamide hydrolysis by N18TG2 cell membranes
Capsaicin	46.9 ± 2.1[a]	>50[a]
C9:0 (pseudocapsaicin)	>50	>50
C16:0 (palvanil)	>50	>50
C18:0	>50	>50
Δ^9-*cis* C18:1 (olvanil)	9.0 ± 2.0[a]	48.0 ± 2.0[a]
Δ^9-*trans* C18:1	>50	>50
$\Delta^{6,9}$-*cis* C18:2 n-6	7.0 ± 2.0	50.5 ± 3.0
$\Delta^{6,9,12}$-*cis* C18:3 n-6	5.0 ± 1.0	49.0 ± 4.2
$\Delta^{6,9,12}$-*cis* C18:3 n-3	8.0 ± 2.0	>50
C20:0	>50	>50
$\Delta^{5,8,11,14}$-*cis* C20:4 n-6 (arvanil)	3.6 ± 0.7	32.0 ± 5.0

Note. The effects are expressed as IC_{50} in μM and are means ± S.D. of three independent experiments carried out in duplicate. N.D., not determined.

[a] Data are from (18).

enates were incubated in 1 ml Tris-HCl 50 mM, pH 9.0, with 25 μM [^{14}C]anandamide for 30 min at 37°C, in the presence or absence of increasing concentrations (5, 10, and 50 μM) of N-AVAMs. After the incubation the amount of [^{14}C]ethanolamine produced from [^{14}C]anandamide hydrolysis was determined as described (14). In both assays the inhibitory effect was expressed as the concentration necessary to achieve 50% inhibition (IC_{50}) and as means ± S.D. of three separate experiments. For these calculations, the amount of [^{14}C]anandamide accumulated into cells during incubations carried out at 4°C, or the amount of [^{14}C]ethanolamine in incubations with no proteins, respectively, were subtracted from total amounts, IC_{50} values were calculated from dose-response curves drawn by using Graph-Pad software.

Binding and cAMP assays. CB1 and CB2-receptor binding affinities of N-AVAMs were determined by means of competition assays followed widely described procedures (18, 22, 24). Membranes from confluent N18TG2 and RBL-2H3 cells, prepared as described in (24) and (22), respectively, were used to determine the displacement by N-AVAMs, tested at various concentrations (0.1, 0.5, 1, 5, 10 or 15 μM), of 300 pM [^{3}H]SR141716A or [^{3}H]WIN55,212-2. Membrane-bound and free radioligands were separated by the filtration procedures described previously (22, 24), and bound radioactivity measured by liquid scintillation counting. The CB1 binding affinities of arvanil, olvanil and the C16:0 analogue, that we named palvanil, in MCF-7 cell membranes were determined by a similar procedure, as described in (23) but using 300 pM [^{3}H]SR141716A. Specific binding was calculated by using 10 μM unlabelled SR141716A, or 10 μM HU-210 (kindly donated by Prof. R. Mechoulam, The Hebrew University, Jerusalem, Israel) when [^{3}H]SR141716A or [^{3}H]WIN55,212-2 was used as radioligand, respectively, and was 35–40% and 16–32% in the two cases. Binding affinities were expressed as Ki calculated, by using the Cheng-Prusoff equation, from IC_{50} values (see above) for the inhibition by N-AVAMs of radioligand binding to membranes. Results are means ± S.D. of three separate experiments. The effects of 5 μM arvanil and olvanil on forskolin-induced cAMP formation in intact N18TG2 cells were determined by means of a cAMP assay kit (Amersham) as advised by the manufacturer and according to the procedure described previously (18).

Assays of VR1-mediated ion currents. Defolliculated *Xenopus laevis* oocytes were injected with 2.5–5 ng VR1 cRNA, synthesized from *MlkI*-linearized VR1 cDNA templates using T7 RNA polymerase (1, 2). Control oocytes were injected with water. 10–14 days after injection, VR1 currents were analyzed by patch-clamp methods as described previously (2). Solution used for recording (on either side of the membrane) comprised 10 mM Tris-HCl, 1 mM EGTA, 150 mM CsCl, 1.0 mM $MgCl_2$, pH 7.4, at room temperature.

Assays for HBCC proliferation and apoptosis. The effect of different concentrations (0.1, 0.5, 1, 2.5, 5) of capsaicin, pseudocapsaicin, arvanil, olvanil, palvanil, RTX, capsazepine and anandamide on the proliferation of MCF-7, EFM-19 and T-47D HBCCs was studied according to the procedures described previously (23). Compounds or vehicle (ethanol) were administered at each change of medium and for 4–6 days after which cells were trypsinized and counted by a haemocytometer. In some experiments, substances were co-incubated with CB1, CB2 and vanilloid receptor antagonists or with ruthenium red, as shown in Fig. 3. In this case, statistically different inhibitory effects were evaluated by means of the unpaired Student's T test. Cell viability was checked by trypan blue. The effect of arvanil on MCF-7 cell apoptosis was determined by analysis of DNA fragmentation after 24 and 72 hour incubation of cells with 1 or 5 μM concentrations of the substance, as described previously ([23] and references therein).

RESULTS

Effect of N-AVAMs on anandamide facilitated diffusion into RBL-2H3 cells. Of the eleven N-AVAMs tested in this study, all the all *cis* unsaturated analogues efficiently inhibited the accumulation of [^{14}C]anandamide into RBL-2H3 cells with the following order of potency: arvanil > 18:3 n-6 > 18:2 n-6, 18:3 n-3 > olvanil (Table 1). The Δ^9-*trans* analogue of olvanil, as well as the completely unsaturated C9:0, C16:0, C18:0 and C20:0 analogues were inactive (IC_{50} > 50 μM).

Effect of N-AVAMs on anandamide hydrolysis by N18TG2 cell membranes. As shown in Table 1, none of the compounds tested, including arvanil, was a potent inhibitor in a FAAH activity assay carried out with [^{14}C]anandamide as the substrate and N18TG2 cell membranes as the source of FAAH (25).

TABLE 2

Effect of N-AVAMs on the Binding of High-Affinity CB1 and CB2 Ligands to Membranes of Cells Selectively Expressing CB1 and CB2 Receptors

	N18TG2 cells [^{3}H]SR141716A	RBL-2H3 cells [^{3}H]WIN55,212-2[c]	MCF-7 cells [^{3}H]SR141716A
Capsaicin	>10[a]	>15	N.D.
C9:0 (pseudocapsaicin)	2.7 ± 1.2	>15	N.D.
C16:0 (palvanil)	3.0 ± 0.5	>15	2.6 ± 0.4
C18:0	3.3 ± 0.7	>15	N.D.
Δ^9-*cis* C18:1 (olvanil)	1.6 ± 0.4[b]	>15[b]	1.0 ± 0.3
Δ^9-*trans* C18:1	4.3 ± 0.3	>15	N.D.
$\Delta^{6,9}$-*cis* C18:2 n-6	1.4 ± 0.2	>15	N.D.
$\Delta^{6,9,12}$-*cis* C18:3 n-6	0.8 ± 0.2	≥10[d]	N.D.
$\Delta^{6,9,12}$-*cis* C18:3 n-3	3.4 ± 0.3	≥10[d]	N.D.
C20:0	>10[a]	>15	N.D.
$\Delta^{5,8,11,14}$-*cis* C20:4 n-6 (arvanil)	0.5 ± 0.2	>15	0.25 ± 0.1
Anandamide	1.9 ± 0.3	0.03	0.85 ± 0.25

Note. The effects are expressed as Ki ± S.D. of three separate experiments carried out in duplicate. Ki were calculated from the corresponding IC_{50} by means of the Cheng-Prusoff equation and using Kd constants previously determined ([18, 22] and D. Melck and V. Di Marzo, unpublished observation). The Ki for anandamide displacement of [^{3}H]SR141716A from N18TG2 cells is 1.9 ± 0.3 μM (18). N.D., not determined.

[a,c] Less than 50% displacement was observed at 10 or 15 μM, respectively.

[b] Data are from (18).

[d] About 50% displacement was observed at 10 μM.

CB1 and CB2 receptor binding affinities of N-AVAMs. Table 2 shows the Ki values for the displacement by the eleven N-AVAMs of [^{3}H]SR 141716A and [^{3}H]WIN55,212-2 from membrane preparations from N18TG2 and RBL-2H3 cells, which selectively express CB1 and CB2 receptors, respectively (22, 24). Although some specific binding was observed for some of the compounds at high concentrations, none of the N-AVAMs tested exhibited Ki values lower than 10 μM for the CB2-containing membranes. On the other hand, most of the compounds inhibited the binding of [^{3}H]SR 141716A to N18TG2 cell membranes, the order of potency being: arvanil > C18:3 n-6 > C18:2 n-6 > olvanil > C18:3 n-3, C18:1 Δ^9-*trans*, C18:0, C16:0, C9:0 > C20:0, capsaicin. Arvanil (Ki = 0.51 μM) was almost 4-fold more potent than anandamide (Ki = 1.9 μM) and exhibited at least a 30-fold selectivity for CB1 *vs.* CB2 receptors. As previously shown for olvanil (18), arvanil behaved as an agonist at CB1 receptors since it inhibited forskolin-induced cAMP formation in intact N18TG2 cells (66.7 ± 2.1% inhibition at 5 μM, n = 3).

Effect of N-AVAMs on VR1-coupled ion currents in Xenopus oocytes. Six long chain N-AVAMs, including olvanil and arvanil, were tested for their ability to elicit membrane currents in inside-out patches excised from *Xenopus* oocytes injected with the VR1 receptor cDNA. At a 2 μM concentration, all compounds elicited ion currents that were reversed by the VR1 antagonist capsazepine (10 μM), but not with Ringer perfusate washout (Fig. 2). No effect was observed in control oocytes injected with water (data not shown). The amplitudes of the currents by the six compounds were comparable, and of the same order of magnitude as those elicited by 2 μM capsaicin or 0.1 μM RTX (data not shown).

Effect of N-AVAMs on HBCC proliferation. The effect of olvanil, arvanil, the C16:0 N-AVAM (that we named palvanil) and pseudocapsaicin on the proliferation of MCF-7, T-47D and EFM-19 human breast cancer cell was studied (Table 3) and compared to the effect of capsaicin, RTX, the vanilloid receptor antagonist capsazepine and anandamide. In EFM-19 cells all compounds inhibited cell proliferation with the following rank of potencies: arvanil > olvanil, capsaicin, pseudocapsaicin > palvanil, RTX, anandamide > capsazepine. Also in MCF-7 and T-47D cells, where a select number of compounds was tested, arvanil was more potent than olvanil and capsaicin, which were more potent than palvanil (Fig. 3a). The effect of capsaicin and anandamide together was not significantly higher than the sum of the effects of the two drugs alone in any of the three lines studied (data not shown). None of the N-AVAMs tested was toxic to cells up to 5 μM. When tested on RBL-2H3 and RBL-1 rat basophilic, DU-145 human prostate, N18TG2 mouse neuroblastoma and J774 mouse monocytic cells, none of the N-AVAMs was active up to 2.5 μM (data not shown). Arvanil did not induce MCF-7 cell apoptosis even after 72 hours and at the higher concentration tested (data not shown). In both MCF-7 and EFM-19 cells, the CB1 receptor antagonist SR 141716A (but not the CB2 antagonist SR144528) significantly attenuated the anti-proliferative effect of anandamide, arvanil and olvanil, but not that of capsaicin and RTX (Fig.

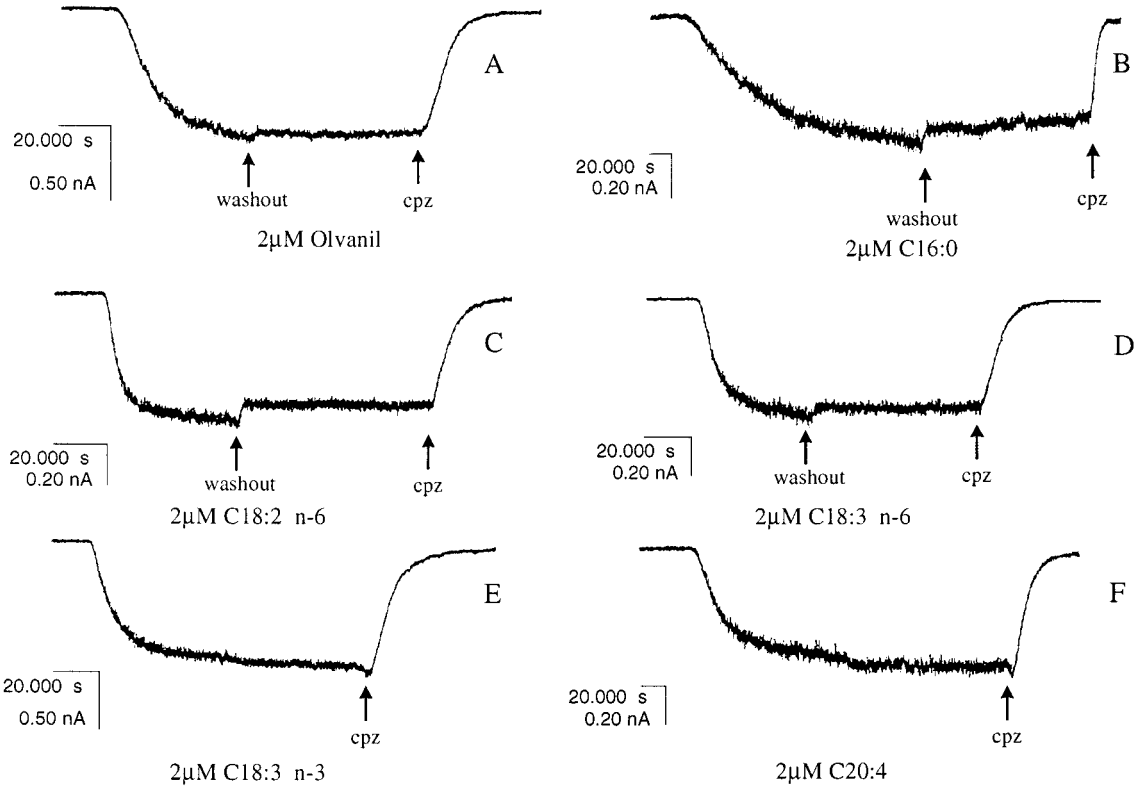

FIG. 2. Effect of N-AVAMs (2 μM) on ion currents in *Xenopus laevis* oocytes injected with VR1 receptor cRNA. The time and ion current scales for each compound are shown. Traces are representative of three separate experiments. The little effect of a water washout is shown in some traces. Little or no response was found with a 200 nM concentration. No response was found with vehicle or with non-transfected oocytes (not shown). Cpz, capsazepine.

3b and data not shown). The competitive vanilloid receptor antagonist capsazepine (0.25 μM) and the ion channel inhibitor ruthenium red (0.2 μM) significantly attenuated the effect of arvanil, olvanil, RTX and capsaicin but not that of anandamide (Fig. 3c and data not shown).

N-AVAMs bind to CB1-like receptors in MCF-7 cell membranes. Membranes from MCF-7 cells contain specific binding sites for the selective CB1 ligand [^{3}H]SR 141716A (Bmax = 121 ± 35 fmol/mg protein, Kd = 3.8 ± 1.5 nM, n = 3, as assessed from Scatchard plots calculated by using increasing concentrations [100–10000 pM] of [^{3}H]SR 141716A). Arvanil, olvanil and palvanil displaced the binding of [^{3}H]SR 141716A with an order of potency identical to that reported above for N18TG2 cell membranes (Table 2) and to that observed for the inhibition of MCF-7 cell proliferation, i.e. arvanil > olvanil > palvanil.

TABLE 3

Effect of N-AVAMs on the Proliferation of Three Human Breast Cancer Cell Lines (MCF-7, EFM-19, and T-47D)

	MCF-7 cells	EFM-19 cells	T-47D cells
Capsaicin	1.05 ± 0.15	0.80 ± 0.05	1.10 ± 0.10
Resiniferatoxin	N.D.	2.50 ± 0.15	N.D.
Capsazepine	N.D.	2.60 ± 0.21	N.D.
C9:0 (pseudocapsaicin)	N.D.	0.85 ± 0.04	N.D.
C16:0 (palvanil)	2.20 ± 0.30	1.00 ± 0.12	1.60 ± 0.15
Δ^9-*cis* C18:1 (olvanil)	1.60 ± 0.18	0.70 ± 0.08	0.75 ± 0.06
$\Delta^{5,8,11,14}$-*cis* C20:4 n-6 (arvanil)	0.40 ± 0.15	0.55 ± 0.06	0.35 ± 0.03
Anandamide	2.30 ± 0.35	1.50 ± 0.30	1.90 ± 0.20

Note. The effects are expressed as IC_{50} in μM, i.e., as the concentration necessary to achieve 50% inhibition of cell proliferation (see legend to Fig. 3), and are means ± S.D. of three independent experiments carried out in duplicate. N.D., not determined.

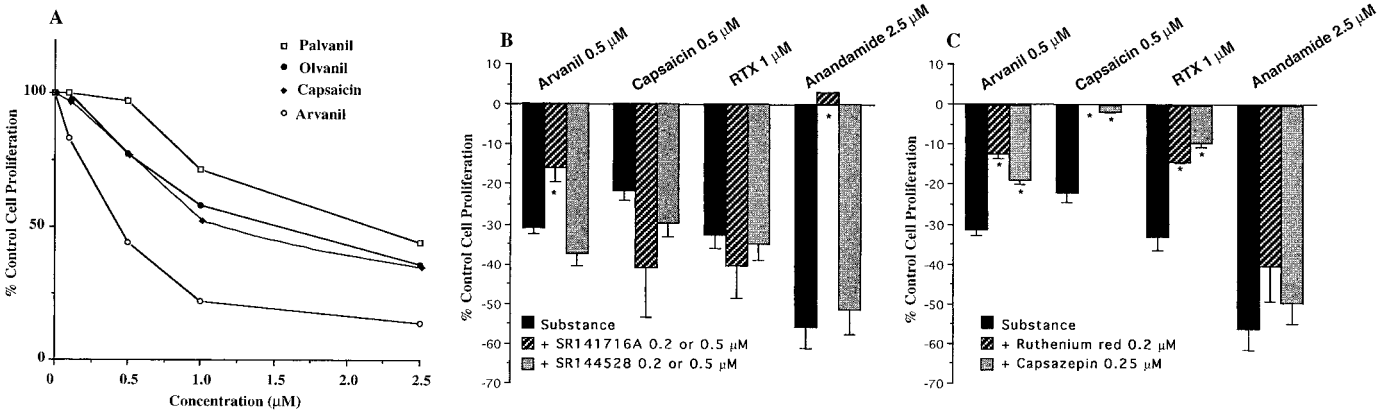

FIG. 3. Effect of N-AVAMs on the proliferation of MCF-7 cells. The effect was expressed as percent cell proliferation, calculated from the following formula: (1 − [control cell number − treated cell number]/[control cell number − initial cell number] × 100. 100% = no effect, 0% = maximal cytostatic effect. (A) Effect of arvanil, olvanil, palvanil and capsaicin on cell proliferation. (B) Effect of cannabinoid receptor antagonists on capsaicin, RTX and arvanil inhibition of cell proliferation. Several concentrations of antagonists were used and the one shown is that exerting the maximal inhibitory effect in each case, i.e. 0.2 μM with capsaicin and arvanil, and 0.5 μM with anandamide. A little (3–9%) albeit significant inhibition of HBCC proliferation was observed with these doses when administered alone. (C) Effect of vanilloid receptor antagonists on capsaicin, RTX and arvanil inhibition of cell proliferation. The effect of capsazepine and ruthenium red alone on HBCC proliferation at the doses shown was negligible. Data are means ± S.D. of three separate experiments carried out in duplicate. Similar results were obtained in EFM-19 cells (Table 3 and data are not shown), where: a) the effect of 1 μM olvanil was almost totally reversed by capsazepine and slightly attenuated by SR 141716A (from −45.8% to −14.6% and −35.6%, respectively); and b) the effect of 0.5 μM arvanil was strongly attenuated by both capsazepine and SR 141716A (from −42.5% to −19.6% and −20.8%, respectively). *, $p < 0.01$.

DISCUSSION

The capsaicin analogue olvanil has been widely studied for its pharmacological properties as an activator of vanilloid receptors and its subsequent vasodilatory and anti-inflammatory actions in vitro, as well as for its possible use as an analgesic agent in vivo (6, 7, 8, 27). Other N-AVAMs have been shown to exhibit a similar pharmacological profile (8, 26). We reported that olvanil potently inhibits the facilitated transport of anandamide into RBL-2H3 cells (18) and binds to CB1 cannabinoid receptors in mouse N18TG2 cells with an affinity comparable to that reported for olvanil binding to vanilloid receptors in rat dorsal root ganglia (27). In this study we attempted to improve the capability of olvanil to inhibit the anandamide transporter and/or activate CB1 receptors without dramatically changing its activity on vanilloid receptors. In order to do so we examined eleven N-AVAMs differing from olvanil for the length or degree of unsaturation of their aliphatic chain. These compounds were tested for: (i) their effect on [^{14}C]anandamide accumulation in RBL-2H3 cells, where an anandamide membrane 'carrier' is present (14), (ii) their potency in competition binding assays with the highly selective CB1 ligand (and antagonist) SR141716A or the synthetic cannabinoid WIN55,212-2 (which is slightly more selective for CB2 than CB1 receptors) (10) in membrane preparations from two cell lines, N18TG2 and RBL-2H3 cells, previously shown to express selectively CB1 and CB2 receptors, respectively (28, 22, 24, 10). Moreover, in order to check their capability to activate vanilloid receptors, we compared the effects of some of the long chain N-AVAMs to that of olvanil (and capsaicin) in a functional assay of VR1 activation.

We found that, of the eleven compounds tested, only the all-*cis* unsaturated analogues potently inhibited anandamide facilitated diffusion. This suggests that the anandamide membrane 'carrier' imposes that at least one *cis* double bond be present in the fatty acyl chain of N-AVAMs for an optimal interaction with the binding site. Indeed, the inhibitory activity of N-AVAMs increased when passing from the mono- to the tri-unsaturated C18 analogues, and arvanil was slightly more potent than the C18:3 n-6 analogue, thus suggesting that the presence of 1,4-diene groups and of 18–20 carbon atoms are important determinants for an optimal binding of N-AVAMs with the 'carrier' protein. Moreover, since arvanil was more potent than AM404 in this model (65.0% and 12.9% inhibition, at 10 μM, Table 1 and [18]), it can be proposed that the presence of a methylene spacer between the 4′-hydroxy-phenyl- and the arachidonate moieties, and/or of a 3′-methoxy-group on the benzene ring, increase the affinity of phenolic anandamide analogues for the anandamide 'carrier'. In view of the fact that none of the N-AVAMs efficiently inhibited anandamide hydrolysis in a FAAH assay carried out here in N18TG2 cells, these data may allow the design of new potent and selective inhibitors of anandamide facilitated transport among the N-AVAM class of compounds.

Most of the N-AVAMs tested in this study displaced the specific binding of [^{3}H]SR141716A from N18TG2

cell membranes, but not that of [^{3}H]WIN55,212-2 from RBL-2H3 cell membranes. Although they are not heterologous expression systems, N18TG2 and RBL-2H3 cells represent two well accepted models for the study of CB1- and CB2-mediated biological actions, and have been previously used to test the binding affinities of CB1 and CB2 agonists (22, 24, 10, 29). In particular, a good correlation among the Kd values of cannabinoid high affinity ligands determined in membranes from N18TG2 cells, mouse brain and cells overexpressing the cDNA for the mouse CB1 receptor was found (24). The CB1 affinities of the active N-AVAMs (Ki = 0.5–4.3 μM) were comparable to—or even higher than—that previously reported for the binding of olvanil to vanilloid receptors in dorsal root ganglia membranes (IC_{50} = 8.8 μM, as determined by using [^{3}H]RTX as the radioligand [27]). In the C18 and, possibly, C20 series, the CB1 affinity constants decreased when increasing the number of 1,4-diene groups, whereas no significant change was observed when shortening a fully saturated C18 fatty acyl chain. The C18:3 n-3 and Δ^9-*trans* C18:1 derivatives and capsaicin were significantly less active than the C18:3 n-6, Δ^9-*cis* C18:1 derivatives and capsaicin were significantly less active than the C18:3 n-6, Δ^9-*cis* C18:1 (i.e. olvanil) and C9:0 (pseudocapsaicin) derivatives, respectively. These data are in agreement with previous structure/activity studies carried out with acylethanolamides containing different fatty acyl chains (30), as well as with the requirement of a saturated terminal chain of at least five carbon atoms for the recently proposed anandamide pharmacophore (31). Our present observations that arvanil, i.e. the 3′-methoxy-4′-hydroxy-benzyl-amide of arachidonic acid, is a weak competitive inhibitor (and, therefore, a poor substrate) of FAAH, and more potent than anandamide as a ligand of CB1 receptors, are in agreement with the previous finding that 4′-hydroxy-phenyl-arachidonoylamides are more metabolically stable than anandamide, and are good ligands for the CB1 receptor only when a methylene spacer is introduced between the 4′-hydroxy-phenyl and the arachidonoyl groups [as in arvanil and *N*-(4′-hydroxy-benzyl)-arachidonoylamine) (32)]. Due to the great difference between the Ki values sometimes observed for the same cannabinoid ligand when using different membrane preparations and radioligands (10), further studies will be required in order to assess whether arvanil can be considered as a relatively potent cannabinoid ligand. The data presented here, however, allow us to propose that, at specific SR141716A/CB1 binding sites, arvanil and the C18:3 n-6 N-AVAM are more potent than anandamide, and exhibit at least 30- and 13-fold selectivity for CB1 over CB2 receptors, respectively. Arvanil, like olvanil (18), behaves as a functional agonist at CB1 receptors since it exhibits a typical CB1-mediated functional response, i.e. the inhibition of the forskolin-induced formation of cAMP in intact N18TG2 cells (10). Since the anti-hyperalgesic activity of cannabinoids is due to activation of CB1 receptors (20, 21), and immune cells involved in inflammatory reactions express both CB1 and CB2 receptor subtypes (10), arvanil may prove to be a potent anti-inflammatory and analgesic compound.

In order to assess if changes in the fatty acyl chain of oleamide would influence its capability to activate vanilloid receptors, we used a functional assay for the recently cloned VR1 receptor carried out in an heterologous expression system for this protein (1). As expected from their pharmacological properties (8, 26), we found that olvanil and five other long chain N-AVAMs were active and behaved qualitatively in a very similar way in this assay. At a concentration previously found to be nearly saturating for capsaicin (1), all compounds produced ion currents of similar amplitudes, whereas at a 10-fold lower concentration little or no effect by N-AVAMs, including capsaicin, was observed (data not shown). The effect of N-AVAMs could only be detected in oocytes injected with VR1 receptor cDNA, and was completely reversed by the vanilloid receptor antagonist capsazepine, thus providing strong evidence for the functional interaction of these compounds with VR1. Unlike capsaicin, however, the effect of long chain N-AVAMs, including olvanil, was not reversed by washout with perfusate solution. This difference may be due to the fact that long-chain N-AVAMs are more lipophilic than capsaicin and, subsequently, more likely to be absorbed on oocyte membranes than the natural vanilloid compound. In support of this hypothesis, two recent studies showed reversibility for the stimulatory effects on TRP and TRPL channels by fatty acids (which are significantly more polar than N-AVAMs) (33), but not by diacylglycerols (which are as lipophilic as N-AVAMs) (34). Interestingly, a very slow return to baseline after wash had been observed also for olvanil-induced currents in rat trigeminal ganglion (6), a tissue which also expresses VR1 (1). The different membrane lipid composition of the cell type or tissue used to test the effect of olvanil may account for some differences in the biological response observed in different assays.

On the basis of the findings described here it is possible to conclude that, of the N-AVAMs tested: (i) as expected, all are functionally active at VR1 receptors, (ii) none is a potent competitive inhibitor of FAAH, and (iii) the C18:3 n-6 analogue and, particularly, arvanil are active at both CB1 receptors and as inhibitors of anandamide facilitated transport; they can, therefore, behave as a cannabimimetic agents by both directly activating CB1 receptors and minimizing the 'carrier'-mediated inactivation of endogenous anandamide. As a direct application of these findings we wanted to investigate the effects of N-AVAMs in a simple and unique model of both anandamide- and capsaicin-like activity, i.e. the inhibition of HBCC proliferation. Anandamide

and synthetic cannabinoids were recently shown to act as anti-mitogenic factors for HBCCs by activating CB1-like receptors (23). In agreement with these findings we found that [^{3}H]SR141716A binding sites, CB1 mRNA and a CB1-immunoreactive protein are expressed in HBCCs (Melck, D., De Petrocellis, L., Orlando, P., Laezza, C., Bifulco, M. and Di Marzo, V., submitted). A non-cannabimimetic substance capable of inhibiting anandamide hydrolysis in HBCCs, oleamide, exerts a weak anti-proliferative action in these cells probably by acting through raised levels of anandamide ([25] and references therein). This shows that both cannabinoid ligands and substances that inhibit the inactivation of endogenous anandamide may give positive responses in this test. No molecular evidence exists, on the contrary, for the involvement of vanilloid receptors in capasicin-induced inhibition of tumoral mammary epithelial cells, which has been associated (35) with inhibition of NADH oxidase and induction of apoptosis. More recent studies, however, have suggested that vanilloid receptors are at least in part involved in the anti-proliferative effects of capsaicin and RTX, for example in glioma cells (36). We found that capsaicin inhibits the proliferation HBCCs while RTX, which is 1–3 orders of magnitudes more active than capsaicin on VR1 receptors (1, 27), was less active in this test. The vanilloid receptor antagonist capsazepine, which does not activate VR1 receptors (1), was also active. However, low doses of capsazepine and of the ion channel blocker, ruthenium red, strongly attenuated the effect of both capsaicin and RTX. Thus, it is possible to suggest that capsaicin and RTX inhibit HBCC proliferation through both vanilloid receptor-independent (e.g. inhibition of NADH oxidase) and -dependent mechanisms. In the latter case, a vanilloid receptor subtype with low affinity for RTX, which was proposed to be present in human tissues (4) and some non-nervous cells (e.g. rat mastocytes [37]), may be involved, analogous to what suggested for the anti-proliferative and apoptotic effects of capsaicin on rat C6 glioma cells (36), which also respond to capsazepine after long-term treatment. When we tested arvanil, olvanil and palvanil on HBCCs, we found that these compounds inhibited proliferation with an order of potency identical to their relative affinity for CB1 receptors in N18TG2 and MCF-7 cell membranes. The anti-proliferative effect of arvanil was about 4- and 2-fold more potent than that of both anandamide and capsaicin, i.e. two substances that preferentially activate CB1 or vanilloid receptors, respectively, and was attenuated in part by SR 141716A, and in part by capsazepine or ruthenium red, at concentrations that totally block the effect of anandamide and capsaicin, respectively. Therefore, these data support our hypothesis that arvanil may behave as a 'hybrid' cannabinoid/vanilloid agonist capable of exploiting this unique property also in intact cells, with subsequent improved biological actions with respect to 'pure' cannabinoid or vanilloid agonists. However, a full molecular characterization of capsaicin anti-proliferative effects on HBCCs must be awaited before definitively concluding that part of arvanil actions in this system are due to activation of VR1 receptors. It is worthwhile noting that this compound, at a 5 μM concentration and after prolonged incubations, did not induce MCF-7 cell apoptosis—an ultimate consequence of NADH oxidase inhibition (35)—which suggests that N-AVAMs may exert non-vanilloid receptor mediated effects in these cells only at doses higher than 5 μM.

In conclusion, we have shown that it is possible to modify the aliphatic chain of olvanil in order to improve both its CB1-binding activity and its capability of inhibiting carrier-mediated anandamide inactivation, while preserving its functional activity at vanilloid receptors. These findings prompt further studies on the possibility of developing potent cannabimimetic N-AVAMs, for example by investigating the effect on their CB1-binding activity of α-methylation or branching of the *n*-pentyl chain, previously shown to greatly improve anandamide cannabimimetic activity (38). This may lead to further vanilloid/cannabinoid 'hybrids' with potential therapeutic use as analgesic, anti-inflammatory, vasodilatory or anti-tumor drugs.

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REFERENCES

1. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) *Nature* **389,** 816–824.
2. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. (1998) *Neuron* **21,** 531–543.
3. Holzer, P. (1991) *Pharmacol. Rev.* **43,** 143–201.
4. Szallasi, A. (1994) *Gen. Pharmac.* **25,** 223–243.
5. Szallasi, A., and Blumberg, P. M. (1996) *Pain* **68,** 195–208.
6. Liu, L., Lo, Y., Chen, I., and Simon, S. A. (1997) *J. Neurosci.* **17,** 4101–4111.
7. Sietsema, W. K., Berman, E. F., Farmer, R. W., and Maddin, C. S. (1988) *Life Sci.* **43,** 1385–1391.
8. Dray, A., and Dickenson, A. (1991) *Pain* **47,** 79–83.
9. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949.
10. Pertwee, R. G. (1997) *Pharmacol. Ther.* **74,** 129–180.
11. Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998) *Trends Neurosci.* **21,** 521–528.
12. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.-C., and Piomelli, D. (1994) *Nature* **372,** 686–691.

13. Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) *Nature* **384,** 83–87.
14. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., and Di Marzo, V. (1997) *J. Biol. Chem.* **272,** 3315–3323.
15. Hillard, C. J., Edgemond, W. S., Jarrahian, A., and Campbell, W. B. (1997) *J. Neurochem.* **69,** 631–638.
16. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997) *Science* **277,** 1094–1097.
17. Maccarrone, M., Van der Stelt, M., Rossi, A., Veldink, G. A., Vliegenthart, J. F. H., and Finazzi-Agro', A. (1998) *J. Biol. Chem.* **273,** 32332–32339.
18. Di Marzo, V., Bisogno, T., Melck, D., Ross, R., Brockie, H., Stevenson, L., Pertwee, R., and De Petrocellis, L. (1998) *FEBS Letts.* **436,** 449–454.
19. Beltramo, M., and Piomelli, D. (1999) *Eur. J. Pharmacol.* **364,** 75–78.
20. Richardson, J. D., Aanonsen, L., Hargreaves, K. M. (1998) *Eur. J. Pharmacol.* **345,** 145–153.
21. Richardson, J. D., Kilo, S., Hargreaves, K. M. (1998) *Pain* **75,** 111–119.
22. Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S. D., and Leon, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3376–3380.
23. De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M., and Di Marzo, V. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8375–8380.
24. Abood, M. E., Ditto, K. E., Noel, M. A., Showalter, V. M., and Tao, Q. (1997) *Biochem. Pharmacol.* **53,** 207–214.
25. Bisogno, T., Katayama, K., Melck, D., Ueda, N., De Petrocellis, L., Yamamoto, S., and Di Marzo, V. (1998) *Eur. J. Biochem.* **254,** 634–642.
26. Janusz, J. M., Buckwalter, B. L., Young, P. A., LaHann, T. R., Farmer, R. W., Kasting, G. B., Loomans, M. E., Kerckaert, G. A., Maddin, C. S., Berman, E. F., *et al.* (1993) *J. Med. Chem.* **36,** 2595–2604.
27. Winter, J., Walpole, C. S. J., Bevan, S., and James, I. F. (1993) *Neuroscience* **57,** 747–757.
28. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) *Nature* **346,** 561–564.
29. Ross, R. A., Murphy, V. L., and Pertwee, R. G. (1997) *Prostagl. Leukotr. Essent. Fatty Acids* **57,** 211, Th16.
30. Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., and Devane, W. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7656–7660.
31. Tong, W., Collantes, E. R., Welsh, W. J., Berglund, B. A., and Howlett, A. C. (1998) *J. Med. Chem.* **41,** 4207–4215.
32. Khanolkar, A. D., Abadji, V., Lin, S., Hill, W. A., Taha, G., Abouzid, K., Meng, Z., Fan, P., and Makriyannis, A. (1996) *J. Med. Chem.* **39,** 4515–4519.
33. Chyb, S., Raghu, P., and Hardle, R. C. (1999) *Nature* **397,** 255–259.
34. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) *Nature* **397,** 259–263.
35. Morre, D. J., Chueh, P. J., and Morre, D. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1831–1835.
36. Biro, T., Brodie, C., Modarres, S., Lewin, N. E., Acs, P., and Blumberg, P. M. (1998) *Brain Res. Mol. Brain Res.* **56,** 89–98.
37. Biro, T., Maurer, M., Modarres, S., Lewin, N. E., Brodie, C., Acs, G., Acs, P., Paus, R., and Blumberg, P. M. (1998) *Blood* **91,** 1332–1340.
38. Ryan, W. J., Banner, W. K., Wiley, J. L., Martin, B. R., and Razdan, R. K. (1997) *J. Med. Chem.* **40,** 3617–3625.